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Ciências

The role of phytochemicals in *Arcobacter butzleri* resistance

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Abstract

Arcobacter butzleri is an emergent pathogen found in a wide range of habitats and hosts, which has developed resistance to several antibiotics. Efflux pumps are an important mechanism of antimicrobial resistance, therefore, the use of efflux pump inhibitors (EPIs) may have the potential to restore *A. butzleri* susceptibility to old antibiotics. Plants have shown the ability to fight off infections despite the moderate antimicrobial action of some phytochemicals, so we aimed to test several bioactive compounds as putative EPIs, evaluating their role in the improvement of antibiotics' performance against *A. butzleri*. To achieve this goal, the tolerance or resistance profile of *A. butzleri* strains regarding phytochemicals and antibiotics was traced through the determination of the minimum inhibitory concentration (MIC); assays of ethidium bromide accumulation were performed to assess the inhibition of the efflux pumps; the MIC of the phytochemicals in the presence of known EPIs was determined to examine the potential role of efflux pumps as resistance mechanism to the phytochemicals; checkerboard assays were made to investigate if the phytochemicals had a synergic interaction with the antibiotics; and finally, quorum sensing inhibition tests were carried out, since this mechanism is a promisor target to fight off bacterial infection.

The determination of the MIC of the phytochemicals demonstrated that none of the compounds had antimicrobial activity at the concentrations tested, except for stilbenes, which MIC ranged from 64 to 512 µg/mL. Ethidium bromide accumulation assays showed that some of the tested phytochemicals presented a fluorescence folding increase higher than the controls, indicating that they may inhibit efflux pumps; however only the stilbenes presented a typical EPI profile. The assessment of the MIC of the phytochemicals in the presence of a sub-inhibitory concentration of EPIs, revealed that the importance of efflux pumps in the bacteria resistance to phytochemicals is dependent on the strain. Several phytochemicals were selected for checkerboard titration assays revealing no synergism with antibiotics, however, several cases of additivity were detected. Quorum sensing assays revealed that resveratrol and pinosylvin were able to inhibit this mechanism.

In conclusion, some of the phytochemicals tested presented potential to reduce *A. butzleri* resistance to antibiotics as demonstrated by the results obtained to resveratrol, pinosylvin and gallic acid, which have shown an additive effect when combined with the antibiotics. According to the ethidium bromide accumulation assay, the additive action of resveratrol and pinosylvin may be associated with efflux pump inhibition. Furthermore, these two stilbenes also possess the capacity to inhibit quorums sensing, suggesting that they may be able to inhibit *A. butzleri* virulence traits.

Keywords

Arcobacter butzleri, resistance, phytochemicals, efflux pump inhibitors, additive interaction.

Resumo alargado

Arcobacter butzleri é um patogéneo emergente normalmente associado a doenças gastrointestinais em humanos e animais, e a problemas reprodutores, nomeadamente abortos, em animais. Como muitos agentes patogénicos, *A. butzleri* tem vindo a desenvolver resistência e multirresistências a vários antibióticos. Considerando que as bombas de efluxo são um importante mecanismo de resistência antimicrobiana, sendo essenciais para o desenvolvimento de multirresistências, a estratégia de usar inibidores de bombas de efluxo para restaurar a suscetibilidade desta bactéria a antibióticos comuns é deveras promissora. Tendo em conta que as plantas estão constantemente expostas a stresses bióticos e abióticos e, apesar de alguns fitoquímicos apresentarem fraca atividade antimicrobiana contra bactérias Gram-negativas, as plantas conseguem combater infeções bacterianas com sucesso através do sinergismo entre compostos, surgindo assim como uma potencial fonte de compostos a explorar. O objetivo deste trabalho foi avaliar a capacidade de 14 fitoquímicos em inibir as bombas de efluxo de *A. butzleri*, e avaliar o seu potencial na melhoria da atividade de vários antibióticos contra esta bactéria.

Para alcançar este objetivo, o perfil antimicrobiano dos fitoquímicos e de vários antibióticos foi avaliado através da determinação da concentração mínima inibitória. Ensaios de acumulação de brometo de etídio foram realizados para determinar a possível inibição das bombas de efluxo pelos compostos em estudo. A concentração mínima inibitória dos fitoquímicos na presença de inibidores de bombas de efluxo conhecidos foi definida, a fim de investigar se as bombas de efluxo são o principal mecanismo de resistência da bactéria aos fitoquímicos. Também foram realizados ensaios de *checkerboard* para avaliar o potencial sinergismo entre os fitoquímicos e antibióticos e por fim também foram realizados ensaios de inibição do *quorum sensing*.

A determinação da concentração mínima inibitória dos fitoquímicos e dos antibióticos revelou que todos os fitoquímicos têm uma concentração mínima inibitória superior a 1024 µg/mL, exceto o resveratrol, o pterostilbeno e o pinosilvino, cujos valores variam entre 64 e 512 µg/mL, para as estirpes em estudo. Os resultados obtidos relativos aos ensaios de acumulação de brometo de etídio mostraram que alguns fitoquímicos, nomeadamente (+)-catequina, (-)-epicatequina, rutina, ácidos cafeico e clorogénico, resveratrol, pterostilbeno e pinosilvino levam a um aumento de fluorescência superior ao aumento de fluorescência verificado para os controlos dos solventes. Isto é, eles levam a uma acumulação de brometo de etídio dentro das células superior aos controlos, o que sugere que estes compostos podem estar a inibir as bombas de efluxo. Porém, somente os estilbenos registaram um aumento de fluorescência superior ao verificado para o inibidor de bombas de efluxo usado como controlo. Estes compostos são também os únicos que apresentam um perfil típico de um inibidor de bombas de efluxo. A fim

de determinar se as bombas de efluxo são um mecanismo relevante de resistência aos fitoquímicos, a concentração mínima inibitória dos fitoquímicos foi determinada na presença de concentrações sub-inibitórias de inibidores de bombas de efluxo para as estirpes de *A. butzleri* mais suscetível (DQ46M1) e mais resistente (CR50-2), de entre as estudadas. Verificou-se que a importância das bombas de efluxo na resistência da bactéria aos fitoquímicos é dependente da estirpe, sendo a mais resistente mais dependente das bombas de efluxo do que a mais suscetível. Com base nos resultados do ensaio da acumulação de brometo de etídio, vários fitoquímicos foram selecionados para testes de *checkerboard*. Os resultados mostraram que várias combinações fitoquímico/antibiótico apresentaram um efeito aditivo, não se observando interação antagonista para nenhuma das combinações avaliadas. Os estilbenos, mais uma vez, foi a classe de fitoquímicos que apresentou os melhores resultados. Por fim, ensaios de inibição do *quorum sensing* foram realizados a fim de determinar se os fitoquímicos têm a capacidade de inibir estes mecanismos de comunicação celular. Os ensaios mostraram que o resveratrol e o pinosilvino conseguem inibir estes sistemas. Assim, uma vez que o *quorum sensing* é fundamental para a regulação de diversos fatores de virulência como é o caso da formação de biofilmes, estes compostos bioativos podem ter o potencial de contribuir para o controlo de *A. butzleri* ao atuar sobre a formação de biofilmes, inibindo-os.

Concluindo, apesar do reduzido potencial antimicrobiano da maioria dos fitoquímicos testados, alguns destes compostos apresentaram potencial no aumento de atividade de antibióticos, como foi o caso do resveratrol, pinosilvino e ácido gálico, os quais mostraram ter uma interação aditiva com os antibióticos. De acordo com o ensaio da acumulação de brometo de etídio, o efeito aditivo apresentado pelo resveratrol e pelo pinosilvino pode estar associado à inibição das bombas de efluxo. Estes dois estilbenos também demostraram a capacidade de inibir o *quorum sensing*, o que sugere que podem ter a capacidade de inibir fatores de virulência associados a *A. butzleri*.

Palavras-chave

Arcobacter butzleri, resistência a antibióticos, fitoquímicos, inibidores de bombas de efluxo, interação aditiva.

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ABC	ATP Binding Cassette
ATP	Adenosine Triphosphate
BHI	Brain-Heart Infusion medium
BRU	Brucella Blood Agar
CCCP	Carbonyl-Cyanide m-Chlorophenylhydrazone
CFU	Colony-Forming Units
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
EPI	Efflux Pump Inhibitor
EtBr	Ethidium Bromide
FDA	Food and Drug Administration
FIC	Fractional Inhibitory Concentration
FICI	Fractional Inhibitory Concentration Index
HIV	Human Immunodeficiency Virus
LB	Luria-Bertani
LPS	Lipopolysaccharide
MATE	Multidrug And Toxic compound Extrusion
MDR	Multidrug Resistance
MFS	Major Facilitator Superfamily
MHB	Müeller-Hinton Broth
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin-Resistant *Staphylococcus aureus*
NMP	1-(1-naphthylmethyl)-piperazine
OM	Outer Membrane

PAβN	Phenylalanine– Arginine β-Naphthylamide
PBS	Phosphate-Buffered Saline solution
QRDR	Quinolone Resistance Determining Region
QSI	Quorum Sensing inhibition
RNA	Ribonucleic Acid
RND	Resistance Nodulation Division
SMR	Small Multidrug Resistance
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
UHT	Ultra-High Temperature
UV	Ultraviolet

Chapter 1- Introduction

1.1. Genus *Arcobacter*

The genus *Arcobacter* is a diverse group of Gram-negative bacteria that, together with the *Campylobacter* and *Sulfurospirillum* genera, constitute the *Campylobacteraceae* family (Collado and Figueras, 2011). Recently, this member of the *Epsilonproteobacteria* class, has been gaining increasing attention since some species are considered emergent pathogens and potential zoonotic agents (Collado et al., 2011; Mansfield et al., 2000).

Currently, this genus is composed of 27 species, the majority isolated in the last decade from several environments and hosts (Table 1).

The first *Arcobacter* was isolated by Ellis et al. in 1977 from bovine foetuses (Fera et al., 2009). However, this genus was only proposed in 1991 to reclassify *Campylobacter cryaerophila* and *Campylobacter nitrofigilis,* two aerotolerant *Campylobacter* species, as *Arcobacter cryaerophilus* and *Arcobacter nitrofigilis,* respectively (Vandamme et al., 1991). One year later, the genus was enlarged with the reclassification of *Campylobacter butzleri* as *Arcobacter butzleri* and the description of the new species *Arcobacter skirrowii* (Vandamme et al., 1992a). *A. butzleri* had originally been isolated in the previous year from humans and animals with diarrhoea (Kiehlbauch et al., 1991).

The name of this genus has Latin roots and means "bow-shaped rod" (Mansfield and Forsythe, 2000). True to its name, *Arcobacter* spp. are small, non-spore forming, curved rods, often helical or S shaped (0.2-0.9 μm wide and 0.5-3 μm long) (Ferreira et al., 2015; Vandamme et al., 1992a), although sometimes cells as long as 20 μm can be found (Mansfield and Forsythe, 2000).

With the exception of *A. anaerophilus*, which is an obligate anaerobe without flagella (Sasi Jyothsna et al., 2013), the members of this group move in darting or corkscrew-like movements due to a polar unsheathed flagellum at one or both ends of the cell (Vandamme et al., 1992a).

This microorganism can grow in aerobic or microaerobic (3-10% oxygen with no hydrogen required) conditions, having an optimal growth temperature of 37°C in microaerophilic conditions and of 30°C in aerobic conditions. Though, *Arcobacter* spp. can grow at higher or lower temperatures, depending on the strain and conditions (Collado and Figueras, 2011; Ferreira et al., 2015; Mansfield et al., 2000; Vandamme et al., 1992a).

Table 1. *Arcobacter* species identified so far and their original sources.

Specie	Source	Reference
Arcobacter nitrofigilis	Roots of *Spartina alterniflora* Loisel (a salt march plant) and in root-associated sediments	(McClung et al., 1983)
Arcobacter cryaerophilus	Faeces, reproductive tracts, aborted foetuses of different farm animals and from milk of cows with mastitis	(Neill et al., 1985)
Arcobacter butzleri	Humans and animals with diarrhoeal disease	(Kiehlbauch et al., 1991)
Arcobacter skirrowii	Preputial fluids of bulls Bovine, porcine, and ovine isolates obtained from aborted foetuses and diarrhoeic faeces.	(Vandamme et al., 1992a)
Arcobacter cibarius	Broiler carcasses in Belgium	(Houf et al., 2005)
Arcobacter halophilus	Hypersaline lagoon in Hawaii	(Donachie et al., 2005)
Arcobacter mytili	Mussels (*Mytilus* sp.) and brackish water in Spain	(Collado et al., 2009a)
Arcobacter thereius	Kidney and liver of Danish pigs' abortions and cloacal content of ducks	(Houf et al., 2009)
Arcobacter marinus	Seawater with seaweeds and Starfish in Korea	(Kim et al., 2010)
Arcobacter trophiarum	Faecal samples taken rectally from fattening pigs in Belgium	(De Smet et al., 2011a)
Arcobacter defluvii	Sewage samples	(Collado et al., 2011)
Arcobacter molluscorum	Mussels (*Mytilus* sp.) and oysters	(Figueras et al., 2011a)
Arcobacter ellisii	Mussels (*Mytilus* sp.)	(Figueras et al., 2011b)
Arcobacter bivalviorum	Mussels (*Mytilus* sp)	(Levican et al., 2012)
Arcobacter venerupis	Clam (*Venerupis pullastra*)	(Levican et al., 2012)
Arcobacter cloacae	Mussels (*Mytilus* sp.) and sewage from the Waste Water Treatment Plant	(Levican et al., 2013)
Arcobacter suis	Pork meat	(Levican et al.,2013)
Arcobacter anaerophilus	Estuarine sediment	(Sasi Jyothsna et al., 2013)
Arcobacter ebronensis	Mussels	(Levican et al., 2015)
Arcobacter aquimarinus	Seawater	(Levican et al., 2015)
Arcobacter lanthieri	Pig and dairy cattle manure	(Whiteduck-Léveillée et al., 2015)
Arcobacter pacificus	Seawater	(Zhang et al., 2016)
Arcobacter faecis	Human waste septic tank	(Whiteduck-Léveillée et al., 2016)
Arcobacter acticola	Seawater on the East Sea in South Korea	(Park et al., 2016)
Arcobacter porcinus	Aborted piglet foetus	(Figueras et al., 2017)
Arcobacter lekithochrous	Molluscan hatchery in Norway	(Diéguez et al., 2017)
Arcobacter haliotis	Molluscan collected in Japan	(Tanaka et al., 2017)

In a broad sense, the exception being *A. pacificus*, all species are oxidase positive, but catalase is only present in some species (Ferreira et al., 2017). Organic acids and amino acids are utilized as carbon sources (Vandamme et al., 1992a).

Campylobacter and *Arcobacter* are morphologically very similar, the key feature to distinguish them is that *Arcobacter* can grow in aerobic conditions and at lower temperatures than the former (Collado and Figueras, 2011). However, with the classification and recognition of new species in recent years, this is not an absolute principle anymore, with, for example, *A. anaerophilus* being an obligate anaerobe (Sasi Jyothsna et al., 2013).

1.2. Clinical relevance of *Arcobacter*

Arcobacter spp. are classified as emergent food and water-borne pathogens, with *A. butzleri*, *A. cryaerophilus*, and *A. skirrowii* being associated with human and animal disease (Vandenberg et al., 2004; Kayman et al., 2012a). In fact, *A. butzleri* and *A. cryaerophilus* have been classified as severe hazards to human health by the International Commission on Microbiological Specifications for Foods (ICMSF, 2002).

Among *Arcobacter* species, *A. butzleri* stands out as the more prevalent in clinical and environmental samples, as well as in food of animal origin (Van den Abeele et al., 2016; Collado and Figueras, 2011; Fernandez et al., 2015).

1.2.1. *Arcobacter* in humans

A. butzleri has been associated with gastrointestinal diseases such as enteritis and colitis, bacteraemia and septicaemia (Van den Abeele et al., 2016; Fera et al., 2010; Fernandez et al., 2015). Furthermore, it has been repeatedly classified as the fourth most common pathogen associated with diarrhoeal illness (Van den Abeele et al., 2014; Collado et al., 2013; Ferreira et al., 2014a; Prouzet-Maulon et al., 2006; Vandenberg et al., 2004).

Although *Arcobacter* spp. have been isolated in asymptomatic hosts (Houf and Stephan, 2007), *A. butzleri* is typically associated with watery diarrhoea, abdominal pain, nausea, vomiting and fever (Arguello et al., 2015; Jiang et al., 2010; Kayman et al., 2012a; Kiehlbauch et al., 1991; Teague et al., 2010; Vandamme et al., 1992b; Vandenberg et al., 2004). Though, there are cases where these symptoms are not all present, as illustrated by an *A. butzleri* outbreak in a school in Italy, where the infected children only reported abdominal pain (Vandamme et al., 1992b). Once again, it is easy to confuse an *Arcobacter* spp. infection with a *Campylobacter* spp. infection as they share many symptoms; however, *Campylobacter jejuni* is usually associated with bloody diarrhoea versus the watery one of *A. butzleri* (Vandenberg et al., 2004).

A study made with infected human colonic epithelial cells (HT-29/B6) concluded that the process by which *A. butzleri* induces diarrhoea is mediated by the reduced expression of tight-junction proteins claudin-1, -5 and -8, which causes an epithelial barrier dysfunction and,

consequently, epithelial apoptosis. This leads to diarrhoea through a leak flux mechanism (Bücker et al., 2009).

Arcobacter spp. has also been associated with a few cases of bacteraemia. *A. butzleri* bacteraemia cases include an 85 year old man with chronic lymphocytic leukaemia (Arguello et al., 2015), a 69 years old woman with acute gangrenous appendicitis (Lau et al., 2002) and a neonate in the United Kingdom (On et al., 1995). On the other hand, *A. cryaerophilus* bacteraemia was diagnosed in an uremic patient with hematogenous pneumonia (Hsueh et al., 1997) and a 7 year old boy that had developed acute respiratory distress and renal failure (Woo et al., 2001). Furthermore, *Arcobacter* spp. was also linked with enteritis (Van den Abeele et al., 2014) and peritonitis (Monzon and Coronel, 2013).

Host characteristics, such as the state of the immune system, may play a role in the development of *A. butzleri* infection and pathogenicity, as studies made in India with human immunodeficiency virus type 1 (HIV-1) infected patients (Kownhar et al., 2007) and in Italy with type 2 diabetic individuals (Fera et al., 2010) showed. Both studies found a higher prevalence of *A. butzleri* in the ill patients versus the control group of healthy subjects. Moreover, a study in Canada found the prevalence of *A. butzleri* in diarrhoeic (56.7%) and non-diarrhoeic (45.5%) individuals very similar (Webb et al., 2016), suggesting that infection only occurs when certain circumstances are met.

In general, *A. butzleri* infections are not very severe, with cases of bacteraemia typically occurring in immunocompromised hosts. However, these infections can persist from a couple of days to a couple of months leading to a loss of life quality and leaving the immune system debilitated (Prouzet-Maulon et al., 2006; Tee et al., 1988; Vandamme et al., 1992b; Vandenberg et al., 2004).

Most laboratories do not use the appropriate conditions for the identification of *Arcobacter* spp., so they tend to be wrongfully classified as campylobacters. As such, the prevalence of *Arcobacter* infections is not truly known (Taylor et al., 1991; Prouzet-Maulon et al., 2006). However, globally, reports from Europe show an A. *butzleri* percentage of 0.07% in healthy patients in Denmark (Engberg et al., 2000), 0.4% in patients suspected of infectious gastroenteritis in the Netherlands (De Boer et al., 2013), 1% in patients suspected of having a *Campylobacter* infection in France (Prouzet-Maulon et al., 2006), 3.5% in hospitalized patients (Vandenberg et al., 2004), 0.7% in stools of patients with enteritis in Belgium (Van den Abeele et al., 2014) and 1.3% in diarrhoeal stools collected from 22 hospitals of Portugal (Ferreira et et al., 2014a). In South Africa two studies were made, one studied a heterogeneous population and had an *Arcobacter* spp. prevalence of 6.2% (Samie et al., 2007), the other analysed diarrhoeic stools obtained from a hospital and had a prevalence of only 0.33%. Additionally, it was reported an *A. butzleri* prevalence in patients with diarrhoea of 1.4% in Chile, (Collado et al., 2013), 0.51% in New Zealand (Mandisodza et al., 2012) and 2.38% in Thailand (Taylor et al., 1991). Lastly, India reported a prevalence of *Arcobacter* spp. of 1.25% (Kownhar et al., 2007).

The discrepancy in the results may be a reflex not only of the diverse prevalence of *Arcobacter* spp. in the different countries, but also due to the different methods of detection used and populations studied (Collado and Figueras, 2011).

There are some reports of travellers that developed *A. butzleri* infections while aboard. For example, a diabetic German man was admitted in the hospital with *A. butzleri* infection three months after visiting Thailand, Singapore and Hong Kong (Lerner et al., 1994), and a man that was returning from an European cruise was hospitalized with *A. butzleri* bacteraemia (Arguello et al., 2015). A larger study also analysed European and US travellers that acquired acute diarrhoea in Mexico, Guatemala and India and reported that 8% of them were hosts to *A. butzleri*. Yet, as other microorganisms were also identified in some of the tourists, the role of *A. butzleri* as the causative agent was not certain (Jiang et al., 2010).

Currently, the precise mechanisms of pathogenicity of *Arcobacter* spp. remains relatively unexplored. Human and animal cell culture *in vitro* assays have shown that several *Arcobacter* species can adhere and invade eukaryotic cells (Fallas-Padilla et al., 2014), and produce toxins that damage host cells (Carbone et al., 2003). *Arcobacter* spp. also seems to be involved in inflammatory processes, as it is possible to find leukocytes (Kayman et al., 2012b; Vandenberg et al., 2004) and lactoferrin (Samie et al., 2007) in stools of patients with *A. butzleri* infection. Also, it was been demonstrated that *A. butzleri* is highly susceptible to human blood serum, being possibly able to activate the complement by an alternative pathway (Wilson et al., 2010).

1.2.2. *Arcobacter* in animals

A. butzleri, *A. cryaerophilus*, and *A. skirrowii* are the species most commonly recovered from animals (Kabeya et al., 2003; On et al., 2002; De Smet et al., 2011a).

Arcobacter spp. has been found in healthy animal hosts (De Smet et al., 2011b; Stirling et al., 2008; Van Driessche et al., 2004), however, they are also associated with diarrhoea (Kiehlbauch et al., 1991; Anderson et al., 1993), mastitis (Logan, 1982), reproductive problems, namely aborts (Oliveira et al., 1997; On et al., 2002; Vandamme et al., 1992a), and a few cases of active colitis (Anderson et al., 1993). One study also reported the development of lesions in the gastric mucosa in piglets infected with *Arcobacter* spp. but it was not possible to definitively link the lesions with the presence of the bacterium (Suarez et al., 1997).

It was been suggested that *Arcobacter* strains associated with infertility could be opportunistic pathogens that infect the foetus after the placenta being compromised as a study found that the strains isolated from reproductively impaired and in normal sows were similar (de Oliveria et al., 1999).

1.3. Distribution and transmission of *Arcobacter*

Arcobacter spp. has been isolated worldwide from healthy and diseased animals and humans, water, food and food processing facilities (Ferreira et al., 2017). The vast distribution of this microorganism is supported by genomic studies, as the analysis of the human strain *A. butzleri* RM4018 shows that a substantial portion of the bacteria's genome is associated with its adaptation to different environmental conditions (Miller et al., 2007).

The most likely route of human contamination is the consumption of contaminated water and food (Miller et al., 2009), though transmission by contact with a human or animal host is also a possibility (Fera et al., 2009; Vandamme et al., 1992b).

1.3.1. Transmission person-to-person

In 1983, in a period of two months, ten children that frequented the same nursey school in Italy, started to suffer from abdominal pain, vomiting and fever. When the children' stools were analysed, it was discovered that not only was *A. butzleri* present in all the samples, but that all the strains shared phenotypic and genotypic characteristics. That, combined with the fact that the other children and staff that used the school dining room did not get sick, plus the conspicuous timing of the infections, all very close together, raised the hypothesis that person-to-person transmission had occur (Vandamme et al., 1992b).

A few years later, it was reported a case of a neonate with *A. butzleri* bacteraemia. This report is important because it was suggested that the infection has been contracted in utero, likely due to a prenatal bleeding experienced by the mother. This was the first study that indicated the possibility of vertical transmission in humans (On et al., 1995).

Venereal transmission of this bacterium has been suggested for animals (Ho et al., 2006a), but no information is available regarding humans.

1.3.2. Distribution and transmission through contact with pets

Arcobacter spp. has also been isolated from the oral cavities and faeces of pets, namely cats and dogs. As such the contact with them and the faecal contamination of the environment has been suggested as a possible route of human infection.

A study performed in Denmark detected *A. butzleri* in the saliva of one cat (12.5%) and seven dogs (58%) (Petersen et al., 2007). In the same year, a study in Chile reported a 3.3% prevalence of *A. butzleri* in the faeces of dogs (Fernández et al., 2007), while, other study in Turkey did not found any isolates in dog's stools (Aydin et al., 2007). In the next year, a study in Belgium found no arcobacters in cats, while only two dogs (0.75%) and five dogs (1.87%) carried arcobacters in the mouth and faeces, respectively (Houf et al., 2008). By contrast, in Italy, it

was detected a high prevalence (78.8%) of *Arcobacter* spp. in cats, of which 77.6% were *A. butzleri* positive. The detection of this microorganism was higher in oral samples than in blood and lymph nodes (76.5% vs 2.3%) (Fera et al., 2009). Recently, a study in Czech Republic tested oral samples from cats and dogs and confirmed the presence of *A. butzleri* in one cat (1.4%) and four dogs (3.7%) (Pejchalova et al., 2017).

1.3.3. Distribution and transmission in farm animals

Several studies have reported the occurrence of *Arcobacter* spp. in healthy farm animal's faeces, namely, cattle (3.6%-39.2) (Van Driessche et al., 2003; Van Driessche et al., 2005; Giacometti et al., 2015; Kabeya et al., 2004; Öngör et al., 2004; Shirzad Aski et al., 2016; Wesley et al., 2000), pigs (7.1-85%) (Van Driessche et al., 2003; Hume et al., 2001; Kabeya et al., 2004; Van Driessche et al., 2004), chicken (14.5-64.3%) (Collado et al., 2009b; Kabeya et al., 2004), goats (10.7%) (De Smet et al., 2011b), sheep (16.1-43.1%) (Van Driessche et al., 2003; Shirzad et al., 2016; De Smet et al., 2011) and horses (15.4%) (Van Driessche et a*l.*, 2003). Being *A. butzleri* the overall most prevalent species (Van Driessche et al., 2004; González et al., 2010; Kabeya et al., 2004; Öngör et al., 2004; Shah et al., 2013), and co-infection with multiple species of *Arcobacter* an usual observation (Van Driessche et al., 2004; Shah et al., 2013). The prevalence of *Arcobacter* can be largely influenced by factors such as the farm where the study was made (reflecting the farm practices), the period of collection of the samples and the methodology used for sampling and isolation (Nieva-Echevarria et al., 2013).

Regarding the transmission of *Arcobacter* spp. among farm animals, it is thought that the main factors for this are the consume of contaminated water (Wesley et al., 2000; Giacometti et al., 2015) and living in a contaminated environment (Van Driessche et al., 2004; 2005; Eifert et al., 2003). Additionally, vertical transmission was also suggested by a study that isolated *Arcobacter* spp. from the amniotic fluid of sows and from the rectal samples of new-born piglets. The similarity between the isolates led the authors to propose that intra-uterine transmission occurred. The same study also detected horizontal transmission from the mother or the environment to the piglets, showing that post-natal contamination occurred (Ho et al., 2006a).

The fact that healthy livestock animals may be a reservoir for *Arcobacter* spp. is a public concern as it was hypothesised that *Arcobacter* spp. are introduced in slaughterhouses by the gut contents of asymptomatic animals leading to co-contaminations that are reflected in the high prevalence of genetic diverse *Arcobacter* spp. isolates in carcasses (Amare et al., 2011; Andersen et al., 2007; Van Driessche and Houf, 2007; Ho et al., 2008; Kabeya et al., 2004).

Besides pets and farm animals, *Arcobacter* spp. has also been found in more exotic or unsuspected animals such as pigeons (Giacometti et al., 2015), ducks (Fernández et al.,2010), pelicans and sparrows (Fernández et al., 2007), raccoons (Hamir et al., 2004), rainbow trout, (Yildiz and Adyn, 2006) white and black rhinoceros, gorillas, alpacas, gazelles, rhea (Wesley and Schroeder-tucker, 2011), lizards, serpents and chelonians (Gilbert et al., 2014).

1.3.4. *Arcobacter* distribution in water and its transmission

Water is suggested as playing a major role in the transmission of *Arcobacter* spp. to animals and humans. In fact, it is estimated that 63% of human *A. butzleri* infections are due to the consumption of contaminated water (Shah and Saleha, 2011).

Arcobacter spp. has been isolated from several water sources such as rivers (Collado et al., 2008, 2010; Fernández et al., 2010; Šilha et al., 2015; Laishram et al., 2016), lakes (Collado et al., 2008), seawater (Fera et al., 2004; Maugeri et al., 2004; Collado et al., 2008), wells (Fong et al., 2007; Rice et al., 1999), sewages and sludge (Collado et al., 2008; McLellan et al., 2011; Merga et al., 2014; Rodriguez-Manzano et al., 2012; Šilha et al., 2015;), drinking water, water that has received tertiary treatments (Jacob et al., 1993; Rodriguez-Manzano et al., 2012) and water used in aquafarming (Xiong et al., 2015). Additionally, it was suggested that the seasons influence the prevalence of *Arcobacter* spp. in water, as it is detected more frequently in the warmer months (Andersen et al., 2007; Collado et al., 2010).

Several reports have established an association between the isolation of *Arcobacter* spp. from water samples and its level of faecal contamination (Collado et al., 2008; Collado et al., 2010; Fong et al., 2007; Merga et al., 2014; Newton et al., 2013), with *A. butzleri* being the dominant species in most studies (Collado et al., 2010; Collado et al., 2008; Merga et al., 2014). It is understood that the inflow of faeces from human (Collado et al., 2008) and animal carriers (Newton et al., 2013; Stampi et al., 1993), transports the bacteria into the sewages and serves as a source of nutrients. That, allied with *Arcobacter*'s ability to survive in harsher environmental conditions than other faecal bacteria (Merga et al., 2014), has led to the high prevalence of this microorganism in the sewage system. Moreover, *A. butzleri* has shown the capacity to adhere and to form biofilms in various materials used in pipes (stainless steel, cooper and plastic) which indicates that it may be able to spread through the water distribution system (Assanta et al., 2002), a point that highlights the importance of the disinfection processes and supports the dissemination of the bacteria through the water system.

A. butzleri is susceptible to chlorination (Rice et al., 1999; Moreno et al., 2004), but the membrane integrity and nucleic acids remained intact for more than five hours, and so, continuous chlorination is recommended to control its spread (Moreno et al., 2004).

Studies made in Spain and South Africa have not found *Arcobacter* spp. in chlorinated drinking water (Diergaardt et al., 2004; Collado et al., 2010). However, other studies have detected this microorganism in non-chlorinated drinking water (Jacob et al., 1993; Rodriguez-Manzano et al., 2012; Shah et al., 2013). Thus, the depuration treatments applied in some water treatment plants are not able to completely remove this pathogen. It is interesting to note that the number of *Arcobacter* spp. isolated from drinking water was much higher than the number of *Campylobacter* spp. found. However, it is not possible to establish if this fact is due to *Arcobacter* resistance to the treatments applied or if it is a reflection of the different optimal growth temperature of these bacteria (Jacob et al., 1993).

Despite the chlorine susceptibility of *Arcobacter* spp., there have been some reported cases of outbreaks related with this bacterium, and so supporting water as a route of contamination. One occurred in 1996 at a Girl Scout camp in Idaho, where the outbreak was associated with the consumption of water from an *A. butzleri* contaminated well when the chlorination system was broken. It was estimated that 81% of the people there became ill with nausea, vomiting, diarrhoea and cramps (Rice et al., 1999). Other case, happened in South Bass Island, Ohio in 2004, affecting many residents and tourists that developed diarrhoea. *Arcobacter* spp. was found, once again, in contaminated wells around the area (Fong et al., 2007). More recently, in 2008, an outbreak of acute gastroenteritis affected residents in Slovenia, where 2.3% of the faecal samples analysed were positive for *A. cryaerophilus*. Assumedly, the water system distribution was contaminated due to the constructions made to build of a new connection (Kopilović et al., 2008).

1.3.5. *Arcobacter* distribution in food and its transmission

The use of sludge and animal manure to fertilize the soil is an old practice. However, it has the side effect of potentially introduce pathogens into the food chain (Udeigwe et al., 2015).The consumption of raw or undercooked contaminated food is another of major route of transmissions suggested to *Arcobacter* spp.(Lappi et al., 2013).

Arcobacter spp. has been found in carcasses and offal of farm animals (beef, pork, poultry, rabbit and lamb) (Rivas et al., 2004; Ho et al., 2006; Šilha et al., 2015), fish (Palareti et al., 2016), mussels (Fernández et al., 2010), raw milk (Giacometti et al., 2015), cheese and fresh (González and Ferrús, 2011) and ready-to-eat (Mottola et al., 2016) vegetables.

Moreover, *Arcobacter* spp. as also been detected, at a higher prevalence than *Salmonella* and *Campylobacter,* in several restaurants popular among tourists in Bangkok. It was determined that, independently of the restaurant, the risk of exposure per meal was 13%, rising to 75% once 10 or more meals are eaten (Teague et al., 2010). *A. butzleri*, particularly, has been identified as the likely etiologic agent of an outbreak of foodborne illness associated with the consumption of roasted chicken served during a wedding reception (Lappi et al., 2013). As mentioned above, *Arcobacter* spp. is frequently found in asymptomatic farm animals, contributing to faecal contamination of the carcasses during evisceration, either directly or using the equipment as an intermediate, and so being an unaccounted contamination risk during slaughter (Ho et al., 2008; Houf et al., 2002; Shah and Saleha, 2011; De Smet et al., 2010).

In food, *Arcobacter* spp. is found more frequently in meat, namely poultry (13.1%-100%) (Atabay et al., 2006; Atabay et al., 1998; Ho et al., 2008; Kabeya et al., 2004; Nieva-Echevarria et al., 2013; Rahimi, 2014; Rivas et al., 2004; De Smet et al., 2010; Villarruel-López et al., 2003), followed by pork (7%-96.4%)(Van Driessche and Houf, 2007; Kabeya et al., 2004; Nieva-Echevarria et al., 2013; Rivas et al., 2004; Villarruel-López et al., 2003), beef (2.2%-37%) (Ho et al., 2006b; Kabeya et al., 2004; Nieva-Echevarria et al., 2013; Rivas et al., 2004; De Smet

et al., 2010; Villarruel-López et al., 2003) and lamb (15%) (Rivas et al., 2004). Being the most prevalent species *A. butzleri*, though *A. cryaerophilus* and *A. skirrowii* are also not uncommon.

Relatively to other animal products, so far, studies indicated that, although breeding hens can be infected with *Arcobacter* spp., there is no contamination of the eggs (Lipman et al., 2008). Regarding dairy products, there are reports of a high prevalence (3.2%-80%) of *Arcobacter* spp. in raw milk (Scullion et al., 2006; Pianta et al., 2007; Ertas et al., 2010; Nieva-Echevarria et al., 2013; Giacometti et al., 2014) and cheese (Serraino et al., 2013; Yesilmen et al., 2014).

Arcobacter spp. has also been isolated from seafood, which consume presents a relevant hazard as this is a food product often eaten undercooked or raw. The bacterium has been found in fish (19%-25%) (Laishram et al., 2016; Rathlavath et al., 2016), clams (100%) (Collado et al., 2009b), shellfish (14.7%-73.3%) (Nieva-Echevarria et al., 2013; Laishram et al., 2016) and mussels (22.7%-41.1%) (Collado et al., 2009a; Fernandez et al., 2001; Maugeri et al., 2000). No arcobacters were found in oysters or frozen shrimps (Collado et al., 2009b). The most prevalent specie isolated was *A. butzleri* (Fernandez, 2001; Collado et al., 2009b; Rathlavath et al., 2016).

Additionally, *Arcobacter* spp. was also found in carrot (Hausdorf et al., 2011) and spinach wash water (Hausdorf et al., 2013), in fresh lettuces (20%) (González and Ferrús, 2011) and ready-to-eat (Mottola et al., 2016) vegetables. These foods are especially dangerous as they are often eaten raw and, especially in the case of the ready-to-eat, not properly washed.

Furthermore, *A. butzleri* is not able to survive in beer (Šilha et al., 2013) or apple and pear purees (Lee and Choi, 2013). High sugar content, acidic pH and the presence of polyphenols and alcohol are some of the factors probably responsible for this (Lee and Choi, 2013; Šilha et al., 2013).

1.3.5.1. Control of *Arcobacter* in food

The treatments that meat is subjected to, in order to be commercialized seems to affect the survival of *Arcobacter* spp., as several studies have showed a decrease of its prevalence. Namely, in the case of chickens, a study isolated *A. butzleri* in 95% of the fresh carcasses, but only in 23% of the frozen carcasses (Atabay et al., 2003). Another study found a prevalence of *Arcobacter* spp. of 96.8% in broiler carcasses pre-scalding, 61.3% in the carcasses pre-chill and only 9.6% in the carcasses post-chill (Son et al., 2007). Concerning pork, a study isolated *Arcobacter* spp. in 96.4% carcasses, but only in 21% of the pork at retail (Van Driessche and Houf, 2007). For beef, a study found that *Arcobacter* spp. has present in 37.4% of the carcasses collected from two slaughterhouses, but after 24 hours of cooling at 7ºC, the percentage of *Arcobacter* spp. isolated lowered significantly (7%) (De Smet et al., 2010). *Arcobacter* spp. has also been found in 9% of minced beef meat at retail (De Smet et al., 2010), as well as in vacuum

packaged chill stored beef (Balamurugan et al., 2013). Moreover, *A. butzleri* is more tolerant to radiation under vacuum in ground pork than *C. jejuni* (Collins et al., 1996).

Relatively to scalding, survival tests also indicate that some *Arcobacter* species are able to survive for several minutes at 52ºC (Ho et al., 2008). It seems that the application of mild heat (50ºC) followed by cold shock (4ºC-8ºC) is more effective than these treatments applied separately (D'Sa and Harrison, 2005).

Regarding milk, it has been shown that, although *A. butzleri* and *A. cryaerophilus* cannot grow, they remain viable in Ultra-High Temperature (UHT), pasteurized and raw milk for six days when stored between 4ºC and 10ºC. In raw milk *A. butzleri* increases when stored at 20ºC. These findings show that, although it is unlikely that *Arcobacter* spp. survives the pasteurization or UHT processes, it is possible that bad hygiene and storage leads to contamination (Giacometti et al., 2014).

Several plant extracts have also shown the capacity to inhibit *Arcobacter* spp. growth, namely the ones from cinnamon, bearberry, chamomile, sage and rosemary (Cervenka et al., 2006). Compounds like cinnamaldehyde, thymol, carvacrol, caffeic and tannic acids, eugenol and resveratrol presented activity against *Arcobacter* spp. (Cervenka et al., 2008; Duarte et al., 2015) Thus, phytochemicals are presented as a viable alternative to the traditional preservatives.

1.4. Antibiotics resistance

Until the commercialization of antibiotics, infections were a major detriment to human health. However, selective pressure exerted by the excessive and inappropriate use of a narrow repertoire of antimicrobials has contributed to the development of bacterial resistance (Okeke et al., 2005).

As many of the antibiotics used in humans are also applied in sub-therapeutic doses to food animals and plant agriculture to promote growth and prevent disease, there is the possibility that human pathogens that have reservoirs in animals, such as *Arcobacter*, will develop resistance to drugs employed in human medicine (Angulo et al., 2004; de Souza and Hidalgo, 1997; Wegener, 2003). Furthermore, the natural human microflora may exchange antibiotic resistance determinants, by horizontal gene transfer with ingested bacteria, as they pass through the colon, enhancing the resistance of these food-borne pathogens and of the bacterial flora (Salyers et al., 2004). The newly acquired resistance phenotypes tends to stabilize and stays ingrained in the bacteria, which means that reducing the use of antibiotics is not enough to reverse the resistance (Barbosa and Levy, 2000).

The increase of antibiotic resistant bacteria coincides with a reduction in the production of new antibiotic molecules. In fact, of the 48 drugs approved by the Food and Drug Administration (FDA) between 1998 and 2003, only 6 (14%) were considered new molecular entities, the other 86% were drugs structurally similar to one or more compounds that are already in the market (Brunton et al., 2011).

Nowadays, the scientific community faces two major challenges in this field: conserving the effectiveness of the existing antibacterial and developing new ones.

1.4.1. Classes of antibiotics

Antibiotics may be produced biosynthetically, by bacteria or fungi in order to kill competing microorganisms or, as is the case of many second and third generation antibiotics, be the result of semisynthetic modifications (Walsh, 2000; Hansen et al., 2003). Antibiotics act by killing the bacteria (bactericidal) or by stopping its growth (bacteriostatic) by inhibiting DNA replication/repair, or protein or cell wall synthesis (Fair and Tor, 2014; Walsh, 2000) (Table 2).

For example, chloramphenicol, a member of the amphenicol class, binds reversibly to the peptidyl transferase centre of the 50S ribosomal subunit preventing its binding to the amino acid–end of tRNA, inhibiting peptide bond formation and, consequently, the elongation step of translation (Brunton et al., 2011). This antibiotic has a broad-spectrum activity and it is fairly used as it is inexpensive (Fair and Tor, 2014). However, there are safety concerns, namely haematological disorders such as aplastic anaemia, bone marrow suppression and leukaemia, as well as neurotoxicity and Grey syndrome (Aminov, 2017).

Erythromycin is a macrolide; this class of antibiotics inhibit protein synthesis by binding reversibly to the 50S ribosomal subunit and causing premature dissociation of peptidyl tRNA from the ribosome. Macrolides are the second most prescribed antibiotic class after the β-lactams, targeting the same range of pathogens but with lesser efficiency against Gram-negative bacteria (Aminov, 2017; Katz and Ashley, 2005).

The tetracycline family is constituted by natural and semisynthetic broad-spectrum agents that have activity against either Gram-positive and Gram-negative bacteria as well as protozoan parasites. They inhibit bacterial protein synthesis by preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor (A) site (Following and Therapy, 2001). Tetracyclines do not exhibit any major adverse effect and are one of the more cheap antibiotics on the market, as such they have been extensively used in human and animal therapy (Following and Therapy, 2001; Roberts, 2005), inclusively in prolonged treatments of non-infectious conditions at sub-therapeutic levels (e.g. acne) (Roberts, 2003).

Table 2. Principal antibiotic classes, their mechanisms of action and year at which they were clinically introduced.

Class	Examples	Start of clinical use	Mechanism of action	Reference
Sulfonamides	Prontosil	1935	Inhibit synthesis of folic acid	(Aminov, 2017)
ß-lactams	Penicillin G	1938	Inhibit cell wall biosynthesis	(Fair and Tor, 2014) (Page, 1984)
Aminoglycosides	Streptomycin	1946	Mistranslation of protein	(Fair and Tor, 2014)
Amphenicols	Chloramphenicol	1948	Inhibit protein synthesis	(Brunton et al.,2011) (Aminov, 2017)
Polymyxins	Colistin	1950	Increased cell membrane permeability	(Falagas and Kasiakou, 2005)
Macrolides	Erythromycin	1952	Inhibit protein synthesis	(Katz and Ashley, 2005)
Tetracyclines	Clortetracycline	1952	Inhibit protein synthesis	(Fair and Tor, 2014) (Following and Therapy, 2001)
Rifamycins	Rifampicin	1958	Inhibit protein synthesis	(Fair and Tor, 2014)
Glycopeptides	Vancomycin	1958	Inhibit cell wall biosynthesis	(Reynolds, 1989)
Quinolones	Ciprofloxacin	1968	Inhibit DNA synthesis	(Oliphant and Green, 2002)
Streptogramins	Pristinamycin	1999	Inhibit protein synthesis	(Cocito et al., 1997) (Fair and Tor, 2014)
Oxazolidinones	Linezolid	2000	Inhibit protein synthesis	(Bozdogan and Appelbaum, 2004)
Lipopeptides	Daptomycin	2003	Disruption of the membrane structural integrity	(Pirri et al., 2009)
Pleuromutilins	Retapmulin	2007	Inhibit protein synthesis	(Brown and Dawson, 2015)
Macrolactones	Fidaxomicin	2011	Inhibit RNA synthesis	(Venugopal and Johnson, 2012)
Diarylquinolines	Bedaquiline	2012	Inhibit ATP synthesis	(Hards et al., 2015)

Ciprofloxacin is a second-generation quinolone. Quinolones inhibit topoisomerases II (DNA gyrase) and IV promoting cleavage of bacterial DNA, quickly killing the cell. Most quinolones favour action upon either DNA gyrase or topoisomerase IV, though some later generation drugs target both. Ciprofloxacin is still one of the better antibiotics against *Pseudomonas aeruginosa* and has also garnered attention for its activity against extremely virulent bacteria such as *Bacillus anthracis* and *Yersinia pestis* (Fair and Tor, 2014; Oliphant and Green, 2002).

1.4.2. *Arcobacter* resistance to antibiotics

Information about the susceptibility of *Arcobacter* spp. is scarce. The most prescribed drugs to treat *Arcobacter* spp. infections are erythromycin or fluoroquinolones, such as ciprofloxacin, though tetracycline, doxycycline, and gentamicin are also considered good alternatives (Shirzad Aski et al., 2016). However, numerous studies report that *Arcobacter* spp. is becoming increasingly resistant to several antibiotic classes.

Regarding human isolates, a ten years long study from Belgium found that 100% of the *A. butzleri* isolates were susceptible to tetracycline and gentamicin, and 96.7% to ciprofloxacin, while 21.3% were resistant to erythromycin and ampicillin (Vandenberg et al., 2006). In New Zealand, it was reported that 100% of the *A. butzleri* species isolated from diarrhoeal faeces were susceptible to ciprofloxacin, 85.7% to erythromycin, 57% to tetracycline and 42.8% to ampicillin (Mandisodza et al., 2012). Moreover, a few years later, a study, also from Belgium, performed in isolates from patients with gastroenteritis illnesses shown that 100% of the *A. butzleri* strains were susceptible to gentamicin, 87% to ciprofloxacin, 86% to tetracycline and 76% to erythromycin, while 90% were resistant to ampicillin and 63% to doxycycline (Van den Abeele et al., 2016).

Studies involving farm animals suggest that tetracycline and gentamicin are effective antibiotics, as resistance to them are relatively low, varying from 0% to 7.4% for tetracycline (Shah et al., 2013; Shirzad Aski et al., 2016) and 0% to 3.7% for gentamicin (Shah et al., 2013; Shirzad Aski et al., 2016); *A. butzleri* is also susceptible to erythromycin (66.7%-100%) (Ünver et al., 2013; Shirzad Aski et al., 2016). Regarding ciprofloxacin one study reported that 100% of the *A. butzleri* strains isolated were susceptible to it (Shirzad Aski et al., 2016), while another point to a resistance of 33.4% (Shah et al., 2013). On the other hand, chloramphenicol, ampicillin and vancomycin are associated with high levels of resistance: 7.4% to 66.7% for chloramphenicol (Shah et al., 2013; Ünver et al., 2013; Shirzad Aski et al., 2016), 55.6% to 84.1% for ampicillin (Shah et al., 2013; Shirzad Aski et al., 2016; Ünver et al., 2013) and 100% for vancomycin (Shirzad Aski et al., 2016; Ünver et al., 2013;).

Considering food samples, several studies made with different kinds of retail meats have reported a high susceptibility of *A. butzleri* to tetracycline (96.6%-100%) (Atabay and Aydin, 2001; Harrass et al., 1998; Kabeya et al., 2004; Rahimi, 2014; Son et al., 2007; Villalobos et al., 2013), erythromycin (87.1%-100%) (Atabay and Aydin, 2001; Kabeya et al., 2004; Villalobos

et al., 2013) ciprofloxacin (100%) (Son *et al.*, 2007), ampicillin (97.7%-100%) (Kabeya et al., 2004; Ferreira et al., 2013) and gentamicin (97%-100%) (Atabay and Aydin, 2001; Son et al., 2007; Abay et al., 2012; Ferreira et al., 2013; Rahimi, 2014). However, numerous cases of resistance to these same antibiotics have also been described. The percentage of isolates resistant to tetracycline is among the lowest (21%) (Zacharow et al., 2015); for erythromycin, the results obtained are vastly different: 4.2% (Son et al., 2007) in the USA and 62% in Poland (Zacharow et al., 2015); regarding ciprofloxacin Portugal reported the highest resistance (55.8%), while other countries reported resistance between 1.6% and 28% of the strains (Villalobos et al., 2013; Rahimi, 2014; Zacharow et al., 2015). The results for chloramphenicol are more controversial varying from 0% to 75%, undoubtedly a reflection of the veterinary practices of each country (Atabay and Aydin, 2001; Ferreira et al., 2013; Harrass et al., 1998; Kabeya et al., 2004; Rahimi, 2014; Villalobos et al., 2013). The resistance reported to ampicillin is particularly high ranging from 57.8% to 87% (Harrass et al., 1998; Atabay and Aydin, 2001; Villalobos et al., 2013; Rahimi, 2014; Zacharow et al., 2015); and lastly, vancomycin is associated with extremely high levels of resistance (95.8%-100%) (Ferreira et al., 2013; Kabeya et al., 2004; Rahimi, 2014). Regarding milk and cheese a study reported that 100% of the *A. butzleri* strains found in these products were resistant to tetracycline and ampicillin. Moreover, 90% of the strains were resistant to vancomycin and 80% to erythromycin (Yesilmen et al., 2014). A work regarding edible bivalve molluscs reported a high percentage of susceptibility: 100% for erythromycin and gentamicin, 96.8% to ciprofloxacin and 54.8% for ampicillin (Collado et al., 2014).

Furthermore, when considering multidrug resistance in *A. butzleri* isolates, a study from Japan on retail meats shown that 56.3% of the strains were resistant to three or more antibiotics (Kabeya et al., 2003), while a study from Malaysia regarding healthy cattle and goats found 20% of the isolates resistant to four or more antibiotics (Shah et al., 2013). In the USA, a study in broiler carcasses reported that 71.8% of the *Arcobacter* spp. isolates were resistant to two or more antibiotics, while only 28.4% of the *Campylobacter* spp. isolates presented that level of resistance (Son et al., 2007).

The differences among studies may reflect the medical or livestock rearing practices of each country or result from the lack of a standardized method for antibiotic susceptibility determination and breakpoint recommendations for *Arcobacter* (Ferreira et al., 2013). Nonetheless, tetracycline and gentamicin have an overall effective action, with erythromycin being a possible alternative antibiotic to clinical and veterinary uses. Nonetheless, ciprofloxacin starts to show an increase in the number of resistant strains reported, especially in Portugal.

1.5. Mechanisms of bacterial resistance

Alexander Fleming, who discovered penicillin, was among the first to warn to the possibility of bacteria developing resistance to antibiotics (Aminov, 2017). In general, the development of resistances are quick, happening in months or years (Zhang et al., 2006).

Bacteria may be intrinsically resistant to certain antibiotics or may acquire resistance by *de novo* mutation or through the acquisition of resistance genes from other microorganisms (Livermore, 2003), this may happen through several genetic mechanisms such as transformation, conjugation or transduction (Tenover, 2006).

Resistance may be achieved by target modification (resulting in an alteration of the sensitivity to the antibiotic), by antibiotic inactivation, by outer membrane permeabilization or due to efflux pumps (reducing the concentration of the antibiotic inside the cell) (Livermore, 2003; Simões et al., 2009).

1.5.1. Target modification

Antibiotic's targets tend to be involved in vital functions of the cell and, as such, cannot be eliminated. However, most antibiotics bind to their targets with high affinity, so a small mutation in the target is enough to hinder the binding between the two. Sometimes the modification needed in the target requires other changes in the cell to compensate the altered characteristics of the target (Spratt and Spratt, 2017).

In *Arcobacter* spp., the only resistance mechanism described regards the resistance to fluoroquinolones and has been associated with a point mutation on the *gyr*A gene, that results in a cytosine to thymine transition within the DNA gyrase subunit GyrA, in the quinolone resistance determining region (QRDR) (Abdelbaqi et al., 2007).

1.5.2. Antibiotic inactivation

This resistance mechanism relies on enzymes that destroy or modify the antibiotics before they can exert its effect (Tenover, 2006). There are three mechanisms that bacteria uses to achieve this: hydrolysis, group transfer and redox mechanisms (Dzidic et al., 2008). A classic example of hydrolysis is the inactivation of the ß-lactam ring in penicillins and cephalosporins by the action of ß-lactamases which bacteria releases into the periplasmic space to intercept the antibiotics before they reach their target in the cytoplasmic membrane (Walsh, 2000). Despite not experimentally validated, the described presence of ß-lactamase genes in *A. butzleri* RM4018 genome indicates that this can be a resistance mechanism to ß-lactam antibiotics (Miller et al., 2007). Also, the addition of certain chemical groups (adenylyl, phosphoryl, or

acetyl groups) to the antibiotic molecules by transferases is enough to modify the antibiotic so it cannot bind to its target. As this strategy requires a co-substrate, it is restricted to the cytoplasm (Dzidic et al., 2008). An example of this, is the resistance of *A. butzleri* RM4018 to chloramphenicol, which is likely due to the presence of a *cat* gene that encodes chloramphenicol *O*-acetyltransferase (Miller et al., 2007), an enzyme that modifies the antibiotic, preventing it from binding to the ribosomes (Shaw, 1967). The last mechanism is the oxidation or reduction of the antibiotics. An example is the oxidation of tetracycline antibiotics by TetX enzyme (Yang et al., 2004).

1.5.3. Outer membrane permeability

Gram-positive bacteria have a cytoplasmic membrane and a thick peptidoglycan cell wall with several layers, while Gram-negative bacteria have an inner and outer membrane externally coated with lipid A, that serves as the anchor for lipopolysaccharide (LPS), with a thin peptidoglycan cell wall in between membranes (Sohlenkamp and Geiger, 2015). The presence of LPS in the outer membrane of Gram-negative bacteria decreases its fluidity, increasing impermeability (Nikaido, 1994). The difference in cell wall constitution is the basis for a general reduced susceptible to antimicrobial agents of Gram-negative bacteria when compared with Gram-positive bacteria (Brunton et al., 2011).

In Gram-negative bacteria, besides the diffusion through the cell membrane, drugs can also penetrate the cell by diffusion through porins (e.g. chloramphenicol, tetracyclines and quinolones) (Delcour, 2009) or by self-promoted uptake, through destabilization of the LPS layer, (e.g. aminoglycosides) (Hancock et al., 1991). As such, alterations in the number, size or selectivity of these channels will modify the diffusion rate of these antibiotic (Delcour, 2009; Nikaido, 1994).

For example, the strain *A. butzleri* RM4018, which lacks the mutation in the *gyr*A gene mentioned above, is susceptible to hydrophilic quinolones (e.g. ciprofloxacin) and resistant to hydrophobic ones (e.g. nalidixic acid). These results suggest that the bacterium has a mechanism of resistance to hydrophobic quinolones at the level of its uptake, namely associated with the membrane permeability or specific efflux pumps (Miller et al., 2007).

1.5.4. Efflux pumps

Efflux pumps are an important mechanism of resistance for both Gram-positive and Gram-negative bacteria, as well as eukaryotic cells (Brunton et al., 2011). They are protein complexes that reside in the membrane and remove unwanted substances from within the cells into the exterior, keeping their intracellular concentration at sub-toxic levels (Walsh, 2000; Webber and Piddock, 2003).

Efflux pumps can be specific for a substrate; however, they can also recognize and expel a wide range of structurally diverse antibiotics with different targets. This characteristic is fundamental for the survival of the bacteria until a more specific resistance mechanism is developed and contributes for the development of multidrug resistance (MDR). Furthermore, they have a role in bacterial pathogenesis, virulence and biofilm formation (Webber and Piddock, 2003; Venter et al., 2015).

Efflux pumps are divided in five superfamilies: Adenosine triphosphate (ATP)-binding cassette (ABC), major facilitator superfamily (MFS), resistance nodulation division (RND), small multidrug resistance (SMR) and the multidrug and toxic compound extrusion (MATE) (da Silva et al., 2011). All these systems use proton motive force (H^+ or Na^+) as an energy source, with the exception of the ABC family that uses ATP hydrolysis (da Silva et al., 2011).

Due to their double membrane, a tripartite pump can be found in Gram-negative bacteria, which consists of an inner membrane protein (responsible for drug selectivity), an outer membrane protein and a periplasmic membrane fusion protein that connects the other two. This system belongs to the RND family (Langton et al., 2005; Venter et al., 2015;).

For example, the multidrug efflux pump (CmeABC), of the RND family, contributes to *C. jejuni*, a closely related bacterium to *A. butzleri* resistance to a range of structurally unrelated compounds such as chloramphenicol, tetracycline, macrolides, fluoroquinolones and ethidium bromide, among others (Pumbwe and Piddock, 2002). Several putative efflux pump genes such as czcB, that codifies a membrane fusion protein related to cation efflux, and czcA, that is associated with heavy metal efflux, have also been described for *A. butzleri* (Miller et al., 2007)

1.6. Phytochemicals

1.6.1. Plants as medicine

The medicinal use of plants has accompanied the human civilization since ancient times, having been used as purgatives, antitussives, sedatives and for the treatment of several maladies such as fever, snakebites and insanity (Croteau et al., 2000). It is interesting to note that some of the plants described in documents almost 6000 years old are still used today in traditional medicine and its active compounds in modern medicine (Paulsen, 2010).

Indeed, despite the accomplishments achieved in the medicinal field, most of the population still relies in traditional medicine (Shah, 2009), with 14-28% of the higher plants being used medicinally (Simões et al., 2009). According to the World Health Organization, plant-based medicine serves as the first line of treatment for 80% of the world's population, especially in developing countries (Kong et al., 2003). Meanwhile, there has been a reawakening in the

interest of modern society in herbal drugs, as it is generally cheaper, easily accessible and thought to have less side effects than some synthetic drugs (Chikezie et al., 2015).

All compounds produced by plants can be classified as primary and secondary metabolites. Primary metabolites, such as carbohydrates, aminoacids and lipids, are produced to aid in the growth and development of the plant, while secondary metabolites are produced in a latter phase to enhance the chances of survival of the plant. Some of these secondary metabolites may influence biological systems, being considered, therefore, bioactive (Azmir et al., 2013). These bioactive, non-nutrient compounds found in plants, known as phytochemicals, are responsible for the medical properties of medicinal plants (Shah, 2009).

Plants produce two kinds of bioactive products to protect themselves from microbial attack: phytoalexins and phytoanticipins. These are not distinguished by differences in their molecular structure but by the circumstances of their production (Vanetten et al., 1994). Therefore, phytoalexins are low molecular weight antimicrobial compounds synthesized *de novo* after the plant tissue has been exposed to microbial infection, while phytoanticipins are low molecular weight antimicrobial compounds present in plants before infection or that are produced after infection from pre-existing constituents. Thus, the same compound may be classified as both phytoalexin and phytoanticipin even in the same plant (Vanetten et al., 1994).

Phytochemicals have been linked in several epidemiological studies to reduced risks of cancer, cardiovascular disease, diabetes and lower mortality rates, and have been shown to have anti-inflammatory, anti-atherosclerotic, anticarcinogenic, antibacterial, antifungal and antiviral activities (Ozkan et al., 2016). In fact, nearly 60% of the antibiotic and anticancer drugs owe their origin either directly or indirectly to natural products (Rao, 2012).

These products are of interest to study because, despite their weak antibiotic activities, comparatively to antimicrobials produced by bacteria and fungi, by using them, plants have been able to fight off infections successfully. Moreover, plant-based antimicrobials can be further modified to enhance its efficacy (Klančnik et al., 2012b; Simões et al., 2009).

1.6.2. Classes of phytochemicals

The organisation of phytochemicals into different classes is not consensual, changing with the intention of the classification. Considering biosynthesis, phytochemicals may be divided in three main categories: terpenes and terpenoids (approximately 25,000 types), alkaloids (approximately 12,000 types) and phenolic compounds (approximately 8000 types) (Azmir et al., 2013). There are four major pathways of synthesis of bioactive compounds: shikimic acid pathway, malonic acid pathway, mevalonic acid pathway and non-mevalonate pathway (Croteau et al., 2000; Rao, 2012; Azmir et al., 2013). Terpenes are produced through the mevalonic acid and non-mevalonate pathways, phenolic compounds are synthesized through shikimic acid and malonic acid pathways, and alkaloids are produced by aromatic amino acids

(from shikimic acid pathway) and by aliphatic amino acids (come from tricarboxylic acid cycle) pathway (Azmir et al., 2013).

Phenolic compounds are characterized by having at least one aromatic ring with hydroxyl groups. This family is constituted by phenolic acids, flavonoids, stilbenes, coumarins and tannins (Liu, 2004). They can be further divided in polyphenols and simple phenols depending on the number of phenol subunits presented. These are compounds usually associated with colour, flavour, growth, reproduction and protection against UV-irradiation, predators and pathogens, being viewed as one of the major classes of natural antimicrobials (Albert et al., 2011; Paulsen, 2010;). Furthermore, they are well known for their antioxidant properties, being the most abundant antioxidants in our diet, of which it is estimated that two thirds are flavonoids and the remaining one third are phenolic acids (Paulsen, 2010).

Phenolic acids are phenols that possess one carboxylic acid functionality (Figure 1) (Robbins, 2003). Phenolic acids are comprised of two groups: hydroxybenzoic acids, such as vanillic, syringic and gallic acids, and hydroxycinnamic acids, such as *p*-coumaric, caffeic, ferulic and chlorogenic acids (Liu, 2004). The basic structure and biosynthetic origin (the amino acid L-phenylalanine) is the same for the two groups, although the number and positions of the hydroxyl groups on the aromatic ring varies (Robbins, 2003). While hydroxybenzoic acids are found only in certain berries and onions; hydroxycinnamic acids are common, being present in flour, coffee, fruit and vegetables (Paulsen, 2010).

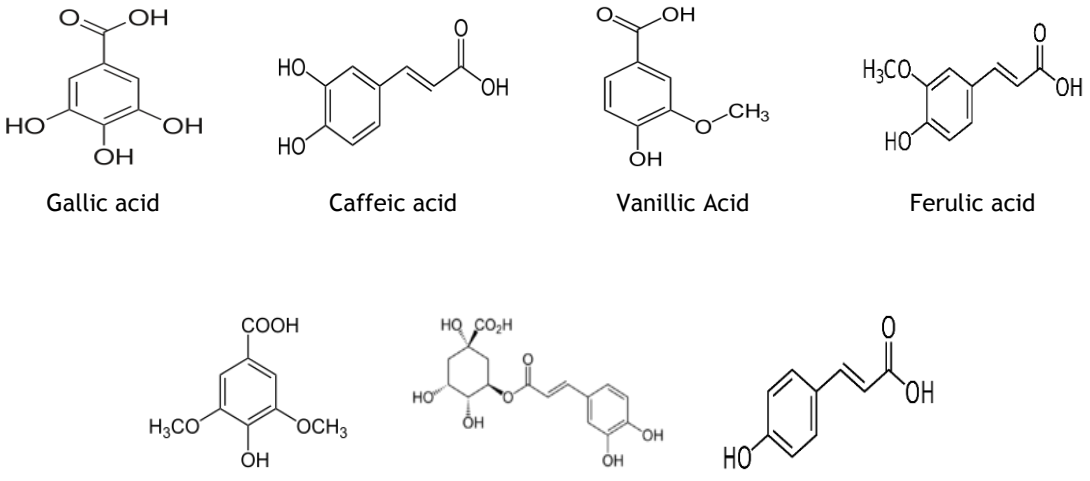


Figure 1. Chemical structure of some phenolic acids.

Vanillic acid is a benzoic acid derivative produced during the synthesis of vanillin from ferulic acid. Studies have shown that vanillic acid has antioxidant, hepatoprotective and anti-

inflammatory activities (Rao, 2012). Moreover, vanillic acid has antibacterial activity against Gram-negative and Gram-positive bacteria such as: *Cronobacter* spp. (Yemiş et al., 2011), *Staphylococcus aureus, Escherichia coli, Pasteurella multocida, Neisseria gonorrhoeae, Proteus mirabilis, Morganella morganni, Staphylococcus epidermidis, Enterococcus faecalis, Listeria monocytogenes* and *Streptococcus agalactia* (Alves et al., 2013).

Caffeic acid is an antioxidant compound known to selectively block the biosynthesis of leukotrienes, molecules involved with asthma and allergic reactions. This phytochemical also has antitumoral activity against colon cancer, and antiviral properties against HIV-1 (Robbins, 2003). Caffeic acid interferes with the stability of the cell membrane and with the metabolic activity of the cells of *S. aureus* (Luís et al., 2014), *Klebsiella pneumoniae* and *S. epidermidis* (Pinho et al., 2015). Moreover, caffeic acid presented a synergistic effect in association with several antibiotics as: norfloxacin in *S. aureus*, imipenem in *E. coli*, and gentamicin and imipenem in *P. aeruginosa* (Lima et al., 2016).

Ferulic acid has many properties: antioxidant, anti-inflammatory, antiviral, antiallergic, hepatoprotective, neuroprotective, vasodilatory, antithrombotic and anticarcinogenic activities (Kumar, 2014). It also increases sperm viability (Kumar, 2014) and absorbs UV-light (protecting the skin) (Ou and Kwok, 2004). Moreover, ferulic acid has antimicrobial action against *E. coli*, *P. aeruginosa*, *S. aureus*, *L. monocytogenes* (Borges et al., 2013), *Bacillus cereus*, *S. epidermidis*, *Salmonella* Typhimurium, *Shigella flexneri* (Fu et al., 2016), *Acetobacter aceti*, *Acetobacter oeni*; *Acetobacter pasteurianus* (Pastorkova et al., 2013) and *Cronobacter sakazakii* (Shi et al., 2016a).

Chlorogenic acid is an ester of caffeic acid and the substrate of oxidation that leads to the browning of food, particularly in apples and potatoes (Liu, 2004). This bioactive compound has anti-inflammatory, antimutagenic (Lou et al., 2011) and antioxidant activities *in vitro*, having a potential protective effect against cardiovascular diseases (Olthof et al., 2000). Chlorogenic acid has antibacterial action against Gram-positive and Gram-negative bacteria, namely *S. aureus* (Luís et al., 2014), *Streptococcus pneumoniae*, *Bacillus subtilis*, *E. coli*, *Shigella dysenteriae* and *S.* Typhimurium (Lou et al., 2011).

It has been suggested that syringic acid has anti-lipid peroxidative, antioxidant and anti-carcinogenesis properties (Periyannan et al., 2017). Also, this phytochemical has hepatoprotective action through the suppression of liver inflammation (Itoh et al., 2010), and is possibly useful in the treatment of diabetes (Krolicka et al., 2013). Additionally, syringic acid has a strong antifungal (Chong et al., 2011) and antibacterial activities against several microorganisms, namely *C. sakazakii* (Shi et al., 2016b), *E. coli* (Zaldivar et al., 1999), *Bacillus* spp., *Acinetobacter* spp., *Coryneforms* spp. and *Enterobacteria* (Moreno et al., 1990).

The phenolic compound *p*-coumaric acid has antioxidant, anti-inflammatory (Zang et al., 2000; Luceri et al., 2007) and neuroprotective activities (Vauzour et al., 2010). *p*-Coumaric acid is able to inhibit lipid peroxidation and to reduce LDL-cholesterol, possibly inhibiting

atherogenesis (Zang et al., 2000), and has a potential protective effect against vascular diseases (Luceri et al., 2007). This phytochemical also presents antimicrobial activity against *E. coli*, *S. aureus* and *B. cereus* (Herald and Davidson, 1983), *Acetobacter aceti*, *A. oeni*, *A. pasteurianus* (Pastorkova et al., 2013) and *K. pneumoniae* (Aziz, 1998).

Gallic acid has antioxidant, antifungal, diuretic, wound healing, antidepressant, anti-inflammatory, anti-diabetic, anti-aging, neuroprotective and cardioprotective properties (Chhillar and Dhingra, 2013; Kateel et al., 2014; Nayeem and Asdaq, 2016; Shahrzad et al., 2001). It as synergic effect with cancer drugs, against lung tumour (Kawada et al., 2001), and is able to inhibit inflammatory allergic reactions regulated by mast cells, having potential benefits in the treatment of asthma and allergic rhinitis (Kim, 2006). Moreover, gallic acid has antimicrobial action against several microorganisms such as: *S. aureus* (Luís et al., 2014), *P. aeruginosa*, *E. coli*, *L. monocytogenes* (Borges et al., 2013), *C. jejuni* and *C. coli* (Sarjit et al., 2015). Gallic acid also shown synergistic effect with norfloxacin and gentamicin against *S. aureus* (Lima et al., 2016).

Flavonoids are low molecular phenolic compounds, which generic structure consists of two aromatic rings linked by three carbons that are usually in an oxygenated heterocycle ring (Figure 2). These compounds may appear as glycosides with more than 80 different sugars having been discovered bound to flavonoids. The differences in the ring containing the oxygen dictates their classification in flavonols (e.g. quercetin and rutin), flavones (e.g. luteolin), flavanols or flavan-3-ols (e.g. catechins and epicatechin), flavanones (e.g. naringenin), anthocyanidins (e.g. cyanidin), and isoflavonoids (e.g. genistein) (Liu, 2004). Their concentrations in plants are dependent of exposure to light, being flavonols the most prevalent members of this class in food (Paulsen, 2010). Moreover, they are known for their antioxidant, anti-inflammatory, anti-carcinogenic and neuro-protective activities (Croteau et al., 2000; Paulsen, 2010). They have remarkable activity against several Gram-positive bacteria, such as *S. aureus, Lactobacillus acidophilus,* and *Actinomyces naeslundii,* and Gram-negative bacteria, such as *C. jejuni, E. coli, Helicobacter pylori, P. aeruginosa Prevotella oralis, Prevotella melaninogenica, Porphyromonas gingivalis,* and *Fusobacterium nucleatum* (Daglia, 2012).

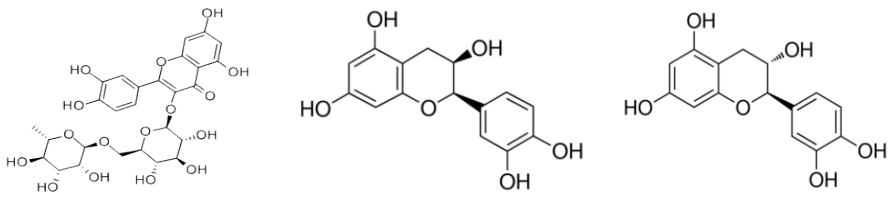
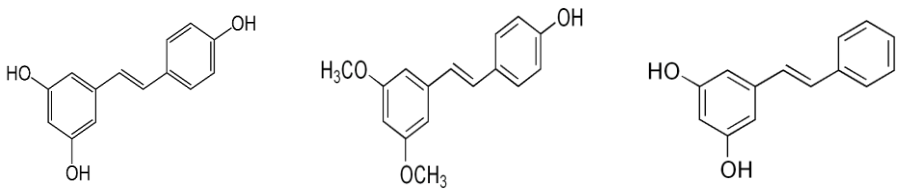


Figure 2. Chemical structure of some flavonoids.

Rutin is able to improve hyperlipidaemia and hyperglycaemia in diabetic animals (Jadhav and Puchchakayala, 2012), has obtaining positive results regarding arthritis (Guardia et al., 2001), has wound healing activity (Nayeem and Karvekar, 2011), and presents gastroprotective effect in experimental lesions induced by ethanol, probably due to its antioxidant properties (La Casa et al., 2000). Furthermore, rutin have antibacterial activity against *B. cereus, P. aeruginosa, K. pneumoniae* (Singh et al., 2008), *S. aureus, B. subtilis, E. coli,* and *K. oxytoca* (Ganeshpurkar et al., 2013). Rutin is also able to synergistically enhance the antibacterial activity of other flavonoids against *B. cereus* and *Salmonella* Enteritidis (Arima et al., 2002).

Catechins have antioxidant, antihypertensive and anti-inflammatory activities (Higdon and Frei, 2003; Yilmaz and Toledo, 2004). They reduce the absorption of lipids in intestine, regulate vascular tone by activating endothelial nitric oxide and suppress platelet adhesion inhibiting thrombogenesis, as such these compounds are beneficial in preventing or treating cardiovascular diseases (Hertog et al., 1993; Velayutham et al., 2008). Catechins have also a relative success in inhibiting the growth of a wide range of Gram-positive and Gram-negative bacteria, such as *Salmonella* Typhi, *Brucella melitensis*, methicillin-resistant *Staphylococcus aureus* (MRSA) and *H. pylori* (Taylor et al., 2005).

Stilbenes are ubiquitously present in plants, being synthesized as a response to microbial infection and to exposure to UV light (Figure 3) (Chen et al., 2017). They have a C6-C2-C6 basic skeleton and consist of two phenyl groups linked by an ethene double bond (Figure 3) (Tsai et al., 2017). This class of bioactive compounds have been attracting attention due to their antioxidant, anti-inflammatory, anticarcinogenic, antidiabetic, anti-dyslipidaemia, cardioprotective and neuroprotective properties (Croteau et al., Tsai et al., 2017). Due to these characteristics, stilbenes have positive effects in a wide range of medical disorders. Of all their properties, antimicrobial and antifungal activities are among the less explored. Microorganisms known to be susceptible to stilbenes include: *B. subtilis*, *S. aureus*, *E. coli*, *P. aeruginosa* (Kumar et al., 2012), *B. cinerea, Cladosporium cuccumerinum, Pyricularia oryzae Cavara, P. viticola* and *Sphaeropsis sapine* (Jeandet et al., 2010). Resveratrol is the best studied stilbene; however, other stilbenes like pterostilbene and pinosylvin have started to gather attention as they appear to have higher bioactive properties (Reinisalo et al., 2015).



Resveratrol Pterostilbene Pinosylvin

Figure 3.Chemical structure of some stilbenes.

Resveratrol is a phytoalexin synthesized from phenylalanine, in reactions mediated by the stilbenes synthase enzyme, as a response to fungi and bacterial attacks (Shah, 2009). Resveratrol can prevent or retard several diseases, including cardiovascular, carcinogenic, and neurodegenerative diseases, as well as increase longevity. Furthermore, resveratrol has anti-inflammatory, antioxidant, antifungal and antimicrobial actions (Paulo et al., 2011a). Bacteria susceptible to this phytochemical include: *B. cereus,* MRSA, *E. faecalis*, *E. coli*, *K. pneumoniae*, *S.* Typhimurium (Paulo et al., 2010; Hwang and Lim, 2015), C. *jejuni, A. butzleri* (Duarte et al., 2015), *A. cryaerophilus* (Ferreira et al., 2014b), *Acetobacter aceti*, *A. oeni, A. pasteurianus* (Pastorkova *et al.*, 2013) and *H. pylori* (Paulo et al., 2011b).

Pinosylvin and pterostilbene share many of the resveratrol biological activities, including anti-cancer, anti-aging, and antimicrobial activities. However, their poor solubility and high sensitivity to external agents, such as air and light, have impaired their proper exploration (Silva et al., 2014). Pinosylvin is known for its strong antifungal activity (Seppänen et al., 2004), but is also possesses antibacterial and antioxidant activities (Jancinova et al., 2012; Koskela et al. 2014; Lopez-Nicolas et al., 2009). Among the microorganisms susceptible to this phytochemical are: *Pseudomonas fluorescens*, *B. cereus*, *Candida albicans* and *Saccharomyces cerevisiae* (Lee et al., 2005; Välimaa et al., 2007).

Pterostilbene is the major antioxidant molecule in blueberries and in the tree *Pterocarpus marsupium*, which is traditionally used to treat diabetes. It has been linked with anti-carcinogenesis, anti-inflammatory and anti-obesity effects (McCormack and McFadden, 2013; Kong et al., 2016; Tsai et al., 2017). Bacteria susceptible to this phytochemical include: *Acetobacter aceti*, *A. oeni*, *A. pasteurianus* (Pastorkova et al., 2013), methicillin-susceptible *S. aureus* (Lee et al., 2017) and MRSA (Yang et al., 2017).

Alkaloids are a diverse group of organic, alcohol-soluble, heterocyclic compounds containing nitrogen. They are produced from more than 20% of the species of flowering plants, usually to ward off predators, as they tend to be very bitter (Compean and Ynalvez, 2014; Croteau et al., 2000; Paulsen, 2010; Rao, 2012). Alkaloids have a significant impact in medicine, since they are known to affect the central nervous system, reduce appetite, act as local anaesthetics and stimulants, are hypertensive agents, vasodilators and possess bactericidal and diuretic action. Moreover, they are used as anti-cancer, anti-arrhythmia and anti-asthma drugs (Chikezie et al., 2015). Examples of alkaloids include codeine, nicotine, morphine, caffeine and pilocarpine (Sawaya et al., 2011; Chikezie et al., 2015).

Pilocarpine is mainly used in the treatment of glaucoma and as a stimulant for sweat and tears (Figure 4) (Sawaya et al., 2011). Although this phytochemical is not known for having a strong antimicrobial action, pilocarpine was able to increase the sensitivity of *S. aureus* to the antibiotics gentamicin and neomycin (Araruna et al., 2012).

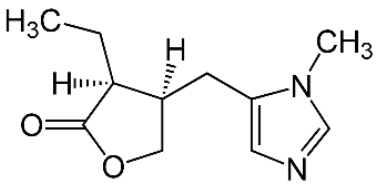


Figure 4. Chemical structure of the alkaloid pilocarpine.

1.6.3. Phytochemicals as inhibitors of efflux pumps

With the reduction of the number of new products in antimicrobial development and the increase resistance to the ones already been commercialized, there is the need to adopt a new perspective in the fight against resistant pathogens. As efflux pumps are one of the major mechanism of resistance of bacteria and even play a role in bacterial pathogenesis, virulence and biofilm formation (Venter et al., 2015), the use of efflux pump inhibitors (EPIs) to block them appears as a good solution to restore the activity of old antibiotics that were becoming ineffective. However, despite being a promising concept, there are no EPIs in clinical use, although several have been described (Venter et al., 2015), especially for Gram-positive bacteria. On the other hand, Gram-negative bacteria, partly due to their thick and lipophilic outer membrane has been a greater challenge (Stavri et al., 2007).

To qualify as an EPI a compound must (1) potentiate the activity of antibiotics that are a substrate for the efflux pumps of the strain in study; (2) should not influence sensitive strains which lack the drug efflux pump; (3) must not potentiate the activity of antibiotics that are not effluxed; (4) must increase the level of accumulation and decrease the level of extrusion of compounds that are substrates of the efflux pump; and (5) must not affect the proton gradient across the inner membrane and not permeabilize the outer membrane (Lomovskaya et al., 2001).

The first EPI identified against RND pumps in Gram-negative bacteria was Phenylalanine-Arginine ß-Naphthylamide (PaßN) initially in *P. aeruginosa* (Lomovskaya et a*l.*, 2001) and latter in *Vibrio cholerae* (Bina et al., 2009). This EPI was also able to restore erythromycin susceptibility in *Campylobacter* spp. with a low-level of resistance (Kurinčič et al., 2012). From PaßN several derivates with lower toxicity and higher stability and solubility where produced. However, these enhancements were not enough for them to be approved for clinical use (Ohene-Agyei et al., 2014).

Another EPI able to modulate Gram-negative bacteria is 1-(1-naphthylmethyl)-piperazine (NMP), which succeeded in reverse MDR in *E. coli* (Kern et al., 2006), *C. jejuni* and *C. coli* (Kurinčič et al., 2012), is able to inhibit RND efflux system in *Vibrio cholerae* (Bina et al., 2009) and potentiates the effect of tetracycline in *Acinetobacter baumannii* (Hancock, 2012).

Carbonyl-cyanide m-chlorophenylhydrazon (CCCP) is an EPI that interferes with the energy level of the bacterial membrane. CCCP was been shown able to potentiate the effect of ciprofloxacin in *K. pneumoniae* (Zhong et al., 2013).

Verapamil is an Ca^{2+} channel blocker, that inhibits efflux pumps by reducing transmembrane potential (Pule et al., 2016). CCCP is an inhibitor of MDR pumps of cancer cells and parasites and also improves the activity of tobramycin (Mahamoud et al., 2007).

Other EPIs able to inhibit the RND family include a series of pyridopyrimidine EPIs specific for the MexAB efflux pump of *P. aeruginosa*, that reached the preclinical stage, but seem to have been halted, and a pyranopyridine EPI, MBX2319, with activity against *Enterobacteriaceae* that is still in the early stages of optimization (Opperman and Nguyen, 2015).

A good approach to search for new EPIs are plants (Venter et al., 2015), as they also use this strategy. For example, the phytochemical berberine for itself is ineffective as an antibacterial, since it is rapidly extruded by efflux pumps, so the plant produces 5'-methoxyhydnocarpin that block the pumps, enhancing berberine action (Stermitz et al., 2000).

Some known plant-originated EPIs for Gram-positive bacteria include reserpine, silybin and carnosic acid (Hemaiswarya et al., 2008; Aparna et al., 2014) ,and for Gram-negative there is geraniol, plumbagin and (−)-epigallocatechin gallate (Venter et al., 2015).

It has been shown that phenolic compounds have a relatively good activity against *Campylobacter* (Klančnik et al., 2012a), a Gram-negative bacterium similar to *Arcobacter*. Moreover, it has been demonstrated that the treatment of *C. jejuni* with antibiotics and phenolic compounds has a synergic effect, partly due to the inhibition of efflux pumps (Oh and Jeon, 2015a), so it is possible that phytochemicals can act as EPIs in *Arcobacter* spp. as well.

Chapter 2 – Aims

A. butzleri is an emergent enteropathogen and potential zoonotic agent that has been developing resistance to several antibiotics, presenting even a multidrug resistance phenotype. Efflux pumps are one of the most relevant mechanisms of antimicrobial resistance, therefore, efflux pump inhibitors (EPIs) represent a possible way to restore the sensitivity of *A. butzleri* to antibiotics. As plants face constant bacterial exposure and are a source of great diversity of compounds, phytochemicals appear as promising potential EPIs.

Therefore, the main objective of this work was to study a selection of phytochemicals to access if they can inhibit efflux pumps in *A. butzleri*, and enhancing the action of several antibiotics. To achieve this aim, several specific objectives were defined:

- To study the antimicrobial properties of the phytochemicals selected against the strains in study;
- To evaluate the action of phytochemicals on the efflux pumps;
- To assess the potential role of efflux pumps in tolerance/resistance to phytochemicals;
- To ascertain the synergic potential between phytochemicals and antibiotics;
- To evaluate if the phytochemicals can inhibit the quorum sensing.

Chapter 3 - Materials and Methods

3.1. Microorganisms

In the long term, the bacteria were preserved in cryogenic tubes at -80ºC in 200 µL of Brain-Heart infusion medium (BHI) (Liofilchem) with 20% of glycerol (José Manuel Gomes dos Santos, LDA). Before performing each assay, bacteria were inoculated in solid medium for 24 hours and subcultured for the same period. At most, the bacteria were subcultured three times. When necessary, the bacterial cultures were temporarily conserved in the fridge.

Four strains of *A. butzleri* isolated from different sources and with distinct antibiotic resistance profiles were used in this study (Table 3). *A. butzleri* was cultured in Tryptic Soy Agar (TSA) at 30ºC in aerobic conditions

Table 3. *Arcobacter butzleri* strains used in this study.

	A. butzleri	
Strain	**Source**	**Resistance profile**
A6-1	Water	Susceptible to ciprofloxacin and erythromycin
DQ46M1	Raw sheep milk	
CR50-2	Chicken meat	Resistant to ciprofloxacin and erythromycin
AB11/11	Slaughterhouse surface	

Additionally, one *C. jejuni* clinical isolate was used as a control (*C. jejuni* 71/09). This strain was cultivated in Brucella Blood Agar (BRU) (Oxoid) for 24 hours at 37ºC in microaerobic conditions.

Chromobacterium violaceum ATCC 12472 was used to perform the quorum sensing inhibition assays. This bacterium was cultivated in Luria-Bertani (LB) agar (Lennox) at 30ºC in aerobic conditions.

3.2. Preparation and storage of the compounds

3.2.1. Phytochemicals

In this work 14 phytochemicals were studied, thirteen polyphenols and one alkaloid (Table 4). Most of these compounds were solubilized in dimethyl sulfoxide (DMSO) (Fluka), except for gallic acid, which was solubilized in distilled water, and pilocarpine nitrate that was solubilized in hydrochloric acid (HCl) 0.1 M. As pilocarpine is not bought pure, it was necessary to have the

nitrate concentration in mind when preparing the solutions to obtain the wanted final concentration of pilocarpine. The phytochemicals solutions were kept frozen until be needed. To perform the assays, working solutions were prepared by dilution of the stock solutions in Tryptic Soy Broth (TSB) (Merck KGaA, Germany), with a final maximum DMSO concentration of 2% (v/v). To prepare the working solutions of the stilbenes (resveratrol, pinosylvin and pterostilbene), TSB with DMSO (for a final maximum DMSO concentration of 2% (v/v)) was heated at 50ºC before the phytochemical solution being added, to facilitate the solubilization.

Table 4. Phytochemicals used in this study.

Phytochemicals	Brand	Solvent used for stock solution:	Concentration of stock solution (mg/mL)
Phenolic acids			
Vanillic acid	Sigma-Aldrich	DMSO	51.2
Caffeic acid	Sigma-Aldrich	DMSO	51.2
Ferulic acid	Sigma-Aldrich	DMSO	51.2
Chlorogenic acid	Sigma-Aldrich	DMSO	51.2
Syringic acid	Sigma-Aldrich	DMSO	51.2
***p*-Coumaric acid**	Sigma-Aldrich	DMSO	51.2
Gallic acid	Sigma-Aldrich	Water	10
Flavonoids			
Rutin	Sigma-Aldrich	DMSO	51.2
(-)-Epicatechin	Fluka	DMSO	51.2
(+)-Catechin	Sigma-Aldrich	DMSO	51.2
Stilbenes			
Resveratrol	TCI Europe N.V.	DMSO	51.2
Pinosylvin	Sequoia Research Products Ltd.	DMSO	51.2
Pterostilbene	Sequoia Research Products Ltd.	DMSO	51.2
Alkaloid			
Pilocarpine nitrate	Fluka	HCl 0.1 M	20.48

3.2.2. Antibiotics

Four antibiotics were used in this work: chloramphenicol (Fluka), tetracycline, ciprofloxacin (Fluka) and erythromycin (Sigma). All antibiotics were solubilized in ethanol 95%, except ciprofloxacin which was dissolved in basified water with sodium hydroxide (NaOH) 1M. Stock solutions of the antibiotics with the concentration of 10 mg/mL were prepared and kept frozen until needed. To perform the assays, working solutions were prepared by dilution of the stock solutions in TSB.

3.2.3. Efflux pump inhibitors

In this study, four known efflux pumps inhibitors were used, namely verapamil (TCI), PaβN (Sigma), CCCP (Acrös Organics) and NMP (Sigma-Aldrich). Stock solutions of verapamil and CCCP were solubilized in DMSO at concentrations of 40 mg/mL and 6.4 mg/mL, respectively, PaβN in distilled water at a concentration of 1 mg/mL and NMP in a water solution with 20% (v/v) DMSO and 20% (v/v) HCl 0.25M at a concentration of 10 mg/mL. The stock solutions were kept frozen until needed. To perform the assays working solutions were prepared by dilution of the stock solutions in TSB (with a final maximum DMSO concentration of 2% (v/v)).

3.3. Growth curves determination

The growth curves of the four *A. butzleri* strains were traced to identify when the cells enter in the end of the exponential growth phase. To do so, the bacteria were inoculated in 20 mL of TSB, with an initial optical density at a wavelength of 620 nm (OD_{620nm}) of 0.05, and incubated with agitation at 30ºC, 100 rotations per minute (rpm). At intervals of two hours, aliquots of 1 mL were taken and, using a spectrophotometer (Shimadzu), its absorbances were measured at 620 nm. This assay was repeated in three distinct days, and the results are shown as the mean ± standard deviation.

3.4. Determination of Minimum Inhibitory Concentration

The antimicrobial activity of an agent can be measured by determining its lowest concentration able to inhibit the bacterial growth, this concentration is defined as the Minimum Inhibitory Concentration (MIC).

The MIC of the 14 phytochemicals, four antibiotics, four EPIs and of ethidium bromide (EtBr) were determined for all the *A. butzleri* strains in study.

The MICs were determined through broth microdilution method, according to the standard M7-A6 by the Clinical and Laboratory Standards Institute with some modifications (NCCLS, 2005). Thus, a series of two-fold dilutions of the compounds was conducted in a 96-well microtiter plate with the final volume of 50 µL per well. The lowest and highest limits of the range of concentrations tested for each compound are shown in Table 5.

Table 5. Lowest and highest limits of the range of concentrations tested for each compound.

Compounds	Lowest concentration tested (µg/mL)	Highest concentration tested (µg/mL)
Phytochemicals		
Vanillic acid	16	1024
Caffeic acid	16	1024
Ferulic acid	16	1024
Chlorogenic acid	16	1024
Syringic acid	16	1024
***p*-Coumaric acid**	16	1024
Gallic acid	16	1024
Rutin	16	1024
(-)-Epicatechin	16	1024
(+)-Catechin	16	1024
Resveratrol	8	512
Pinosylvin	4	256
Pterostilbene	4	256
Pilocarpine	16	1024
Antibiotics		
Chloramphenicol	2	128
Ciprofloxacin	0.006	1
Tetracycline	1	64
Erythromycin	0.5	32
Efflux pump inhibitors		
Verapamil	12.5	800
PaβN	2.5	160
CCCP	4	256
NMP	12.5	800
EtBr	0.5	32

The bacterial cell suspension of each strain was prepared by suspension of several colonies in an isotonic saline solution (NaCl 0.85%) (Fluka) and the turbidity was adjusted to 0.5 McFarland units using a densitometer (BioSan, DEN-1B). The adjusted suspension was then diluted in TSB and 50 µL of the diluted bacterial suspension was added to each well, to give a final concentration of approximately $5x10^5$ CFU/mL. In all assays three controls were introduced: a growth control prepared with medium and bacterial diluted suspension; a medium sterility control, only with culture medium; and a compound sterility control, with the phytochemicals or EPIs diluted on TSB, which was also used to compensate the colour of the compounds (blank control). The plates were then incubated for 48 hours at 30ºC in aerobic conditions and the bacterial growth was evaluated visually (through analysis of turbidity) and confirmed by measuring the OD_{620nm} using a microplate reader (EZ Read 400 Microplate Reader, Biochrom)

and the Galapagos Expert software (it was considered that bacterial growth occurred when OD_{620nm}>0.05). MIC was defined as the lowest concentration of the compound able to visibly inhibit bacterial growth (Richard, Lynn and C., 2007).

The assays were carried out in duplicate and at least three independent experiments were performed.

3.5. Determination of Minimum Inhibitory Concentration of phytochemicals in the presence of EPIs

The MIC values of the phytochemicals in the presence of sub-inhibitory concentrations (one quarter of the MIC) of EPIs was determined. The assay was based on the method of Ohene-Agyei et al. (2014) with slight modifications.

Firstly, the phytochemicals were added to a 96-well microplate and serially diluted (1:2) with TSB in the same range of final concentrations as in the previous assay (Table 5) with a final volume of 25 µL per well. Then, the solutions on the wells were, once again, diluted by two-folds by the addition of 25 µL of the solution of the efflux pump inhibitors (four-fold concentrated regarding the final concentration). Lastly, the inoculum that was prepared as described previously in section 3.4 was added to a final concentration of approximately $5x10^5$ CFU/mL by well in a final volume of 100 µL.

Control wells were prepared with the bacterial diluted suspension, to confirm bacterial growth (positive control), with only the culture medium (sterility control) and with the bacterial suspension in the presence of the EPI, to assess if the EPI is not inhibiting bacterial growth.

The plates were incubated for 48 hours at 30ºC in aerobic conditions. Afterwards, bacterial growth was evaluated visually (through analysis of turbidity) and confirmed by measuring OD_{620nm} using a microplate reader- Bio Rad xMark™ Microplate Absorbance Spectrophotometer.

3.6. Ethidium bromide accumulation assays

To assess if the phytochemicals were inhibiting the efflux pumps, ethidium bromide accumulation assays were performed. The assay was based on the method of Ferreira et al. (2014b) with modifications.

Firstly, the most susceptible and the most resistant *A. butzleri* strains and *C. jejuni* 71/09 were cultured to mid to late exponential phase of growth. Thus, *A. butzleri* strains were cultured in 20 mL of TSB, initiating the culture with a 24 hours culture in solid medium and starting with an initial OD_{620nm} of 0.05, and incubating at 30ºC and 100 rpm in aerobic conditions for five hours. Relatively to the *C. jejuni* strain, the same process was executed but the bacterium was inoculated in Müeller-Hinton broth (MHB) (Liofilchem) and incubated at 37ºC whit an agitation

of 100 rpm in microaerobic conditions for six hours, being the culture initiated with an initial OD_{620nm} of 0.05.

Afterwards, the cells were harvested by centrifugation at 13 400 mg for six minutes (miniSpin Eppendorf), washed one time with Phosphate-buffered saline solution (PBS) and the cellular deposit was resuspended in PBS. The optical density at 620 nm of the solution was adjusted to 0.4. Fifty µL of the inoculum was pipetted into a 96-well black polystyrene microplate (Greiner Bio-One) with a clear flat bottom, to a final OD_{620nm} of 0.2 and the plate was then incubated for ten minutes at 30ºC. Once the incubation time was over, the phytochemicals were added at 1/2×, 1/4×, 1/8× and 1/16× MIC.

Two solvent controls (PBS and DMSO) were prepared, as well as, one positive control (the EPI CCCP at 32 µg/mL). Lastly, ethidium bromide (Fluka) was added to each well at a concentration of 2 µg/mL and the fluorescence was measured in a fluorimeter (spectra MAX, Gemini EM) (excitation 530 nm, emission 600 nm) at intervals of one minute for thirty minutes. The fluorescence of the compounds and the autofluorescence of cells were first analysed, using wells with the tested concentrations of the several phytochemicals and wells with the inoculum without ethidium bromide.

The assays were carried out in triplicate and three independent experiments were performed.

3.7. Checkerboard assays

To study the potential synergism between the phytochemicals and antibiotics, checkerboard tests were performed adapted from Duarte et al. (2012).

The test was undertaken in 96-wells microtiter plates, firstly the antibiotic was added to the plate and series of two-fold dilutions in TSB were made horizontally from right to left to a volume of 50 µL, so that in the first column the final concentration of the antibiotic was 4×MIC. In another plate, the phytochemical was added to the wells and a series of two-fold dilutions in TSB were made vertically. The range of concentrations tested were dependent of the solubility of the compounds.

When the two plates were prepared, 50 µL of the phytochemical solutions were transferred to the correspondent well in the microplate with the antibiotic so that the solutions were diluted by two folds. The column A contained the phytochemicals alone, and the row 1 had only the antibiotic. To fulfil the volume, 50 µL of medium were added.

The inoculum was prepared by suspension of several colonies in NaCl 0.85% and turbidity was adjusted to 0.5 McFarland units, as described previously in section 3.4. After the cellular suspension was diluted 1:67 in TSB, 50 µL were added to each well to a final volume of assay of 150 µL.

A growth control consisting of bacterial inoculum diluted in TSB and a sterility control consisting of only medium were also included.

The plates were incubated for 48 hours at 30ºC in aerobic conditions and bacterial growth was evaluated visually (through analysis of turbidity) and confirmed by measuring the OD_{620nm} using a microplate reader- Bio Rad xMark™ Microplate Absorbance Spectrophotometer.

For *C. jejuni* 71/09, the assay was performed in the same way; however, using as medium MHB, and with incubation occurring at 37ºC in microaerobic conditions.

The results were calculated and expressed in terms of the Fractional Inhibitory Concentration Index (FICI) that corresponded to the sum of the Fractional Inhibitory Concentration (FIC) of each compound. The FIC, in turn, is calculated by dividing the MIC of the drug in combination by the MIC of the drug alone. If the FICI is lesser or equal to 0.5, the combination is considered synergic; if the FICI stands between 0.5 and 1 inclusive, the results are considered additivity; if the FICI is superior to 1 but inferior or equal to 4, it is classified as not having interaction; and if the FICI is higher than 4 it means that the compounds have an antagonistic reaction with each other (Sopirala *et al.*, 2010).

3.8. Quorum sensing inhibition by phytochemicals

To assess if the phytochemicals can inhibit the quorum sensing, a phenomenon that allow bacteria to communicate and regulate several physiological activities, quorum sensing inhibition assays were performed.

To do so, a bacterial suspension of *C. violaceum* ATCC 12472 was obtained by aerobic growth (16 hours) in LB broth (Liofilchem) at 30ºC and 250 rpm. The $OD_{620\ nm}$ of the bacterial suspension was adjusted to 1 and it was used to inoculate LB agar plates. A cotton swab was used to spread the bacterial suspension in the agar plate without overlaps. The process was repeated in total three times rotating the plate in a 60-degree angle in between. To finalize, the swab was passed on the sides of the plates. Sterile discs (6 mm diameter) were impregnated with 20 µL of a solution of the phytochemicals with a concentration of 51.2 mg/mL, as such the discs placed onto the inoculated plates had 1.024 mg of phytochemical. The plates were then incubated for 24 hours at 30ºC. DMSO and HCl 1M were used as a solvent control.

To measure the quorum sensing inhibition, it was necessary to examine if there was inhibition of the production of violacein pigment around the disc (an area that is colourless, but where there exists cell growth). The diameter (mm) of this area was calculated as the total diameter (the sum of the diameter of pigment and cell growth inhibition – D1) minus the diameter of the cell growth inhibition zone (D2). The experiments were performed in three independent days (Luís et al., 2016).

Chapter 4 - Results and discussion

4.1. *A. butzleri'* susceptibility to antimicrobial agents

To assess the susceptibility of the *A. butzleri* strains in study to antibiotics, the MIC of each antibiotic was determined. To do so, the traditional method of broth microdilution was used, thus, serial dilutions of the antibiotics were made in microtiter plates, so each well had a different concentration of the compound before adding the inoculum of the strain being tested. After incubation, the lowest concentration with no visible growth was considered the MIC.

All the antibiotics chosen have a target inside the cell, so their efflux could potentially be an effective mechanism of resistance for *Arcobacter* spp., as it is for other Gram-negative bacteria (Borges-Walmsley et al., 2003). The four *A butzleri* strains selected were chosen due their resistance profile which was determined in previous works which had in consideration the breakpoints of *Campylobacter* spp.: A6-1 and DQ46M1 are susceptible to two of the antibiotics chosen (ciprofloxacin and erythromycin), while Ab11/11 and CR50-2 are resistant to these antibiotics.The results obtained can be seen on Table 6.

Table 6. Minimum inhibitory concentration of the antibiotics for the four *Arcobacter butzleri* strains in study.

	MIC (µg/mL) of antibiotics			
Antibiotics	***A. butzleri* A6-1**	***A. butzleri* DQ46M1**	***A. butzleri* CR50-2**	***A. butzleri* AB11/11**
Tetracycline	4	4	2	4
Chloramphenicol	32	16	32	32
Erythromycin	8	4	32	16
Ciprofloxacin	0.0625	0.0625	32	16

Breakpoints used for resistance: tetracycline ≥ 16 µg/mL, chloramphenicol ≥ 32 µg/mL, erythromycin ≥ 32 µg/mL and ciprofloxacin ≥ 4 µg/mL. Breakpoints used for susceptibility: tetracycline ≤ 4 µg/mL, chloramphenicol ≤ 8 µg/mL, erythromycin ≤ 8 µg/mL and ciprofloxacin ≤ 1 µg/mL.

As there are no well-defined breakpoints for *Arcobacter* spp. the interpretation of the results can be difficult. In this work, the classification of the strains as susceptible or resistant was based on the breakpoint values suggested for *Enterobacteriaceae* (NCCLS, 2005). Using that classification system, the *A. butzleri* strain DQ46M1 stands out as the overall most susceptible strain, as it is susceptible to tetracycline, erythromycin, and ciprofloxacin, and has an intermedium resistance to chloramphenicol; while the *A. butzleri* strain CR50-2 is, overall, the most resistant one, as it is resistant to chloramphenicol, erythromycin, and ciprofloxacin.

The *A. butzleri* strains A6-1 and DQ46M1 were susceptible to ciprofloxacin and erythromycin, while CR50-2 was resistant to both antibiotics, as expected. The *A. butzleri* strain Ab11/11, considering the breakpoints defined in this work, was resistant to ciprofloxacin but had an intermediate resistance to erythromycin. The differences in classification are due to the use of different breakpoints. For the strains tested in this study, tetracycline was the most efficient antibiotic, as all of strains were susceptible to it.

Table 7. Minimum inhibitory concentration of the antibiotics being studied for the *Campylobacter jejuni* 71/09 strain (used as control in following assays).

	MIC (µg/mL)			
	Tetracycline	**Chloramphenicol**	**Erythromycin**	**Ciprofloxacin**
***C. jejuni* 71/09**	0.125	4	2	0.125

Breakpoints used for resistance: tetracycline ≥ 16 µg/mL, chloramphenicol ≥ 32 µg/mL, erythromycin ≥ 32 µg/mL and ciprofloxacin ≥ 4 µg/mL. Breakpoints used for susceptibility: tetracycline ≤ 4 µg/mL, chloramphenicol ≤ 8 µg/mL, erythromycin ≤ 8 µg/mL and ciprofloxacin ≤ 1 µg/mL.

Furthermore, the MIC of the antibiotics for *C. jejuni* 71/09 was also determined as this microorganism was used as a control in the synergism and ethidium bromide accumulation assays that followed (Table 7). Using the *Campylobacter spp.* breakpoint defined by the National Antimicrobial Resistance Monitoring System (CDC, 2010), this strain is susceptible to all the antibiotics.

To assess if the phytochemicals selected presented antimicrobial activity against *A. butzleri*, their MIC was determined through the same method used to determine this parameter for the antibiotics.

Phenolic compounds are able to inhibit the growth of several pathogens of the human gastrointestinal tract, such as *B. cereus*, *H. pylori* and *Salmonella,* among others (Nohynek et al., 2006). Yet, in this study, as can be seen in Table 8, most of the phytochemicals had no antimicrobial action at the concentrations tested, against *A. butzleri.* Due to solubility problems, the range of concentrations could not be risen. However, for the purposes of this work, these results are positive as the aim is not to treat the infection, but to use the phytochemicals to inhibit efflux pumps, enhancing the antimicrobial activity of the antibiotics.

Table 8. Minimum inhibitory concentration of the fourteen phytochemicals under evaluation for the four *Arcobacter butzleri* strains in study.

	MIC (µg/mL)			
	A. butzleri strains			
Phytochemicals	**A6-1**	**DQ46M1**	**CR50-2**	**AB11/11**
(+)-Catechin	>1024	>1024	>1024	>1024
(-)-Epicatechin	>1024	>1024	>1024	>1024
Rutin	>1024	>1024	>1024	>1024
Gallic acid	>1024	>1024	>1024	>1024
Caffeic acid	>1024	>1024	>1024	>1024
Vanillic acid	>1024	>1024	>1024	>1024
Ferulic acid	>1024	>1024	>1024	>1024
Syringic acid	>1024	>1024	>1024	>1024
***p*-Coumaric acid**	>1024	>1024	>1024	>1024
Chlorogenic acid	>1024	>1024	>1024	>1024
Pilocarpine	>1024	>1024	>1024	>1024
Resveratrol	256	256	256	512
Pterostilbene	128	64	64	128
Pinosylvin	128	128	128	128

Several phenolic compounds here tested, namely *p*-coumaric and caffeic acids (Oh and Jeon, 2015b), chlorogenic, ferulic and syringic acids (Klančnik et al., 2012a), vanillic and gallic acids (Klančnik et al., 2012a; Oh and Jeon, 2015b) have demonstrated activity against *Campylobacter* spp., a bacterium closely related to *A. butzleri*. Nonetheless, the weak antimicrobial action here observed is not completely unexpected as *A. butzleri* is a Gram-negative bacterium and, generally, phytochemicals have a better antimicrobial action against Gram-positive bacteria, due to the different structure of their cell walls (Metsämuuronen and Siren, 2014).

In the present work, stilbenes were the only class of phytochemicals with antimicrobial action, with resveratrol showing the highest MIC values: 512 µg/mL for *A. butzleri* Ab11/11 strain and 256 µg/mL for all the other strains; and pterostilbene and pinosylvin having slightly lower MICs: 64 µg/mL and 128 µg/mL, and 64 µg/mL, respectively.

The MIC obtained here for resveratrol in *A. butzleri* (256 and 512 µg/mL) is marginally higher than what was previously found for this species (100 µg/mL) (Ferreira et al., 2014b; Duarte et al., 2015), which may be related with the high heterogeneity found among *A. butzleri* strains (Ferreira et al., 2013). A study testing resveratrol activity against several Gram-negative and Gram-positive bacteria has shown antibacterial activity of this compound against Gram-positive bacteria with a bacteriostatic mode of action; however no antimicrobial activity was found for the Gram-negative bacteria tested (Paulo et al., 2010). By comparison, *A. butzleri* seems to be more susceptible to resveratrol than other Gram-negative bacteria (e. g. *S.* Typhimurium and

P. aeruginosa), showing, however, higher MICs than the ones obtained to *H. pylori* (MIC= 50 µg/mL) (Paulo et al., 2011b), *C. jejuni* (MIC= 100 µg/mL) and *C. coli* (MIC= 50 µg/mL) (Duarte et al., 2015).

A study that compared pinosylvin and resveratrol antimicrobial activity, have found that in concordance with the results obtained in this work, pinosylvin has a slightly better activity than resveratrol, as it was able to inhibit the growth of *S.* Typhimurium in approximately 60%, while resveratrol inhibit it in approximately 50% (Plumed-Ferrer et al., 2013). The MIC obtained in this work for pinosylvin (128 µg/mL) is close to the results reported for *E. coli* (250 µg/mL) (Lee et al., 2005).

Pterostilbene has shown antimicrobial activity against Gram-positive and Gram-negative pathogens, such as *S. aureus* (25 µg/mL), *E. coli* (50 µg/mL), *P. aeruginosa* (25 µg/mL) (Lee et al., 2017) and MRSA (15.63 µg/mL and 32.25 µg/mL) (Ishak et al., 2016). The results for the Gram-negative bacteria are slightly lower than the ones obtained in this work (64 µg/mL and 128 µg/mL), with *E. coli* being the closest.

Literature suggests that resveratrol generally shows a lower antimicrobial activity than its derivatives (Chalal et al., 2014) and that is indeed observed in this work. Differences of antimicrobial action may be due to the fact that resveratrol is more hydrophilic, which hinders the diffusion across the cell membranes, or due to the methylated hydroxyphenyl groups in pterostilbene structure, that are known to increase biocidal activity of phenolics (Pastorkova et al., 2013).

Several members of the alkaloid class are able to inhibit Gram-positive and Gram-negative bacteria (Cushnie et al., 2014). However, in this study, pilocarpine does not demonstrate antimicrobial action against *A. butzleri*. Works with *S. aureus* and *E. coli* also noted the lack of antimicrobial activity of this phytochemical (Araruna et al., 2012). Interestingly, a study have shown that no microorganism inoculated in eye drops containing pilocarpine survived more than two hours (similarly to what happen in the eye drops with gentamicin), though no definitive correlation was established between these results and the presence of pilocarpine (Akinkunmi, 2013).

Table 9. Minimum inhibitory concentration of gallic acid for the control strain *Campylobacter jejuni* 71/09.

	MIC (µg/mL)
Phytochemicals	***C. jejuni* 71/09**
Gallic acid	128

The MIC of gallic acid for the control *C. jejuni* strain 71/09 was determined (Table 9). For this microorganism, the range of concentrations tested was enough to determine the MIC of this phytochemical (128 µg/mL) which was actually lower than what was reported for other *C. jejuni* strains (512 µg/mL-1024 µg/mL) (Oh and Jeon, 2015b).

These assays revealed that all the phytochemicals, except the stilbenes, had no antimicrobial action against *A. butzleri* at the concentrations tested, which is an expected result for a Gram-negative bacterium. This result, does not hinders the potential role of phytochemicals as EPIs.

4.2. Phytochemicals as efflux pump inhibitors for *A. butzleri* strains

To investigate if the phytochemicals are targeting efflux pumps, ethidium bromide accumulation assays were performed. The ethidium bromide is a common substrate for most of the efflux pumps, which emits a weak fluorescence in aqueous solutions (outside the cells) but becomes strongly fluorescent when concentrated in the periplasm of Gram-negative bacteria or in the cytoplasm of Gram-positive bacteria (Geall and Blagbrough, 2000; Rodrigues et al., 2011). As such, if the phytochemicals can inhibit efflux pumps, the levels of ethidium bromide inside the cell will rise, which will be reflected in an increase of fluorescence.

Before carrying out the assay, the MIC of ethidium bromide had to be determined so that a sub-inhibitory concentration could be defined (Table 10).

Table 10. Minimum inhibitory concentration of ethidium bromide for the *Arcobacter butzleri* strains being studied.

	MIC (µg/mL)			
	A. butzleri **A6-1**	***A. butzleri*** **DQ46M1**	***A. butzleri*** **CR50-2**	***A. butzleri*** **AB11/11**
Ethidium bromide	16	16	16	8

Since it is important that the cells are still viable for the assay, the growth curves of all the strains had also to be traced. As can be seen in Figure 5, all the strains enter in the stationary phase at around six hours, so the cells were harvested at five hours to assure that they were collected during the exponential phase.

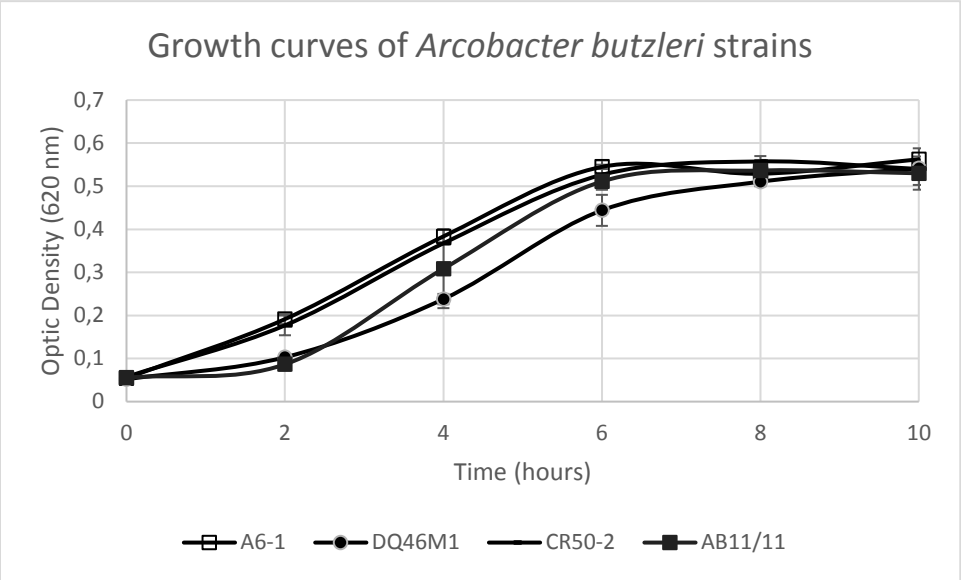


Figure 5.Growth curves of all *Arcobacter butzleri* strains.

The most resistant (CR50-2) and most susceptible (DQ46M1) *A. butzleri* strains were chosen to carry out the assay. After the cells have been washed, the cellular suspension adjusted and left at 30ºC for ten minutes to stabilize, the phytochemicals, at several sub-inhibitory concentrations, and the ethidium bromide, at a concentration of 2 µg/mL, were added and the fluorescence started to be measured. As most of the phytochemicals had a MIC superior to 1024 µg/mL, the sub-inhibitory concentrations chosen for these compounds were 1024 µg/mL, 512 µg/mL, 256 µg/mL and 128 µg/mL. For all the other phytochemicals that had a defined MIC the concentrations selected were 1/2x, 1/4x, 1/8x and 1/16x MIC.

The folding increase of fluorescence was determined by calculating the ratio of the fluorescence at each minute by the fluorescence at time 0. As can be seen in Figure 6, regarding *A. butzleri* DQ46M1, the phytochemicals that led to the highest increase of fluorescence at time 30 minutes were pterostilbene (see also Appendix, Figure 21 A and B), resveratrol (see also Appendix, Figure 20 A and B) and pinosylvin (see also Appendix, Figure 22 A and B). These results suggest that these phytochemicals may be inhibiting the efflux pumps, leading to the retention of ethidium bromide inside the cell and consequently to an increase of fluorescence. Therefore, they were selected for further studies of synergistic activity with antibiotics, by the performance of checkerboard assays.

The other phytochemicals in study led to a fluorescence folding increase lower than the EPI control. However, some achieve a fluorescence folding superior to 1.5 (higher than the solvent controls), and as such they were also selected for synergistic activity assays. These phytochemicals were: (-)-epicatechin (Appendix, Figure 9 A and B), (+)-catechin (Appendix, Figure 10 A and B), rutin (Appendix, Figure 12 A and B), caffeic acid (Appendix, Figure 14 A and B) and chlorogenic acid (Appendix, Figure 17 A and B).

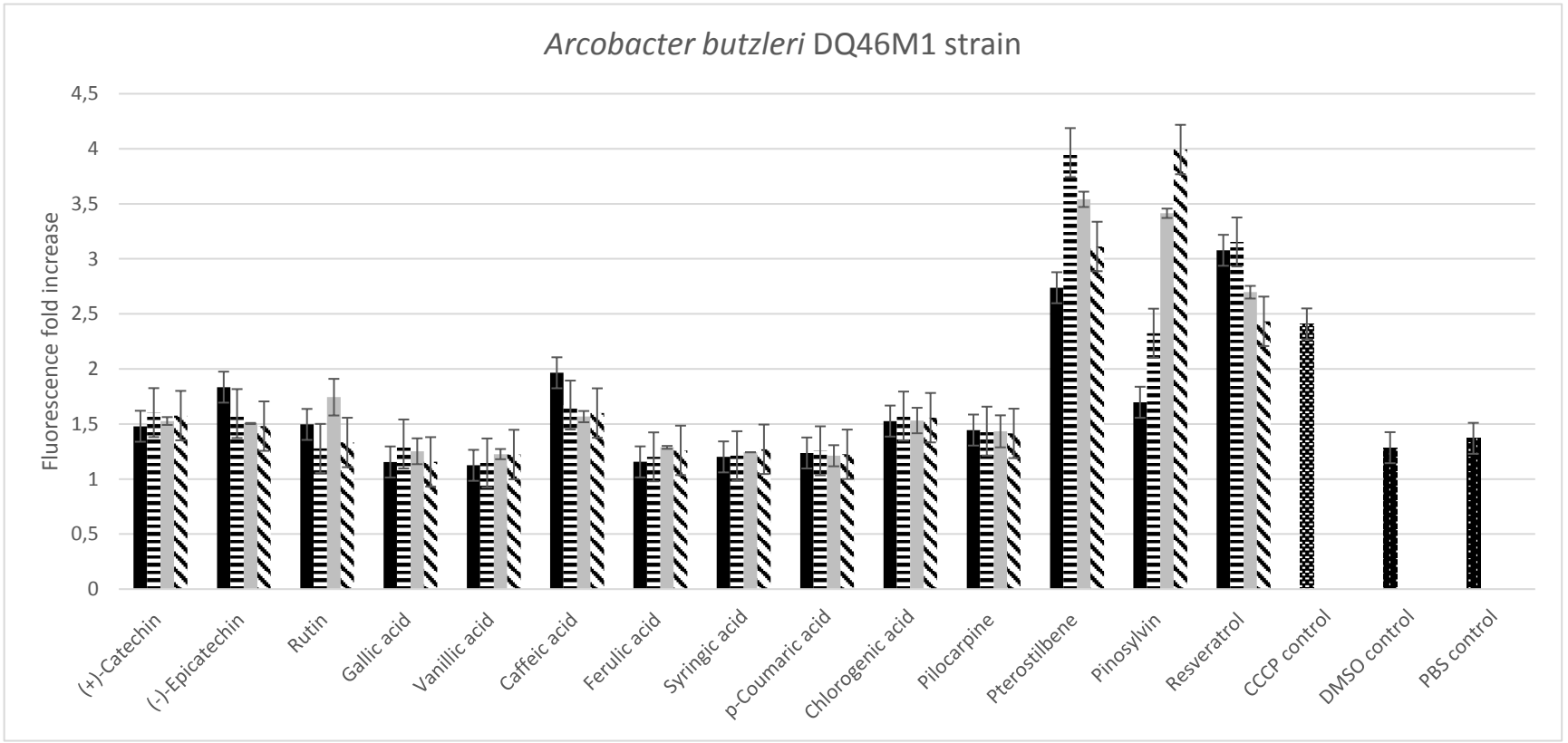


Figure 6.Fluorescence folding increase measured at 30 minutes for *Arcobacter butzleri* DQ46M1 strain in the presence of sub-inhibitory concentrations of the phytochemicals. The highest concentration tested (black) was 128 µg/mL for resveratrol, 64 µg/mL for pinosylvin, 32 µg/mL for pterostilbene and 1024 µg/mL for the other compounds; the second highest concentration (horizontal stripes) was 64 µg/mL for resveratrol, 32 µg/mL for pinosylvin, 16 µg/mL for pterostilbene and 512 µg/mL for the rest; the third highest concentration (grey) was 32 µg/mL for resveratrol, 16 µg/mL for pinosylvin, 8 µg/mL for pterostilbene and 256 µg/mL for the other phytochemicals and the lowest concentration tested (diagonal stripes) was 16 µg/mL for resveratrol, 8 µg/mL for pinosylvin, 4 µg/mL for pterostilbene and 128 µg/mL for the other phytochemicals. DMSO and PBS were used as solvent controls and CCCP at 32 µg/mL was used and the EPI control.

Furthermore, despite the minor increase in ethidium bromide accumulation associated with gallic acid (Appendix, Figure 11 A and B), this compound was also selected for assays of synergism with antibiotics, as it is frequently described as having synergism with several antibiotics (Lima et al., 2016; Oh and Jeon, 2015a; Sanhueza et al., 2017).

The other phytochemicals: vanillic acid (Appendix, Figure 13 A and B), syringic acid (Appendix, Figure 16 A and B), ferulic acid (Appendix, Figure 15 A and B), *p*-coumaric acid (Appendix, Figure 18 A and B) and pilocarpine (Appendix, Figure 19 A and B), were not submitted to further studies, as they did not lead to an increase of fluorescence higher than the solvent controls, which suggests that they are not affecting the efflux pumps activity.

Regarding the most resistant *A. butzleri* strain (CR50-2) (Figure 7) only pterostilbene (Appendix, Figure 35 A and B), pinosylvin (Appendix, Figure 36 A and B) and resveratrol (Appendix, Figure 34 A and B) had a fluorescence folding increase superior to 1.5. Furthermore, the reading of the variation of the fluorescence through time (Appendix, Figures 9-36 A) in both strains, shows that most phytochemicals do not have the typical profile of ethidium bromide accumulation as CCCP shows: an initial increase of fluorescence until reaching a plateau. That fact may suggest that for most phytochemicals, the accumulation of ethidium bromide may not be due to the inhibition of the efflux pumps. Stilbenes, however, not only show the highest increase in fluorescence, but also have an ethidium bromide accumulation profile similar to CCCP, especially pinosylvin (Appendix, Figure 34 to 36 A), implying that they are in fact inhibiting the efflux.

Of all the phytochemicals, pterostilbene is one which behaviour changed more markedly from one strain to the other. In the *A. butzleri* DQ46M1 strain, pterostilbene lead to one of the highest rates of ethidium bromide accumulation. However, in the *A. butzleri* CR50-2 strain, only the highest concentrations could achieve a fluorescence folding superior to the solvent controls. This is probably because the kind of efflux pump that this phytochemical affects are not as expressed in this strain, as it is in the *A. butzleri* DQ46M1 strain.

As efflux pumps are fundamental for the survival of the bacteria and the development of multidrug resistances (Webber and Piddock, 2003; Venter et al., 2015). It is logic to assume that *A. butzleri* CR50-2, being the most resistant strain, must have an overexpression of efflux pump systems. As such, if the phytochemicals are inhibiting efflux pumps, the results should be more visible in this strain, which is not the case (see Figures 23 to 36 A and B). Such may be related to the types of efflux pumps being inhibited by the phytochemicals. As the ethidium bromide was added at a low concentration, if the phytochemicals only inhibit specific efflux pumps, the activity of the remain efflux pumps may be enough to keep the compound outside the cell.

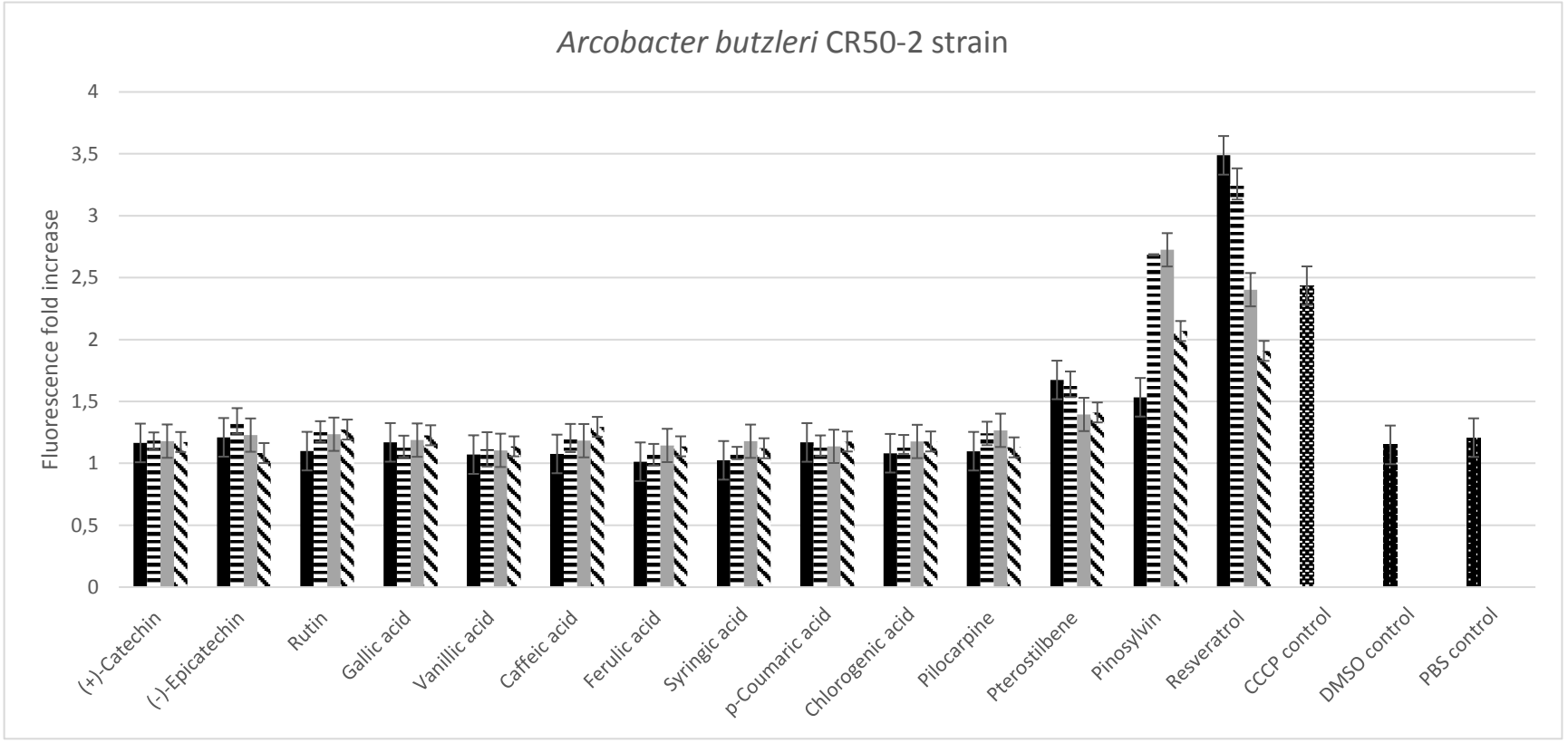


Figure 7. Fluorescence folding increase measured at 30 minutes for *Arcobacter butzleri CR50-2* strain in the presence of sub-inhibitory concentrations of the phytochemicals. The highest concentration tested (black) was 128 µg/mL for resveratrol, 64 µg/mL for pinosylvin, 32 µg/mL for pterostilbene and 1024 µg/mL for the other compounds; the second highest concentration (horizontal stripes) was 64 µg/mL for resveratrol, 32 µg/mL for pinosylvin, 16 µg/mL for pterostilbene and 512 µg/mL for the rest; the third highest concentration (grey) was 32 µg/mL for resveratrol, 16 µg/mL for pinosylvin, 8 µg/mL for pterostilbene and 256 µg/mL for the other phytochemicals and the lowest concentration tested (diagonal stripes) was 16 µg/mL for resveratrol, 8 µg/mL for pinosylvin, 4 µg/mL for pterostilbene and 128 µg/mL for the other phytochemicals. DMSO and PBS were used as solvent controls and CCCP at 32 µg/mL was used and the EPI control.

It is of note that for some phytochemicals the fluorescence folding increase obtained was not proportional with the concentrations been used. That is, lower concentrations reflected a superior increase in fluorescence that higher concentration. This may be happening due to a problem of solubility, especially in the case of the stilbenes that have a remarkable tendency to precipitate. Despite this, the order of difference between concentrations is not significant, not affecting the interpretation of the results.

Overall, stilbenes were the phytochemicals with the more promisor results, appearing to be modulating the efflux pumps in both strains. Even though, only stilbenes should be classifiable to further tests with the *A. butzleri* CR50-2 strain, the phytochemicals selected for the *A. butzleri* DQ46M1, were used for synergism assays with antibiotics for both strains.

As gallic acid is described to reduce the expression of CmeABC, a RND-type efflux pump in *C. jejuni*, lowering the MIC of ciprofloxacin 8 to 16 times and erythromycin 4 to 16 times (Oh and Jeon, 2015a), its ability to inhibit efflux pumps was also tested for *A. butzleri*. As can be seen in Figure 8, the two highest concentrations had a folding increase superior to 1.5, but inferior to the EPI used as control, which may imply that the inhibition of the efflux pumps could not be the only mechanism responsible for the synergism reported.

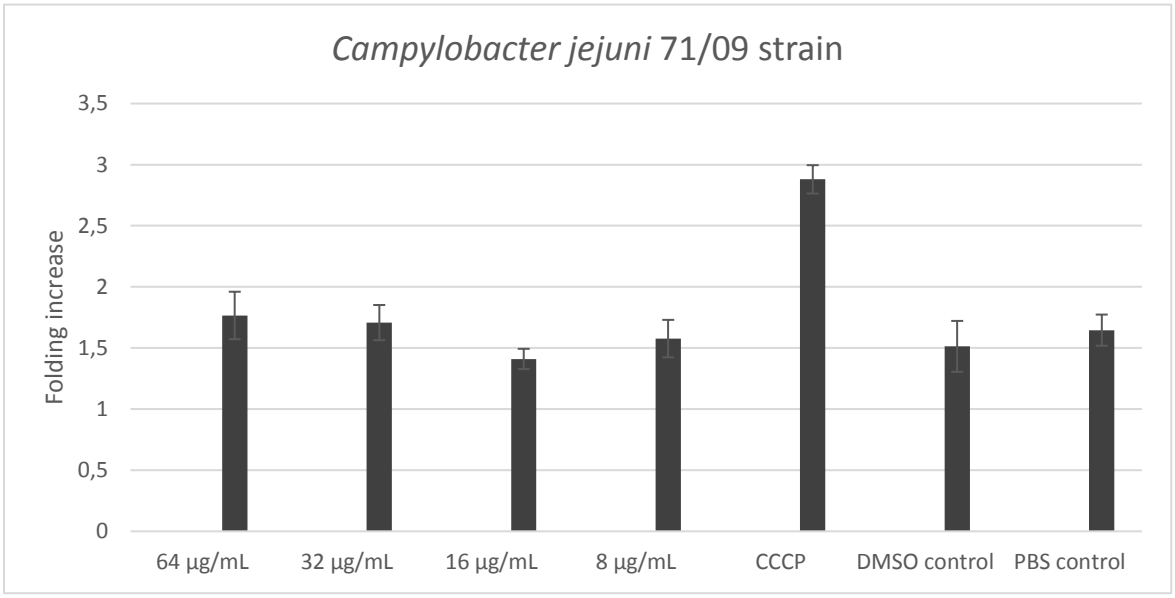


Figure 8. Fluorescence folding increase measured at 30 minutes for *Campylobacter jejuni* 71/09 in the presence of sub-inhibitory concentrations of gallic acid (1/2 MIC, 1/4 MIC, 1/8 MIC and 1/16 MIC). CCCP at 32 µg/mL was the EPI used as control.

4.3. Efflux pumps as a resistance mechanism against the phytochemicals

To assess if the efflux pumps are involved in the resistance of *A. butzleri* strains to the phytochemicals, their MIC in the presence of sub-inhibitory concentrations (1/4xMIC) of well-known efflux pumps inhibitors was determined. Thus, the MIC of several efflux pumps were firstly determined, so that sub-inhibitory concentrations could be selected in the following assays (Table 11). The selected EPIs (PaβN, NMP and CCCP) have previously demonstrated to be able to modulate *Campylobacter* spp. resistance; however, verapamil has not shown the same effect (Klančnik et al., 2012b).

Table 11. Minimum inhibitory concentration of four known efflux pumps inhibitors for the four *Arcobacter butzleri* strains in study.

	MIC (µg/mL)			
EPIs	***A. butzleri* A6-1**	***A. butzleri* DQ46M1**	***A. butzleri* CR50-2**	***A. butzleri* AB11/11**
PaβN	20	80	40	80
Verapamil	>800	>800	>800	>800
CCCP	128	128	64	128
NMP	200	200	200	200

As can be seen in Table 11, PaβN, an EPI that acts on RND family efflux pumps (Lomovskaya et al., 2001), is the more active EPI against the *A. butzleri* strains in study with MICs ranging from 20 to 80 µg/mL that are considered sub-inhibitory for *Campylobacter* spp. (Klančnik et al. 2012b; Kurinčič et al., 2012).

For the realization of the assays, the most susceptible (DQ46M1) and the most resistant (CR50-2) *A. butzleri* strains were selected. Nine phytochemicals were chosen to be tested: three stilbenes, three flavonoids and three phenolic acids. These phytochemicals are the same that were selected for synergism assays.

In the case of the *A. butzleri* DQ46M1 strain (Table 12), the MIC of all the phenolic acids, except for gallic acid in the presence of PAβN, which lowered at least by half, remained unchanged as far as the range of concentrations tested can show. Concerning the stilbenes, all suffered a variation of their MICs in the presence of the EPIs, with the exception of pterostilbene in the presence of verapamil. Pinosylvin was the phytochemical most affected with the MIC decreasing two to 16 folds; the MIC of resveratrol lowered two to four times and pterostilbene had its MICs cut by half.

Table 12. Minimum inhibitory concentration of several phytochemicals in the presence of sub-inhibitory concentrations of efflux pump inhibitors for the *Arcobacter butzleri* DQ46M1.

	MIC (µg/mL) in presence of efflux pumps inhibitors							
	+PaßN	Fold decrease	**+Verapamil**	Fold decrease	**+CCCP**	Fold decrease	**+NMP**	Fold decrease
(+)-Catechin	>1024	-	>1024	-	>1024	-	>1024	-
(-)-Epicatechin	>1024	-	>1024	-	>1024	-	>1024	-
Rutin	>1024	-	>1024	-	>1024	-	>1024	-
Gallic acid	1024	≥2	>1024	-	>1024	-	>1024	-
Caffeic acid	>1024	-	>1024	-	>1024	-	>1024	-
Chlorogenic acid	>1024	-	>1024	-	>1024	-	>1024	-
Resveratrol	64	4	64	4	128	2	64	4
Pterostilbene	32	2	64	-	32	2	32	2
Pinosylvin	8	16	64	2	16	8	32	4

Overall, PaßN was the EPI associated with the most relevant variations of the MICs. From these results, it can be inferred that the resistance mechanism to stilbenes, especially pinosylvin, is associated with efflux pumps of the RND family. CCCP led to a considerable reduction of the MIC of pinosylvin (reduction of eight times). This EPI exerts its activity by dissipating the proton gradient, that drives most of the efflux pumps, across the inner membrane (Dreier and Ruggerone, 2015). Thus, this EPI can inhibit most of the efflux pumps, including the RND family.

Table 13. Minimum inhibitory concentration of several phytochemicals in the presence of sub inhibitory concentration of efflux pump inhibitors for the *Arcobacter butzleri* strain CR50-2.

	MIC (µg/mL) in presence of efflux pumps inhibitors							
	+PaßN	Fold decrease	**+Verapamil**	Fold decrease	**+CCCP**	Fold decrease	**+NMP**	Fold decrease
(+)-Catechin	64	≥32	>1024	-	>1024	-	>1024	-
(-)-Epicatechin	512	≥4	>1024	-	1024	≥2	1024	≥2
Rutin	256	≥8	>1024	-	1024	≥2	>1024	-
Gallic acid	128	≥16	512	≥4	256	≥8	512	≥4
Caffeic acid	256	≥8	512	≥4	>1024	-	>1024	-
Chlorogenic acid	128	≥16	>1024	-	>1024	-	>1024	-
Resveratrol	64	4	32	8	64	4	64	4
Pterostilbene	16	4	64	-	16	4	64	-
Pinosylvin	16	8	128	-	64	2	128	-

In the case of the *A. butzleri* CR50-2 (Table 13), the MIC of all the phytochemicals decreased in the presence of PaβN, being (+)-catechin the most affected, with its MIC decreasing at least 32 times. Globally, CCCP also made the MIC of all phytochemicals decrease, with exception of (+)-catechin and caffeic and chlorogenic acids. Verapamil and NMP did not influenced the MIC of several compounds. Verapamil only lowered the MIC of resveratrol and gallic and caffeic acids and NMP of (-)-epicatechin, resveratrol and gallic acid. That may be because verapamil, though able to inhibit MDR pumps by interfering with the proton motive force, has as a target the ATP-dependent multidrug transporters (Pule et al., 2016) that are not the principal pump in Gram-negative bacteria. NMP, on the other hand, does have activity on RND type efflux pumps, but its action has been mainly demonstrated in *E. coli* (Marchetti et al., 2012).

Overall, the influence of the EPIs in the MIC of the phytochemicals was not as strong as what has been described in the literature. For *A. butzleri* it has been reported that the MIC of resveratrol in the presence of PaβN lowered 16 times and for *A. cryaerophilus* the MIC decreased four times (Ferreira et al., 2014b). While the results for *A. cryaerophilus* are identical to what was found here for both strains, the results reported for *A. butzleri* are closer to the results here obtained for pinosylvin. Other studies in *C. jejuni* found that the MIC of chlorogenic acid decreased 128 times in the presence of PaβN and 0.5 times when in the presence of NMP; while the MIC of gallic acid decreased more than 32 folds when in the presence of PaβN and 16 when in the presence of NMP (Klančnik et al., 2012a). Although the results reported for chlorogenic acid when in the presence of PaβN were much more marked than the variation found here (the MIC of the compound lowered 16 folds in the most resistant strain), the results described for this phytochemical in the presence of NMP and for gallic acid in the presence of PaβN are very close to the results obtained in this work.

Based on the MICs in the presence of PaβN, which led to a decrease of the MIC for *A. butzleri* CR50-2 of all the phytochemicals, it can be suggested that the *A. butzleri* CR50-2 strain overexpresses RND efflux pumps and that this efflux systems are associated with resistance to the phytochemicals. As efflux pumps showed to be especially relevant in the resistance to stilbenes, it may be the case that these compounds and ethidium bromide are competing substrates for RND efflux pumps, leading to the accumulation of ethidium bromide inside the cell.

4.4. Evaluation of synergistic interaction between phytochemicals and antibiotics

Plant extracts have shown synergism with antibiotics against several bacterial species, which opens the possibility for new treatments for infectious diseases (Nascimento *et al.*, 2000). Considering plants extracts activity and the previously reported interaction of phytochemicals with antibiotics against *Campylobacter* (Oh and Jeon, 2015a), allied with the potential of resveratrol as a putative EPI in *A. butzleri* and *A. cryaerophilus* (Ferreira et al., 2014b), further studies were taken to understand the potential synergetic interaction between antibiotics and several phytochemicals against *A. butzleri*.

So, to assess how the phytochemicals and the antibiotics interact with each other, checkerboard titration assays were performed. This method allows to test the effect of the combination of several concentrations of both compounds in *A. butzleri* growth.

Table 14. Fractional Inhibitory Concentration Index and correspondent classification of the effect of the combination phytochemical-antibiotic in *Arcobacter butzleri* DQ46M1 strain.

	FICI of the combination phytochemical/antibiotic			
Phytochemicals	Chloramphenicol	Tetracycline	Erythromycin	Ciprofloxacin
(+)-Catechin	2.0	2.0	2.0	≤2.0
(-)-Epicatechin	1.5	1.5	2.0	≤2.0
Rutin	2.0	2.0	≤2.0	1.5
Gallic acid	**1.0**	**≤0.6**	**≤1.0**	≤2.0
Caffeic acid	2.0	≤2.0	≤2.0	2.0
Chlorogenic acid	1.5	2.0	1.5	2.0
Resveratrol	**1.0**	**1.0**	**1.0**	**1.0**
Pterostilbene	**≤1.0**	2.1	≤2.1	≤2.1
Pinosylvin	**1.0**	**≤1.0**	**≤1.0**	**≤1.0**

The combinations phytochemical/antibiotic highlighted in bold correspond to additive interaction combinations, the others were classified as indifferent interactions.

Relatively to the most susceptible *A. butzleri* strain, DQ46M1 (Table 14), most of the combinations showed no interaction (indifference). Nonetheless, the combinations of gallic acid with tetracycline and erythromycin, pterostilbene with chloramphenicol, as well as pinosylvin and resveratrol with all the antibiotics, presented an additive effect. It is of note that none of the phytochemicals tested had an antagonistic interaction with the antibiotics.

Table 15. Fractional Inhibitory Concentration Index and correspondent classification of the combination phytochemical-antibiotic in *Arcobacter butzleri* CR50-2 strain.

	FICI of the combination phytochemical/antibiotic			
Phytochemicals	Chloramphenicol	Tetracycline	Erythromycin	Ciprofloxacin
(+)-Catechin	≤1.5	1.5	≤1.5	2.0
(-)-Epicatechin	≤1.5	1.5	**≤0.6**	1.5
Rutin	**0.6**	2.0	2.0	2.0
Gallic acid	**≤0.8**	**0.8**	**≤1.0**	≤1.1
Caffeic acid	1.5	≤2	≤1.3	2.0
Chlorogenic acid	**0.6**	1.5	**1.0**	2.0
Resveratrol	**≤0.6**	**1.0**	≤1.5	**1.0**
Pterostilbene	≤1.1	2.1	1.1	≤1.1
Pinosylvin	**1.0**	**1.0**	1.1	**1.0**

The combinations phytochemical/antibiotic highlighted in bold correspond to additive interaction combinations, the others were classified as indifferent interactions.

Relatively to the most resistant *A. butzleri* strain, CR50-2, there are a high number of additive interaction. Only (+)-catechin, caffeic acid and pterostilbene showed an indifference effect with all antibiotics, while resveratrol, pinosylvin and gallic acid showed additive effect with three of the antibiotics; chlorogenic acid had an additive effect with chloramphenicol and erythromycin; (-)-epicatechin and rutin only had additivity with one antibiotic, erythromycin and chloramphenicol, respectively (Table 15). Once again, none of the phytochemicals tested had an antagonistic effect.

Some of the phytochemicals in study have been associated with synergic or additive effects before. In *C. jejuni*, it was reported that caffeic acid, at a lower range of concentrations (0-256 μg/mL) than those used in this study, had synergistic interaction with ciprofloxacin and erythromycin (Oh and Jeon, 2015a). Comparatively, in this study, caffeic acid showed an indifferent effect with all the antibiotics for both strains evaluated. A study with MRSA tested caffeic acid for its synergism with antibiotics and obtained comparable results to the ones here presented - indifference (Kyaw et al., 2012).

Despite the results obtained for the previous accumulation assay, where gallic acid was not pointed as presenting a relevant role as an EPI, this compound was one of the phytochemicals with the best results in checkerboard assay, presenting an additive interaction with several antibiotics. Other study, using MRSA strains, has obtained comparable results for gallic acid, classifying most of its combinations as additive and occasionally as indifferent (Kyaw et al., 2012). For *P. aeruginosa*, this phytochemical also showed synergism with tetracycline and, in one strain, additivity with ciprofloxacin (Jayaraman et al., 2010). This may suggest that gallic acid enhances the activity of several antibiotics by other mechanisms than the inhibition of efflux pumps. This phytochemical may be acting on other targets or even on multiple targets,

besides the efflux pumps, a feature observed for other phytochemicals such as quercetin that both increases inner bacterial permeability and inhibits the enzyme ATPase in *E. coli* (Simões et al., 2009).

Flavonoids, such as quercetin and combinations of rutin and morin, have been shown to present additive or synergic interaction with several antibiotics in MRSA strains (Amin et al., 2015). Rutin also presented an additive interaction with tetracycline and ciprofloxacin for *P. aeruginosa* (Jayaraman et al., 2010), what did no happen here, though it did have additive effect with chloramphenicol in *A. butzleri* CR50-2 strain. Chlorogenic acid had an additive effect with ciprofloxacin and erythromycin in *A. butzleri* CR50-2 strain which was also observed in *S. aureus, P. aeruginosa*, *Ent. aerogenes* and *E. coli* (Hemaiswarya and Doble, 2010).

In literature, pterostilbene have shown synergism with gentamicin in *P. aeruginosa, E.coli* and *S. aureus* (Lee et al., 2017). In this study, gentamicin was not tested, since it is one of the antibiotics to which higher levels of susceptibility can be found for *A. butzleri*; however, pterostilbene showed additive interaction with chloramphenicol.

Table 16. Fractional Inhibitory Concentration Index and correspondent classification of the combination gallic acid-antibiotic in *Campylobacter jejuni* 71/09i strain.

	FICI of the combination phytochemical/antibiotic			
	Chloramphenicol	Tetracycline	Erythromycin	Ciprofloxacin
Gallic acid	**≤1.0**	≤1.5	≤1.1	**≤0.7**

The combinations phytochemical/antibiotic highlighted in bold correspond to additive interaction combinations, the others were classified as indifferent interactions.

A control assay with *C. jejuni* and gallic acid was carried out in order to assess if the synergism reported in other works (Oh and Jeon, 2015a) could be obtained here too (Table 16). It showed that this phytochemical has additive effect with chloramphenicol and ciprofloxacin and no interaction with tetracycline and erythromycin, although the FICI of the last is close to what is considered an additive effect. These results are not as positive as the synergic results reported previously for gallic acid with ciprofloxacin and erythromycin in *C. jejuni* (Oh and Jeon, 2015a), what may be due to the fact that this study is using a different clinical strain with a different genetic background.

The fact that some of the phytochemicals had additive effect with the antibiotics when associated with the results obtained from the ethidium bromide accumulation assays, give weight to the suggestion that some of the phytochemicals, particularly gallic acid, contribute to the enhancement of the antibiotics activity not through the inhibition of the efflux pumps, but more likely due to the permeabilization of the membrane. This suggestion is sustained by the literature that reports that some phenolic compounds weaken outer membrane of the

Gram-negative bacteria (Alakomi et al., 2007), with pinosylvin being able to damage the outer membrane of *Salmonella*, increasing susceptibility to novobiocin (Plumed-Ferrer et al., 2013) and gallic and *p*-coumaric acids being able to increase membrane permeability and accumulation of ciprofloxacin in *C. jejuni* (Oh and Jeon, 2015a).

Overall, the stilbenes were the only ones that had a noteworthy influence on the activity of efflux pumps of *A. butzleri, associated with* additive interaction with several antibiotics.

4.5. Quorum sensing inhibition

Several bacteria can communicate intercellularly by a set of mechanisms that are collectively named quorum sensing systems. Quorum sensing contributes to motility, biofilm formation, virulence, and colonization (Plummer, 2012). These characteristics make quorum sensing inhibition a promising strategy to fight bacterial infections (Singh *et al.*, 2009). Several plant extracts and phytochemicals have quorum sensing inhibition properties (Adonizio et al., 2006; Singh et al., 2009; Cushnie et al., 2014).

To test the phytochemicals, quorum sensing inhibition assays with the biosensor strain *C. violaceum* ATCC 12472 were carried out. This bacterium produces a purple pigment, violacein, regulated by quorum sensing (Adonizio et al., 2006). As such, quorum sensing inhibition (QSI) is detected by an inhibition of violacein production. When QSI is evaluated in a qualitative manner in solid medium, it is translated in the formation of a ring of colourless but viable cells around a disc impregnated with the test compound. QSI can be calculated by subtracting the diameter of cell growth inhibition (D2) to the total diameter (pigment and cell growth inhibition) around the disc (D1).

As can be seen in Table 17, only (+)-catechin, (-)- epicatechin, pinosylvin and resveratrol were able to inhibit the quorum sensing. The results obtained for (+)-catechin are supported by a study that found that this phytochemical has a negative effect on the expression quorum sensing regulatory genes on *P. aeruginosa* (Vandeputte et al., 2010). The results obtained for resveratrol (8.0 ±1.0 mm) are similar to what is described in the literature (8.5 ±0.75 mm) (Duarte et al., 2015). A study shown that extracts of apple peels have quorum sensing inhibition ability and that rutin, (-)epicatechin and caffeic acid are the most abundant phenolic compounds present (Fratianni et al., 2011). Although rutin and caffeic acid have not presented quorum sensing inhibition in this work, (-)-epicatechin had a pigment inhibition ring with a diameter of 2.0 ±0.9 mm.

Table 17. Screening of phytochemicals for quorum sensing inhibition using *Chromobacterium violaceum* ATCC 12472.

	Diameter (mm)		
Phytochemicals	D1	D2	QSI (D1-D2)
(+)-Catechin	12.1±0.6	9.3±0.8	2.8±0.2
(-)-Epicatechin	11.7±1.2	9.7±0.7	2.0±0.9
Rutin	6.0±0.0	6.0±0.0	0.0±0.0
Gallic acid	6.0±0.0	6.0±0.0	0.0±0.0
Caffeic acid	12.0±0.8	12.0±0.8	0.0±0.0
Vanillic acid	13.0±0.6	13.0±0.6	0.0±0.0
Ferulic acid	12.4±1.0	12.4±1.0	0.0±0.0
Syringic acid	15.9±0.9	15.9±0.9	0.0±0.0
***p*-Coumaric acid**	14.6±1.2	14.6±1.1	0.0±0.0
Chlorogenic acid	7.8±0.9	7.8±0.9	0.0±0.0
Resveratrol	21.7±0.1	13.7±0.9	8.0±1.0
Pilocarpine	6.3±0.6	6.3±0.6	0.0±0.0
Pterostilbene	7.5±0.3	7.5±0.1	0.0±0.0
Pinosylvin	33.4±0.3	24.2±0.8	9.2±1.1
Negative control (DMSO)	6.0±0.0	6.0±0.0	0.0±0.0

The level of inhibition of (+)-catechin, (-)- epicatechin is not very remarkable, but resveratrol and pinosylvin are promisor agents.

As these stilbenes can modulate the activity of efflux pumps and inhibit quorum sensing mechanisms, they may be interesting compounds to further explore, namely regarding biofilm formation inhibition and perhaps the production of virulence factors (Christiaen et al., 2014).

Quorum sensing is vital to organize biofilm formation (Plummer, 2012), while efflux pumps are an important resistance and survival mechanism for cells when they are organized in biofilms (Soto, 2013). So, it is possible that resveratrol and pinosylvin, that target these two mechanisms, will be able to inhibit the formation or participate in the destruction of *A. butzleri* biofilms. If that is the case, these phytochemicals may be useful in the control of this bacterium.

Chapter 5 - Conclusions and future perspectives

This work allowed for a better understanding regarding the ability of several phytochemicals to inhibit *A. butzleri'* efflux pumps and interact with antibiotics activity. The principal conclusions taken from this work were:

- The determination of the MICs of the phytochemicals showed that none of the compounds, with exception of the stilbenes, presented antimicrobial action against *A. butzleri* in the range of concentrations tested. These results do not interfere with the potential role of phytochemicals as EPIs;

- Globally, the study of the accumulation of ethidium bromide in the cells in the presence of sub-inhibitory concentrations of the phytochemicals revealed that (+)-catechin, (-)-epicatechin, rutin, caffeic and chlorogenic acids, resveratrol, pterostilbene and pinosylvin, showed an inhibitory effect on the efflux pumps activity when compared with controls. In the case of the stilbenes, this inhibitory action was stronger than the observed for EPI control;

- Efflux pumps relevance in the resistance of the bacterium to the phytochemicals is dependent on the strain. The *A. butzleri* CR50-2 strain may overexpress efflux pumps of the RND family which can be associated with resistance to the phytochemicals;

- Among the phytochemicals tested, several compounds showed additive interaction with the antibiotics, with none presenting an antagonistic effect. Stilbenes were the phytochemicals with the more relevant effect;

- Resveratrol and pinosylvin had the ability to inhibit the quorum sensing. These two phytochemicals were also among the compounds with the better results in the ethidium bromide accumulation assays, as such they may be of interest for further studies.

This work was the first step in the research of efflux pump inhibitors for *A. butzleri*. In the future, it will be important to complement this study and assess if the phytochemicals can permeabilize the membrane or if they have action in a strain lacking efflux pumps. It would also be of interest to expand the study to a higher number of strains.

Furthermore, it would be beneficial to test other stilbenes as this phytochemical has obtained good results in the synergism assays.

Lastly, it would be relevant to study the impact of pinosylvin and resveratrol on biofilm formation.

Chapter 6 – Bibliography

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Appendix 1

This appendix shows the graphics associated with point 2 of chapter 4 (Results and discussion) regarding the accumulation of ethidium bromide in the first 30 minutes after being added to the cells in the presence of sub-inhibitory concentrations of the phytochemicals.

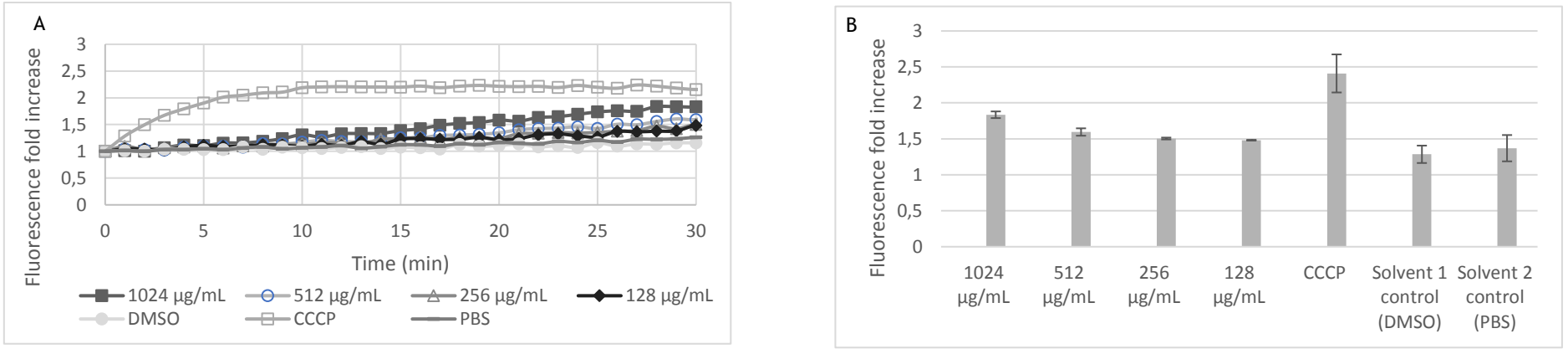


Figure 9. Ethidium bromide accumulation assay for DQ46M1 strain in the presence of sub-inhibitory concentrations of (-)-epicatechin over 30 minutes (A) and at 30 minutes (B).

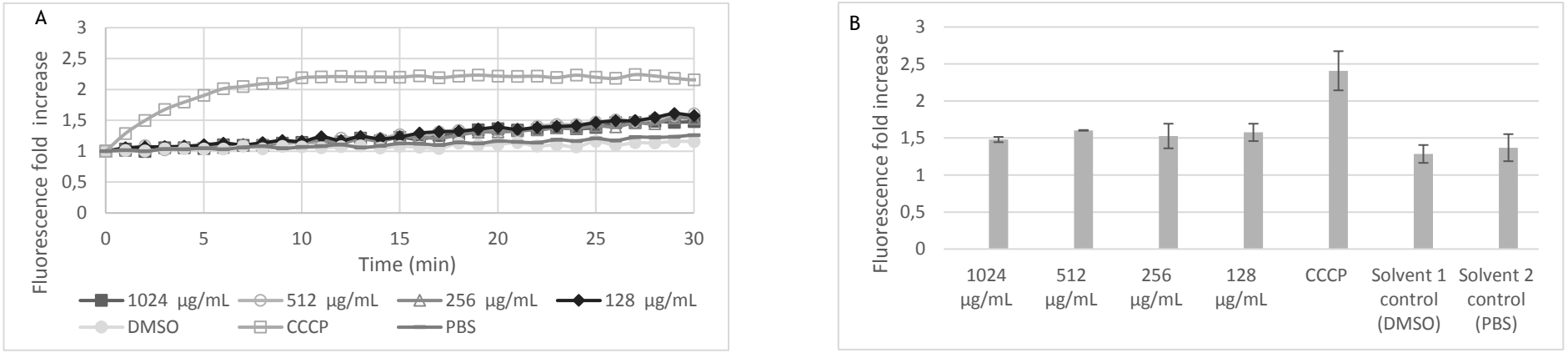


Figure 10. Ethidium bromide accumulation assay for DQ46M1 strain in the presence of sub-inhibitory concentrations of (+)-catechin over 30 minutes (A) and at 30 minutes (B).

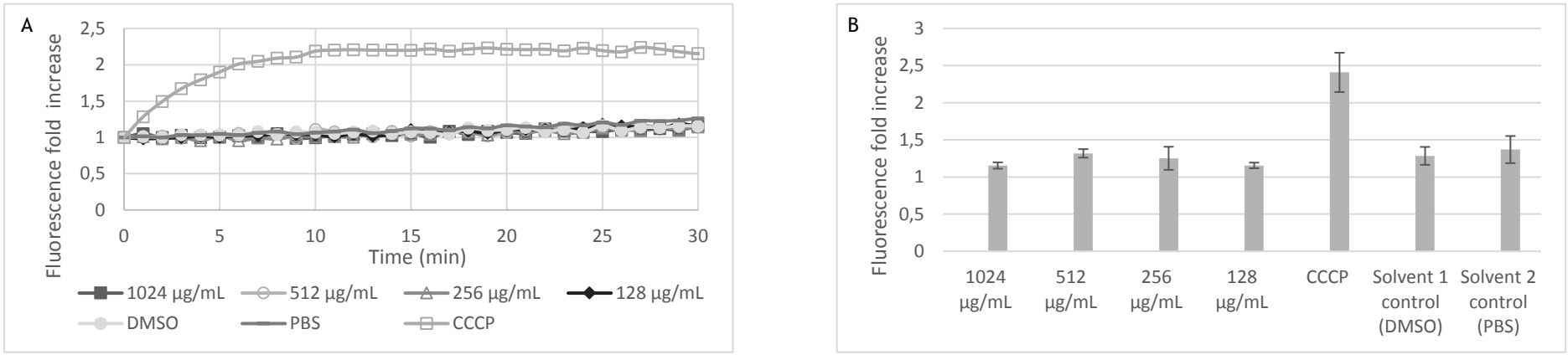


Figure 11. Ethidium bromide accumulation assay for DQ46M1 strain in the presence of sub-inhibitory concentrations of gallic acid over 30 minutes (A) and at 30 minutes (B).

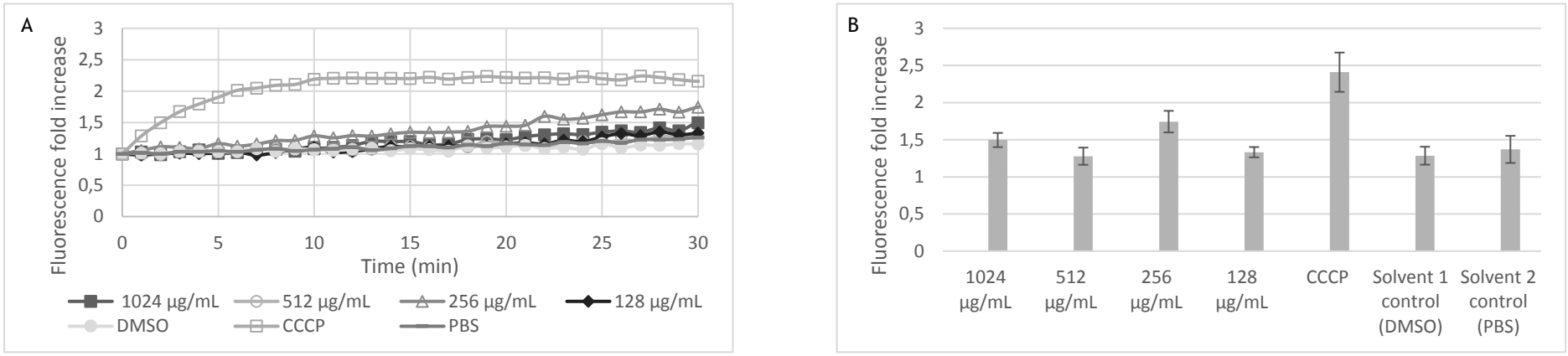


Figure 12. Ethidium bromide accumulation assay for DQ46M1 strain in the presence of sub-inhibitory concentrations of rutin over 30 minutes (A) and at 30 minutes (B).

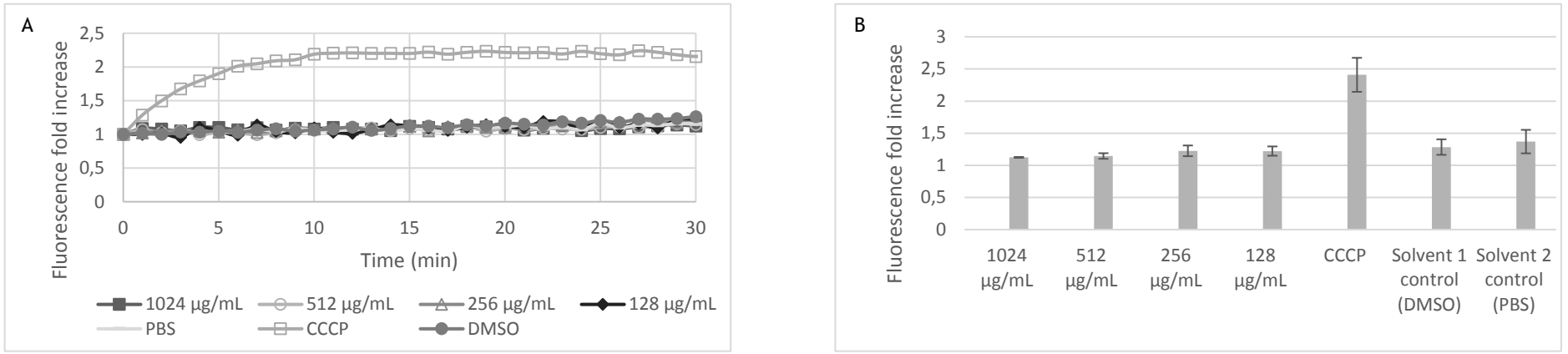


Figure 13. Ethidium bromide accumulation assay for DQ46M1 strain in the presence of sub-inhibitory concentrations of vanillic acid over 30 minutes (A) and at 30 minutes (B).

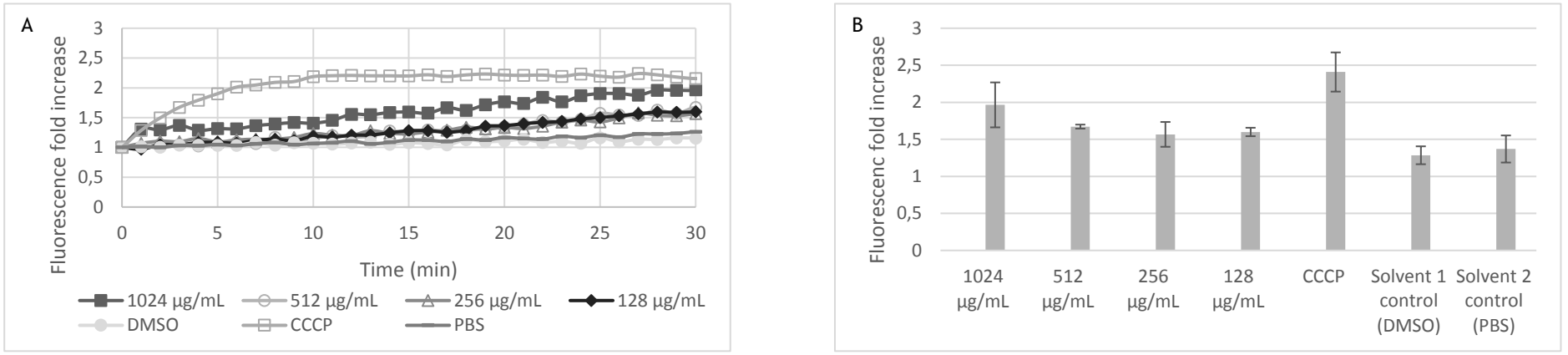


Figure 14. Ethidium bromide accumulation assay for DQ46M1 strain in the presence of sub-inhibitory concentrations of caffeic acid over 30 minutes (A) and at 30 minutes (B).

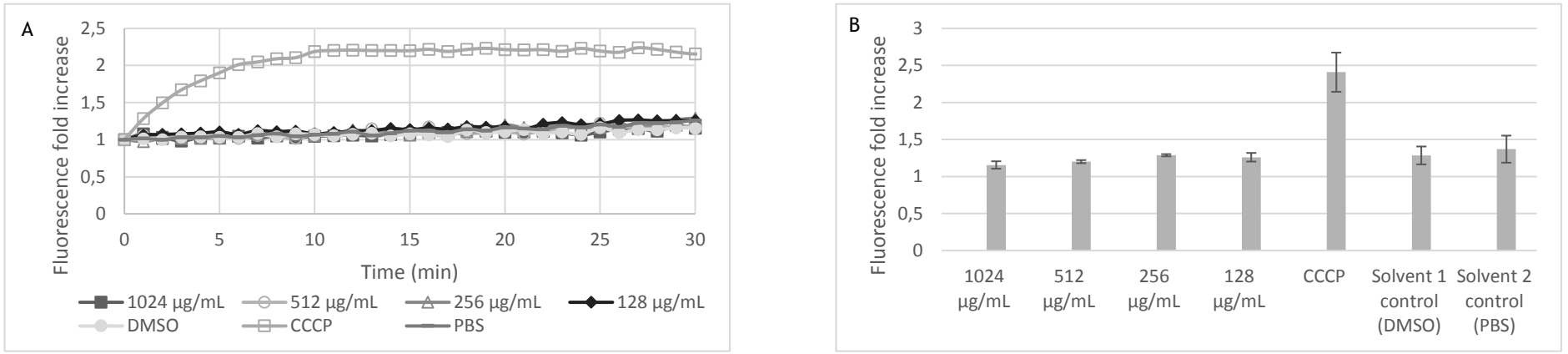


Figure 15. Ethidium bromide accumulation assay for DQ46M1 strain in the presence of sub-inhibitory concentrations of ferulic acid over 30 minutes (A) and at 30 minutes (B).

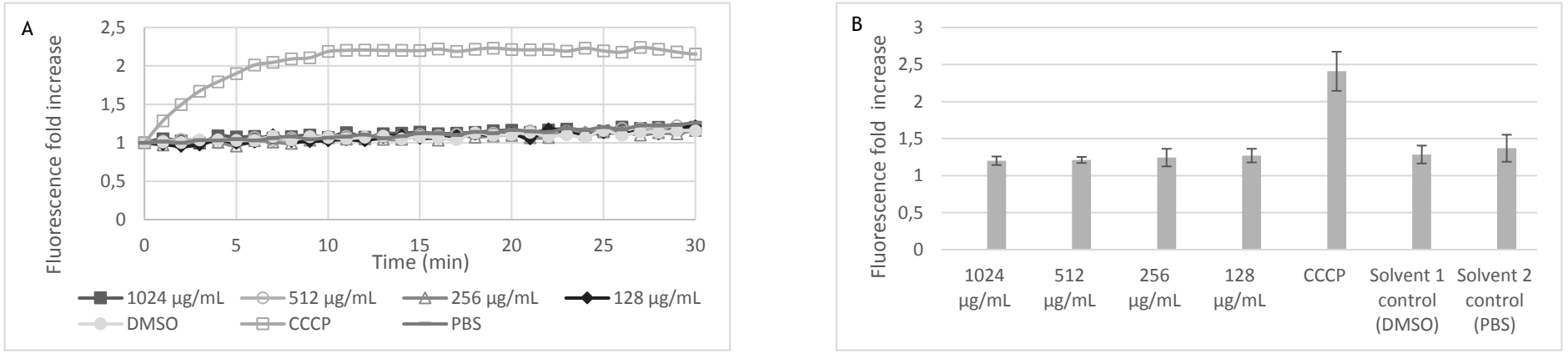


Figure 16. Ethidium bromide accumulation assay for DQ46M1 strain in the presence of sub-inhibitory concentrations of syringic acid over 30 minutes (A) and at 30 minutes (B).

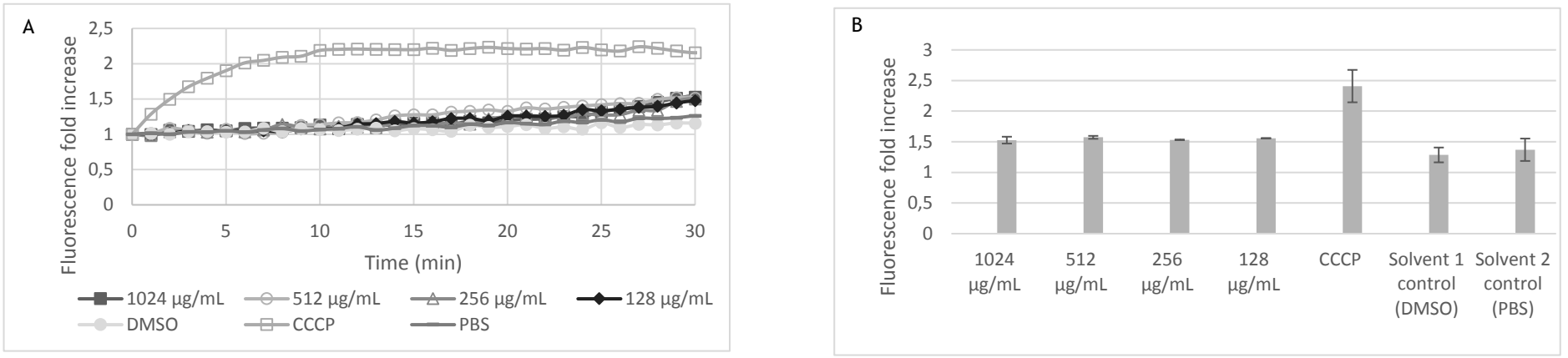


Figure 17. Ethidium bromide accumulation assay for DQ46M1 strain in the presence of sub-inhibitory concentrations of chlorogenic acid over 30 minutes (A) and at 30 minutes (B).

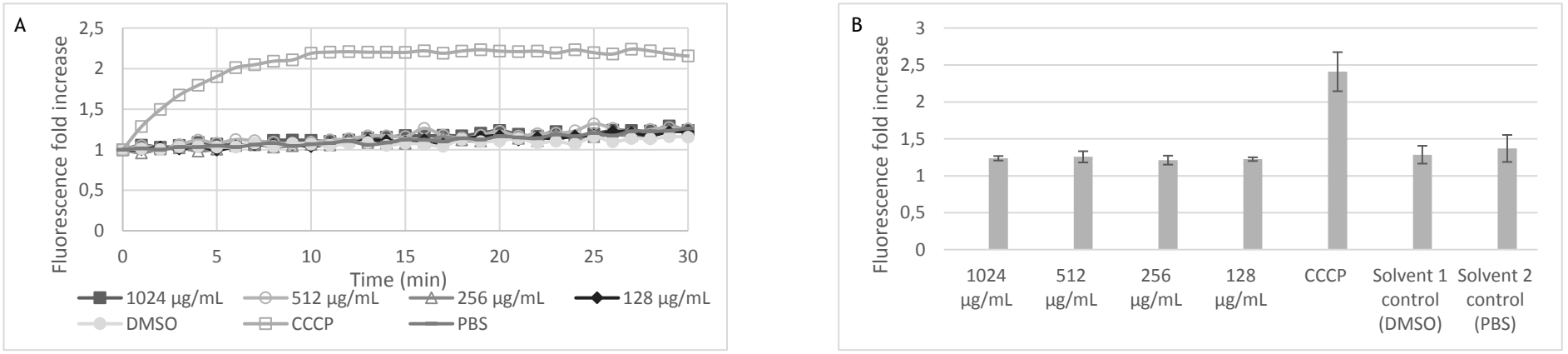
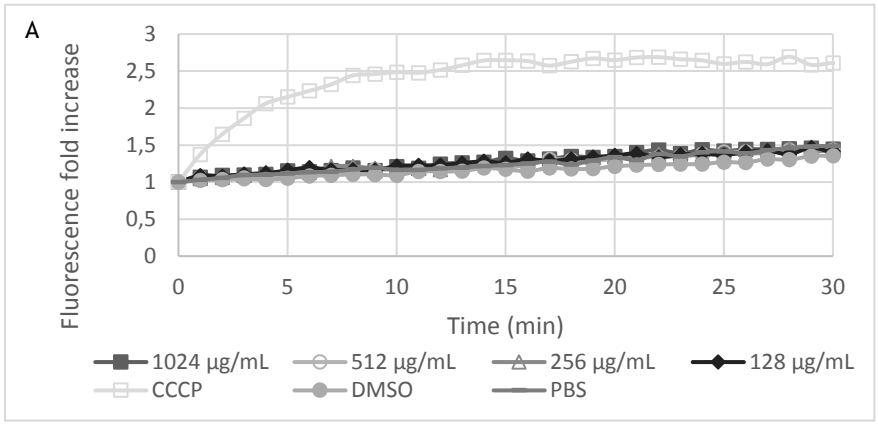


Figure 18. Ethidium bromide accumulation assay for DQ46M1 strain in the presence of sub-inhibitory concentrations of *p*-Coumaric acid over 30 minutes (A) and at 30 minutes (B).



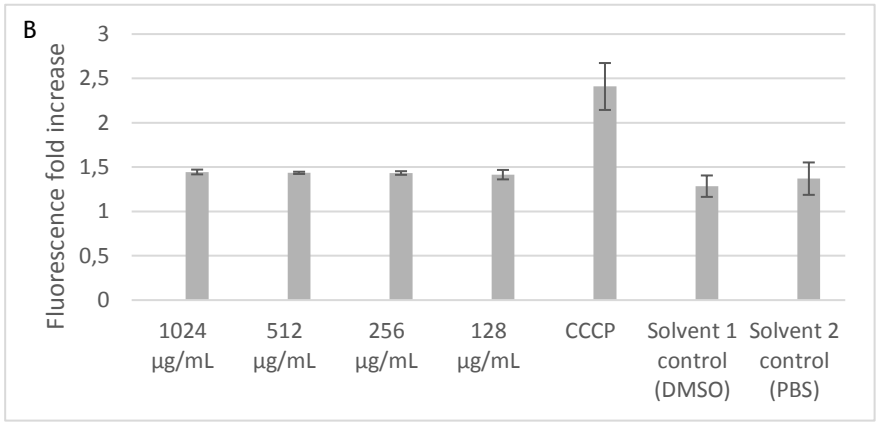
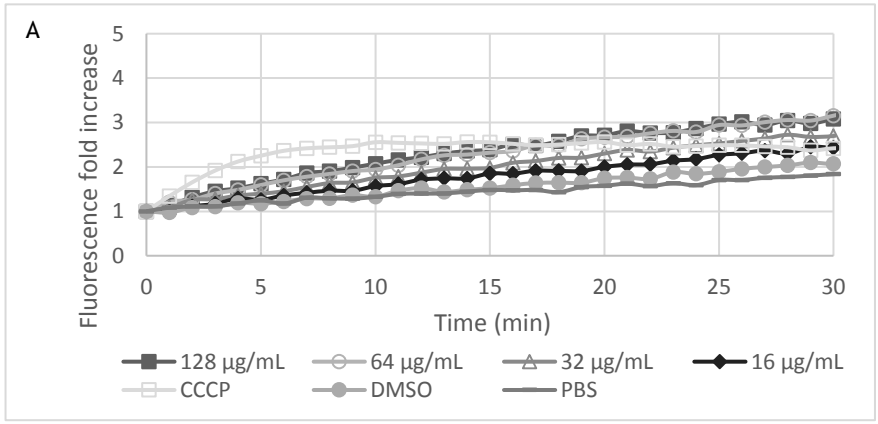


Figure 19. Ethidium bromide accumulation assay for DQ46M1 strain in the presence of sub-inhibitory concentrations of pilocarpine over 30 minutes(A) and at 30 minutes (B).



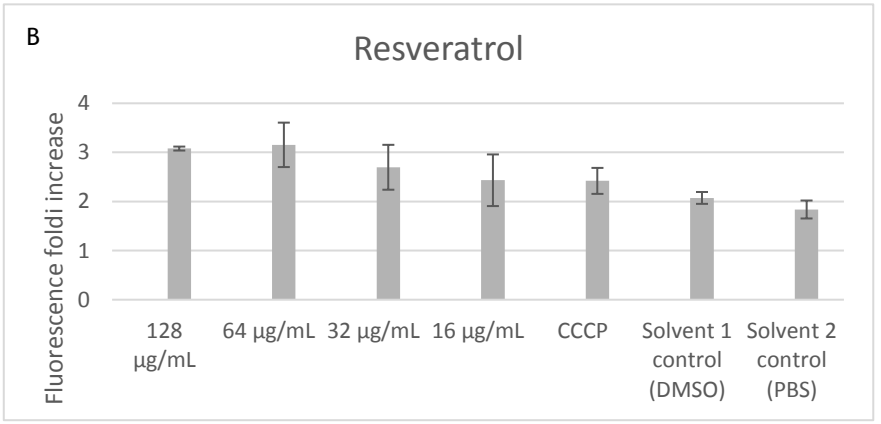


Figure 20. Ethidium bromide accumulation assay for DQ46M1 strain in the presence of sub-inhibitory concentrations of resveratrol over 30 minutes (A) and at 30 minutes (B).

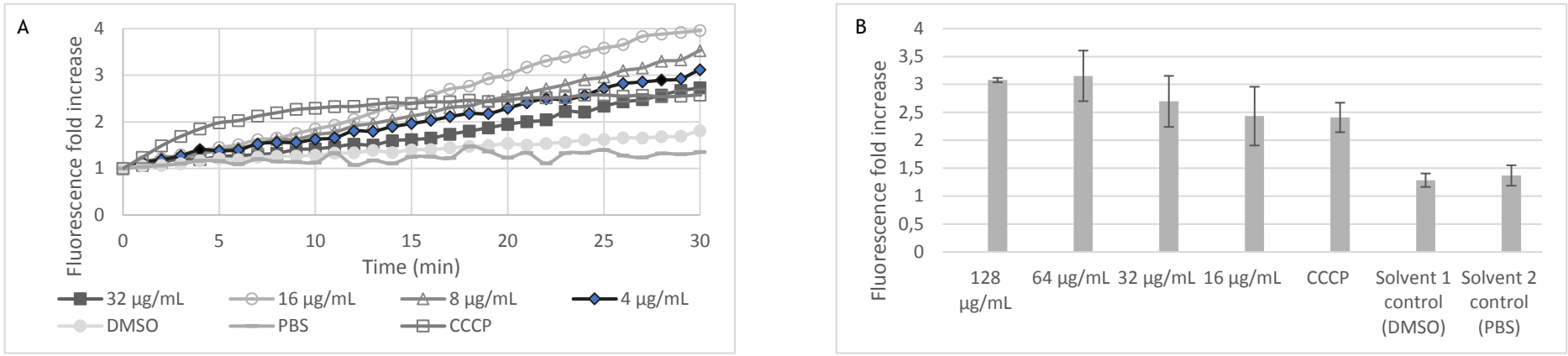


Figure 21. Ethidium bromide accumulation assay for DQ46M1 strain in the presence of sub-inhibitory concentrations of pterostilbene over 30 minutes (A) and at 30 minutes (B).

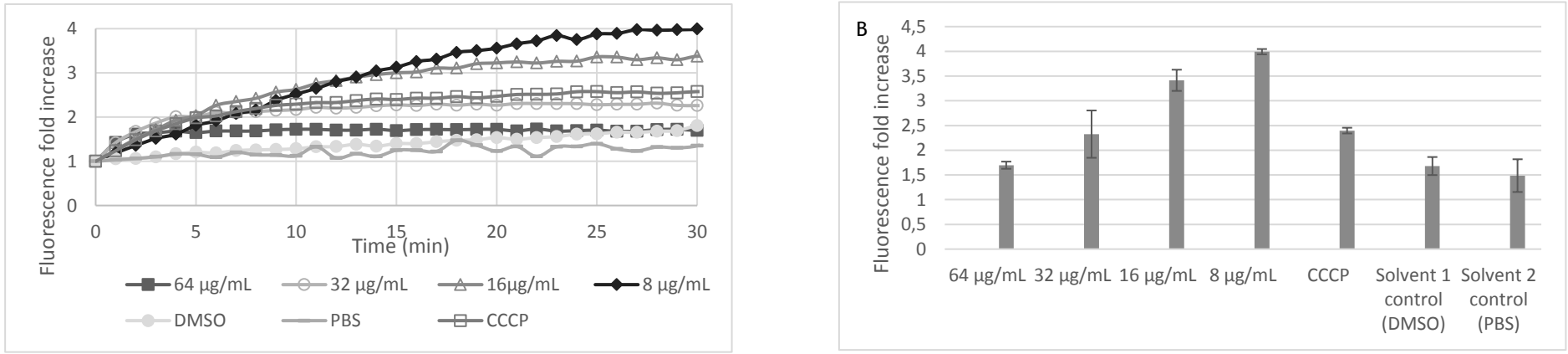


Figure 22. Ethidium bromide accumulation assay for DQ46M1 strain in the presence of sub-inhibitory concentrations of pinosylvin over 30 minutes(A) and at 30 minutes (B).

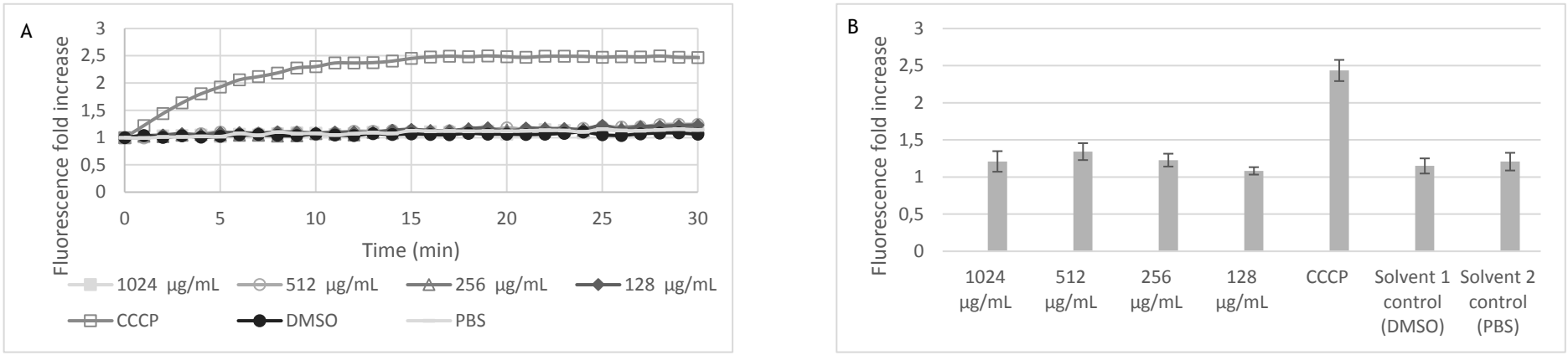


Figure 23. Ethidium bromide accumulation assay for CR50-2 strain in the presence of sub-inhibitory concentrations of (+)-catechin over 30 minutes(A) and at 30 minutes (B).

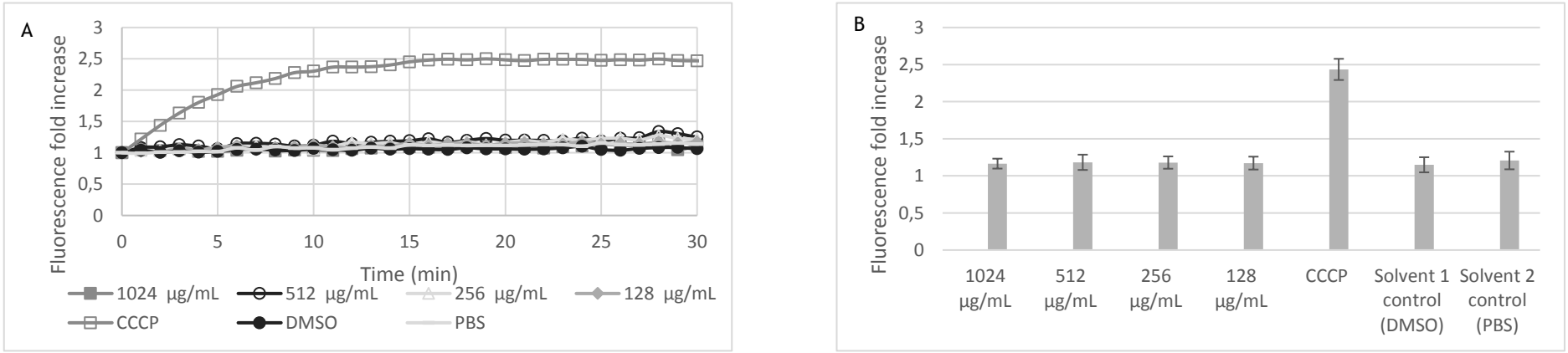


Figure 24. Ethidium bromide accumulation assay for CR50-2 strain in the presence of sub-inhibitory concentrations of (-)-epicatechin over 30 minutes (A) and at 30 minutes (B).

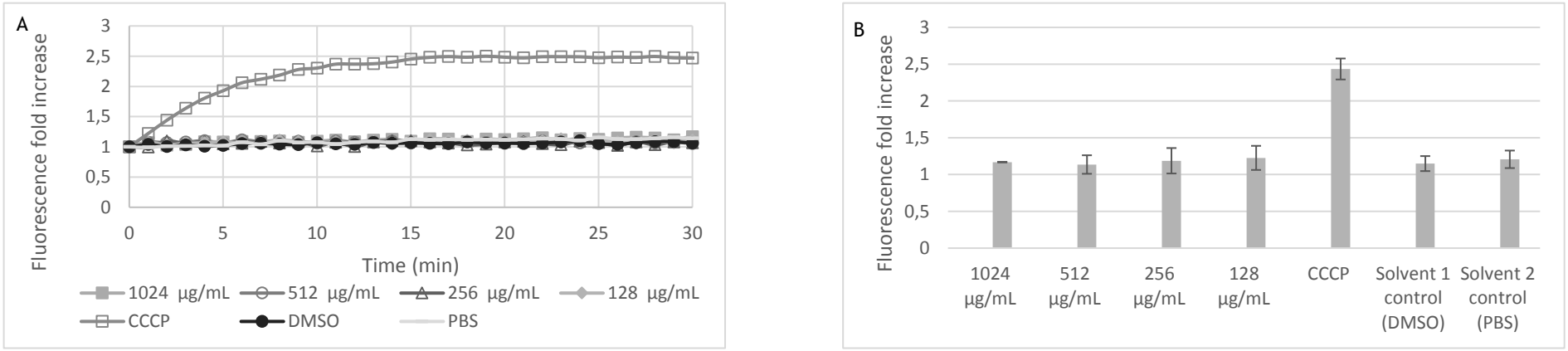


Figure 25. Ethidium bromide accumulation assay for CR50-2 strain in the presence of sub-inhibitory concentrations of gallic acid over 30 minutes(A) and at 30 minutes (B).

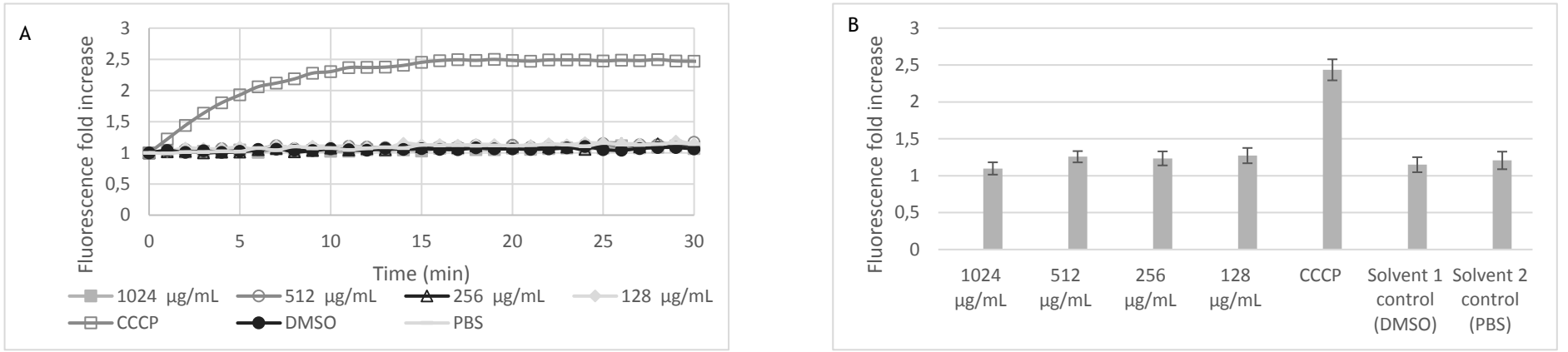


Figure 26. Ethidium bromide accumulation assay for CR50-2 strain in the presence of sub-inhibitory concentrations of rutin over 30 minutes (A) and at 30 minutes (B).

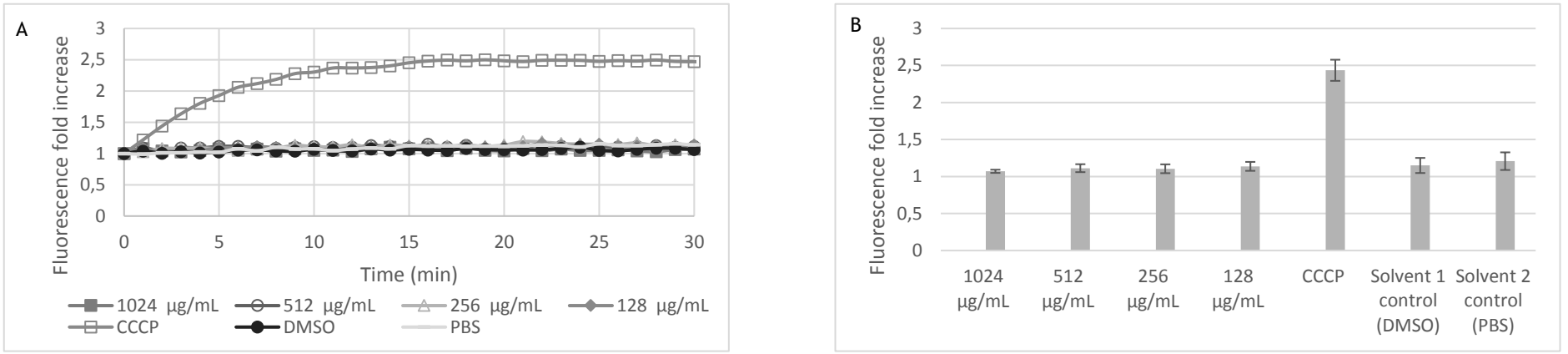


Figure 27. Ethidium bromide accumulation assay for CR50-2 strain in the presence of sub-inhibitory concentrations of vanillic acid over 30 minutes (A) and at 30 minutes (B).

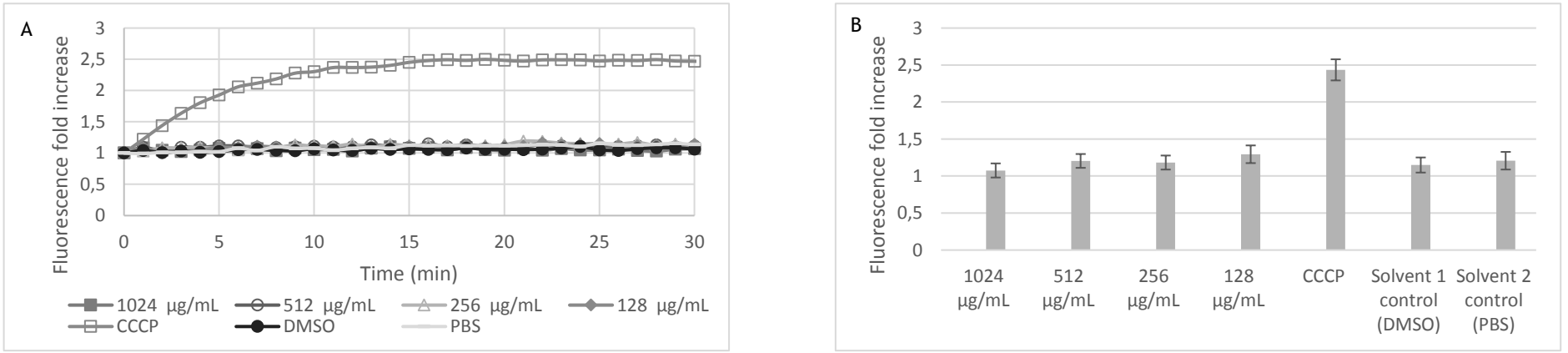


Figure 28. Ethidium bromide accumulation assay for CR50-2 strain in the presence of sub-inhibitory concentrations of caffeic acid over 30 minutes(A) and at 30 minutes (B).

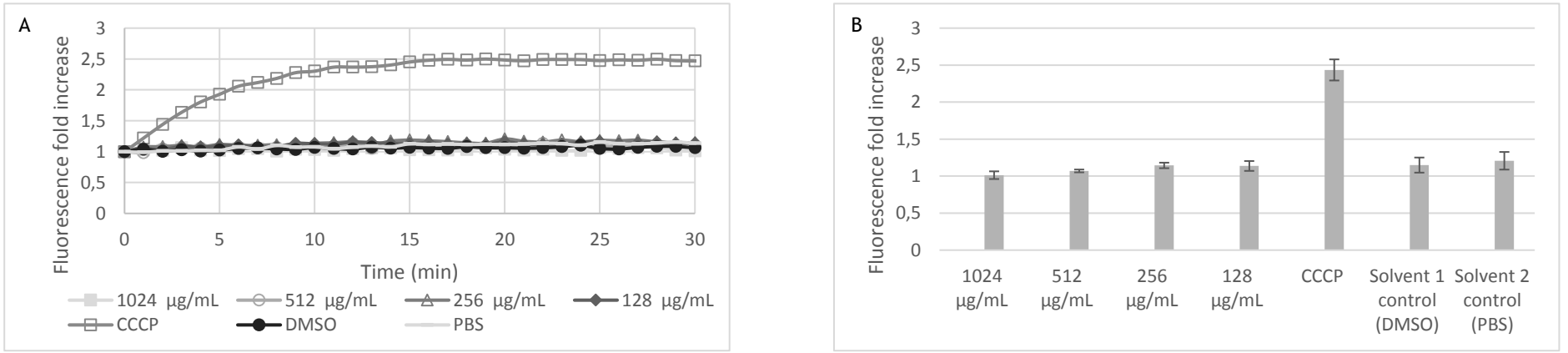


Figure 29. Ethidium bromide accumulation assay for CR50-2 strain in the presence of sub-inhibitory concentrations of ferulic acid over 30 minutes (A) and at 30 minutes (B)

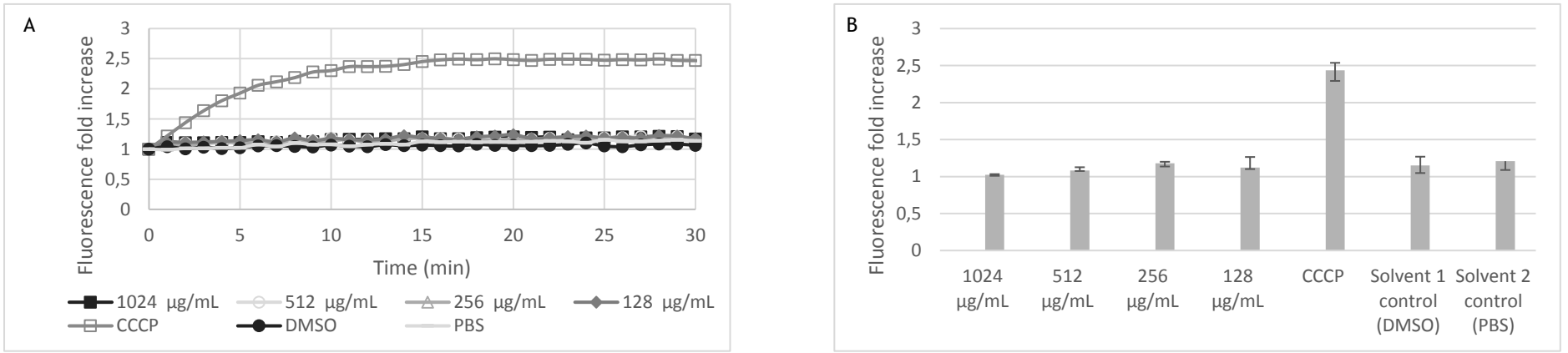


Figure 30. Ethidium bromide accumulation assay for CR50-2 strain in the presence of sub-inhibitory concentrations of syringic acid over 30 minutes (A) and at 30 minutes (B)

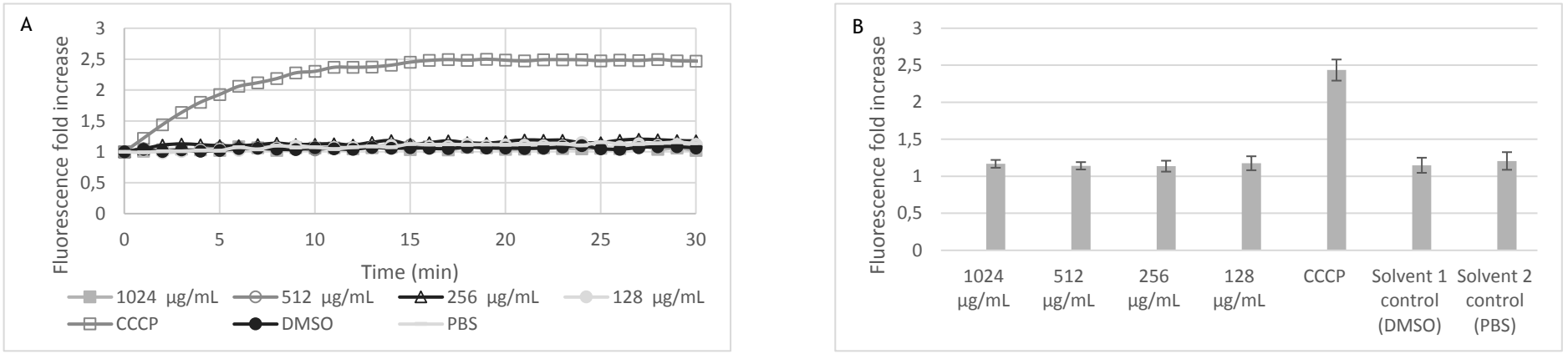


Figure 31. Ethidium bromide accumulation assay for CR50-2 strain in the presence of sub-inhibitory concentrations of *p*-Coumaric acid over 30 minutes (A) and at 30 minutes (B)

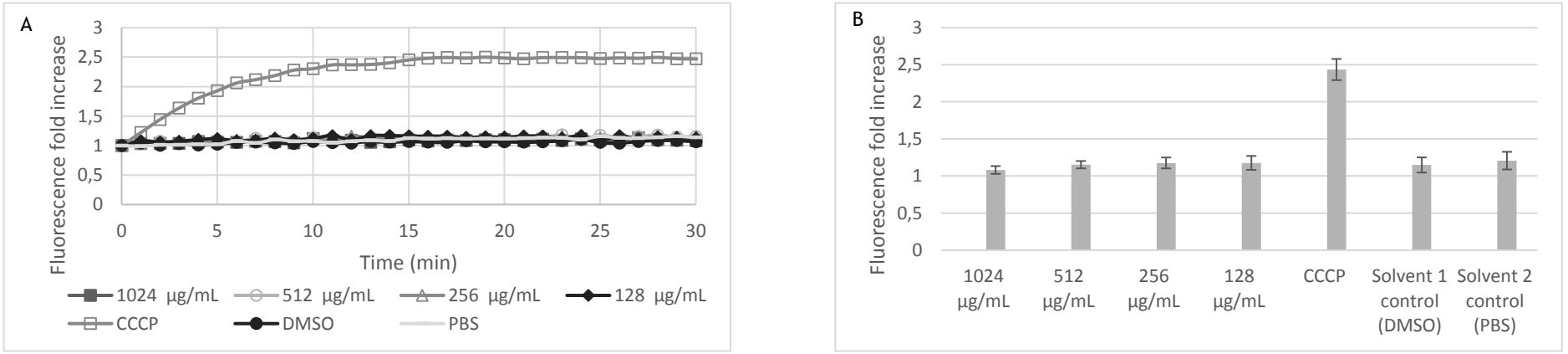


Figure 32. Ethidium bromide accumulation assay for CR50-2 strain in the presence of sub-inhibitory concentrations of chlorogenic acid over 30 minutes(A) and at 30 minutes (B).

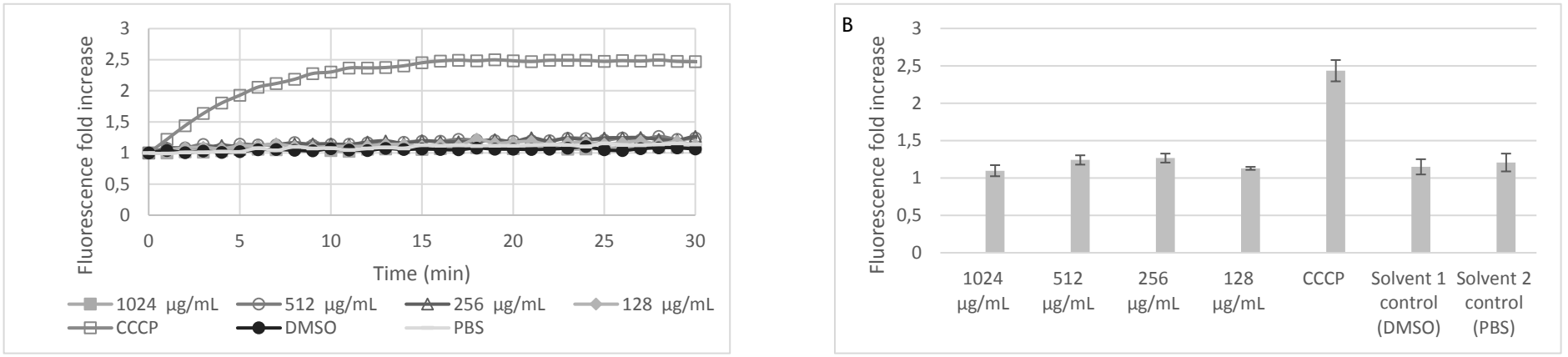


Figure 33. Ethidium bromide accumulation assay for CR50-2 strain in the presence of sub-inhibitory concentrations of pilocarpine over 30 minutes (A) and at 30 minutes (B).

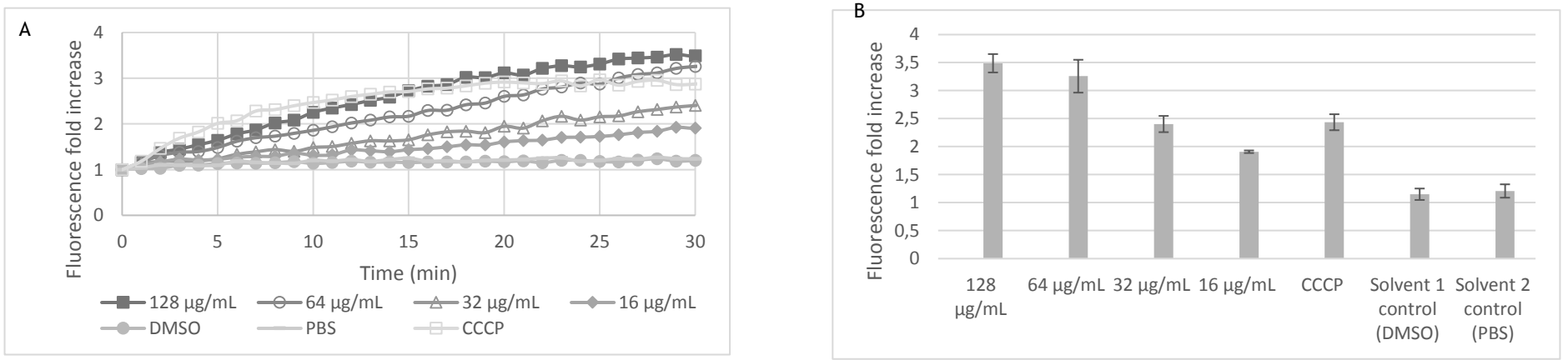


Figure 34. Ethidium bromide accumulation assay for CR50-2 strain in the presence of sub-inhibitory concentrations of resveratrol over 30 minutes (A) and at 30 minutes (B).

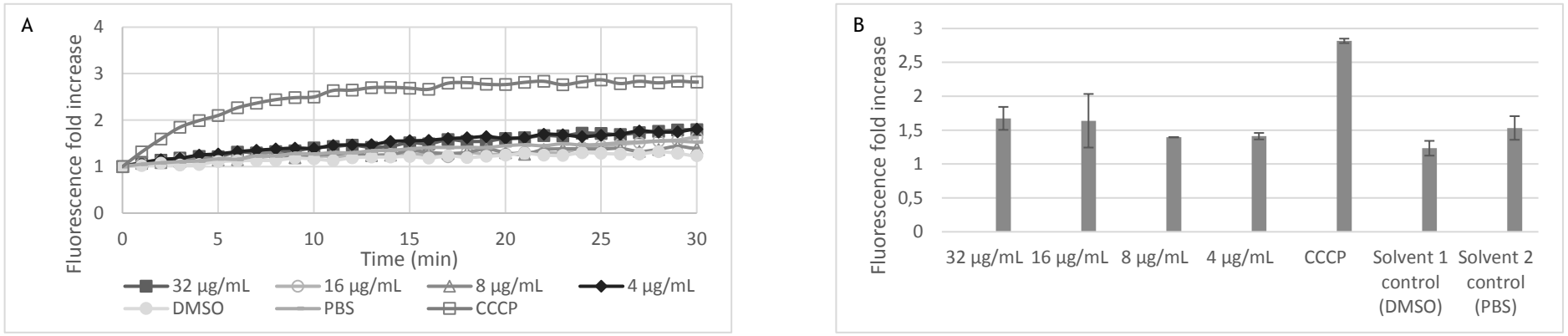


Figure 35. Ethidium bromide accumulation assay for CR50-2 strain in the presence of sub-inhibitory concentrations of pterostilbene over half an hour (A) and at 30 minutes (B).

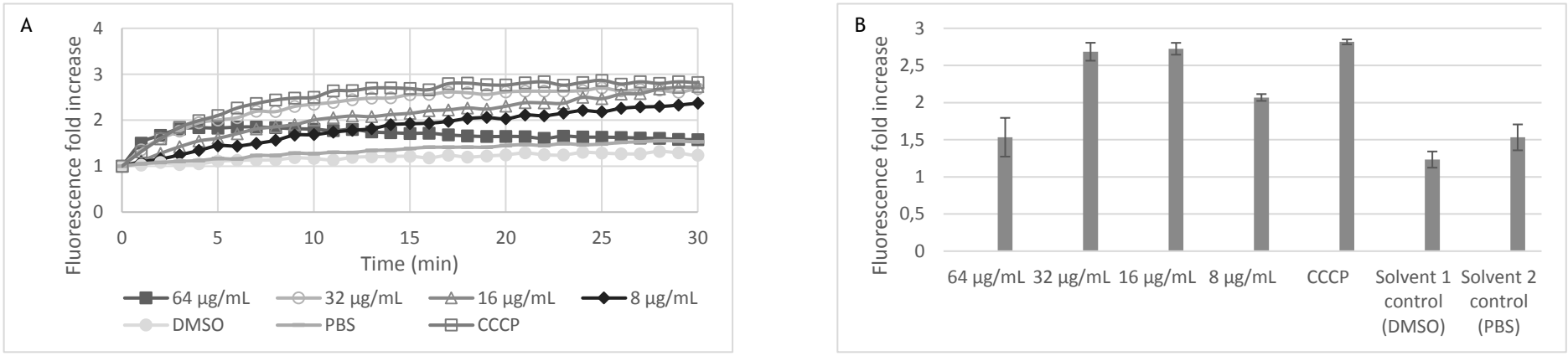


Figure 36. Ethidium bromide accumulation assay for CR50-2 strain in the presence of sub-inhibitory concentrations of pinosylvin over 30 minutes (A) and at 30 minutes (B).

Appendix 2

The work here presented has result in:

Oral communication XII annual CICS-UBI Symposium, Covilhã (2017): **Sousa V.C.**, Luís Â., Domingues F., Ferreira S., The role of phytochemicals in *Arcobacter butzleri* resistance to antibiotics.

6 THE ROLE OF PHYTOCHEMICALS IN ARCOBACTER BUTZLERI RESISTANCE TO ANTIBIOTICS

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CICS-UBI – Health Sciences Research Centre, University of Beira Interior, 6200-001 Covilhã, Portugal.

Arcobacter butzleri is an emergent pathogen found in a wide range of habitats and hosts, that has developed resistance to several antibiotics. Efflux pumps are an important mechanism of antimicrobial resistance, therefore, the use of efflux pump inhibitors (EPIs) can be potentially used to overcome the decreasing sensitivity of *A. butzleri* to antibiotics. Plants have been gaining attention as they face constant bacterial exposure and are a source of great diversity of compounds. Thus, we aimed to test several phytochemicals as putative EPIs, evaluating their role in the improvement of antibiotics' performance against *A. butzleri*. The minimum inhibitory concentration (MIC) of 12 phytochemicals and four antibiotics was determined. All phytochemicals had a MIC higher that 1024 µg/mL, except resveratrol which MIC ranged from 256-512 µg/mL, depending on the strain. Ethidium bromide accumulation assays showed that some of the tested phytochemicals presented a fluorescence folding increase higher than the control, indicating that they may inhibit the efflux pumps, however only resveratrol presented a typical EPI profile. MIC determination was performed for phytochemicals in the presence of EPIs showing that efflux pumps do not play a key role in bacterial resistance to phytochemicals, except for resveratrol. Despite the lack of activity as EPIs of most of the tested compounds, 7 phytochemicals (catechin, epicatechin, rutin, resveratrol and caffeic, chlorogenic and gallic acids) were still selected for checkerboard titration assays revealing no synergism with antibiotics. In conclusion, the tested phytochemicals presented a low potential to decrease the resistance of *A. butzleri* to antibiotics.

Keywords: *Arcobacter butzleri*, **Phytochemicals, Antibiotics Resistance, Efflux Pumps Inhibitors.**

Poster presentation at the II International Congress on Health Sciences Research towards innovation and entrepreneurship: Trends in Biotechnology for Biomedical Applications, Covilhã (2017): Sousa V.C., **Luís Â.**, Domingues F., Ferreira S., Phytochemicals as potential efflux pump inhibitors in *Arocbacter butzleri*

II International Congress in Health Sciences Research
Abstracts Book

P10. PHYTOCHEMICALS AS POTENTIAL EFFLUX PUMP INHIBITORS IN *ARCOBACTER BUTZLERI*

Sousa, VC. Luís, Â. Domingues, F. Ferreira, S.

CICS-UBI – Health Sciences Research Centre, University of Beira Interior, Covilhã, Portugal

Arcobacter spp. has been isolated from a wide range of habitats and hosts such as water, food (vegetal and animal origin), and food processing facilities. Some members of this genus are considered emerging pathogens and possible zoonotic agents with *A. butzleri* being classified as a serious hazard to human health by the International Commission on Microbiological Specifications for Food. The inappropriate use of antibiotics has led to a spread of resistant pathogenic bacteria, being *A. butzleri* resistant to several antibiotics. Efflux pumps are one of the most relevant mechanisms of antimicrobial resistance, therefore, efflux pump inhibitors (EPIs) represent a possible way to overcome the decreasing sensitivity of *A. butzleri* to antibiotics. As plants face constant bacterial exposure and are a source of great diversity of compounds, phytochemicals appear as promising potential EPIs.

The aim of this study was to test the potential of several phytochemicals as putative EPIs and ascertain if its co-administration with antibiotics could enhance their performance. The minimum inhibitory concentration of 12 phytochemicals and 4 antibiotics was determined and the resistance profile of the strains was traced. Ethidium bromide accumulation assays were performed to evaluate the potential inhibition of efflux pumps by the phytochemicals. The evaluated phytochemicals showed no or low antibacterial activity, one of the stipulated criteria to qualify a compound as an EPI. Several of the tested phytochemicals showed an increase of the level of accumulation of ethidium bromide, as an efflux pump substrate, and so the compounds presenting a fluorescence folding increase higher than that of the efflux pump inhibitor carbonyl cyanide 3-chlorophenylhydrazone were selected for checkerboard titration. Despite the observed ethidium bromide accumulation, no synergism between phytochemicals and antibiotic usually effluxed was found.