University of São Paulo
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Unravelling the potential of citrus essential oils derived from citrus processing as an alternative antimicrobial feed additive in pigs

Carmen Milagros Sinche Ambrosio

Thesis presented to obtain the degree of Doctor in Science. Area: Food Science and Technology

Piracicaba
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Food Engineering

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Profª. Dra. **CARMEN JOSEFINA CONTRERAS CASTILLO**

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For my beloved family
and friends

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Carmen M.S. Ambrosio
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CONTENTS

RESUMO GERAL

Desvendando o potencial dos óleos essenciais cítricos derivados do processamento de citros como um aditivo alternativo antimicrobiano na alimentação animal

Os citros são os frutos mais cultivados no mundo e os óleos essenciais (OE) são o principal subproduto das indústrias de processamento de sucos de cítricos. Recentemente, os OEs surgiram como uma alternativa promissora ao uso de antibióticos na produção suína devido as suas propriedades antimicrobianas. Isso como resultado da proibição total de uso de antibióticos como aditivo melhorador de desempenho pela União Europeia desde 2016 visando combater o aumento da resistência a antibióticos em animais e humanos. Devido à grande disponibilidade dos OEs de citros no mercado global sua aplicação na alimentação de suínos poderia se tornar interessante e financeiramente viável. Assim, o objetivo desta tese foi desvendar a atividade antibacteriana seletiva dos OEs cítricos comerciais, bem como sua composição química relacionada a essa atividade, visando a aplicação potencial dos OEs de citros como alternativa aos antibióticos na alimentação de suínos. Esta tese é dividida em cinco estudos, dos quais os três primeiros investigaram a atividade antibacteriana seletiva *in vitro* de seis OEs de citros comerciais, o possível mecanismo de ação subjacente à atividade seletiva dos OEs cítrico e a composição química, visando desvendar os compostos dos OE de citros envolvidos no desempenho antibacteriano seletivo. O estudo seguinte, foi relacionado à microencapsulação de um OE de citros usando uma matriz de amido modificado e quitosana utilizando a técnica de *spray-drying*. Foram avaliadas as propriedades físicas, morfológicas e atividade antimicrobiana das microcápsulas do OE de citros. No último estudo, avaliou-se o efeito de um OE de citros microencapsulado na microbiota intestinal de suínos usando um sistema de fermentação *in vitro*. O resultado da presente tese mostrou o potencial antibacteriano seletivo dos OEs de citros, ou seja, um efeito antibacteriano mais forte sobre bactérias patogênicas do que sobre bactérias benéficas, causando maiores alterações na cinética normal de crescimento de bactérias patogênicas do que nas bactérias benéficas. O provável mecanismo relacionado à ação antibacteriana seletiva de um OE de citros pode ser descrito como uma alteração mais pronunciada da permeabilidade e da integridade da membrana citoplasmática, assim como na estrutura externa da bactéria patogênica do que da bactéria benéfica. A caracterização da composição química dos OE de citros permitiu inferir que os compostos menores presentes nesses OEs estariam envolvidos em conferir a atividade antibacteriana seletiva, principalmente os monoterpenos oxigenados, como carvona, *cis*-carveol, *trans*-carveol, *cis*-p -Mentha-2,8-dien-1-ol e *trans*-p-Mentha-2,8-dien-1-ol. Além disso, os resultados do experimento de fermentação *in vitro* mostraram que um OE de citros microencapsulado alterou a microbiota intestinal de suínos, causando a redução da diversidade microbiana do íleo e do colón, e mudando sua composição bacteriana. Portanto, esta tese fornece novas informações sobre o efeito antimicrobiano dos OEs de citros, como uma alternativa potencial aos antibióticos na produção de suínos.

Palavras-chave: Óleos essenciais de citros; Microbiota intestinal de suínos; Melhorador de desempenho; Microencapsulação

GENERAL ABSTRACT

Unravelling the potential of citrus essential oils derived from citrus processing as an alternative antimicrobial feed additive in pigs

Citrus is the most grow fruit around the world and essential oils (EOs) are the main by-product of Citrus-juice processing industries. Lately, EOs have emerged as a promising alternative to antibiotic use in pig production due to their antimicrobial properties. This as a result of the full restriction of antibiotics' use as feed additive by the European Union since 2016 to counter the increase of antibiotic resistance in animals and humans. Due to the vast availability of citrus EOs in the global market, from a practical standpoint, their application in pig feed could turn interesting and financially feasible. Thus, the aim of this thesis was to unravel the selective antibacterial activity of commercial citrus EOs, as well as their chemical composition related to this activity, targeting citrus EOs' potential application as an alternative to antibiotics in pig feed. This thesis is divided into five studies, of which the three first studies investigated the in vitro selective antibacterial activity of six commercial citrus EOs, the possible mechanism of action underlying citrus EOs' selective activity and the chemical composition to unravel citrus EOs' compounds involved in conferring that selective antibacterial performance. The next study was related to the microencapsulation of a citrus EO using a modified starch-chitosan matrix technique by spray-drying. Physical properties, morphology and antimicrobial activity of the citrus EO microcapsules were evaluated. In the last study, the effect of a microencapsulated citrus EO on pig gut microbiota using a stage of art in vitro fermentation was studied. The result of the present thesis showed the potential of selective antibacterial activity of citrus EOs, that is, a stronger antibacterial effect on pathogenic bacteria than beneficial bacteria by causing higher disturbances of the normal growth kinetics of pathogenic bacteria than beneficial bacteria. The probable mechanism related to the selective antibacterial action of a citrus EO can be described as altering more remarkably the permeability and integrity of the cytoplasmic membrane as well as the external structure of a pathogenic bacterium than a beneficial bacterium. The characterization of the chemical composition of citrus EOs allowed to infer that minor compounds present in these EOs would be involved in conferring their selective antibacterial activity, these would be mainly oxygenated monoterpenes such as carvone, *cis*-carveol, *trans*-carveol, *cis*-p-Mentha-2,8-dien-1-ol and *trans*-p-Mentha-2,8-dien-1-ol. In addition, on the basis of an *in vitro* fermentation set-up, results showed that a microencapsulated citrus EO shifted pig gut microbiota in a way that reduced ileal and colonic diversity and altered their composition. Therefore, this thesis provides new information about the antimicrobial effect of citrus EOs as a potential alternative to antibiotics in pig production.

Keywords: Citrus essential oils; Pig gut microbiota; Growth promoter: Microencapsulation

1. INTRODUCTION AND THESIS OUTLINE

Since 1950, antibiotics at sub-therapeutic levels have been widely used in the feed of food-producing animals as growth promoter agents and for disease prevention (Guardabassi and Kruse, 2009). Thus, antibiotics became an essential component to improve animal health and keep animal well-being. The mechanism whereby antibiotics showed growth promoting effects, despite it has not been fully elucidated yet, was explained as having direct effect on the animal gut microbiota by decreasing competition for nutrients, reducing microbial metabolites production that depress growth and fighting pathogens associated with subclinical diseases (Dibner and Richards, 2005; Zeineldin et al., 2019). These effects have been translated in an increase of average daily gain and consequently an improvement of feed conversion ratio in animals (Cromwell, 2002; Teillant et al., 2015). Therefore, antibiotics use positively contributing to the productivity of animal production, such as pig production (Teillant et al., 2015).

In intensive pig production, pigs from early life, that is, at around 3-4 weeks after their birth, have received antibiotics in their feed to control the negative impact of weaning (Cromwell, 2002). Weaning is a critical period of pig's life due to the sudden dietary, social, and environmental changes that piglets face (Gresse et al., 2017), since they are removed from the sow, adapted to new environments, mixed with other piglets and changed from a sow's milk diet to a solid based-grain diet (Rhouma et al., 2017). Consequently, these changes leave an unbalanced microbiota and intestinal inflammation, turning piglets highly susceptible to gastrointestinal infections by enteric pathogens, such as enterotoxigenic *Escherichia coli*, which triggers a diarrhea status known as post-weaning diarrhea (PWD) (Fairbrother et al., 2005; Rhouma et al., 2017). The PDW is an important disease that affect pig production worldwide, since it increase the piglet mortality and morbidity, decrease considerably the animal growth rate and increase the necessity of medication, thus leaving economic losses for this sector (Gresse et al., 2017; Rhouma et al., 2017). Several antibiotics have been administrated in the feed for the control of PWD and to promote pig growth, such as beta-lactams (amoxicillin), tetracyclines (Oxytetracycline, Chlortetracycline), Sulphonamides + Diaminopyrimidines (trimethoprim + sulfamethoxazole), Aminoglycosides (neomycin) , polymyxins (colistin) and Halquinol. (Burch et al., 2008; Hall et al., 2017; Kempf et al., 2013)

Nonetheless, the long-term use of antibiotics as growth promoters in pig production and other livestock animals has been associated with selection of antibiotic-resistant bacteria and the increase of antimicrobial resistance genes in the gut of these animals (Zeineldin et al., 2019). This has turned a significant public health concern due to the possibility of spreading from animal-to-human antibiotic resistance (Marshall and Levy, 2011). Studies

have indicated that propagation can occur by direct contact with farm animals, or indirectly, through the food chain by consumption of food animal products or through environmental pathways such as contaminated water and animal waste application to farm field (da Costa et al., 2013; Marshall and Levy, 2011; Tang et al., 2017). Currently, the emergence of plasmid-mediated colistin resistance (*mcr*-1 gene) in gram-negative bacteria, such as *E. coli*, from food-producing animals and humans (Liu et al., 2016) has turned on the alarms globally, since colistin is considered the last source of antibiotic therapy to treat infection caused by multidrug-resistant gram-negative bacteria in humans (Walkty et al., 2009). Thus, concern regarding the potential loss of colistin effectiveness in human medicine arose. Evaluation of the impact of antibiotic resistance has estimated that the global burden of deaths attributable to this threat reached 700,000 lives in 2016 (low estimate) and this figure would reach 10 million deaths by 2050, if unless action is taken over the world. (O' Neill, 2016).

In view of this threat, the banning of antibiotics administration to food-producing animals as growth promoters has been established in European Union countries since 2006 by means of the Regulation (EC) No. 1831/2003 in order to counter the antibotics resistance increase. Recently, some other countries have started to regulate the use of some antibotics, mainly colistin, for this purpose, such as Brazil (MAPA, 2016), China (Walsh and Wu, 2016), Japan (Food Safety Commission of Japan, 2017), South Korea, New Zealand (Johnson, 2010; Liu and Liu, 2018) and United State (Brüssow, 2017), with more countries expected to follow. Consequently, the withdrawal of antibiotics has challenged the productivity of the pig production sector and other livestock production. Thus, to keep productivity and to fulfill the market pressure for more natural and safe meat, the search for alternatives to replace antibiotics became a real necessity.

In this scenery, phytogenic as essential oils (EOs) have gained in the last two decades more attention as a natural alternative to antibiotics. EOs are aromatic-volatile oily liquids extracted from plant material such as flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots (Burt, 2004). They comprise very complex mixtures containing more than 60 different compounds at quite different concentrations (Bakkali et al., 2008; Burt, 2004). These compounds confer to EOs their several biological properties, whereby, EOs have turned a promising alternative to antibiotics. Reports have shown that EOs, specifically, for their antimicrobial, antioxidant, and anti-inflammatory properties can exert beneficial effects on the pig gut ecosystem, such as stimulation of digestive fluids secretion and nutrient absorption, improve intestinal morphology, reduce pathogenic stress, modulate intestinal microbiota, exert antioxidant effects and reduce inflammation. As consequence of these effects a growth promotion in pigs is observed (Omonijo et al., 2018; Stevanović et al., 2018; Zeng et al., 2015). The modulation of the intestinal microbiota by the antimicrobial

effect of EOs has been the main reason to add EOs in pig feed (Omonijo et al., 2018). It has been remarked that beneficial modulation by EOs would comprise the suppression of pathogenic bacteria to reduce their effects whereas not compromising beneficial bacteria (Ouwehand et al., 2010; Si et al., 2006). Interestingly, a group of EOs from *Citrus* plant were found for presenting that selective antibacterial spectrum after an *in vitro* screening of 28 several EOs extracted from different plants (Ambrosio et al., 2017). This result was part of the findings of my Master study. Thus, this thesis was based on the interest to study more deeply the selective antibacterial activity showed by citrus EOs, thinking it as a potential alternative to antibiotics.

It is also important to highlight that the interest in study citrus EOs in this thesis was even more supported by the following reason: Citrus EOs are a common by-product of citrus-juice processing industries and they are the most produced and commercialized type of EOs over the world (Iwabuchi et al., 2010). Thus, there is a considerable availability of these oils in the global market. The annual production of citrus EOs reaches > 60,000 tons per year and they have a comparatively low price ($9-10/Kg) regarding other EOs. (Lange, 2015). Among citrus EOs. orange EO is the most produced one, being Brazil the major producer and exporter country of this oil (Barbieri and Borsotto, 2018; Iwabuchi et al., 2010). To 2018, the worldwide orange EO production reached 54.9 mil tons (United Nations, 2019), of which Brazilian production was accountable for 26.98 mil tons (Citrus Br, 2019).

In addition another reason why citrus EOs have attracted attention for this thesis is their safety to be used as an additive in food applications as they are generally recognized as safe (GRAS) by the United State-Food and Drug Administration (Tisserand and Young, 2014). Therefore, considering the mentioned aspects, citrus EOs could be an economic, eco-friendly and natural alternative to synthetic antibiotics used as growth promoters.

Thesis outline

As introduced above (**Chapter 1**), citrus EOs, by-products of an orange-juice industry, were characterized by their antimicrobial activity related to their chemical composition, targeting their potential application as an alternative to antibiotics in pig production. For that, the thesis was organized into three parts as shown in Figure 1.1. In the first part, the selective antibacterial activity of six commercial citrus EOs and their chemical composition (**Chapter 2**), as well as the mechanism of action underlying this selective antibacterial activity (**Chapter 3**) were studied. In addition, fractionalization of a citrus EOs (**Chapter 4**) was performed to unravel EO compounds involved with the selective antibacterial activity and antioxidant activity. The second part was related to the use of the spray-drying technique for microencapsulating a citrus EO (**Chapter 5**). Physical properties,

morphology and antimicrobial activity of the citrus EO microcapsules were evaluated. In the third part, the effect of the microencapsulated citrus EO (in Chapter 4) on pig gut microbiota using a stage of art in vitro fermentation was studied (**Chapter 6**).

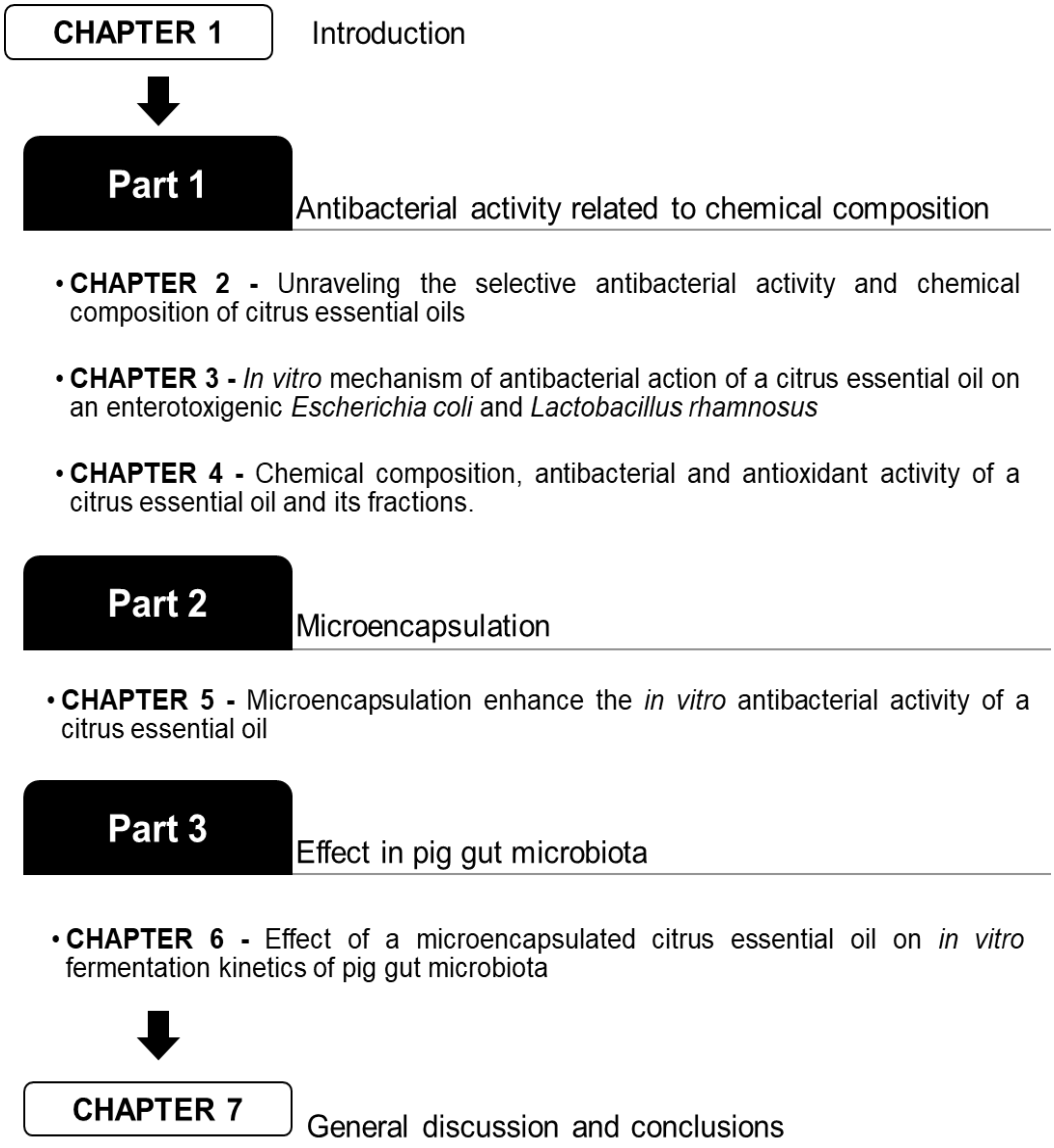

Figure 1.1. Flow-chart explaining thesis organization

The general aim of this thesis was to unravel the selective antibacterial activity of commercial citrus EOs, as well as their chemical composition related to this activity, targeting citrus EOs's potential application as an alternative to antibiotics in pig feed. For that, some specific objectives were intended:

- To evaluate the selective antibacterial activity of six commercial citrus EOs on enterotoxigenic *E. coli* strains as model of pathogenic bacteria and *Lactobacillus*

species as model of beneficial bacteria, as well as to determine the chemical composition of these citrus EOs.

- To investigate the *in vitro* antibacterial mechanism of action underlying the selective antibacterial activity of a citrus EO.
- To fractionate a citrus EO and to characterize the chemical composition, antibacterial and antioxidant activities of citrus EO and its separated fractions.
- To microencapsulate a citrus EO using a modified starch-chitosan matrix and to evaluate physical properties, morphology and antimicrobial activity of microcapsules.
- To evaluate the effects of a microencapsulated citrus EO on pig gut microbiota by means of an *in vitro* fermentation model.

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2. UNRAVELING THE SELECTIVE ANTIBACTERIAL ACTIVITY AND CHEMICAL COMPOSITION OF CITRUS ESSENTIAL OILS [1]

Abstract

Post-weaning diarrhea (PWD) is an often disease affecting piglets. It is caused mainly by enterotoxigenic *Escherichia coli* (ETEC) colonization in pig gut. Antibiotics has been used to prevent, combat and control PWD and its negative impact on the productivity of pig breeding sector. Nonetheless, antibiotics due to their wide antibacterial spectrum also can reach beneficial gut bacteria, such as *Lactobacillus*. Lately, essential oils (EOs) have emerged as a potential alternative to using antibiotics in animal breeding because of their effect on bacterial growth. Commonly, citrus EOs are by-products of food industry and the availability of these EOs in the worldwide market is huge. Thus, six commercials citrus EOs were evaluated on ETEC strains, as model of pathogenic bacteria, and on *Lactobacillus* species, as models of beneficial bacteria. In overall, citrus EOs exhibited a selective antibacterial activity with higher effect on pathogenic bacteria (ETECs) than beneficial bacteria (*Lactobacillus)*. Brazilian orange terpenes (BOT) oil presented the highest selective performance and caused higher disturbances on the normal growth kinetic of ETEC than on *Lactobacillus rhamnosus*. The action was dose-dependent on the maximal culture density (*A*) and the lag phase duration (λ) of the ETEC. The highest sub-inhibitory concentration (0.925 mg/mL) extended the λ duration to ETEC eight times (14.6 h) and reduced *A* in 55.9%. For *L. rhamnosus*, the λ duration was only extended 1.6 times. Despite the fact that limonene was detected as the major compound, the selective antibacterial activity of the citrus EOs could not be exclusively attributed to limonene since the presence of minor compounds could be implicated in conferring this feature.

Keywords: Citrus essential oils; ETEC; *Lactobacillus*; Pig breeding; Animal feed; Limonene

2.1. Introduction

The incidence of post-weaning diarrhea (PWD) is a serious problem in the worldwide pig industry, causing severe economic losses due to increased pig morbidity and mortality, decreased animal growth rate and increased need for medication to treat animals (Fairbrother et al., 2005; Gresse et al., 2017). PWD is caused mainly by enterotoxigenic *Escherichia coli* (ETEC) that is highly present in the gastrointestinal tract of affected pigs. This pathotype is characterized by production of adhesins, which intermediate bacterial adherence to the intestine (Fairbrother et al., 2005; Rhouma et al., 2017), and toxins that lead to hypersecretion of water and electrolytes (Amezcua et al., 2002; Nataro and Kaper, 1998). The presence of ETEC in the environment is an important transmission factor since

[1] Chapter adapted from:
Ambrosio, Carmen M.S., Ikeda, N.Y., Miano, A.C., Saldaña, E., Moreno, A.M., Stashenko, E., Contreras-Castillo, C.J., Da Gloria, E.M., 2019. Unraveling the selective antibacterial activity and chemical composition of citrus essential oils. Sci. Rep. 9, 17719.
doi:10.1038/s41598-019-54084-3

they can survive protected in the manure for about 6 months. Furthermore, *E. coli* multiply rapidly and can reach up to 10^9 CFU per gram of feces, with the infection produced dependent on the degree of bacterial colonization (Dubreuil et al., 2016).

To control PWD outbreaks caused by enterobacteria as ETECs, antibiotics have frequently been included in the diet of weaned piglets as treatment, preventive measure, or growth promoter. However, continuous antibiotic use has been suggested as one cause of the emergence and worldwide dissemination of resistant bacteria. For example, currently, there is great concern about the emergence of plasmid-mediated colistin resistance (*mcr*-1) in *E. coli*, a resistance mechanism to an important antibiotic class in human health, polymyxins, which was largely used in pig production as growth promoter (Liu and Liu, 2018; Liu et al., 2016; Rhouma et al., 2016), but banned its use for this purpose by the European Union legislation (EU, 2003) since 2006, and recently, by China (Walsh and Wu, 2016), Japan (Food Safety Commission of Japan, 2017)and Brazil legislations (MAPA, 2016). However, therapeutic use of colistin is still allowed following the current recommendation of the European Medicine Agency, which restrict the rational use of colistin to treat clinical cases in livestock animals (European Medicines Agency, 2016). Moreover, the wide spectrum activity of some antibiotics can affect gut microbiota since they can kill or inhibit both pathogenic and beneficial bacteria. Thus, longtime antibiotic use can provoke decreased microbiota diversity and increased chances of pathogens colonizing the gut (Gresse et al., 2017). On the other hand, it is well known that *Lactobacillus* is the major group of beneficial bacteria presented in pig gut microbiota, which has been identified as an important group of bacteria able to prevent gut diseases (Dowarah et al., 2017). Possible *Lactobacillus* mechanisms to fight post-weaning infections in piglets have been described as (i) the direct inhibition of pathogen growth and its virulence by secretion of antimicrobial metabolites as bacteriocins, (ii) the modulation of microbiota composition and its activity and, (iii) the stimulation of the host immune system and improvement of intestinal barrier integrity (Gresse et al., 2017). In this scenario, finding an antimicrobial feed additive with a selective antibacterial activity, high spectrum activity on pathogenic bacteria and a reduced or not effect on beneficial bacteria like *Lactobacillus* would be very desirable. In the last decade, phytogenic compounds like essential oils (EOs) have received more attention as potential alternatives to replace antimicrobial growth promoters (AGP) in animal production due to their known biological properties: antimicrobial, antioxidant and anti-inflammatory (Diaz-Sanchez et al., 2015; Zeng et al., 2015). A few studies have reported that some EOs can suppress pathogenic bacteria while stimulating beneficial microorganisms such as *Lactobacillus* in the pig gut (Si et al., 2006; Simitzis, 2017). Specifically, the citrus EOs, which are by-products of orange juice production (Fisher and Phillips, 2008), could be an excellent alternative for that purpose since they have shown good potential to fight pathogenic bacteria

such as *Listeria* spp. (Friedly et al., 2009), *Salmonella* spp. (O'Bryan et al., 2008), *E. coli*, *Staphylococcus aureus* and *Bacillus cereus* (Chanthaphon et al., 2008). Furthermore, the use of citrus EOs in animal feed could become feasible since there is a huge availability of these oils in the worldwide market. Therefore, the aim of this study was to evaluate the selective antibacterial activity of six commercial citrus EOs on ETEC strains isolated from pig gut and on two *Lactobacillus* species belonging to ATCC, as well as to determine the chemical composition of these citrus EOs.

2.2. Material and methods

2.2.1. Essential oils supply

Six citrus commercial EOs were used in the study. These were by-products from orange juice production and were supplied by a factory from São Paulo State, Brazil. The oils were named by the factory as follows: Orange oil phase essence (OOPE), Orange peel oil (OPO), Brazilian orange terpenes (BOT), Tahiti lime oil phase (TLOP), Orange peel oil five fold (OPOFF) and Citrus terpenes (CT). Once the samples were received, they were kept in amber bottles under refrigeration (4°C) until use.

2.2.2. Bacterial strains

The evaluated bacterial strains in this research were four ETECs strains and two *Lactobacillus* species. The ETECs strains were isolated from pig gut and provided by The Swine Heath Laboratory of the Department of Preventive Veterinary Medicine and Animal Health from FMVZ-São Paulo University: *E. coli* U7 (K88+/ LT+/STb+), *E. coli* U21 (K88+/LT+/STb+/F18+/Sta+), *E. coli* U23 (LT+/STb+/F18+) and *E. coli* U25 (LT+/STb+/F18+/Sta+). The two *Lactobacillus* species were standard cultures from the American Type Culture Collection (ATCC), *L. plantarum* ATCC 8014 and *L. rhamnosus* ATCC 7469. ETECs strains were cultivated in Tryptic Soy Agar-Difco (TSA, Difco^{TM}) at 37ºC for 18-20 h and *Lactobacillus* species in MRS (Man, Rogosa and Sharpe agar, Difco^{TM}) agar at 30°C for 48 h. After activation, the bacteria were sub-cultured in Brain-Heart Infusion broth or MRS both (Difco^{TM}) supplemented with 15% of glycerol. After incubation, they were stored at -20ºC until their use.

2.2.3. Antibacterial activity

Screening by disc diffusion

All EOs were initially screened by disc diffusion method, following the standard protocol M02-A11 from the Clinical and Laboratory Standards Institute(CLSI, 2012a). EO solutions were prepared at 90% (v/v), using acetone as an emulsifier to improve dispersion. ETECs strains (*E. coli* U7, U21, U23 and U25) were grown on TSA agar and *Lactobacillus* species on MRS agar. Isolated colonies of each bacterium were transferred to tubes containing sterile saline solution (0.85%) until reaching an optical density within 0.08 to 0.1 abs, at 625 nm, which corresponds to 0.5 McFarland standard, therefore containing ~1-2 x 10^8 CFU/mL(CLSI, 2012a). After this, Mueller Hinton (MH) agar plates (*E. coli*) and MRS agar plates (*Lactobacillus* spp.) were inoculated and the bacterial inoculum spread. Seven microliters of each EO solution (90% v/v) were placed on 6-mm diameter sterile paper discs (Whatman Nº 3), which was transferred to the inoculated agar plates. Three discs with the same EO solution were placed in each plate; one disc of colistin (15 µg/disc) was used as a positive control, and one disc of acetone (10 µL/disc) was used as a negative control since its non-antimicrobial activity was proved. Then, the agar plates were incubated at 37ºC for 24 h (*E. coli*) and at 30°C for 48 h (*Lactobacillus* spp.). Inhibition zone diameters (IZD) were measured after incubation with the aid of a caliper rule. The experiment was carried out in three independent replicates.

The EO presenting the highest IZDs for the four ETECs strains and the lowest IZDs for the two *Lactobacillus* species was considered as the best selective EO between pathogenic and beneficial bacteria and was thus selected for further investigation. Referring to the bacterial strains, the most resistant ETEC strain and the most sensitive *Lactobacillus* specie to the activity of EOs were selected to continue with the study.

Determination of Minimal Inhibitory Concentration (MIC)

The determination of the MIC of the selected EO was performed by microdilution assay in a 96-well microplate following the standard protocol M07-A9 from the Clinical and Laboratory Standards Institute, with some modifications(CLSI, 2012b). For the assay, the standard inoculum was prepared in sterile saline (0.85% w/v) from living colonies of the selected bacteria above contained in plates of TSA agar (*E. coli*) or MRS agar (*Lactobacillus* spp.) at the optical density equivalent to 0.5 McFarland Standard (0.08-0.13 at 625 nm) as described previously. Subsequently, this inoculum was diluted at 1:100 to obtain an inoculum of 10^6CFU/mL (final inoculum). The EO stock solution was prepared at 29.6 mg/mL (3.29%)

with MH or MRS broth using Tween 80 as emulsifier. From the stock solution, two-fold serial dilutions were made in a range from 14.80 to 0.116 mg/mL along the Y-axis of the microplate. Twenty microliters from the final inoculum were added to each well containing 180 µL of several EO concentrations, being the final volume in each well of 200 µL and bacterial population of approximately 10^5 CFU/mL. The following controls were used: culture medium control (200 µL of MH or MRS broth); growth control (180 µL of MH or MRS broth + 20 µL of inoculum); Tween 80-emulsifier control (200 µL of MH or MRS broth with Tween 80) and growth control containing the emulsifier (180 µL of MH or MRS broth with Tween 80 + 20 µL of inoculum). Finally, microplates were incubated in a microplate reader (Vitor™ X3, PerkinElmer) at 37ºC for 24 h for *E. coli* and at 30°C for 36 h for *Lactobacillus* spp.

The MIC was established as the lowest EO concentration that inhibited visible bacterial growth. The existence or not of bacterial growth was evaluated by construction of survival curves and by resazurin test at the end of the incubation period. The lowest concentration that did not produce detectable absorbance values (at 600 nm) until the end of incubation was considered as the MIC obtained by survival curves. For resazurin test, 25 µL of resazurin (R7017; Sigma-Aldrich) solution at 0.0135% m/v were used per well. Thus, after visual inspection the presence of viable cells was evidenced through a change in the resazurin color from blue resazurin to pink resofurin (Pereira et al., 2014), after further incubation at 37°C (*E. coli*) or 30°C (*Lactobacillus spp.*) for 1h. Assays were carried out in triplicate in three independent replicates.

Bacterial growth modeling and calculation of kinetics parameters

Bacterial growth kinetics (or survival curves) for each tested EO concentration was built from absorbance readings at 600nm of the wells of the microplate configured as above, carried out every hour during the total incubation period, 24 h for *E. coli* and 36 h for *Lactobacillus* spp. Bacterial growth kinetics were modeled using the Gompertz model modified by Zwietering *et al*.(Zwietering et al., 1990) (Eq. (1)), since this model considers the three main biological parameters of bacterial growth. The data were fitted to the mathematical model with a confidence level of 95% using the Levenberg–Marquardt algorithm in Statistica 12.0 (StatSoft, Inc., Tulsa, OK, USA) software.

$$y = A \exp\left(-\exp\left(\frac{\mu_{max} \cdot e}{A}(\lambda - t) + 1\right)\right) \qquad \text{Eq. (1)}$$

Where: $\boldsymbol{y}$ represents the relative population size against time, the A, μ_{max} and λ are the three parameters that described three phases of the bacterial growth curve (Zwietering et

al., 1990). The asymptote *A* is the maximal bacterial culture density ($OD_{600\ nm}$), μ_{max} represents the maximum specific growth rate (h^{-1}) and it is the tangent of the log phase curve, λ is the lag phase duration (h) and is defined as the x-axis intercept of this tangent. *e* represents the number e= 2.7183.

Finally, the goodness of fit for the model was measured based on the mean square error (MSE) and on the corrected determination coefficient (corrected R^2) for each set of data.

Minimal Bactericidal Concentration (MBC)

The determination of MBC was performed from wells containing EO concentrations where there was no visible bacterial growth. So, an aliquot of 100 µL was taken from each well and seeded in MH or MRS agar. Plates were incubated for 24 h at 37ºC for *E. coli* and for 48 h for *Lactobacillus* spp. The MBC was defined as the lowest concentration of EO able to cause total bacterial death, represented by the visible absence of colonies on the agar plates.

2.2.4. Chemical composition of essential oils

The chemical composition characterization of the EOs was performed by gas chromatography coupled with mass spectrometry (GC/MS) using non-polar and polar columns.

The analysis on non-polar column was carried out using an Agilent Technology gas chromatograph 6890 Plus Series (Santa Clara, CA, USA) coupled to a selective Mass Spectrometry Detector 5973 and an Auto Sampler 7893. A fused-silica capillary column DB-5MS (J&W Scientific, Folsom, CA, USA) of 60 m × 0.25 mm id x 0.25 µm of film thickness coated with 5%-phenyl polydimethylsiloxane was used. The oven temperature was set as follows: initial oven temperature was held at 45°C for 5 min, then raised to 150ºC at 4ºC/min for 2 min, one more time raised to 250°C at 5°C/min, and finally to 300°C at 10°C/min, which was kept for 60 min. The injector temperature was 250°C, 2.0 µL of samples diluted in dichloromethane was injected in the “split” mode at a ratio of 30:1. EIMS, electron energy was 70 eV. The mass detector operated in full scan mode in the range of 40 to 350 m/z. The temperature of the ion source and transfer line was 230°C and 285°C, respectively. Helium gas was used as the carrier gas with an inlet pressure of 16.97 psi. The retention index (RI) was calculated for all the volatile compounds using a homologous series of C7–C30 n-alkanes (49451-U Sigma-Aldrich), according the linear equation of Van den Dool and Kratz(Van Den Dool and Kratz, 1963).

The analysis on polar column was carried out using an Agilent Technology gas chromatograph 7890ª Plus Series (Palo Alto, CA, USA) coupled to a selective Mass Spectrometry Detector 5975C. A fused-silica capillary column DB-WAX (J&W Scientific, Folsom, CA, USA) of 60 m × 0.25 mm i.d x 0.25 µm of film thickness coated with polyethylene glycol was used. The oven temperature was set as follows: initial oven temperature was held at 50°C for 5 min, then raised to 150ºC at 4ºC/min for 7 min and finally to 230°C at 4°C/min, which was kept for 40 min. The injector temperature was 250 °C, 2.0 µL of samples diluted in dichloromethane was injected in the "split" mode at a ratio of 30:1. EIMS, electron energy was 70 eV. Helium gas was used as the carrier gas with an inlet pressure of 16.91 psi. Mass detector operated in full scan mode in the range of 40 to 350 m/z. The retention index (RI) was calculated for all the volatile compounds as described above.

The identification of the components was performed by comparing their RI and mass spectra with data published in the literature(Adams, 2007; Babushok et al., 2011) and in the computer libraries (NIST 107 and WILEY 8).

2.2.5. Data analysis

The IZD data from the initial screening was evaluated by analysis of variance (ANOVA) followed by the Tukey test for pairwise comparison at 5% of significance using XLSTAT (Addinsoft, New York, NY, USA). Based on this, the susceptibility of ETECs strains and *Lactobacillus* species was determined. Also, principal component analysis (PCA) using the correlation matrix was performed based on IZDs means using XLSTAT (Addinsoft, New York, NY, USA).

Furthermore, an ANOVA to detect significant differences in the growth kinetics parameters *A*, λ and μ_{max} of *E. coli* and *Lactobacillus* spp. after exposure to EO concentrations was performed ($p<0.05$) using R software. If significant differences were detected in those parameters, as effect of the EO concentrations, the behavior of each parameter was modeling by nonlinear regression.

In addition, the multiple factor analysis (MFA) was performed on polar and non-polar data (two tables) to describe and contrast the chemical composition profile of the six citrus EOs obtained by GC-MS, with this analysis running in the XLSTAT software.

2.3. Results

2.3.1. Antibacterial activity

Screening by disc diffusion

The antibacterial activity screening of the six citrus EOs on the ETECs and *Lactobacillus* species is shown in Table 2.1. The association of inhibition zone diameter (IZD) means of six citrus EOs, when evaluated on ETECs and *Lactobacillus* species, by principal component analysis (PCA), showed that the first principal component explained 96.62%, and the second component 2.80% of the total variance (Fig. 2.1). Therefore, a good representation of antibacterial activity of these citrus oils was obtained. The IZD data showed that all the citrus oils had high antibacterial activity on all the ETECs, while low activity on the two *Lactobacillus* species was observed (Table 2.1). Consequently, it is possible to highlight these citrus oils as having a selective antibacterial activity. Furthermore, in contrast to the antibiotic colistin, an antibiotic that presented a selective performance (antibacterial activity on ETECs and no activity on *Lactobacillus* species), the citrus EOs showed superior performance on ETECs. Looking at the PCA (Fig. 2.1), four of the six citrus EOs, BOT, OOPE, CT and OPO, were the most selective oils since a closer association of their antibacterial activity with ETECs than with *Lactobacillus* species was observed. IZDs of these oils on ETECs were > 18 mm. However, from these four citrus EOs, BOT stood out by exhibiting the best selective antibacterial activity ($p<0.05$), since it presented the largest IZDs to all ETECs and considerable low IZDs ($p<0.05$) to the two evaluated *Lactobacillus* species. Therefore, this oil was selected to continue the study.

Regarding the susceptibility of ETECs, *E. coli* U7 was the most sensitive strain ($p<0.05$) to the activity of the citrus EOs, since the largest IZDs were observed for this ETEC. Conversely, *E. coli* U21 was the least sensitive or the most resistant ETEC, since were gotten the lowest IZDs ($p<0.05$) on this bacterium. In the case of *Lactobacillus* species, it was observed that *L. plantarum* was the more resistant beneficial bacterium to the citrus EOs activity, since lowest IZDs were gotten on this bacterium. This behavior of the sensitivity for the bacteria tested is also represented in the PCA (Fig. 2.1), where the farthest association of *E. coli* U21 to the EOs is observed, and the closer association of *L. rhamnosu*s than of *L. plantarum* to EOs can be observed. Therefore, *E. coli* U21 was selected as the most resistant ETEC and *L. rhamnosus* selected as the most sensitive beneficial bacterium to the antibacterial activity of the citrus oils.

Table 2.1. Antibacterial activity of the citrus essential oils on ETECs strains isolated from pig gut and *Lactobacillus* strains*.

	Strain	***E. coli* U7**			***E. coli* U21**			***E. coli* U23**			***E. coli* U25**		
Essential oil**		IZD**	PC**	% I	IZD**	PC**	% I	IZD**	PC**	% I	IZD**	PC**	% I
1	BOT	27.4 ± 0.8$^{ab, A}$	17.4 ± 0.2	157.4	20.1 ± 0.3$^{a, C}$	12.4 ± 0.3	161.6	22.8 ± 0.9$^{a, B}$	13.3 ± 0.2	171.0	25.4 ± 1.5$^{a, AB}$	15.6 ± 0.7	162.4
2	TLOP	24.3 ± 1.1$^{bc, A}$	15.3 ± 1.8	158.7	12.5 ± 1.7$^{c, B}$	12.4 ± 0.4	100.8	14.9 ± 0.8$^{b, B}$	12.9 ± 0.1	115.6	22.3 ± 2.5$^{a, A}$	16.7 ± 1.2	133.9
3	OPO	23.8 ± 1.4$^{c, A}$	16.1 ± 1.8	148.2	18.1 ± 0.2$^{b, B}$	12.5 ± 0.5	144.6	20.8 ± 1.6$^{a, AB}$	12.8 ± 0.4	162.9	22.2 ± 1.6$^{a, AB}$	16.4 ± 0.3	135.6
4	OPOFF	13.4 ± 0.9$^{d, A}$	17.1 ± 0.5	78.3	9.3 ± 0.9$^{d, B}$	12.7 ± 0.1	73.2	10.1 ± 0.4$^{c, B}$	12.6 ± 0.1	80.2	13.3 ± 0.9$^{b, A}$	15.6 ± 1.2	85.4
5	OOPE	25.7 ± 0.6$^{abc, A}$	16.9 ± 0.6	151.1	19.2 ± 0.5$^{ab, B}$	12.6 ± 0.5	152.4	22.3 ± 1.5$^{a, AB}$	13.0 ± 0.5	172.7	22.9 ± 1.3$^{a, A}$	15.8 ± 0.4	144.7
6	CT	28.6 ± 2.2$^{a, A}$	17.7 ± 1.1	161.9	19.8 ± 0.8$^{ab, B}$	12.1 ± 0.6	163.0	22.1 ± 1.2$^{a, B}$	12.8 ± 0.7	172.4	22.5 ± 0.7$^{a, B}$	15.8 ± 0.5	142.0

***OOPE = Orange oil phase essence, OPO = Orange peel oil, BOT = Brazilian orange terpenes, TLOP = Tahiti lime oil phase, OPOFF = Orange peel oil five fold and CT = Citrus terpenes.

** IZD: Inhibition zone diameter; PC: Positive control (Colistin); %I: inhibition of essential oil in relation to colistin.

* Values are means ± Standard Deviation (SD) of triplicate determinations expressed in mm including 6 mm of paper disk.

a-b: Mean values within a column having different superscripts are significantly different (essential oils) by the least significant difference Tukey test ($p < 0.05$).

A-B: Mean values within a row having different superscripts are significantly different (strains) by the least significant difference Tukey test ($p < 0.05$).

Table 2.1. Continued

	Strain	***L. rhamnosus* ATCC 7469**			***L. plantarum* ATCC 8014**		
Essential oil***		IZD**	PC**	% I	IZD**	PC**	% I
1	BOT	9.9 ± 0.3$^{bc, A}$	6.0 ± 0.0	165.7	8.8 ± 0.4$^{ab, B}$	6.0 ± 0.0	161.6
2	TLOP	8.5 ± 0.2$^{d, A}$	6.0 ± 0.0	141.3	8.0 ± 0.3$^{b, A}$	6.0 ± 0.0	133.9
3	OPO	9.1 ± 0.3$^{cd, A}$	6.0 ± 0.0	151.8	8.4 ± 0.4$^{ab, B}$	6.0 ± 0.0	140.2
4	OPOFF	6.0 ± 0.0$^{e, A}$	6.0 ± 0.0	100.0	6.0 ± 0.0$^{c, A}$	6.0 ± 0.0	100.0
5	OOPE	10.4 ± 0.5$^{ab, A}$	6.0 ± 0.0	173.2	8.8 ± 0.1$^{ab, B}$	6.0 ± 0.0	146.8
6	CT	10.9 ± 0.5$^{a, A}$	6.0 ± 0.0	182.4	9.1 ± 0.4$^{a, A}$	6.0 ± 0.0	151.0

***OOPE = Orange oil phase essence, OPO = Orange peel oil, BOT = Brazilian orange terpenes, TLOP = Tahiti lime oil phase, OPOFF = Orange peel oil five fold and CT = Citrus terpenes.

** IZD: Inhibition zone diameter; PC: Positive control (Colistin); %I: inhibition of essential oil in relation to colistin.

* Values are means ± Standard Deviation (SD) of triplicate determinations expressed in mm including 6 mm of paper disk.

a-b: Mean values within a column having different superscripts are significantly different (essential oils) by the least significant difference Tukey test ($p < 0.05$).

A-B: Mean values within a row having different superscripts are significantly different (strains) by the least significant difference Tukey test ($p < 0.05$).

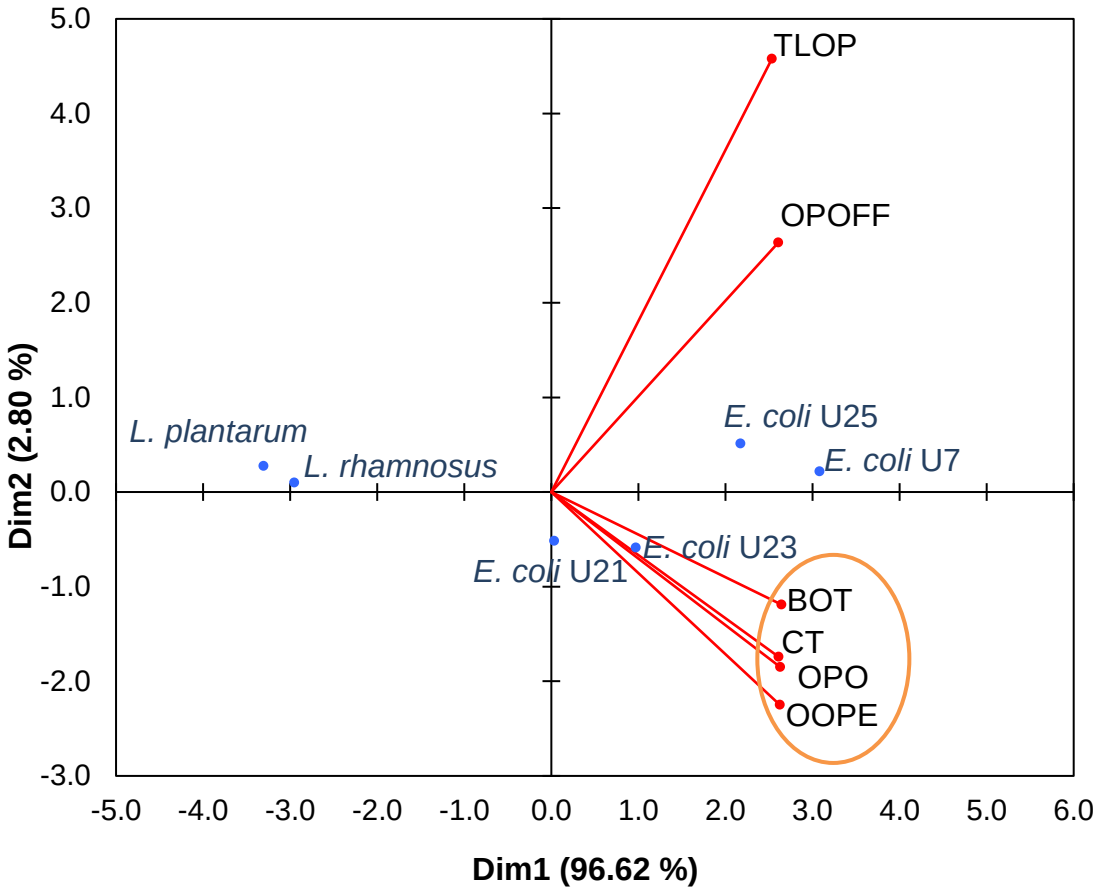

Figure 2.1. Principal component analysis (PCA) of six citrus EOs based on their antibacterial activity on ETECs strain and *Lactobacillus* species. OOPE = Orange oil phase essence, OPO = Orange peel oil, BOT = Brazilian orange terpenes, TLOP = Tahiti lime oil phase, OPOFF = Orange peel oil five fold and CT = Citrus terpenes.

Determination of Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC)

The MIC and MBC determinations were made only using the most selective EO, which was BOT, the most resistant *E. coli* strain (*E. coli* U21), and most sensitive *Lactobacillus* species (*L. rhamnosus*), as determined in the screening phase. The MIC and MBC values for BOT, as determined by survival curves and resazurin test, are shown in Table 2.2. The MIC for *E. coli* U21 was 1.85 mg/mL (Fig. 2.2a), which was also the MBC for this bacterium. The MIC for *L. rhamnosus* was 3.70 mg/mL (Fig. 2.2b) and the MBC was 7.40 mg/mL (Table 2). Thus, these results reaffirm the selective antibacterial activity of this citrus oil, since to totally inhibit the growth of the beneficial bacterium *L. rhamnosus* required an EO concentration equivalent to twice the MIC observed for *E. coli* U21, and to kill it necessitated a concentration of four times the *E. coli* U21 MIC. Therefore, pathogenic bacterium was more sensitive to BOT than beneficial bacterium

Table 2.2. MIC and MBC for Brazilian orange terpenes (BOT)

Bacterial strain	Brazilian Orange Terpens	
	MIC (mg/mL) *	MBC (mg/mL)
E. coli U21	1.85	1.85
L. rhamnosus	3.70	7.40

*Determined by survival curves and resazurin test

Bacterial growth modeling and calculation of kinetic parameters

The curves representing the bacterial growth kinetic for *E. coli* U21 and *L. rhamnosus*, when they were exposed to different concentrations of the BOT, are shown in Fig 2.2. The Gompertz model modified by Zwietering *et al*. (Zwietering et al., 1990) was used to fit the data of the bacterial growth (Eq. 1). The parameters obtained of this model for both bacteria are shown in Table 2.3.

Table 2.3. Calculated parameters* of Modified Gomperzt Model (Eq. (1) (Zwietering et al., 1990)) for each evaluated concentration of Brazilian orange terpenes (BOT)

C_{EO}[1]	***E. coli* U21**				***L. rhamnosus* ATCC 7469**			
(mg/mL)	**A ($OD_{600\ nm}$) [a]**	**μ_{max} (h^{-1}) [b]**	**λ (h) [a]**	**R^2**	**A ($OD_{600\ nm}$) [b]**	**μ_{max} (h^{-1}) [b]**	**λ (h) [a]**	**R^2**
14.80	-	-	-	-	-	-	-	-
7.40	-	-	-	-	-	-	-	-
3.70	-	-	-	-	-	-	-	-
1.85	-	-	-	-	1.143 ± 0.027	0.165 ± 0.005	26.61 ± 2.19	0.99
0.925	0.289 ± 0.016	0.078 ± 0.004	16.88 ± 0.97	0.99	1.192 ± 0.047	0.215 ± 0.104	23.85 ± 3.95	0.99
0.463	0.400 ± 0.054	0.058 ± 0.018	8.16 ± 1.31	0.99	1.210 ± 0.040	0.197 ± 0.089	20.24 ± 3.37	0.99
0.231	0.437 ± 0.060	0.072 ± 0.008	3.04 ± 0.57	0.98	1.216 ± 0.032	0.202 ± 0.048	19.55 ± 2.34	0.99
0.116	0.400 ± 0.053	0.097 ± 0.006	2.58 ± 0.45	0.98	1.210 ± 0.028	0.204 ± 0.035	19.35 ± 1.77	0.99
0.00	0.655 ± 0.049	0.073 ± 0.012	2.30 ± 0.55	0.99	1.176 ± 0.054	0.148 ± 0.008	16.61 ± 1.94	0.99

*A = maximal bacterial culture density (OD600 nm), μ_{max} = the maximum specific growth rate (h^{-1}), λ = the lag phase duration (h)

(-) Undetermined parameters due to total inhibition.

[1] Concentration of essential oil

[a] There are significant differences in the growth kinetics parameters after exposure to essential oil concentrations ($p<0.05$).

[b] No significant differences were observed ($p<0.05$).

The survival curves or growth kinetics of *E. coli* U21 (Fig. 2a) showed that the four highest EO concentrations totally inhibited the growth of this bacterium and the growth was only observed for concentrations equal to or under 0.925 mg/mL of BOT oil. In the case of *L. rhamnosus* (Fig 2.2b), bacterial growth was observed up to the concentration of 1.85 mg/mL of BOT and the three highest concentrations caused complete inhibition of this bacteria. Furthermore, we observed that EO concentration was able to provoke higher disturbances on the normal growth kinetic of *E. coli* U21 than *L. rhamnosus*. The modified Gompertz model allowed us to evaluate these disturbances with more accuracy through the three main

biological parameters that it considers: maximal bacterial culture density (A), maximum specific growth rate (μ_{max}) and lag phase duration or adaptation time (λ).

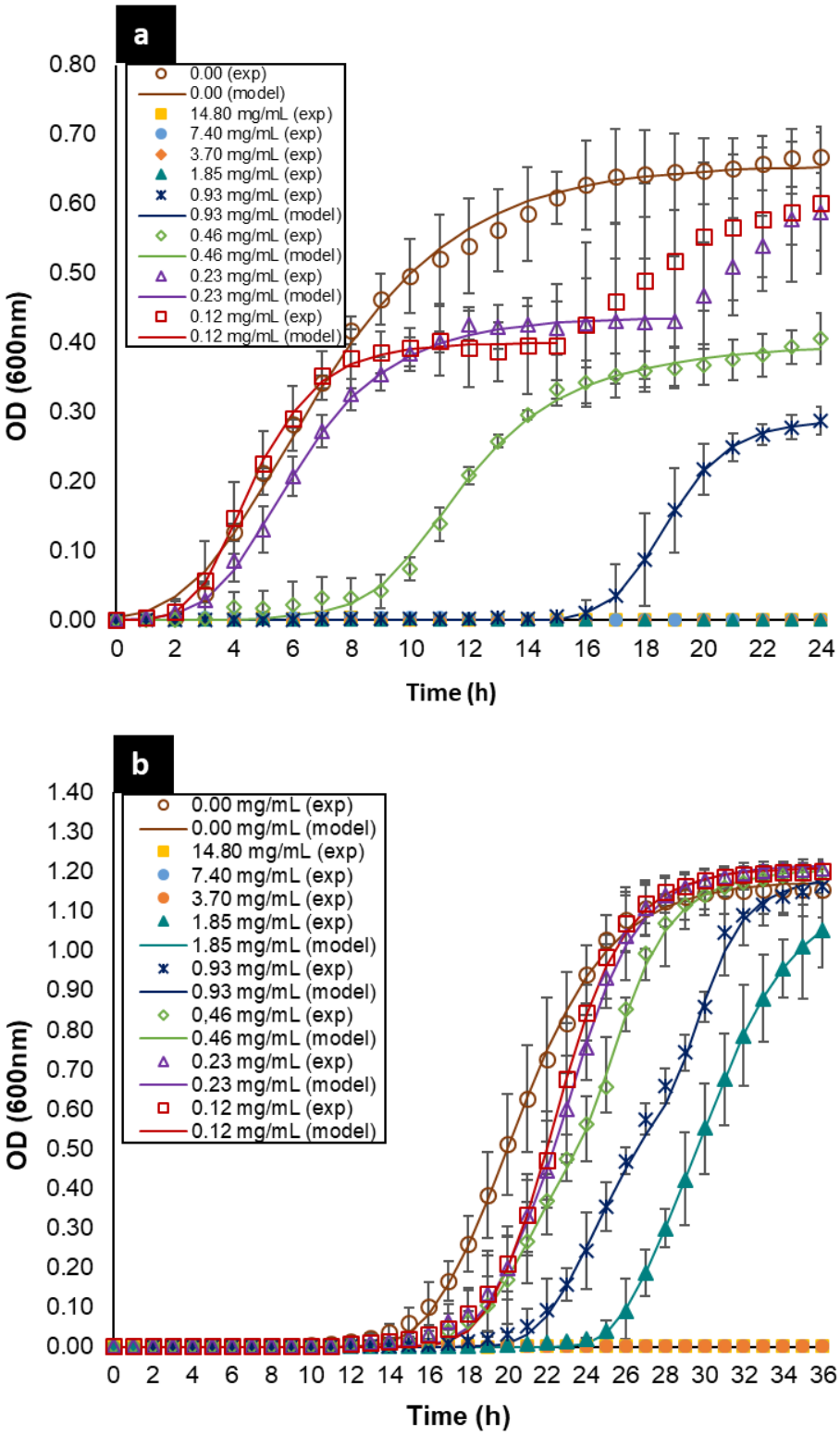

Figure 2.2. Bacterial growth kinetic as function of BOT concentrations of *E. coli* U21 (a) and *L. rhamnosus* (b). The dots are the experimental values; the vertical bars are the standard deviation and the curves are the modified Gompertz model (Eq. (1)). BOT = Brazilian orange terpenes

The values of parameter *A* were significantly affected (*p<0.05*) for *E. coli* U21, since *A* was greatly reduced as the EO concentration was increased (Table 2.3). For instance, *A* was reduced in 38.9% at the lowest concentration and in 55.9% at the highest subinhibitory concentration (0.925 mg/mL), in contrast with the control (0 mg/mL of BOT). The parameter *A* had an inverse sigmoidal behavior as function of the EO concentration (Fig. 2.3a) and the mathematical function that describes this behavior to *E. coli* U21 is shown in Eq. (2), which had a good fit (R^2 = 0.91). Regarding *L. rhamnosus*, this parameter was not affected by EO concentrations (Table 2.3), no significant differences were detected (*p<0.05*), and it was considered the average in the general model of this bacterium, which was A_{LR} = 1.191 ± 0.028.

$$A_{EC} = 3.47 + 4.46(C_{EO})^{1.5} - 2.83.e^{(C_{EO})} \qquad (2)$$

The maximal growth rate, that is, the parameter μ_{max} for both bacteria, *E. coli* U21 and *L. rhamnosus*, was not affected by the EO concentrations since no significant differences were detected among the concentrations tested (*p<0.05*). Consequently, it was considered an average growth rate of each bacterium for the general model, 0.076 ± 0.014 h^{-1} to *E. coli* U21 and 0.189 ± 0.026 h^{-1} to *L. rhamnosus*. As observed, the growth rate was quite higher for *L. rhamnosus* than *E. coli* U21.

Regarding parameter λ, which is the lag phase duration, we observed that for both bacteria this parameter increased as the EO concentration was increased. For *E. coli* U21, λ was slightly longer than control at the three lowest EO concentrations, but from 0.463 mg/mL to above, the lag phase was notably increased. For instance, λ increased approximately eight times at the highest subinhibitory concentration of BOT oil (0.925 mg/mL), in contrast with the control (0.00 mg/mL). Therefore, this parameter had an exponential behavior as function of the EO concentration (Fig. 2.3b) and the mathematical function that describes it is shown in Eq. (3), which had a good fit (R^2 = 0.97). For *L. rhamnosus*, λ only suffered a significant increase, approximately 1.6 times at the highest effective subinhibitory BOT concentration (1.85 mg/mL). The behavior of λ to this beneficial bacterium was lineal as function of the EO concentration (Fig. 2.4); the mathematical function that describes it is shown in Eq. (4) and it had a good fitting (R^2 = 0.93).

$$\lambda_{EC} = 2.52e^{2.06(C_{OE})} \qquad (3)$$

$$\lambda_{LR} = 4.971C_{OE} + 18.06 \qquad (4)$$

Finally, the modified Gompertz model to describe the bacterial growth kinetics as function of EO concentration ($0.00 < C_{EO} < 14.80$ mg/mL) and time of exposure (in hours), is shown in Eq. (5) for *E. coli* U21, and in Eq. (6) for *L. rhamnosus*. Additionally, the model for *E. coli* U21 was plotted in 3D and is shown in Fig. 2.3c. The surface obtained highlights the shifts in the bacterial growth kinetic of *E. coli* U21 as function of initial EO concentration.

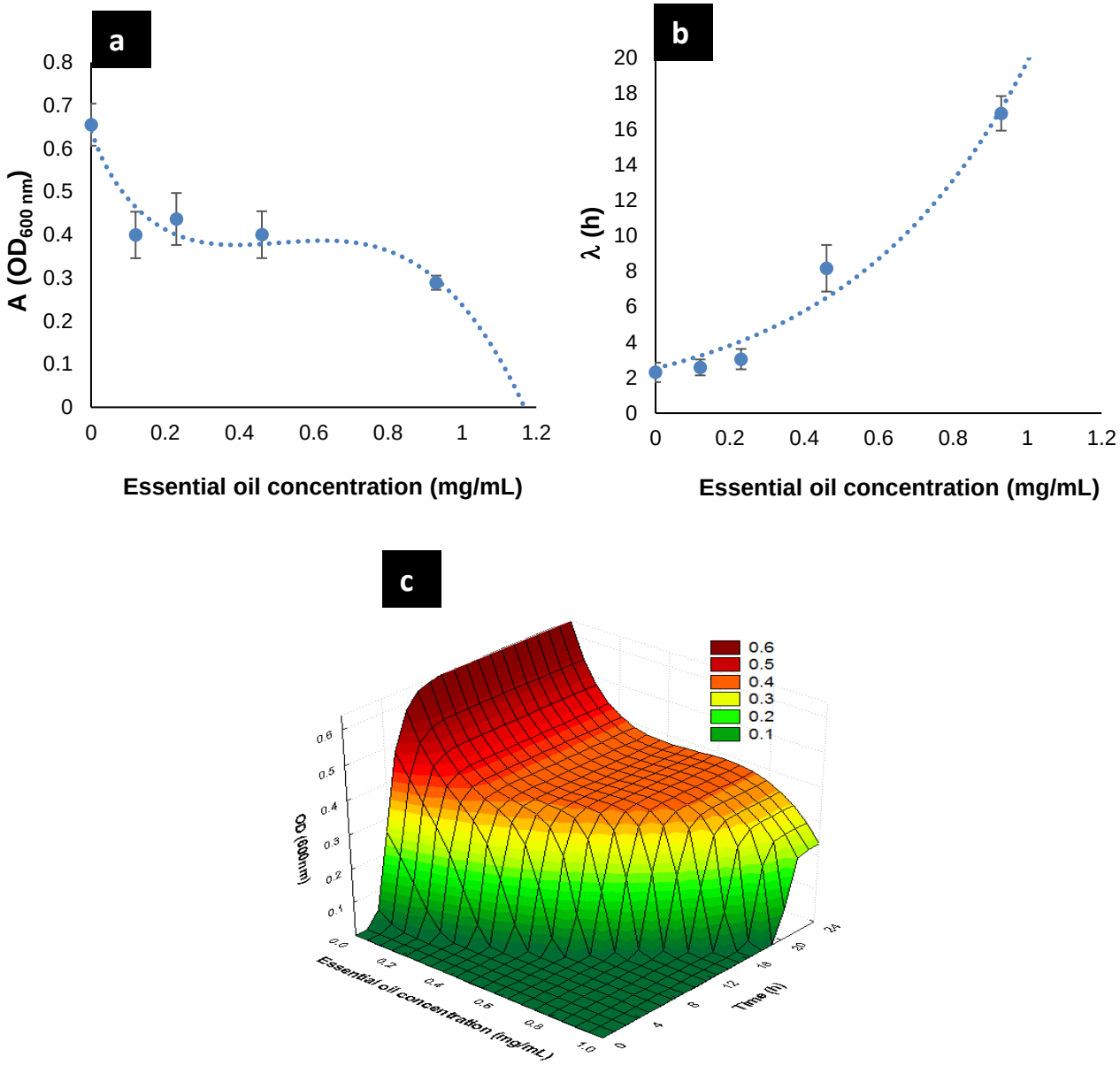

Figure 2.3. Parameters *A* (a) and λ (b) of the modified Gompertz model (Eq. (1)) to *E. coli* U21 as function of the BOT concentration. The dots are the experimental values; the vertical bars are the standard deviation and the curves are the model of Eqs. (2) and (3), respectively. (c) General model that describes the bacterial culture density of *E. coli* U21 as function of the time of treatment and the BOT concentration applied (Eq. (5)). BOT = Brazilian orange terpenes.

$$y_{EC\ (t,C_{EO})} = (3.47 + 4.46(C_{EO})^{1.5} - 2.83.e^{(C_{EO})}) \exp\left(-\exp\left(\frac{0.076e\left(2.52e^{2.06(C_{EO})} - t\right)}{3.47+4.46(C_{EO})^{1.5}-2.83.e^{(C_{EO})}} + 1\right)\right) \quad (5)$$

$$y_{LR\ (t,C_{EO})} = 1.191 \exp\left(-\exp\left(\frac{0.1896e(4.971C_{EO}+18.06 - t)}{1.191} + 1\right)\right) \quad (6)$$

Therefore, evaluation of growth bacterial kinetic parameters confirmed that the citrus oil, BOT, had a stronger effect on the pathogenic bacterium than on the beneficial bacterium, provoking higher disturbances in its growth kinetics. This proves the selective antibacterial features of the BOT oil.

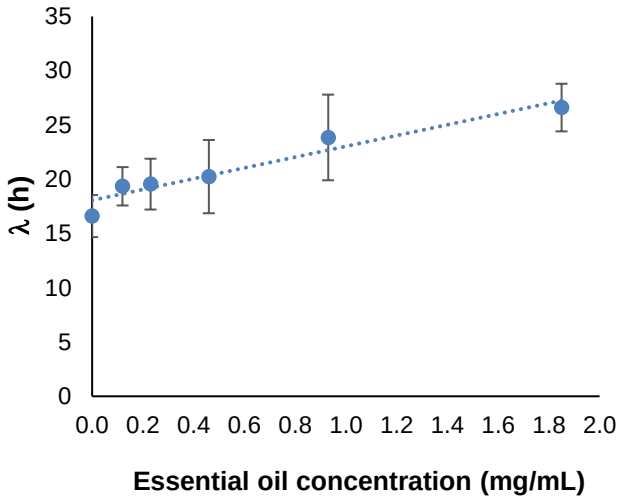

Figure 2.4. Parameters λ of the modified Gompertz model (Eq. (1)) to *L. rhamnosus* as function of the BOT concentration. The dots are the experimental values; the vertical bars are the standard deviation and the curves are the model of Eq. (4). BOT = Brazilian orange terpenes

2.3.2. Chemical composition characterization of essential oils

The chemical composition of the six citrus EOs is shown in Table 2-S1 (Supplementary information). Overall, the identification of the chemical composition of each citrus EO by both columns was quite similar, as is observed in the individual factor map of the MFA (Fig. 2.5a), the polar and non-polar identification points were very close to each other. In addition, a good representation of the chemical composition data was obtained, since the first dimension of the MFA explained 53.89% and the second dimension explained 33.84% of the total variance. For the six citrus oils, limonene was detected as the major compound; however, TLOP had a negative association with this compound in both dimensions (Fig. 5b), since it presented the lowest relative amount of limonene (46.53%/47.46%).

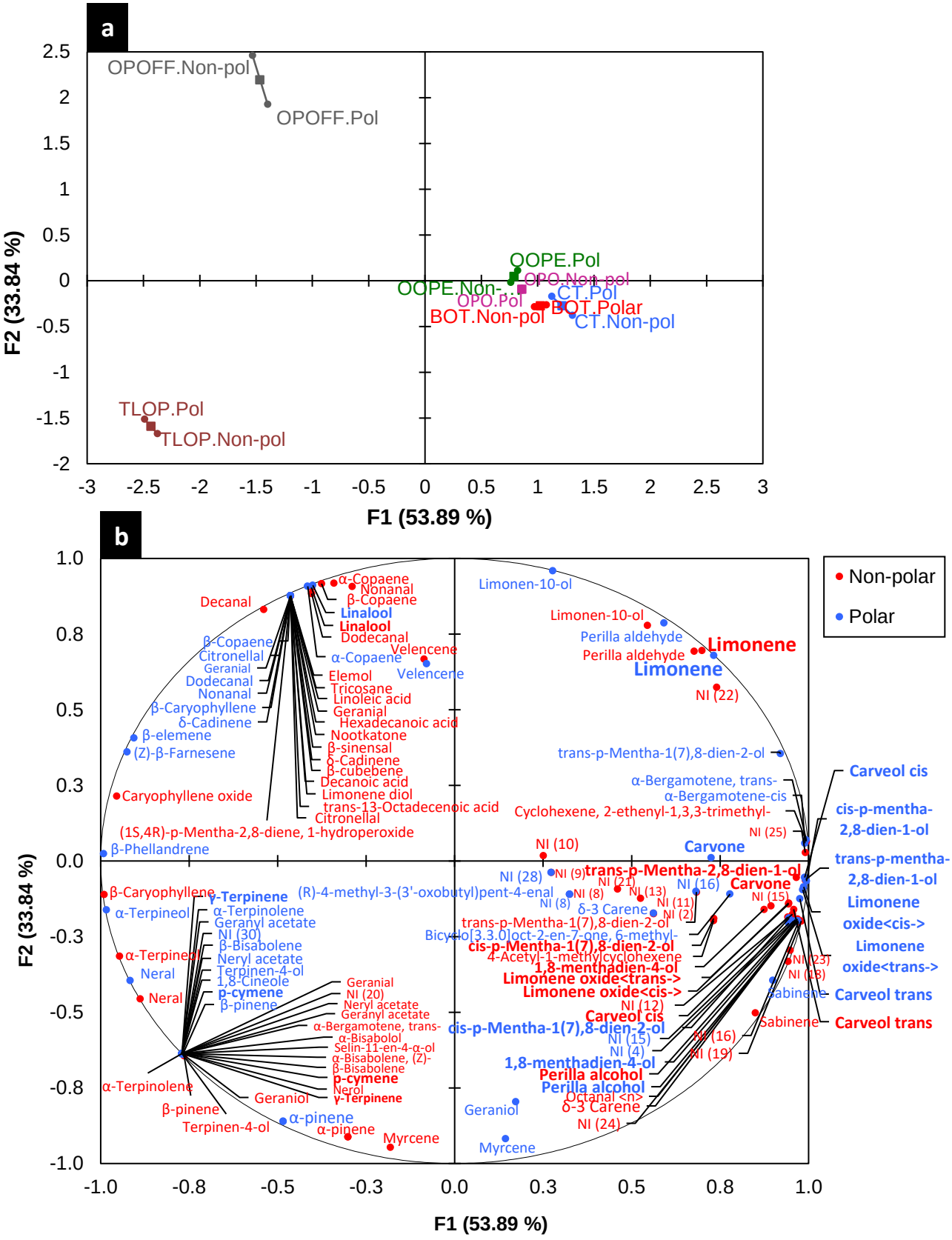

Figure 2.5. Multiple factor analysis (MFA) of the chemical composition profile of six citrus commercial EOs. The individual factor map of the overall chemical composition profiles by polar and non-polar identification (a) and biplot of the detailed chemical composition profile (b). OOPE = Orange oil phase essence, OPO = Orange peel oil, BOT = Brazilian orange terpenes, TLOP = Tahiti lime oil phase, OPOFF = Orange peel oil five fold and CT = Citrus terpenes.

Moreover, it was observed that BOT, CT, OOPE and OPO had a positive association with limonene in the two dimensions of the MFA and OPOFF had that positive association in the second dimension, showing all these EOs presented higher amount of limonene (Fig. 2.5b). However, OPOFF presented a fully different chemical profile than BOT, CT, OOPE and OPO oils (Fig. 2.5a) and it was the oil that presented the weakest antibacterial activity. BOT, CT, OOPE and OPO oils presented a very close chemical composition profile and they were characterized by having the highest selective antibacterial activity. Therefore, this allows us to suggest that the antibacterial activity of the citrus EOs could not be attributed specifically to limonene. Minor common compounds such as *cis*-limonene oxide, *trans*-carveol, carvone*, trans*-limonene oxide, *cis*-p-Mentha-2,8-dien-1-ol, *trans*-p-Mentha-2,8-dien-1-ol, perrilla alcohol, *cis*-carveol, *cis*-p-Mentha-1(7),8-dien-2-ol and 1,8-menthadien-4-ol (Fig. 2.5b) were detected in these four citrus EOs and they were either present exclusively in these four EOs or in higher amounts than TLOP and OPOFF oils. Therefore, these minor compounds could be implicated to confer the selective and highest antibacterial activity of BOT, CT, OOPE and OPO oils.

In addition. we observed that TLOP and OPOFF had a different chemical profile. The TLOP profile is shown in the third quadrant of the MFA (Fig. 2.5b); besides limonene, this oil had a high amount of p-cymene and γ-terpinene. The OPOFF profile is shown in the second quadrant of the MFA (Fig. 2.5b), and linalool was detected as second major compound in it.

2.4. Discussion

Citrus EOs are one of the main by-products of the orange juice industry and could have several applications besides cosmetology and food industry. Citrus oils have been reported as having antimicrobial properties (Fisher and Phillips, 2008; Friedly et al., 2009; O'Bryan et al., 2008; Settanni et al., 2012), but testing their antimicrobial activity on bacteria affecting animal farms has not been greatly mentioned. *E. coli* causing post-weaning diarrhea in piglets during their early life is a critical problem for the pig industry (Gresse et al., 2017). The antibacterial activity of commercial citrus EOs evaluated in this study on *E. coli* strains isolated from pig gut and on *Lactobacillus* bacteria showed that, in overall, citrus EOs presented a selective antibacterial activity, having higher activity on pathogenic bacteria *E. coli* than on beneficial bacteria *Lactobacillus* spp. The selectivity towards bacterial pathogens by EOs or EO compounds rather than beneficial bacteria have been already reported (Ambrosio et al., 2017; Ouwehand et al., 2010; Si et al., 2006), EOs or single EO compounds were shown to have higher inhibitory effect on *E. coli* O157:H7, *E. coli* K88, *S. typhimurium* than on *Lactobacillus* and *Bifidobacterium* spp. (Si et al., 2006). As an alternative to AGPs, the selectivity of citrus oils could be an important feature of antimicrobial

spectrum, which commonly has not been considered to conventional AGPs long used, since the aim is to have an effect on the pig gut. Thus, current search for antimicrobial substances as alternatives to an AGP should consider selectivity aspects between pathogenic and beneficial gut bacteria. The lower effect on beneficial bacteria by AGP and its potential alternatives (such as citrus EOs) would be desirable since beneficial bacteria such as *Lactobacillus* spp. contribute to fighting pathogen colonization in the gut and preventing gut infections. Thereby, reinforcing gut microbiota and contribute to improve animal health (Ouwehand et al., 2010).

The capability of citrus EOs to inhibit pathogenic bacteria has been well reported in several studies. For instance, tangerine EO (*Citrus reticulata*) was reported as having an inhibitory effect on *S. aureus*, *Bacillus subtilis*, and *Pseudomonas aeruginosa* (Yi et al., 2018). Another study proved the high effectiveness of the lemon EO (*Citrus limon* L. Burm) to inhibit several strains of *Listeria monocytogenes*, *S aureus* and *Salmonella enterica* associated with foodborne diseases (Settanni et al., 2012) Also, it has been reaffirmed the anti-salmonella (O'Bryan et al., 2008) and anti-listeria (Friedly et al., 2009) activities of several commercial citrus oils. Specifically, the antibacterial activity of citrus oils on *E. coli* has been investigated. Tangerine EO (*Citrus reticulata*) was shown to be effective to produce an inhibition of 14.6±1.1mm on *E. coli* (Yi et al., 2018). A total inhibition of *E. coli* growth by this oil was found at 1.96 mg/mL (Mandal and Mandal, 2016). A close value to this MIC was found for BOT oil in our study (1.85 mg/mL = 0.21%v/v). Other citrus EOs such as bergamot, orange and lemon were also effective to produce inhibitions ≥ 18 mm on *E. coli* O157, but full inhibition of the growth of this bacterium was reported at higher MICs than the BOT oil, between 0.5 - 1.0 %v/v (Fisher and Phillips, 2006). Similarly, another study reported higher MICs than the BOT to mandarin and lemon EOs on *E. coli*, 5 and 30 µL/mL, respectively (Espina et al., 2011). In addition, the EO of sweet orange (*Citrus sinensis* Osbeck) has presented an inhibitory effect on *E. coli* at 18.8 µL/mL (Tao et al., 2009), also considered as a high MIC in contrast to the BOT oil. Conversely, the non-effectiveness of several citrus oils to fight *E. coli* affecting animals, such as *E. coli* associated with poultry colibacillosis, has been reported (Ebani et al., 2018). In comparison, the citrus EOs tested in our study were quite effective in treating *E. coli* affecting pigs.

Furthermore, some studies highlighted that citrus EOs have higher effectiveness to inhibit Gram-positive pathogenic bacteria than Gram-negative pathogenic bacteria (Fisher and Phillips, 2006; J. jing Guo et al., 2018). However, in our study, the opposite was observed, since the Gram-negative *E. coli* was more sensitive than the Gram-positive *Lactobacillus* spp. to the activity of citrus oils. The difference in the sensitivity to EOs between these two groups of bacteria has been hypothesized to be the consequence of differences in the cell wall structure, since Gram-positive bacteria lack an outer membrane

(OM), which Gram-negative bacteria have. This OM contains lipopolysaccharides (LPS) with polar ends (O-polysaccharides) and transmembrane channels (porins), which permit the passage of hydrophilic solutes and make difficult for hydrophobic compounds to diffuse such as EOs components into the cell. Therefore, this would allow Gram-negative bacteria be more resistant to EOs (Nazzaro et al., 2013). Nonetheless, the antibacterial spectrum of EOs depends on the specificity of the functional groups of EO compounds to single or multiple targets. Some EO compounds have the ability to disintegrate the OM of Gram-negatives as *E. coli*, release the material associated to this membrane and penetrate the cell, provoking a disruptive effect (Helander et al., 1998). Probably, the compounds present in citrus EOs may have this ability due to their higher effectiveness observed on this Gram-negative bacterium, *E. coli*. On the other hand, the antibacterial activity of citrus oils on Gram-positive beneficial bacteria has been little reported. Orange, lemon, mandarin and grapefruit EOs had a low inhibitory effect on *Lactobacillus sakei* and *Lactobacillus curvatus*, exhibiting the orange oil the lowest effect on these bacteria (12.8±0.5 and 13.5±0.2 mm, respectively) (Viuda-Martos et al., 2008). The authors demonstrated that the inhibitory effect of these four oils was dose-dependent causing inhibition of those *Lactobacillus* species only at the highest concentrations tested (Viuda-Martos et al., 2008). This was also noticed in our study, where the citrus oils exhibited IZDs lower than 11 mm to *Lactobacillus* species. Moreover, *L. rhamnosus* was inhibited at a high BOT concentration and killed even at an upper concentration, thus showing BOT had a low antibacterial activity on *L. rhamnosus*. Although general structures and biosynthesis pathways among Gram-positive bacteria are conserved, some Gram-positive bacteria, such as *Lactobacillus* spp., could show low sensitivity to antimicrobials, such as EOs, since the cell wall of Gram-positive lactic acid bacteria (LAB) as *Lactobacillus* spp. possess unique properties that could confer intrinsic resistance to some antimicrobial agents (Chapot-Chartier and Kulakauskas, 2014). For instance, the intrinsic resistance to antibiotics of some *Lactobacillus* (e.g. to vancomycin) would be related to the fact of having a D-lactate instead of D-alanine as the last amino acid in the peptidic chain of the peptidoglycan layer of their cell wall (Chapot-Chartier and Kulakauskas, 2014; Gueimonde et al., 2013), which would avoid the antibiotic binds to the peptidic chain and cause the inhibition of these bacteria (Campedelli et al., 2018).

Additionally, it was observed that BOT oil caused higher disturbances on the growth kinetics of *E. coli* than *L. rhamnosus*, significantly affecting its maximal culture density and the lag phase duration. Both parameters were dose-affected and changed as function of the BOT concentration. The higher dose-dependent effect of some EOs and single EO compounds on the growth kinetic of *E. coli* than on *Lactobacillus* spp. has already been observed (Ouwehand et al., 2010). Oregano, thyme and rosemary EOs, carvacrol, eugenol and thymol provoked higher reduction on the maximal culture density of *E. coli* strains than

Lactobacillus fermentum and *Lactobacillus reuteri* with increasing of the concentration of EOs/EO compounds. (Ouwehand et al., 2010). Also, the dose-dependent effect of carvacrol to extend the lag phase of *E. coli* by increasing the concentration of this compound has been proved (Burt et al., 2005). In addition, some combinations of EOs have been reported as more efficient to cause an increase of the *E. coli* lag phase. For instance, combinations of oregano with basil EOs and oregano with lemon balm EOs were able to significantly increase the *E. coli* lag phase, approximately 7.4 h and 3.6 h longer, respectively, compared to when oregano EO was used alone (Gutierrez et al., 2008). Regarding *L. rhamnosus*, in our study, we observed only the lag phase duration was extended as the BOT concentration was increased. This effect on *L. rhamnosus* has been previously observed with the oil of *Melaleuca armillaris*, which additionally reduced the growth rate and final culture density with increasing of the EO concentration (Hayouni et al., 2008). Therefore, this oil had a higher dose-dependent effect on the growth kinetic parameters of *L. rhamnosus* than the citrus oil (BOT) tested in our study. Moreover, a recent study observed that *Eucalyptus globulus* and *Pimenta pseudocaryophyllus* EOs presented a dose-dependent effect on the lag phase of *L. rhamnosus* as well; however, *P. pseudocaryophyllus* oil caused higher extension of this parameter in comparison to *E. globulus* oil at the same sub-MICs (Ambrosio et al., 2018).

Limonene has been shown as a major compound of citrus EO composition and most of their biological activities have been attributed to this compound. All citrus oils evaluated in our study presented limonene as the major compound. However, the mismatching between limonene content and antibacterial activity of these oils suggested that their antibacterial activity cannot be attributed exclusively to limonene. Some studies have already reported the lack of antibacterial activity of limonene individually tested. For instance, the pompia EO (*Citrus limon var. pompia*), which presented limonene as major compound (28%), at a concentration of 256.3 mg/mL, presented an antibacterial effect on several pathogenic bacteria, but when limonene was evaluated alone, it did not exhibit any antibacterial effect (Fancello et al., 2016). Thus, this proved that limonene would not be the compound responsible for the antibacterial activity observed for this oil. Nonetheless, coexisting minor compounds in citrus oils could contribute to conferring the antibacterial property of these oils. In mandarin EO, compounds like octanal, decanal, citral, citronellal, linalool, α-sinensal and thymol were suggested as possible collaborators to the antibacterial activity, when this oil (with 56.8% of limonene) was tested against Gram-negative and Gram-positive bacteria (Yi et al., 2018). Other minor compounds, belonging to oxygenated monoterpenes class, such as 4-terpineol, α-terpineol, *cis*-geraniol, β-citral, nerol and α-citral, might also be implicated in conferring the antibacterial activity of citrus oils, since they have been detected in high amounts in the composition of the citrus EO that presented high antibacterial activity (Settanni et al., 2012). Minor oxygenated monoterpenes compounds (cis-limonene oxide,

trans-carveol, carvone, *trans*-limonene oxide and perrilla alcohol) were also detected in the group of the most selective citrus oils of our study. Possibly, these compounds might play an important role in conferring the selective antibacterial activity of citrus EOs. In addition, an orange cold pressed EO rich in limonene (85.3%) presented an antibacterial activity on *E. coli* ten times higher than limonene alone, and even minor compounds, such as linalool, pinene and terpineol, presented a higher activity than limonene (Q. Guo et al., 2018).

Furthermore, the antibacterial activity of limonene has been shown to be variable and depending on its stereoisomeric form present in the EO. The (-) stereoisomer of limonene could inhibit *E. coli* at a lower concentration (8 mg/mL) than the (+) stereoisomer (11 mg/mL). On the other hand, limonene alone has been proved to stimulate the growth of beneficial bacteria as *L. fermentum*, instead of having any inhibitory effect on it (Ouwehand et al., 2010). Likewise, limonene has been reported as not effective to inhibit several *Lactobacillus* species including *L. rhamnosus* ATCC 7469 (Fancello et al., 2016), the bacteria also evaluated in our study, and which showed be the more resistant to the antibacterial activity of the citrus oils proved. Therefore, it would be possible to infer that limonene could collaborate with the selective activity of citrus oils when it is present in the gut, promoting the beneficial bacteria while other minor compounds could act in inhibiting pathogenic bacteria. It has been reported that in an EO, major and minor components probably act in synergism to confer the biological properties of the EO (Bakkali et al., 2008). When an EO compound is proved, individually, its effect may differ from the effect that this compound may have in combination with the other compounds inside the EO. Thus, it would be recommended the use of the whole EO instead of single EO compounds, since every compound inside an EO could exert a different mechanism of action on the bacteria cell (Swamy et al., 2016), and this could reduce the chance to bacteria develop easily resistance to the EO. Contrariwise, bacteria could develop a rapid and easy mechanism of resistance to a single EO compound, as in the case of an antibiotic, which consist of a single compound. Several mechanisms of action of EOs have been proposed in the literature. The mechanism of action comprises a series of events on the bacterial cells. Initially, they can destabilize the cellular architecture, leading to the breakdown of membrane integrity and thus increased permeability of cellular constituents. This disrupts many cellular activities, including energy production, membrane transport, and other metabolic regulatory functions (Swamy et al., 2016). In addition, EOs can alter the membrane fatty acids composition, the membrane proton motive force and affect proteins in the cytoplasmatic membrane. Additionally, EOs can interfere with the quorum sensing activity, decreasing the proteolytic activity, biofilm formation, and virulence factors expression and their functions, as well as to affect the metabolome of bacteria (Nazzaro et al., 2013).

2.5. Conlcusions

Our study highlights as an important feature of antimicrobial spectrum the selectivity between pathogenic and beneficial gut bacteria, which should be considered when searching for antimicrobial substances as alternatives to conventional AGPs, since the aim is to have an effect on the pig gut. Our study has proved, by a screening, MIC determination, and growth kinetic parameters evaluation, the selective antibacterial activity of citrus EOs on *E. coli* and *Lactobacillus* spp., thus suggesting these EOs as potential alternatives to AGPs. Consequently, based on the selective performance and the huge viability in the global market of citrus EOs, the possible application of these oils in the pig production sector could turn feasible. In addition, chemical composition characterization showed that minor compounds present in these citrus oils would be implicated in conferring their selective activity, instead of limonene, the major present compound, playing this role exclusively. Finally, our results motivate further research to clarify, for instance, the possible mechanism of action that citrus oils would have on pathogenic and beneficial bacteria as well as their direct effect on the pig gut and on the microbiota resident in it.

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Supplementary table

Table 2-S1. Chemical composition of the six citrus essential oils*.

Compounds[1]	LRI_c[2]		LRI_L[3]		BOT %[6]		CT %[6]		OOPE %[6]		OPO %[6]		TLOP %[6]		OPOFF %[6]	
	NP[4]	P[5]	NP	P	NP	P	NP	P	NP	P	NP	P	NP	P	NP	P
NI	922	-	-	-	-	-	-	-	-	-	0.08	-	-	-	-	-
Tricyclene	923	-	923.2	-	-	-	0.06	-	-	-	-	-	-	-	-	-
α-Thujene	927	-	931	-	-	-	-	-	-	-	-	-	0.35	-	-	-
α-pinene	936	1020	939	1025.4	0.45	0.73	0.87	1.07	0.60	0.75	0.60	0.85	1.52	2.51	-	0.17
Sabinene	975	1118	976	1122	0.32	0.47	0.38	0.48	0.35	0.44	0.35	0.52	0.16	0.24	-	0.14
β-pinene	981	1106	980	1100	0.10	0.13	0.10	-	0.09	-	-	-	8.45	10.73	-	-
Myrcene	989	1158	991	1160.2	0.85	1.41	0.85	1.22	0.86	1.12	0.97	1.38	1.17	1.48	0.58	0.77
Octanal	1002	1285	1002.8	1287.2	0.20	0.01	0.16	-	0.14	-	0.18	-	-	-	-	-
α-Terpinene	1003	-	1018	-	-	-	-	-	-	-	-	-	0.09	-	-	-
δ-3 Carene	1011	1146	1011	1146.8	0.11	0.14	0.15	0.17	0.12	-	0.11	-	-	-	-	-
NI	1028	-	-	-	0.29	-	0.30	-	-	-	-	-	-	-	-	-
p-cymene	1028	1267	1024.3	1270	-	-	-	-	-	-	-	-	11.82	12.01	-	-
β-Phellandrene	-	1208	-	1209.3	-	0.10	-	0.08	-	0.09	-	0.13	-	0.34	-	0.28
1,8-Cineole	-	1211	-	1211.1	-	-	-	-	-	-	-	-	-	0.21	-	-
Limonene	1037	1202	1031	1198.2	78.65	79.38	74.23	79.89	76.68	80.61	79.27	81.18	46.53	47.46	79.95	82.07
γ-Terpinene	1062	1243	1062	1245	-	-	-	-	-	-	-	-	7.65	8.19	-	-
α-Terpinolene	1088	1280	1088	1282	-	-	-	-	-	-	-	-	0.55	0.55	-	-
Linalool	1099	1538	1098	1543.3	0.28	0.36	0.31	0.34	0.85	0.86	0.59	0.81	0.36	0.39	2.37	3.02
Nonanal	1104	1390	1102	1391.5	-	-	-	-	0.10	-	0.11	-	-	-	0.29	0.27
NI	1104	-	-	-	-	-	0.06	-	-	-	-	-	-	-	-	-
Fenchol	-	1580	-	1588	-	-	-	-	-	-	-	-	-	0.10	-	-
trans-p-Mentha-2,8-dien-1-ol	1125	1622	1123	1639	0.89	2.08	1.14	1.97	0.98	1.73	0.71	1.75	-	0.20	0.20	0.56
NI	-	1633	-	-	-	0.19	-	0.15	-	0.14	-	0.14	-	-	-	-
cis-p-Mentha-2,8-dien-1-ol	-	1664	-	1652.1	-	1.39	-	1.62	-	1.40	-	1.37	-	0.15	-	0.41
4-Acetyl-1-methylcyclohexene	1134	-	1139.3	-	0.11	-	0.13	-	-	-	0.09	-	-	-	-	-
cis-Limonene oxide	1137	1446	1134	1450.5	1.95	1.89	2.38	2.13	1.97	1.69	1.43	1.64	0.36	0.33	0.50	0.61
trans-Limonene oxide	1140	1459	1139	1461.6	1.64	0.90	2.17	1.08	1.73	0.89	1.32	0.84	0.30	0.22	0.42	0.33
NI	1143	1466	-	-	-	-	-	-	-	-	-	-	0.20	0.12	-	-
NI	1150	-	-	-	-	-	0.08	-	-	-	-	-	-	-	-	-

Citronellal	1152	1475	1153	1475.3	-	-	-	-	-	-	-	-	-	-	0.32	0.34
NI	1153	-	-	-	-	-	0.08	-	-	-	-	-	-	-	-	
Octanoic Acid	1164	-	1177	-	-	-	0.18	-	-	-	-	-	-	-	-	-
2-cyclohexen-1-one, 4-(1-methylethenyl)-	1172	-	-	-	-	-	0.14	-	-	-	-	-	-	-	-	-
Nonanol	-	1650	-	1655.7	-	-	-	-	-	-	-	-	-	-	-	0.17
1,8-menthadien-4-ol	1183	1680	1189	1681	0.17	0.46	0.28	0.46	0.28	0.38	0.13	0.32	-	-	-	-
Cryptone	-	1674	-	1674.8	-	0.13	-	-	-	-	-	-	-	-	-	-
Terpinen-4-ol	1186	1601	1177	1601.2	-	-	-	-	-	-	-	-	2.24	2.39	-	-
p-Cymen-8-ol	1189	-	1183.9	-	-	-	-	-	-	-	-	-	0.09	-	-	-
trans-p-Mentha-1(7),8-dien-2-ol	1192	1789	1180.5	1791	-	0.27	0.26	0.25	0.23	0.23	0.23	0.27	-	-	-	0.18
NI	1192	1732	-	-	0.25	2.75	-	-	-	-	-	-	-	-	-	
α-Terpineol	1199	1692	1189	1694	-	-	-	-	-	-	-	-	1.85	1.54	0.68	0.83
NI	1200	-	-	-	-	-	-	-	-	-	0.43	-	-	-	-	
octyl acetate	-	1471	-	1474.6	-	-	-	-	-	-	-	-	-	-	-	0.11
NI	1201	-	-	-	-	-	-	-	0.56	-	-	-	-	-	-	-
NI	1201	-	-	-	0.44	-	0.56	-	-	-	-	-	-	-	-	-
NI	1204	-	-	-	0.65	-	0.84	-	0.85	-	0.86	-	-	-	-	-
Decanal	1205	-	1204	-	-	-		-	-	-	-	-	0.20	-	2.29	-
NI	1210	-	-	-	-	-	0.16	-	-	-	0.12	-	-	-	-	-
trans-Carveol	1222	1828	1217	1836.3	1.82	2.29	2.19	2.17	1.69	1.84	1.33	1.65	0.11	0.14	0.22	0.37
Nerol	1225	1791	1228.9	1794.6	-	-	-	-	-	-	-	-	0.21	0.18	-	-
cis-p-Mentha-1(7),8-dien-2-ol	1233	1879	1233	1894.9	0.14	0.16	0.17	0.16	-	0.13	0.16	0.20	-	-	-	-
cis-Carveol	1236	1858	1229	1854.4	0.76	1.05	0.96	1.00	0.87	0.85	0.56	0.81	-	-	-	0.21
Neral	1239	1676	1240	1678.5	-	-	-	-	-	-	-	-	1.90	1.77	0.41	0.50
Carvone	1249	1732	1243	1733.6	1.65	0.62	1.84	2.95	1.51	2.62	1.38	2.81	-	0.27	0.33	0.75
Geraniol	1249	1839	1255	1839.3	-	0.30	-	0.24	-	0.22	-	0.25	0.22	0.27	-	0.19
NI	1261	-	-	-	-	-	-	-	-	-	-	-	0.10	-	-	-
Geranial	1267	1724	1270	1725	-	-	-	-	-	-	-	-	1.69	-	0.29	0.55
1,5-Hexadiene, 2,5-dimethyl-3-methylene-	1271	-	-	-	-	-	0.05	-	-	-	-	-	-	-	-	-
NI	1274	1746	-	-	0.11	0.43	0.14	0.40	0.19	0.30	0.13	0.26	-	-	-	-
cis-Carvone oxide	1279	-	1263	-	-	-	0.10	-	-	-	-	-	-	-	-	-
Perilla aldehyde	1281	1781	1273.4	1793.9	0.24	0.25	0.18	0.21	0.20	0.21	0.23	0.22	-	-	0.23	0.28
NI	1289	1947	-	-	0.45	-	0.51	0.47	0.37	0.43	0.47	0.42	-	-	-	
Limonen-10-ol	1294	1985	1239	1979	0.15	0.17	0.22	0.16	0.16	0.20	0.17	0.19	-	-	0.23	0.32
NI	-	1979	-	-	-	0.08	-	-	-	-	-	-	-	-	-	-

(R)-4-methyl-3-(3'-oxobutyl)pent-4-enal	-	1947	-	-	-	0.55	-	-	-	-	-	-	-	-	-	-
Perilla alcohol	1303	1995	1296.3	2006.6	0.26	0.19	0.38	0.17	0.28	0.16	0.28	0.14	-	-	-	-
Cyclohexene, 2-ethenyl-1,3,3-trimethyl-	1308	-	-	-	1.48	-	1.51	-	1.28	-	1.45	-	0.15	-	0.62	-
Limonene dioxide	1312	-	1294	-	-	-	0.07	-	-	-	-	-	-	-	-	-
(1S,4R)-p-Mentha-2,8-diene, 1-hydroperoxide	1322	-	-	-	-	-	-	-	-	-	-	-	-	-	0.33	-
NI	1322	-	-	-	1.41	-	1.38	-	1.18	-	1.25	-	0.26	-	-	-
NI	1335	-	-	-	0.96	-	1.02	-	0.86	-	0.94	-	-	-	-	-
NI	1347	-	-	-	-	-	-	-	-	-		-	0.24	-	-	-
Limonene diol	1349	-	1321	-	-	-	-	-	-	-		-	-	-	0.27	-
NI	1349	-	-	-	-	-	0.17	-	-	-	0.33	-	-	-	-	-
NI	1355	-	-	-	0.25	-	0.33	-	0.36	-	0.40	-	-	-	0.32	-
Neryl acetate	1357	1716	1365	1718.1	-	-	-	-	-	-		-	2.57	1.98	-	-
Decanoic acid	1360	-	1375.5	-	-	-	-	-	-	-		-	-	-	0.35	-
NI	1361	-	-	-	1.52	-	1.47	-	1.12	-	1.31	-	0.19	-	-	-
NI	1367	-	-	-	0.27	-	0.26	-	0.24	-	0.21	-	-	-	-	-
Geranyl acetate	1376	1745	1381	1751	-	-	-	-	-	-	-	-	0.72	0.48	-	-
NI	1377	-	-	-	1.18	-	1.18	-	0.91	-	1.09	-	-	-	0.36	-
α-Copaene	1385	1498	1377	1491	-	-	-	-	0.14	0.30	0.10	0.34	-	-	0.45	2.59
β-Bourbonene	1394	1525	1384.2	1523.2	-	-	-	-	-	-	-	-	0.11	-	-	0.13
β-cubebene	1396	1542	1386.6	1541.7	-	-	-	-	-	-	-	-	-	-	0.59	0.23
β-elemene	1396	1592	1390.4	1590.9	-	-	-	-	-	-	-	-	0.11	0.10	-	0.15
Dodecanal	1409	1708	1407	1711.5	-	-	-	-	-	-	0.19	-	-	-	0.76	0.71
cis-α-Bergamotene	1420	1553	1414.5	1559.1	-	0.63	-	0.64	-	0.59	-	0.54	0.16	0.16	-	0.32
β-Caryophyllene	1433	1603	1428	1598.5	-	-	-	-	-	-	-	-	0.41	-	0.24	0.33
trans-α-Bergamotene	1440	1560	1434.5	1575.7	-	0.36	-	0.39	-	0.33	-	0.31	1.98	-		0.13
β-Copaene	1442	1599	1432	1579.8	-	-	-	-	0.10	-	0.11	-	-	-	0.52	0.48
α-Himachalene	1444	-	1445.1	-	-	-	-	-	-	-	-	-	0.08	-	-	
cis-β-Farnesene	1454	1657	1445.9	1651.4	-	-	-	-	-	-	-	-	0.11	0.10	-	0.13
β-Santalene	1468	-	1462	-	-	-	-	-	-	-	-	-	0.16	-	-	-
trans-β-Farnesene	1492	-	1455.9	-	-	-	-	-	-	-	-	-	0.11	-	-	-
β-Selinene	1496	-	1485	-	-	-	-	-	0.11	-	-	-	-	-	-	-
cis-α-Bisabolene	1503	-	1503.1	-	-	-	-	-	-	-	-	-	0.36	-	-	-
Velencene	1504	1721	1491	1728.6	-	-	-	-	1.84	1.21	0.31	0.18	-	-	1.57	0.99
β-Bisabolene	1511	1723	1509	1727.5	-	-	-	-	-	-	-	-	3.07	3.60	-	-

δ-Cadinene	1526	1753	1523.2	1763.3	-	-	-	-	-	-	-	-	-	-	0.40	0.41
α-7-Epi-selinene	1534	-	1540	-	-	-	-	-	0.09	-	-	-	-	-	-	-
Elemol	1557	-	1549	-	-	-	-	-	-	-	-	-	-	-	0.26	-
Caryophyllene oxide	1597	1987	1583	1986.2	-	-	-	-	0.10	-	-	-	0.31	0.18	0.32	-
NI	1639	-	-	-	-	-	-	-	-	-	-	-	0.20	-	-	-
α-Selin-11-en-4-ol	1672	-	1654.9	-	-	-	-	-	-	-	-	-	0.21	-	-	-
NI	1683	-	-	-	-	-	-	-	-	-	-	-	0.12	-	-	-
α-Bisabolol	1694	-	1683	-	-	-	-	-	-	-	-	-	0.23	-	-	-
β-Sinensal	1697	-	1694	-	-	-	-	-	-	-	-	-	-	-	0.42	-
Nootkatone	1822	-	1823	-	-	-	-	-	-	-	-	-	-	-	0.35	-
Hexadecanoic acid	1955	-	1968.4	-	-	-	-	-	-	-	-	-	-	-	0.79	-
Linoleic acid	2127	-	2128.9	-	-	-	-	-	-	-	-	-	-	-	0.45	-
trans-13-Octadecenoic acid	2132	-	-	-	-	-	-	-	-	-	-	-	-	-	0.70	-
Eicosane	2259	-	-	-	-	-	-	-	-	-	-	-	-	-	0.18	-
Tricosane	2298	-	2300	-	-	-	-	-	-	-	-	-	-	-	0.27	-
Docosane	2373	-	-	-	-	-	-	-	-	-	-	-	-	-	0.15	-
Bicyclo[3.3.0]oct-2-en-7-one, 6-methyl-	-	1694	-	-	-	0.12	-	0.13	-	0.28	-	0.26	-	-	-	-
NI	-	1708	-	-	-	-	-	-	-	-	-	0.21	-	-	-	-
NI	-	1571	-	-	-	-	-	-	-	-	-	-	-	0.17	-	-
NI	-	1587	-	-	-	-	-	-	-	-	-	-	-	1.44	-	-
TOTAL					100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

*OOPE = Orange oil phase essence, OPO = Orange peel oil, BOT = Brazilian orange terpenes, TLOP = Tahiti lime oil phase, OPOFF = Orange peel oil five fold and CT = Citrus terpenes.

[1] Identification by GC/MS using non-polar and polar columns

[2] LRI_C: Linear retention index (Calculated)

[3] LRI_L: Linear retention index (Literature)

[4] NP: non-polar column DB-5MS

[5] P: polar column DB-WAX

[6] Relative amounts of the identified compounds based on the area of each peak in the chromatogram.

NI: Not identified compound

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3. *IN VITRO* MECHANISM OF ANTIBACTERIAL ACTION OF A CITRUS ESSENTIAL OIL ON AN ENTEROTOXIGENIC *Escherichia coli* AND *Lactobacillus rhamnosus*[2]

Abstract

Citrus essential oils (EOs) are well recognized for their antimicrobial properties. These oils, common by-product of citrus processing industries, could be an excellent alternative to synthetic antibiotics used to fight pathogenic bacteria affecting food-producing animals and their spreading through the food chain. Nevertheless, it is important a further understanding about the mechanism of antibacterial action displayed by citrus EOs. Therefore, this study investigated the *in vitro* mechanism of action of a commercial citrus EO, Brazilian orange terpenes (BOT), on an enterotoxigenic *Escherichia coli* (ETEC) and on *Lactobacillus rhamnosus*. initially, bacteria were exposed sequentially to BOT every 3 h (three times) at sub-MICs and results showed that sequential exposure to BOT provoked a higher reduction of bacteria viability than a single exposure and the reduction of ETEC viability was higher compared to that of *L. rhamnosus*. Then, evaluation of the BOT effects on the cell membrane permeability and integrity, indicated that BOT oil increased the membrane permeability and possibly caused disruptive effects on the integrity of bacterial cells as reflected by a significant increase of the relative electric conductivity (electrolytes leakage) and the release of essential cell constituents. These effects were more obvious with increasing the BOT concentration and, interestingly, BOT effects were more pronounced on the ETEC than on *L. rhamnosus* cells. This was ratified by scanning electron microscopy (SEM), which showed more noticeable morphological damages and disturbances on ETEC cells than *L. rhamnosus* cells when BOT concentration was increased. Finally, limonene was detected as the major compound in BOT by polar/non-polar GC-MS (78.65% / 79.38%).

Keywords: Pig breeding; Antibiotic substitutes; Food safety; Essential oils: Lactobacilli

3.1. Introduction

Enterotoxigenic *Escherichia coli* (ETEC) infection, also called postweaning enteric colibacillosis, is the most common disease affecting pig health in early life. At weaning period, the undeveloped gut microbiota, the sudden dietary and environmental changes are key factors to turn piglets highly susceptible to ETEC infection, which lead to a diarrhea status in these animals (Fairbrother et al., 2005; Gresse et al., 2017). The main virulence factors of ETECs are the adhesins, which allow ETECs to adhere to receptors on the small intestine epithelium, and the enterotoxins that they produce (the heat-labile toxin (LT) and heat-stable toxin (ST)), which increase the fluids and electrolytes secretion of the small intestine epithelial cells (Nagy and Fekete, 2005). As results of ETEC infection, piglets often

[2] Chapter adapted from:
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decrease weight gain and can suffer a sudden death, which produces significant losses to the pig breading sector. For this reason, since the 1950s, antibiotics administration has become a normal practice. However, besides of their therapeutic use, antibiotics have also been used as growth promoting agents and, therefore, has been continuously added to the pig feed in order to improve growth (Teillant et al., 2015).

Nowadays, it is well known that probably there is a relationship between the growing emergence and spreading of antibiotic-resistant bacteria with the overuse of antibiotics as growth promoter agents in farm animals, which has turned as a public health threat (Vanderhaeghen and Dewulf, 2017). A study has revealed that there is a high correlation between the use of several antibiotics and the prevalence of resistance to them in gut commensal bacteria from farm animals (Chantziaras et al., 2014). Subsequently, other studies have reported key evidence of transferring antibiotic resistance from farm animals to humans (Marshall and Levy, 2011a). Furthermore, it has been found that farm animals and farm workers, such as pig farmers, habitually are harbors of a high percentage of resistant bacteria in their gut, probably, as consequence of the direct contact. This fact turns these people a conduit to introduce resistant genes to the community (Aubry-Damon et al., 2004; Hummel et al., 1986). In the same way, the consumption of food animal products (fermented, minimally processed, or raw foods) from farm animals fed with antibiotics could represent another direct or indirect way of transferring resistant bacteria and their genes to humans (Economou and Gousia, 2015; Marshall and Levy, 2011b), such as *E. coli* resistant strains (Lei et al., 2010; Liu et al., 2016; Martínez-Vázquez et al., 2018). In view of this threat and to keep up the productivity of pig breeding sector due to the prohibition of the use of antibiotics as growth promoters since 2006 by the European Union Regulation (EU, 2003), the search for alternatives to replace or to reduce the use of antibiotics has become a real necessity.

Essential oils (EOs) have emerged as promissory alternative to synthetic antibiotics because of their well-known antimicrobial properties. The application of citrus EOs, one of the most produced and commercialized type of EOs, could become financially feasible because of the huge offer of these oils in the global market. Currently, Brazil is one of the largest producing countries. Specifically, Brazil is the major orange oils exporters globally (Barbieri and Borsotto, 2018; Iwabuchi et al., 2010). To date, in 2017 and 2018, Brazilian exportations of orange EOs reached 27.17 and 26.95 mil tons, respectively (Citrus Br, 2019; United Nations, 2019).

On the other hand, lactobacilli are normal inhabitant and the most abundant bacteria in pig gut. They play an important role in the pig gut health as lactobacilli are able to combat pathogen colonization through competitive exclusion, production of antibacterial compounds and by regulating the intestinal mucosa immunity by increasing antibody levels (Dowarah et al., 2017; Yang et al., 2015). However, when either antibiotics or EOs are administrated to

pigs, they also can reach these beneficial gut bacteria and affect their growth and functions. Nonetheless, it has been reported that citrus EOs present a selective antibacterial activity, causing lower effects on the growth kinetics of *Lactobacillus* sp. than ETECs (Ambrosio et al., 2019). Hence, it is important a further understanding of the possible mechanism underlying the mode of antibacterial action of citrus EOs on *Lactobacillus,* but also on pathogenic such as ETECs. Therefore, the aim of this study was to investigate the *in vitro* antibacterial mechanism of action of a commercial citrus EO, Brazilian orange terpenes, on an ETEC and on a *Lactobacillus* strain.

3.2. Material and methods

3.2.1. Essential oils supply

A commercial citrus EOs was used in this study, Brazilian orange terpenes (BOT), which was a by-product from orange juice production and was supplied by a factory located in São Paulo State, Brazil. The sample was kept in amber bottles under refrigeration (4 °C) until use.

3.2.2. Bacterial strains

An ETEC strain and a *Lactobacillus* species were evaluated in this study as models of pathogenic and beneficial bacteria, respectively. The ETEC strain, *E. coli* U21 (K88+/LT+/STb+/F18+/Sta+), was isolated from pig gut and provided by The Swine Heath Laboratory of the Department of Preventive Veterinary Medicine and Animal Health from School of Veterinary Medicine and Animal Science from the University of São Paulo, Brazil. *Lactobacillus rhamnosus* ATCC 7469 was purchased from American Type Culture Collection (ATCC). The ETEC strain was cultivated in Tryptic Soy Agar-Difco (TSA) at 37 ºC for 18-20 h and *L. rhamnosus* was grown in MRS agar (Man, Rogosa and Sharpe agar) at 30 °C for 48 h. After activation, the bacteria were sub-cultured in Brain-Heart Infusion (BHI) broth or MRS both supplemented with 15% v/v of glycerol. After incubation, they were stored at -20 ºC until their use.

3.2.3. Antibacterial mechanism

For evaluating the antibacterial mechanism of BOT, in view of the proved antibacterial activity of this EO as reported by (Ambrosio et al., 2019), the Minimal Inhibitory Concentration (MIC) of this EO on *E. coli* U21 was considered as the basis for this study.

Reduction of Cell viability after Sequential exposure (SE) to EO

The effect of the sequential exposure (SE) to the BOT oil on the bacterial cell viability was evaluated in order to simulate *in vitro* the periodic exposure that gut bacteria would undergo every time that pigs fed on diets containing the EO this was carried out following the approach described by Ambrosio et al. (2018). The SE was carried out considering the MIC of BOT for *E. coli* U21 (1.85 mg/mL) that was previously determined by Ambrosio et al. (2019). For this assay, *E. coli* U21 and *L. rhamnosus* (both at ~10^6 CFU/mL) were exposed, sequentially, every 3 h to sub-MICs (MIC/4 and MIC/2) of the BOT oil. In total, bacteria underwent three exposures every 3 h. This means that every 3 h the BOT solution (at either MIC/4 or MIC/2) with bacteria was centrifuged, the supernatant (BOT solution) discarded and the BOT solution renewed on the remaining bacterial population decanted after centrifugation. Therefore, after each exposure (every 3 h: first = 0–3 h, second = 3–6 h, third = 6–9 h) an aliquot was taken, and bacterial viability was measured by CFU counting from 10-fold serial dilutions in sterile peptone and plated on MH agar (*E. coli* U21) or MRS agar (*L. rhamnosus*). In order to compare the effect of the SE with a single exposure on bacterial cell viability, a single exposure assay was performed separately. Both bacteria received only a single dose of the BOT oil solution (either MIC/4 or MIC/2) at the beginning of the experiment, which was left to react for 9 h. Nevertheless, an aliquot was taken every 3 h and the bacterial cell viability was measured by CFU counting as described above, to evaluate the reduction produced by the single exposure at 3 h, 6 h and 9 h. Controls (without BOT oil) were done for the sequential exposure and single exposure procedures. Three independent replicates were performed.

Permeability of cell membrane

The permeability of bacterial cell membrane was expressed in terms of the relative electric conductivity according to the method described by Diao et al. (2014) with slight modifications. For this assay, *E. coli* U21 was cultured in BHI broth and *L. rhamnosus* was cultured in MRS broth and incubated at 35 °C for 11 h. Then, bacteria suspensions were centrifuged at 5000 ×g for 10 min and washed with 5% glucose solution until their electric conductivities was near to that of the 5% glucose solution itself, and they were the case for isotonic bacteria. The electric conductivity was measured with an electrical conductivity meter. BOT was added to a 5% glucose solution at three different concentrations (2×MIC, MIC, MIC/2) and the electric conductivities of the mixtures (BOT solutions) were measured and denoted as L_1. Then, the different concentrations of BOT were added to the isotonic bacteria solutions (*E. coli* U21 or *L. rhamnosus*) and were mixed. Thereafter, the samples were incubated at 35 °C for 9 h, and

the conductivities were measured after 1 h, 3 h, 6 h and 9 h, and denoted as L_2. The conductivity of bacteria in 5% of glucose solution treated in boiling water for 5 min was used as control and denoted as L_0. The permeability of the bacterial cell membrane was calculated according to the Equation 1 (Diao et al., 2014; Zhang et al., 2016):

$$Relative\ electric\ conductivity\ (\%) = \frac{L_1 - L_2}{L_0} \times 100$$

Integrity of cell membrane

The integrity of cell membrane of *E. coli* U21 and *L. rhamnosus* was evaluated by measuring the release of cell constituents including proteins and reducing sugars into the supernatant of cell suspensions according to the method described by Diao et al. (2014) with some modifications. Bacterial cells from 45 mL working culture of tested bacteria (cultured overnight) were collected by centrifugation at 5000 ×g for 15 min, washed three times and resuspended in 5 mL of phosphate buffered saline (PBS, pH 7.4). Forty-five milliliters of BOT at different concentrations (MIC/2, MIC, 2×MIC) were added to the bacterial cell suspensions, shacked and incubated at 35 °C for 6 h. Then, the suspensions were centrifuged for 5 min at 10000 ×g and the levels of proteins and reducing sugars in the supernatant were determined according to the Bradford's method (Bradford, 1976) and Somogyi-Nelson method, respectively (Nelson, 1944). Untreated sample (no EO addition) was used as a control.

Scanning electron microscopy (SEM)

To determine the morphological changes that the different concentrations of BOT oil provoked on *E. coli* U21 and *L. rhamnosus* cells, SEM was performed. The bacterial cells were grown either in BHI (*E. coli* U21) or MRS (*L. rhamnosus*) for 11 h at 3 5°C, centrifuged and resuspended in different BOT concentrations (MIC/2, MIC, 2×MIC). In addition, a control treatment was performed without EO treatment. Next, the bacterial cells suspensions were incubated at 35 °C for 3 and 6 h, respectively. After incubation, suspensions were centrifuged at 5000 ×g for 10 min, the bacterial cells (pellet) were washed twice with PBS and fixed in 2.5% glutaraldehyde (v/v) for 24 h at 4°C. Thereafter, the cells were dehydrated by a sequential exposure at ethanol concentrations 30%, 50%, 70%, 90% and 100%. Then, cells were dried at “critical point” in liquid CO_2 under 95 bar, and samples were gold-covered (30 nm of layer) by cathodic spraying. Finally, the morphology of the bacterial cells was

observed using a scanning electronic microscope (LEO 435 VP, Leo Electron Microscopy Ltd., Cambridge, England).

3.2.4. Chemical composition

The chemical composition profile of BOT was determined by gas chromatography coupled with mass spectrometry (GC/MS) using non-polar and polar columns as described by (Ambrosio et al., 2019).

3.2.5. Data analysis

All experiments, except for the SEM, were performed in triplicate. Data were expressed as the mean ± SD. One-way analysis of variance (ANOVA) and Tukey's test for pairwise comparison at 5% of significance were used to detect significant differences between EO concentrations and time of treatment using R software. Student t-test was performed in order to detect significant differences between bacteria using R software.

3.3. Results

3.3.1. Antibacterial mechanism

Reduction of Cell viability after Sequential exposure (SE) to EO

The comparison of the sequential exposure and single exposure to BOT oil on *E. coli* U21 and *L. rhamnosus* using sub-MIC is shown in Figure 3.1. The single exposure results showed only a bacteriostatic effect on *E. coli* U21 within 6 h of treatment at either MIC/2 or MIC/4, and a loss of this effect was observed afterward (Fig. 3.1A and 3.1C). When *E. coli* U21 was exposed sequentially to BOT, a drastic reduction of its viability was observed in contrast to a single exposure. In fact, MIC/4 and MIC/2 decreased the *E. coli* U21 population in ~2.9 log and ~4.5 log, respectively, at the end of the trial (9 h). In addition, a dose-dependent effect in the sequential exposure trial was found in a way that MIC/2 caused higher reduction on *E. coli* U21 viability than MIC/4 from the second exposure. MIC/4 only caused a reduction of ~1.3 log after the second exposure (3-6 h). Conversely, MIC/2 already reduced the initial *E. coli* U21 population in ~2.1 log after the second exposure (3-6 h) and in ~3.0 log after the third exposure (6-9 h). Regarding the *L. rhamnosus* viability, the sequential exposure to BOT

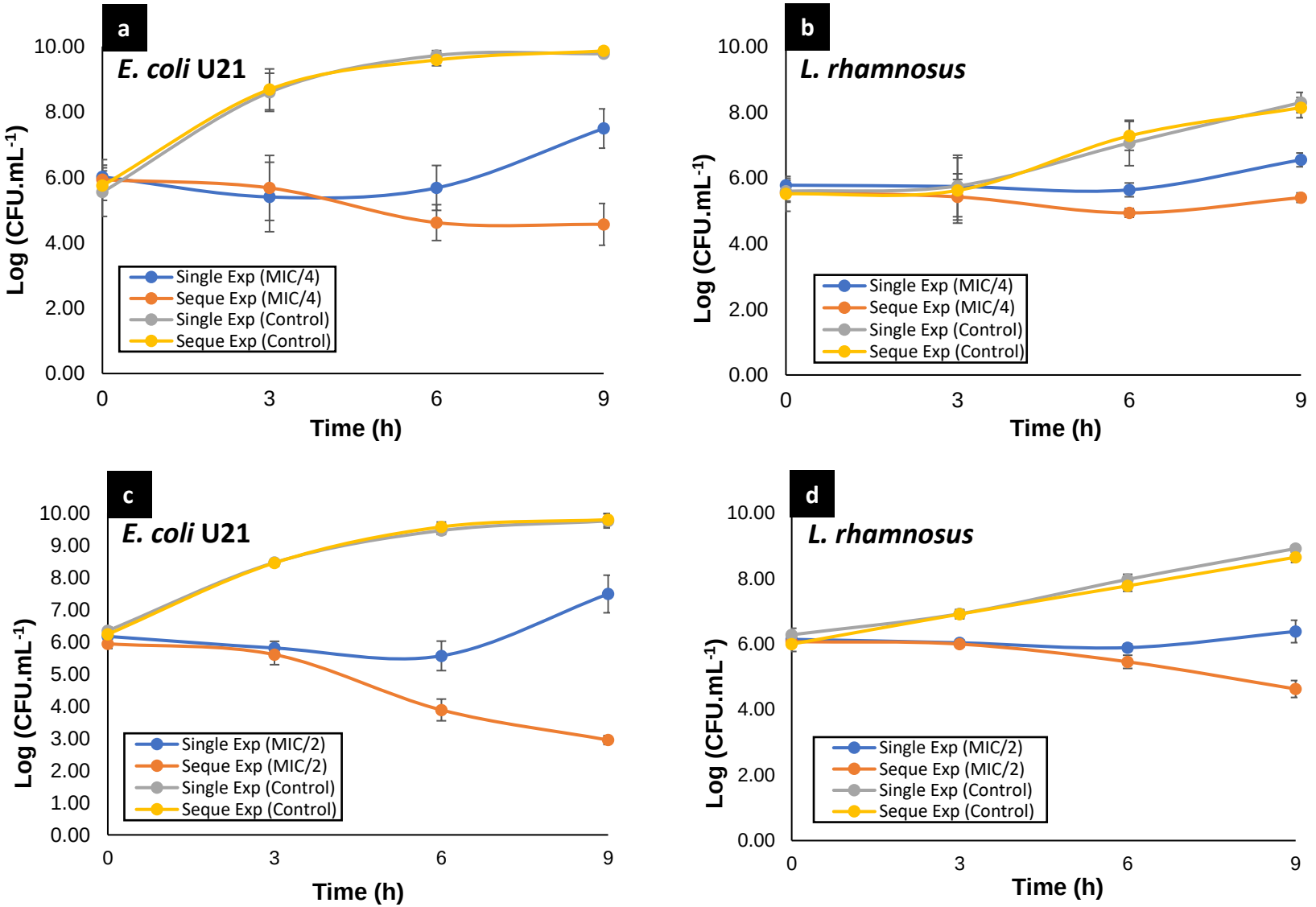

Figure 3.1. Effect of sequential exposure to BOT oil on *E. coli* U21 (A, C) and *L. rhamnosus* (B, D) at MIC/4 and MIC/2 in contrast to a single exposure.

provoked slightly higher reduction compared to the single exposure, (Fig. 3.1B and 3.1D). At the end of the trial (9 h), MIC/4 and MIC/2 reduced the *L. rhamnosus* population in ~1.1 log and ~1.7 log, respectively. Thus, the sequential exposure effect was lower on this bacterium than on *E. coli* U21. As observed in figures 3.1B and 3.1D, there was also a dose-dependent effect on the reduction of *L. rhamnosus* viability in a way that MIC/2 caused slightly higher reduction than MIC/4. For instance, after the second exposure MIC/4 and MIC/2 reduced ~0.6 log of the initial *L. rhamnosus* population and the population was not decreased after subsequent exposures by MIC/4, but MIC/2 caused an additional reduction of ~0.8 log after the third exposure (6-9 h).

Therefore, the sequential exposure to BOT oil was higher efficient in reducing the bacterial viability compared to a single exposure. The sequential exposure had a higher effect on *E. coli* U21 compared to *L. rhamnosus*. These results suggest that probably the application of sub-MICs to the animal feed could be suitable to decrease pathogenic bacteria without noticeable effects on beneficial bacteria in the pig gut, since gut bacteria would be in contact with the antimicrobial agent every time that animals ingest their feedstuffs.

Permeability of cell membrane

Relative electric conductivity was evaluated to express permeability changes of *E. coli* U21 and *L. rhamnosus* cell membranes as an effect of BOT treatment (Fig. 3.2). Firstly, a slight increase of the relative electric conductivity in the control treatment of both bacteria was observed, probably, associated with the normal lysis and death of bacteria. In overall, results revealed that the electric conductivity for *E. coli* U21 was higher in contrast to *L. rhamnosus*. Furthermore, electric conductivity increased over the exposition time to BOT oil and there was a significant dose-dependent increase of the electric conductivity for *E. coli* U21 ($p < 0.001$). Thus, higher relative electric conductivity was found at the highest BOT concentration. It is noteworthy that when *E. coli* U21 was exposed to MIC/2 and MIC after 6 h no further increase of the electrical conductivity was observed. However, 2×MIC increased the electrical conductivity in the course of time. Regarding *L. rhamnosus*, there was no significant effect on the electric conductivity in response to the BOT concentrations ($p < 0.001$). However, BOT oil increased the electric conductivity compared to the control. Moreover, there was a decrease in the electric conductivity up to 6 h of treatment with the BOT oil. This effect is probably related to the viability recovery of *L. rhamnosus*. Therefore, BOT oil affected the membrane permeability of both bacteria, but provoked higher effect on *E. coli* U21 than on *L. rhamnosus* at the EO concentrations tested and with increasing the treatment time. Thus, meaning that BOT oil was able to produce higher cell lysis, followed by

higher leakage of intracellular components, such as electrolytes (Na^+, K^+, Ca^{2+}, and others) and, consequently, higher death of the *E. coli* U21 cells than *L. rhamnosus* cells.

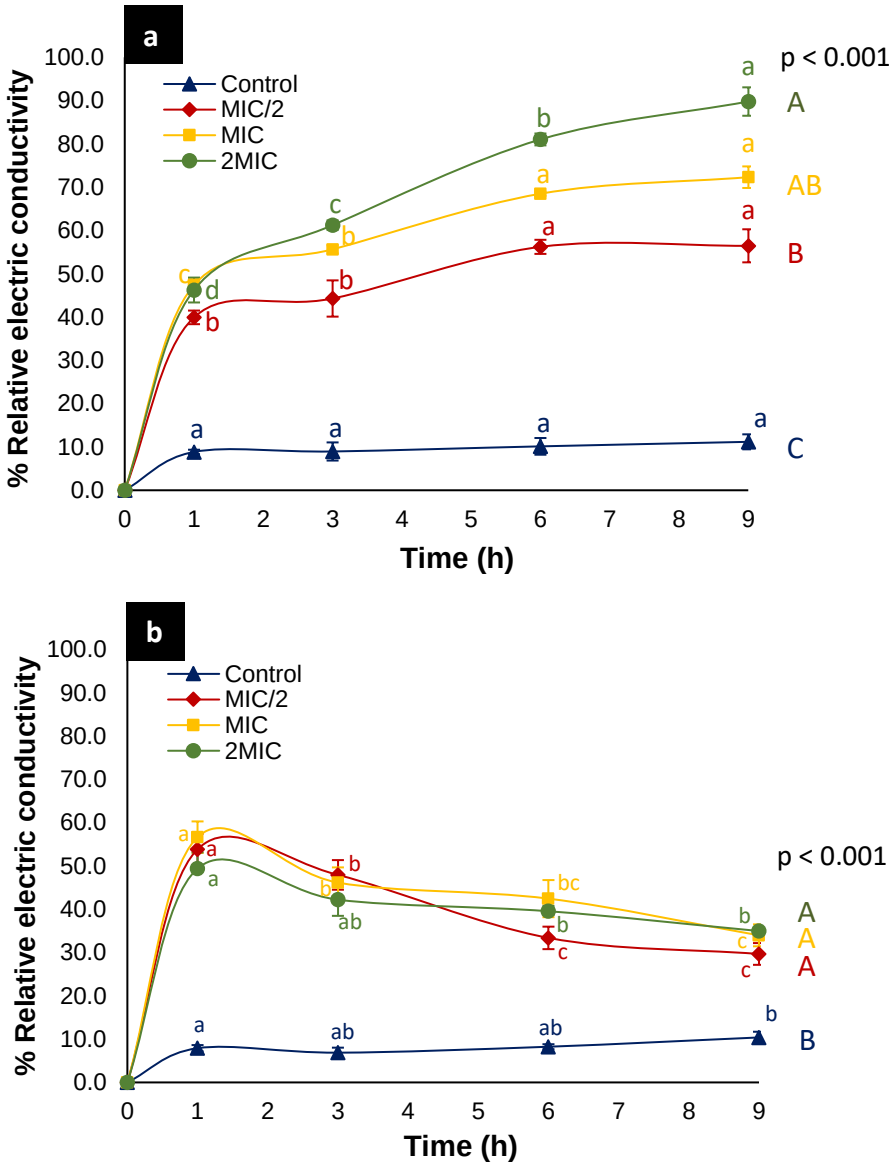

Figure 3.2. Effects of BOT oil on cell membrane permeability of *E. coli* U21 (A) and *L. rhamnosus* (B).

Integrity of cell membrane

The integrity of cell membrane in response to the treatment with BOT oil was determined by measuring the release of cell constituents, e.g. reducing sugars and proteins, in the supernatant of *E. coli* U21 and *L. rhamnosus,* which is shown in Table 3.1. The results revealed that the release of reducing sugars and proteins increased significantly as the BOT concentration was increased. However, BOT oil caused a significant higher release of

reducing sugars (p = 0.016) for *E. coli* U21 compared to *L. rhamnosus*. For *E. coli* U21, BOT oil increased significantly the release of reducing sugars by 3.9, 2.4 and 1.6 times at 2×MIC, MIC and MIC/2, respectively. For *L. rhamnosus*, BOT oil increased the release of reducing sugars by 2.4 and 1.2 times at 2×MIC and MIC, respectively. Regarding to release of proteins, no significant (p = 0.085) difference was observed between *E. coli* U21 and *L. rhamnosus*. Nonetheless, compared to the control, the release of proteins was increased significantly by 7.1, 3.2 and 1.8 times at 2×MIC, MIC and MIC/2 in *E. coli* U21, respectively. Regarding *L. rhamnosus*, BOT oil increased the release of proteins by 3.8, 2.0 and 1.2 times at 2×MIC, MIC and MIC/2, respectively. Therefore, these results show irreversible damages of the cytoplasmic membrane in response to the BOT oil treatment.

Table 3.1. The effect of the Brazilian orange terpenes oil on the release of cell constituents of tested bacteria*.

BOT level	Proteins (µg/mL)				Reducing sugar (µg/mL)			
	E. coli U21		*L. rhamnosus* ATCC 7469		*E. coli* U21**		*L. rhamnosus*** ATCC 7469	
Control	16.9±0.6		38.7±5.0		94.5±4.7		106.9±15.8	
2×MIC	120.8±11.8	(7.1±0.5)^{a}	142.3±20.0	(3.8±0.9)^{a}	371.2±18.5	(3.9±0.2)^{a}	256.1±9.5	(2.4±0.4)^{a}
MIC	54.1±3.9	(3.2±0.3)^{b}	77.9±1.7	(2.0±0.3)^{b}	226.1±19.3	(2.4±0.2)^{b}	124.0±20.5	(1.2±0.3)^{b}
MIC/2	29.9±1.9	(1.8±0.1)^{c}	46.9±5.3	(1.2±0.2)^{b}	150.6±8.4	(1.6±0.1)^{c}	77.2±8.9	(0.7±0.1)^{b}

* Values are means ± Standard Deviation (SD) of triplicate determinations. Values in brackets are the ratios of cell constituent 'release regarding to the control.
a-b: Mean values within a column having different superscripts are significantly different (BOT concentrations) by the least significant difference Tukey test (p < 0.05).
*indicate significant difference between bacteria (p < 0.05) based on Student's t-test.

Scanning electron microscopy (SEM)

E. coli U21 and *L. rhamnosus* were treated with BOT oil at MIC/2, MIC and 2×MIC for 3 h and 6 h, then morphological and physical changes were observed by SEM. Fig. 3.3 shows the SEM images of treated and untreated bacteria (Control). The imagens illustrated a higher destructive effect of BOT oil on *E. coli* U21 compared to *L. rhamnosus* cells. In comparison with the control, *E. coli* U21 treated with BOT, cells presented morphological changes, such as deformation, wrinkling and wilting, shrinking, corrugation, attachment to other cells, membrane break and collapse (Fig. 3.3 on the left side), which could have caused the leakage of cell constituents and genetic material. These changes were more evident with the increasing of BOT concentrations and treatment length. Regarding *L. rhamnosus*, no remarkable morphological changes were observed compared to the control. However, *L. rhamnosus* cells were slightly wrinkled, corrugated and in some cases, cells were perforated, minced and broken when the highest BOT concentration (2×MIC) was tested. Therefore,

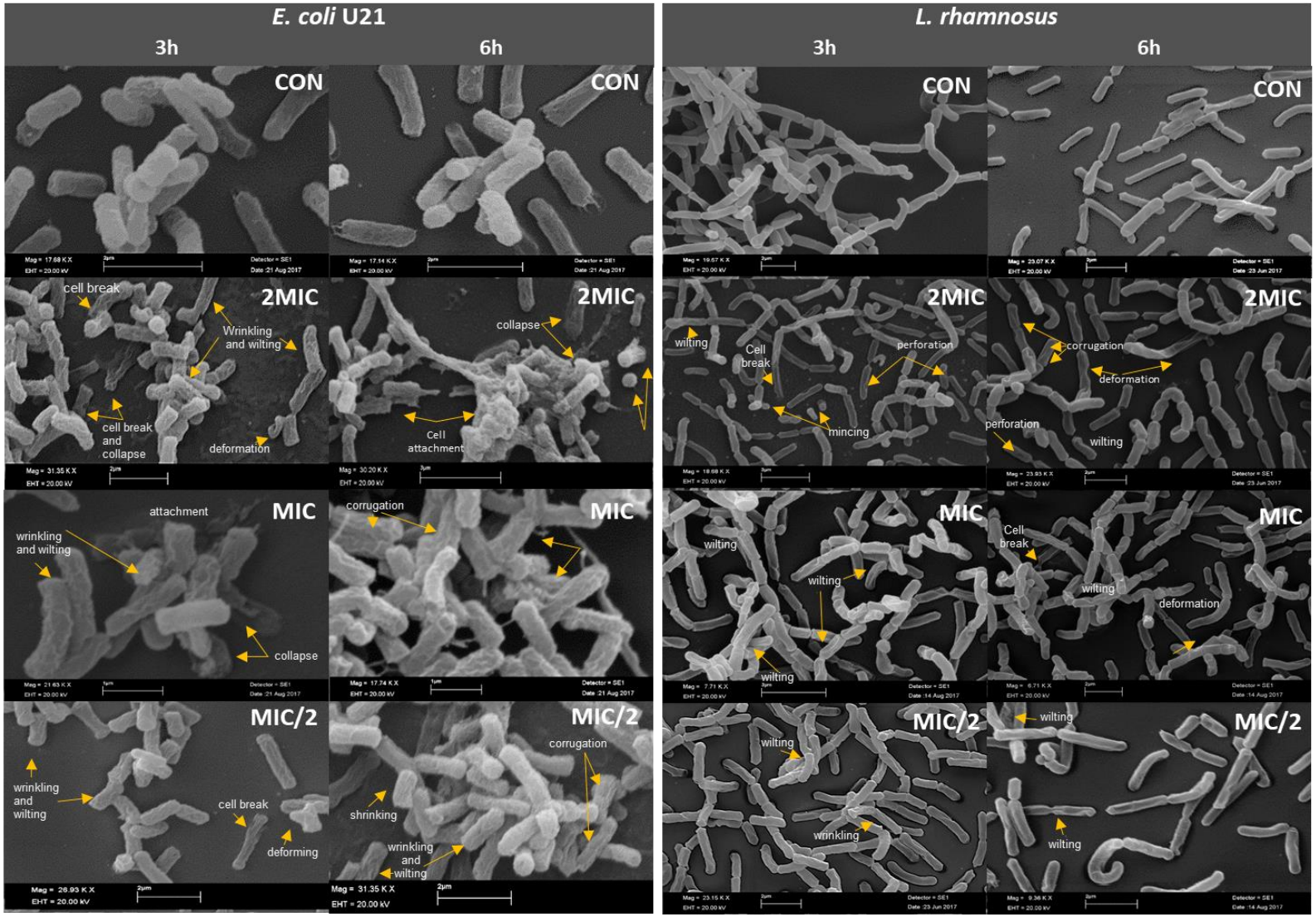

Figure 3.3. Scanning electron microphotographs of *E. coli* U21 and *L. rhamnosus* untreated (control) and treated with different BOT concentrations (MIC/2, MIC and 2×MIC) during 3 and 6 h.

SEM observations showed that BOT oil might cause noticeable higher severe effects on the cell envelope (cell wall and cytoplasmic membrane) of *E. coli* U21 compared to the effects on *L. rhamnosus*. This result supports the observations of permeability and integrity of cell membrane for both bacteria.

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Chemical composition of BOT oil

The chemical composition profile of BOT oil is shown in the Table 3.2 (data of this table were part of supplementary material by (Ambrosio et al., 2019). Results using polar and non-polar column showed that limonene (78.65%, 79.38%) was the major compound present in the BOT oil followed by minor compounds such as *cis*-limonene oxide, *trans*-limonene oxide, *trans*-carveol, carvone, *trans*-p-mentha-2,8-dien-1-ol, myrcene, *cis*-p-mentha-2,8-dien-1-ol and *cis*-carveol, among others.

Table 3.2. Chemical composition Brazilian orange terpenes essential oil*.

Compounds[1]	LRI_c[2]		LRI_L[3]		BOT %[6]	
	NP[4]	P[5]	NP	P	NP	P
α-pinene	936	1020	939	1025.4	0.45	0.73
Sabinene	975	1118	976	1122	0.32	0.47
β-pinene	981	1106	980	1100	0.10	0.13
Myrcene	989	1158	991	1160.2	0.85	1.41
Octanal	1002	1285	1002.8	1287.2	0.20	0.01
δ-3 Carene	1011	1146	1011	1146.8	0.11	0.14
NI	1028	-	-	-	0.29	-
β-Phellandrene	-	1208	-	1209.3	-	0.10
Limonene	1037	1202	1031	1198.2	78.65	79.38
Linalool	1099	1538	1098	1543.3	0.28	0.36
trans-p-Mentha-2,8-dien-1-ol	1125	1622	1123	1639	0.89	2.08
NI	-	1633	-	-	-	0.19
cis-p-Mentha-2,8-dien-1-ol	-	1664	-	1652.1	-	1.39
4-Acetyl-1-methylcyclohexene	1134	-	1139.3	-	0.11	-
cis-Limonene oxide	1137	1446	1134	1450.5	1.95	1.89
trans-Limonene oxide	1140	1459	1139	1461.6	1.64	0.90
1,8-menthadien-4-ol	1183	1680	1189	1681	0.17	0.46
Cryptone	-	1674	-	1674.8	-	0.13
trans-p-Mentha-1(7),8-dien-2-ol	1192	1789	1180.5	1791	-	0.27
NI	1192	1732	-	-	0.25	2.75
NI	1201	-	-	-	0.44	-
NI	1204	-	-	-	0.65	-
trans-Carveol	1222	1828	1217	1836.3	1.82	2.29
cis-p-Mentha-1(7),8-dien-2-ol	1233	1879	1233	1894.9	0.14	0.16
cis-Carveol	1236	1858	1229	1854.4	0.76	1.05
Carvone	1249	1732	1243	1733.6	1.65	0.62
Geraniol	1249	1839	1255	1839.3	-	0.30
NI	1274	1746	-	-	0.11	0.43

cis-Carvone oxide	1279	-	1263	-	-	-
Perilla aldehyde	1281	1781	1273.4	1793.9	0.24	0.25
NI	1289	1947	-	-	0.45	-
Limonen-10-ol	1294	1985	1239	1979	0.15	0.17
NI	-	1979	-	-	-	0.08
(R)-4-methyl-3-(3'-oxobutyl)pent-4-enal	-	1947	-	-	-	0.55
Perilla alcohol	1303	1995	1296.3	2006.6	0.26	0.19
Cyclohexene, 2-ethenyl-1,3,3-trimethyl-	1308	-	-	-	1.48	-
NI	1322	-	-	-	1.41	-
NI	1335	-	-	-	0.96	-
NI	1355	-	-	-	0.25	-
NI	1361	-	-	-	1.52	-
NI	1367	-	-	-	0.27	-
NI	1377	-	-	-	1.18	-
cis-α-Bergamotene	1420	1553	1414.5	1559.1	-	0.63
trans-α-Bergamotene	1440	1560	1434.5	1575.7	-	0.36
Bicyclo[3.3.0]oct-2-en-7-one, 6-methyl-	-	1694	-	-	-	0.12
TOTAL					100.00	100.00

*Data published as part of supplementary material in (Ambrosio *et al.*, 2019)
[1] Identification by GC/MS using non-polar (DB-5MS) and polar (DB-WAX) columns
[2] LRI_C: Linear retention index (Calculated)
[3] LRI_L: Linear retention index (Literature)
[4] NP: non-polar column DB-5MS
[5] P: polar column DB-WAX
[6] Relative amounts of the identified compounds based on the area of each peak in the chromatogram.
NI: Not identified compound

3.4. Discussion

Citrus EOs antibacterial activity has been investigated over the years focusing their applications in food and cosmetic industries. However, citrus EOs might be used in animal production, for instance in pig breeding sector to treat bacteria affecting pigs, such as *E. coli*. The antibacterial activity of citrus EOs (orange and lemon) initially was reported in the 60's on food spoilage bacteria (Subba et al., 1967), but citrus oils have also been potentially effective against *Sallmonella* spp, *Listeria* spp., *S. aureus* and *Pseudomonas* spp. (Friedly et al., 2009; Guo et al., 2018; Mitropoulou et al., 2017; O'Bryan et al., 2008). Thus, citrus oils have exhibited a broad antibacterial spectrum. Specifically, the anti-*E. coli* activity of citrus EOs was firstly observed by Dabbah et al. (1970), in which the EOs obtained from orange, lemon and lime were the most effective to inhibit the growth and to reduce *E. coli* population. Similarly, other citrus oils, such as bitter orange (*Citrus aurantium*), sweet orange (*Citrus sinensis*), lemon (*Citrus limon*) and mandarin (*Citrus reticulata*) were highly effective to inhibit *E. coli* growth (Bourgou et al., 2012) at MICs ranging from 25-40 µg/mL (Frassinetti et al., 2011). Nevertheless, the antibacterial activity of citrus oils on *E. coli* has been shown to be

citrus plant species-specific (Thielmann, Muranyi, & Kazman, 2019). The BOT oil evaluated in this study, previously has been proved for presenting an anti-ETEC activity and reduced activity on *Lactobacillus* sp. (Ambrosio et al., 2019). Consequently, considering BOT oil as an EO with a selective antibacterial spectrum.

The results found herein highlight the possible mechanism of action of BOT oil behind its inhibition or killing effect on the enterotoxigenic *E. coli* U21 and *L. rhamnosus*. Initially, the results of a sequential exposure to BOT oil (3 times at sub-MICs) showed a higher reduction of the bacteria viability compared to a single exposure, exerting the sequential BOT exposure a higher reduction of *E. coli* U21 viability compared to *L. rhamnosus.* This result is in-line with an earlier study conducted by Ambrosio et al. (2018), in which sequential exposure to *Eucayptus globulus* EO at 3.7 mg/mL (sub-MIC) provoked higher reduction of *E. faecalis* viability than the single exposure, and the effect on the viability reduction of this bacterium was higher compared to *L. rhamnosus*. Herein, a single exposure to BOT only provoked a bacteriostatic effect on *E. coli* U21 viability until 6 h of treatment at MIC/2 or MIC/4. Previous results of a single exposure to clove and rosemary EOs at their MIC/2 showed the bacteriostatic effect of these oils on *E. coli* viability until 5 h of treatment, with the subsequent recovery of *E. coli* growth afterwards (Fu et al., 2007). Normally, the effectiveness of an antimicrobial is determined based on a single exposure. However, gut bacteria frequently undergo sequential exposure to the antimicrobial effect through the constant ingest of the feedstuff. Moreover, sequential exposure to EO every 3 h can simulate the gastric transit time of weaned piglets (Davis, Illum, & Hinchcliffe, 2001; Snoeck et al., 2004), in which the stomach digesta containing the antimicrobial agent (antibiotic or EO) might reach bacteria in the small intestine. Thus, our results proved the differential effect on bacteria viability between sequential and single exposures to citrus EO. Based on this fact, the application of an antimicrobial agent at sub-MICs may be sufficient and effective to be dosed for pigs, since a higher noticeable effect on bacteria viability was found, targeting mainly pathogenic bacteria viability.

The effect of EOs on loss of bacterial viability is consequence of alterations and damages provoked by EOs on the bacterial cell structure and functionality. The mechanism of action of EOs involves a series of events on the cell surface and inside the cytoplasm of the bacterial cell (Nazzaro et al., 2013). These events are mediated by biochemical reactions (Swamy et al., 2016) produced by interactions between the chemical compounds in the EO and the components of the bacterial cell wall (Hyldgaard et al., 2012). Depending on the type of bacteria, Gram-positive or Gram-negative, the cell wall structure differs, which could determine the effect of an EO. Because of the lipophilic nature of EOs, it has been hypothesized that EOs can easily penetrate the cell wall of Gram-positive bacteria compared to cell wall of Gram-negative bacteria. This fact may be associated with the chemical

composition of the membrane: while the cell wall of Gram-positive bacteria is mainly composed by a peptidoglycan layer (90-95%), the cell wall of Gran-negative bacteria contains not only the peptidoglycan layer but also an outer membrane with polar constituents (porins and lipopolysaccharides), which would prevent and limit the penetration of non-polar compounds (i.e. EOs) into the bacterial cell (Nazzaro et al., 2013). Conversely to this, the BOT oil, in general, presented a higher antibacterial effect on the Gram-negative *E. coli* U21 than on the Gram-positive *L. rhamnosus*. After an EO destabilizes and crosses the bacterial cell wall, the EO compounds have the ability to insert into the cytoplasmic membrane, disrupt the phospholipid bilayer and bind to membrane proteins, disturbing then the normal membrane functionality (Juven et al., 1994). Bacterial cytoplasmic membrane results to be the first target of EOs. Cytoplasmic membrane provides a permeability barrier to the passage of small ions (electrolytes), which are essential to facilitate cell membrane functions (Diao et al., 2014). When the leakage of electrolytes is increased, the electric conductivity of bacterial cell suspension treated with EOs also increases, thus indicating disruption of the permeability barrier of the cytoplasmic membrane (Zhang et al., 2016). Results based on the relative electric conductivity showed that increasing BOT concentrations and treatment length caused increasing changes on membrane permeability of bacteria. However, this effect was more pronounced on *E. coli* U21. It has been reported that EOs alter membrane permeability by destroying the electron transport system, thus increasing the permeability to electrolytes such as K^+, H^+, Ca^{2+} and Na^+ (Hyldgaard et al., 2012). Earlier results performed by Zhang et al. (2016) showed that the membrane permeability of *E. coli* was increased when increased concentrations (MIC=1 mg/mL and 4MIC=4 mg/mL) of cinnamon EO and treatment length were adopted. Additionally, it has been reported that cinnamon EO increased rapidly the permeability of *E. coli* by 30-40% after 1 h of treatment and by 40-60% after 9 h of treatment, depending on the EO concentration tested. In a similar way to this previous findings, in our study, the BOT oil increased the membrane permeability of *E. coli* U21 by 40-50% and 50-90% at the same treatment lengths depending on the BOT concentrations (MIC/2=0.93, MIC=1.85 and 2×MIC=3.7 mg/mL). Likewise, it has been reported that the citrus oil from *Citrus medica* L. var. *sarcodactylis* increased the membrane permeability of *E. coli* to 37.04 and 46.05% at its MIC and 2×MIC, respectively (Li et al., 2019). Furthermore, limonene, the major compound in citrus EOs, has been found for having the ability to provoke >90% membrane permeabilization of *E. coli* after 1 h of treatment, with the corresponding more than 2 log of cell viability inactivation in the study by Espina et al. (2013). In addition, other compounds found in EOs, such as carvacrol and eugenol (10 mM), increased the membrane permeability of *E. coli* and *L. sakei*, but eugenol rendered higher permeabilization effect on *E. coli* than on *L. sakei* (Gill and Holley, 2006). A similar result was found for the BOT oil, which exerted higher permeabilization effect on *E. coli* U21 than on *L. rhamnosus*. Nevertheless,

the *Zataria multiflora* EO was found for increasing significantly the membrane permeability (75-90%) of *L. curvatus* (Ziaee et al., 2018).

EOs not only influence alterations on membrane permeability, also can lead to the breakdown of the cytoplasmic membrane integrity, increasing the leakage of vital-internal cell constituents to the extracellular environment (Nazzaro et al., 2013; Swamy et al., 2016). Thus, the apparent disruptive effect of BOT on membrane integrity has been elucidated by analyzing the cell constituents' release - proteins and reducing sugars. The results revealed that BOT oil caused a higher apparent release of specific cytoplasmic material from *E. coli U21* cells than from *L. rhamnosus* cells. A probable reason for this effect may be related to the fact that BOT oil could have affected the cytoplasmic membrane integrity and produced irreversible damages of a higher number of *E. coli* U21 cells compared to *L. rhamnosus* cells. The integrity of the cytoplasmic membrane is essential for the survival of bacteria since it is an important factor for the biological activities taking place into the bacterial cells (Nazzaro et al., 2013). The cell constituent leakage of *E. coli* U21 and *L. rhamnosus* in response to the BOT oil treatment was dose dependent. Proteins are key structural components of bacterial cells and are largely distributed in the cytoplasm and membranes. The release of proteins from *E. coli* by the effect of an EO has been previously observed. For instance, *Amomum tsaoko* EO increased the release of proteins from *E. coli* cells in 7.1 and 15.6 times at MIC (3.13 mg/mL) and 2×MIC (6.25 mg/mL), respectively (Guo et al., 2017). These results were higher compared to the data obtained in our current research. Similarly, other EOs, such as cinnamon, *Trachyspermum copticum*, Spanish oregano, Chinese cinnamon, savory and *Kaempferia pandurata,* have triggered the release of proteins from *E. coli*, *E. coli* O157:H7 and *E. coli* K1.1 (an ETEC serotype). Thus, these results altogether indicate the ability of EOs to disrupt the membrane integrity of these bacterial strains (Huang et al., 2017; Miksusanti et al., 2017; Oussalah et al., 2006; Zhang et al., 2016). In addition, the release of reducing sugars through the damaged cytoplasmic membrane of *E. coli* U21 and *L. rhamnosus* cells in response to the BOT oil treatment was observed. This observation is in-line with previous reports regarding the effects of EOs obtained from fennel seed (*Foeniculum vulgare* Mill.) and clove buds (*Syringa yunnanensis*), on *Salmonella dysenteriae* and *S. aureus* (Guo et al., 2017; Xu et al., 2016). Regarding the effect of EOs on membrane integrity of beneficial bacteria, a study conducted by Ziaee et al. (2018) has shown that *Zataria multiflora* EO affected the membrane integrity of *L. curvatus* as confirmed by the high losses of nucleic acids and proteins. In our study, the highest concentration of BOT produced only 2.4 times more release of proteins from *L. rhamnosus* cells. Similarly, vanillin, the major compound in vanilla EO, was found for affecting more remarkably the cytoplasmic membrane integrity of *E. coli* cells compared to *L. plantarum* cells (Fitzgerald et al., 2004).

EOs have several targets on the bacterial cell, particularly they affect bacterial membranes and cytoplasm, but also can disturb the normal morphology of the bacterial cells. EO exposure have evidenced that bacterial cells, in some cases, undergo severe deformations as consequence of the lysis and damages on cell wall. SEM observations showed that the BOT oil altered the normal morphology of the regular-rod shaped *E. coli* U21 cells. The same effect was observed for *L. rhamnosus* cells but with a lesser extent. Noticeable alterations on the external structure of *E. coli* U21 cells in response to BOT treatment was observed, such as severe damages, collapse and cell breakdown to debris with leakage of inner dense cellular content. These effects were produced in a dose-dependent manner. These data are in agreement with the findings obtained by Li et al. (2019), the EO from *Citrus medica* L. var. *sarcodactylis* caused more severe morphological changes (irregular shape and shriveled) and destruction of *E. coli* cells at 2×MIC than at the MIC of this oil. Likewise, studies testing other types of EOs, such as from oregano, clove, cinnamon, savory and *Amomum tsaoko* also have shown that *E. coli* and *E. coli* O157:H7 cells underwent important morphological changes and drastic damages after exposure to EOs (Guo et al., 2017; Oussalah et al., 2006; Rhayour et al., 2003; Zhang et al., 2016). In addition, scientific evidences have shown that single EO compounds, such as carvacrol, eugenol, thymol, limonene and cinnamaldehyde can change the fatty acid profile of the cell envelope (cell wall and cytoplasmic membrane) of *E. coli* O157:H7 and other Gram-negative bacteria, which would be implicated with damages, alterations or disintegration of the cell envelope structure as it was evidenced by SEM (Di Pasqua et al., 2007). Regarding to the effect of EOs on the morphology and structure of beneficial bacteria, the study conducted by Ziaee et al. (2018) showed that *L. curvatus* cells became swollen, corrugated and severely damaged after treatment with *Zataria multiflora* Boiss EO. The BOT oil showed to cause slight corrugation, wilting, mince and cell breakdown of *L. rhamnosus* in our present study.

Therefore, our results showed that the BOT oil alters the permeability and disrupts the integrity of the cytoplasmic membrane, with the subsequent leakage of essential cellular constituents. In addition, the BOT oil induced morphological alterations and reduced the viability of *E. coli* U21 cells more remarkably compared to *L. rhamnosus*. A citrus EO is composed of roughly 400 chemical compounds (Mahato et al., 2017), which may suggest that each compound may impact on the viability of bacterial strains by means of different mechanisms of action. For instance, the mechanism of *E. coli* inactivation by limonene has been reported by comprising the permeabilization of the outer membrane of *E. coli* by targeting phospholipids, lipopolysaccharides or β-sheet proteins, gaining access to the periplasmic space and cytoplasmic membrane (Espina et al., 2013). This permeabilization effect by limonene, it has been also attributed to changes on the lipid profile of the *E. coli* cell envelop, increasing the caproic acid content (54.5%), leading to alterations on the bacterial

cell surface (Di Pasqua et al., 2007). Similarly, other citrus EO compounds, such as carvone and carveol, also detected in the BOT oil, have been found able to change the hydrophobicity, surface charge, and membrane integrity of *E. coli* cells with the subsequent K^+ leakage (Lopez-Romero et al., 2015). In view of this, from a practical standpoint, due to the diverse of compounds in an EO and the different antibacterial mechanism of action that every compound could exert, the use of a whole EO would facilitate the adoption of a natural approach to combat pathogens compared to the use of a single isolated EO compound. Besides of the already discussed, EOs could exert other mechanisms to inactivate bacteria, such as interrupt the DNA/RNA synthesis and function, affect the intermediary metabolism and interfere with the intercellular communication (quorum sensing) by decreasing the biofilm formation and the virulence factors expression (Nazzaro et al., 2013; Radulovic et al., 2013).

3.5. Conclusions

In conclusion, results of this study showed that the probable mechanism underlying the selective antibacterial action of the citrus EO, BOT, on *E. coli* U21 and *L rhamnosus* can be described as altering the permeability and integrity of their cytoplasmic membrane, as well as their external structure (normal morphology), but in a higher degree for *E. coli U21* than for *L. rhamnosus.* Moreover, the sequential exposure to BOT at lower levels than the MIC may be effective to reduce the bacterial viability compared to a single exposure, with remarkable higher reduction effect on *E. coli* U21 viability. Therefore, citrus EOs, a common by-product of the food industry, could be a potential alternative to the antibiotic use in pig breeding sector to combat pathogens as enterotoxigenic *E. coli*. However, because of the complex composition of citrus EOs it is probable that there are other mechanisms of antibacterial action of citrus EOs and this should be the focus of future research.

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4. CHEMICAL COMPOSITION, ANTIBACTERIAL AND ANTIOXIDANT ACTIVITY OF A CITRUS ESSENTIAL OIL AND ITS FRACTIONS[3]

Abstract

Citrus are the most grow fruit around the world, and essential oils (EOs) are the main by-product of *Citrus* processing industries. In addition to food/beverage and cosmetic applications, citrus EOs could also potentially be used as an alternative to antibiotics in food-producing animals, such as pigs. This would be possible due to the reported selective antibacterial activity of citrus EOs on pathogenic and beneficial bacteria. Nonetheless, the chemical composition associated with EO bioactivity is well-known. Thus, this study was focused on fractionating a commercial citrus EO, Brazilian orange terpenes (BOT), and on the characterization of the chemical composition, antibacterial and antioxidant activities of the whole EO and its separated fractions. By means of fractional vacuum distillation, BOT oil was separated into four fractions: F1, F2, F3 and F4 by collecting them at regular intervals of 10 min. Results showed that the three first fractions were characterized by having a high concentration of the major compound, limonene (86.98/87.36 - 91.83/93.2% by polar/non-polar GC-MS), even higher than the whole BOT oil. Conversely, the F4 presented a larger amount of BOT's minor compounds such as carvone, *cis*-carveol, *trans*-carveol, *cis*-p-Mentha-2,8-dien-1-ol and *trans*-p-Mentha-2,8-dien-1-ol and a very low amount of limonene (0.96/0.61%). In addition, others minor 26/15 (non-polar/polar GC-MS) compounds were detected exclusively in the F4 fraction. Antibacterial activity results showed that F4 was the only fraction that exhibited this activity, which was characterized by selective, higher activity on pathogenic bacterium than on beneficial bacterium. But F4 activity was lower than the BOT oil. Similarly, F4 displayed the highest antioxidant activity among the fractions, which was equivalent to the whole BOT oil ($p<0.05$). Thus, these results indicate that probably those minor compounds detected in F4 would be more involved in conferring the biological activities for this fraction and consequently for the whole BOT oil, instead of the major compound, limonene, playing this role exclusively. However, the synergistic interaction between major and minor compounds would determine the resulting biological activities of BOT oil.

Keywords: Fractional distillation; Carvone; Cis/trans-carveol; Limonene; *E. coli*

4.1. Introduction

Citrus are the most grow fruit around the world and their processing results in a large amount of waste (peel, seeds and pulp) and by-products (Dosoky and Setzer, 2018). Essential oils (EO) are the main by-product of *Citrus* processing industries and they have been the most popular source of flavors (aromas) and fragrances of commercial value, principally for the cosmetic, food and beverage industries (Sawamura, 2010; Tranchida et al., 2012). This is due to citrus EOs being categorized and generally recognized as safe (GRAS)

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by the United State-Food and Drug Administration (Tisserand and Young, 2014) and also because they harbor several biological properties, which have turned them into an economic, eco-friendly and natural alternative to synthetic preservatives and antioxidants for these industries (Mahato et al., 2019).

A citrus EO is a mixture of more than 200 compounds, of which 85-99% are volatile compounds and 1-15% are non-volatile compounds (Fisher and Phillips, 2008). The volatiles are mainly monoterpene and sesquiterpene hydrocarbons, their oxygenated derivatives, aliphatic aldehydes, alcohols and esters. The non-volatile portion is comprised of hydrocarbons, sterols, fatty acids, waxes, carotenoids, coumarins, psoralens, and flavonoids (Tranchida et al., 2012). However, the monoterpene limonene is the major compound in citrus EOs, and may range from 32 to 98% (Mahato et al., 2019). The qualitative and quantitative composition of citrus EOs directly determines their features and biological properties (Dosoky and Setzer, 2018), such as their antimicrobial and antioxidant activities. Some studies have indicated a possibility that the major compound of citrus EOs, limonene, could be responsible for conferring the biological properties of these oils (Erasto and Viljoen, 2008; Espina et al., 2013), but other authors have suggested that the consortia between major and minor components of EOs would be involved in conferring their biological properties (Bakkali et al., 2008).

In addition to the applications mentioned, citrus EOs could also have another potential application related to meeting the urgent demand for alternatives to substitute antibiotics in food-producing animals, such as pigs. During the last 15 years, there has been a heightened concern about the impact to public health of the growing emergency of antibiotic-resistance superbugs, since the overuse of antibiotics in the raising of food-producing animals has been suspected as one of the main factors contributing to this matter (Lillehoj et al., 2018). As a measurement to counter this concern, antibiotics use, and more specifically, their dietary application as growth promoters in food-producing animals, has been banned within the European Union since 2006 (EU, 2003). Nonetheless, recently, some other countries have adopted restrictions regarding the use of some antibiotics for this purpose such as China, Brazil, Japan, South Korea, New Zealand (Food Safety Commission of Japan, 2017; Johnson, 2010; Liu and Liu, 2018; Walsh and Wu, 2016) and United State (Brüssow, 2017). Interestingly, a feature that might stand out citrus EOs as a promising alternative is their reported potential selective antibacterial activity, presenting a stronger antibacterial action on pathogenic than on beneficial bacteria that occur in the pig gut (Ambrosio et al., 2017, 2019b). Selectivity toward pathogenic bacteria by EOs rather than beneficial bacteria has been highlighted as a desirable antimicrobial spectrum feature for candidate compounds as possible alternatives to antibiotics (Omonijo et al., 2018; Ouwehand et al., 2010; Si et al., 2006). In addition, dietary supplementation with EOs could

be a nutritional strategy to prevent oxidative stress and damage to the organism of food-producing animals, since it is well known that EOs harbor sources of natural antioxidants that can effectively inhibit oxidative reactions (Simitzis, 2017), thus preventing the formation of free radicals at localized sites of the animal organism and also on the derived animal products (Omonijo et al., 2018; Simitzis, 2017). In this context, understanding what compounds contained in citrus EOs are associated with their selective antibacterial activity and antioxidant capacity is critical to their future development. Fractional distillation is a unit operation that assists in the separation of EO components by volatility differences (Silvestre et al., 2016). The large number of compounds in an EO can be separated into a range of isolated fractions. Moreover, vacuum application of fractional distillation allows for a separation avoiding degradation of thermolabile EO compounds, since the system operates at low temperature (Falcão et al., 2012). In this process, the more volatile compounds are vaporized, followed by intermediate ones, and so on. The less volatile compounds remain in the boiler (Falcão et al., 2012). Consequently, fractional distillation using a vacuum system would be a helpful tool to separate the compounds of a citrus EO and test their biological properties. Therefore, the aim of this study was to fractionate a commercial citrus EO, Brazilian orange terpenes (BOT) using a vacuum distillation system and to characterize the chemical composition, antibacterial and antioxidant activities of BOT and its separated fractions.

4.2. Material and methods

4.2.1. Essential oils supply

A commercial citrus EO was used in this study, Brazilian orange terpenes (BOT), which was a by-product of orange juice processing and was supplied by a factory in São Paulo State, Brazil. Once the sample was received, it was kept in amber bottles under refrigeration (4°C) until use. Then, this oil was used as the core material for fractional distillation.

4.2.2. Fractionation of citrus essential oil

The BOT oil was fractionated in a batch vacuum distillation system using a BR–800 Micro fractional distillation apparatus (BR instruments, USA) equipped with a Teflon spinning band distillation column (20 cm x 7 mm) of 30 theorical plates and covered with a silvered vacuum jacket. The system had an automatic reflux valve (solenoid), coupled at the top of the distillation column, and a condenser (coupled to a condensing bath VWR 1160s, USA)

with a connecting arm linked to a vacuum system and to a fraction collector. This latter instrument allows for the automatic fraction collection according to vapor temperature. The system pressure was regulated by a high-performance vacuum pump (RV3-Edwards, USA) and was monitored by a dual capacitance manometer (PDR 2000-MKS Instruments, USA). To start the fractional distillation, 20 g of the BOT oil was weighted in a round bottom flask (50 mL) equipped with a thermowell, which allows for to introduction of a temperature probe for controlling the EO temperature during boiling. In addition, the temperature at the top of the fractionating column was also monitored by a thermocouple. The initial temperature in the boiling flask (T_1) and at the top of the fractionating column (T_2) were 27 and 23 °C, respectively. Heating of the boiling flasks was performed in a gradual way, thus T_1 and T_2 were constantly monitored. Distillation started once T_1 reached 54°C (boiling temperature of BOT) and a first drop of condensed vapor appeared at the end of the condenser. Heat increasing was stopped and maintained at the point where T_1 and T_2 were stabilized (approximately 40 min later). Then, the first fraction was collected at: T_1= 90°C; T_2 = 51°C; pressure = 17.5 mbar. After 10 min, collection of the first fraction was finished and immediately following, collection of the second fraction began. Thus, in this experimental protocol, the fractions of BOT oil were collected at regular intervals of 10 min. The remaining fraction in the boiling flask was considered as the bottom fraction, the terpeneless one.

4.2.3. Chemical composition of essential oils

The chemical composition characterization of the BOT oil and its fractions was performed by gas chromatography coupled with mass spectrometry (GC/MS) using non-polar and polar columns as described by (Ambrosio et al., 2019b).

The analysis on the non-polar column was carried out in an Agilent Technology gas chromatograph 6890 Plus Series (Santa Clara, CA, USA) coupled to a selective Mass Spectrometry Detector 5973 and an Auto Sampler 7893. A capillary column composed of a fused-silica DB-5MS (J&W Scientific, Folsom, CA, USA) of 60 m × 0.25 mm id x 0.25 µm thick film coated with 5%-phenyl polydimethylsiloxane was used. The oven temperature was set as follows: initial temperature was held at 45°C for 5 min, then raised to 150ºC at 4ºC/min for 2 min, then raised to 250°C at 5°C/min, and finally to 300°C at 10°C/min, which was maintained for 60 min. The injector temperature was 250°C, with 2 µL of the sample diluted in dichloromethane being injected in “split” mode at a ratio of 30:1. The EIMS, electron energy was 70 eV. The mass detector operated in full scan mode with a range from 40 to 350 m/z. The temperature of the ion source and transfer line were 230°C and 285°C, respectively. Helium gas was used as the carrier gas with an inlet pressure of 16.97 psi. The retention index (RI) was calculated for all the volatile compounds using a homologous series

of C7–C30 n-alkanes (49451-U Sigma-Aldrich), according the linear equation of Van den Dool and Kratz (Van Den Dool and Kratz, 1963).

The analysis of the polar column was carried out in an Agilent Technology gas chromatograph 7890a Plus Series (Palo Alto, CA, USA) coupled to a selective Mass Spectrometry Detector 5975C. A fused-silica capillary column DB-WAX (J&W Scientific, Folsom, CA, USA) of 60 m × 0.25 mm i.d x 0.25 µm thick film coated with polyethylene glycol was used. The oven temperature was set as follows: initial oven temperature was held at 50°C for 5 min, then raised to 150ºC at 4ºC/min for 7 min and finally to 230°C at 4°C/min, which was maintained for 40 min. The injector temperature was 250 °C, with 2 µL of the sample diluted in dichloromethane being injected in “split” mode at a ratio of 30:1. The EIMS, electron energy was 70 eV. Helium gas was used as the carrier gas with an inlet pressure of 16.91 psi. The mass detector operated in full scan mode ranging from 40 to 350 m/z. The retention index (RI) was calculated for all the volatile compounds as described above.

The identification of the components was performed by comparing their RI and mass spectra with data published in the literature (Adams, 2007; Babushok et al., 2011) and in computer libraries (NIST 107 and WILEY 8).

4.2.4. Antibacterial activity

Bacterial strains

An ETEC strain and a *Lactobacillus* specie were evaluated in this study as models of a pathogenic bacterium and a beneficial bacterium of occurrence in the pig gut, respectively. The ETEC strain, *E. coli* U21 (K88+/LT+/STb+/F18+/Sta+), was isolated from pig gut and provided by The Swine Heath Laboratory of the Department of Preventive Veterinary Medicine and Animal Health, School of Veterinary Medicine and Animal Science at the University of São Paulo, Brazil. *Lactobacillus rhamnosus* ATCC 7469 was purchased from American Type Culture Collection (ATCC). The ETEC strain was cultivated in Tryptic Soy Agar-Difco (TSA) at 37 ºC for 18-20 hours and *L. rhamnosus* was grown in MRS (Man, Rogosa and Sharpe) agar at 30 °C for 48 hours. After activation, bacteria were sub-cultured in a Brain-Heart Infusion (BHI) or MRS, both supplemented with 15% v/v of glycerol. After incubation, they were stored at -20 ºC until their use.

Determination of Minimal Inhibitory Concentration (MIC)

The determination of the MIC of BOT oil and its fractions was performed by microdilution assay in a 96-well microplate following the standard protocol M07-A9 from the

Clinical and Laboratory Standards Institute (CLSI, 2012), with some modifications. For the assay, the standard inoculum was prepared in sterile NaCl solution (0.9 % w/v) from living colonies of the selected bacteria, contained in plates of either TSA agar (*E. coli*) or MRS agar (*L. rhamnosus*.) at the optical density equivalent to the 0.5 McFarland Standard (0.08-0.13 at 625 nm). Subsequently, the standard inoculum was diluted at 1:100 to obtain an inoculum of 10^6 CFU/mL (final inoculum). The stock solution of the BOT and each fraction were prepared at 14.8 mg/mL (1.65% v/v) with Mueller Hinton (MH) or MRS broth using Tween 80 as emulsifier. From each stock solution, two-fold serial dilutions were made ranging from 14.80 to 0.116 mg/mL. Twenty microliters of the final inoculum were added to each well containing 180 µL of several concentrations of the BOT or its fractions. The final volume in each well was thus 200 µL per well and the bacterial population was ~10^5 CFU/mL. The following controls were used: culture medium control (200 µL of MH or MRS broth); growth control (180 µL of MH or MRS broth + 20 µL of inoculum) and growth control containing the emulsifier (180 µL of MH or MRS broth with Tween 80 + 20 µL of inoculum). Finally, microplates were incubated at 37ºC for 24 h for *E. coli* and at 30°C for 36 h for *L. rhamnosus*. The MIC was established as the lowest BOT/fractions concentration that inhibited visible bacterial growth supported by resazurin test at the end of the incubation period. For the resazurin test, 25 µL of resazurin (R7017; Sigma-Aldrich) solution at 0.0135% m/v were used per well. Then, after visual inspection, the presence of viable cells was evidenced through a change in the resazurin color from blue resazurin to pink resorufin (Pereira et al., 2014) after 1h of further incubation. Assays were carried out in triplicates, with three independent repetitions.

Minimal Bactericidal Concentration (MBC)

The determination of MBC was performed from microplate wells containing concentrations of BOT oil or its fractions where there was no visible bacterial growth. An aliquot of 100 µL was taken from each well and seeded in MH or MRS agar. Then, plates were incubated for 24 h at 37ºC for *E. coli* U21 and for 36 h for *L. rhamnosus*. The MBC was defined as the lowest concentration of BOT/fractions able to cause total bacterial death, represented by the visible absence of colonies on the agar plates.

4.2.5. Antioxidant capacity

Reagents

Fluorescein (FL), 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox C), Butil hidroxitolueno (BHT) and α-tocopherol were purchased from Sigma (Sigma-Aldrich, USA). An FL stock solution (8.2 $x10^{-4}$ mM) was made in a 75 mM potassium phosphate buffer solution (PBS, pH 7.4) and was kept at 4 °C. The AAPH stock solution was prepared in PBS (75 mM, pH 7.4) at a final concentration of 153 mM and was kept in an ice bath. Trolox standard was prepared at 206 µM in PBS (75 mM, pH 7.4), which was the stock solution. From this stock solution, serial dilutions in PBS (75 mM, pH 7.4) were made to reach 10.32, 20.62, 41.23, 61.85, 82.46 and 103.08 µM, which were the working concentrations for the Trolox calibration curve. BHT and α-tocopherol solutions were prepared at 1 mg/mL in PBS as well.

ORAC assay

The antioxidant capacity of the BOT oil and its fractions was evaluated by the oxygen radical absorbance capacity (ORAC) assessment following the approach used by Ou et al. (2001), with some modifications. The assessment was carried out using a Spectrophotometer Modulus™ II Microplate reader, Model 9310-011 (Turner Biosystems, California, USA). For the assessment, FL was used as the fluorescent probe and AAPH as a peroxyl radical generator. BHT and α-tocopherol were used as reference antioxidant compounds.

The BOT oil and its fractions were prepared as follows: 10 mg were accurately weighted, dissolved in 1 mL of methanol and well shaken. After appropriate dilution in PBS (75 mM, pH 7.4), the BOT/fractions working solutions for the analysis were established. Reactions were carried out in a 96-well black microplate, loading 25 µL of the BOT/fractions working solutions and 150 µL of FL solution (8.2 $x10^{-4}$ mM) into the wells of the microplate. The microplate was pre-incubated at 37°C for 5 min under stirring into the reader, then 25 µL of AAPH (153 mM) was rapidly added to each well using a multichannel pipet. The microplate was then immediately placed back into the reader, shaken and the reaction was initiated. Thus, the final reaction mixture was 200 µL and the reduction in fluorescence was recorded by reading fluorescein excitation at 490 nm and emission at 570 nm, every 2.2 minutes, for 88.6 min. The microplate was automatically shaken prior to each reading. Positive controls - BHT and α-tocopherol - were run in the microplate too. A blank with FL and AAPH using PBS instead of the antioxidant compounds (BHT, α-tocopherol or EO/fractions) were also set in

the microplate. Furthermore, the six Trolox calibration concentrations (10.32, 20.62, 41.23, 61.85, 82.46 and 103.08 µM) were placed in the microplate. All reaction mixtures were prepared in octuplicates, and three independent assays were performed for each sample. All fluorescence measurements were expressed relative to the initial reading, and plotted vs time (x-axis), generating a typical decay curve. The area under the fluorescence decay curve (AUC) was calculated using the following equation:

$$AUC = 1 + \sum_{i=2.2}^{i=88.6} \frac{f_i}{f_0} \qquad (1)$$

where f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time *i*. The ORAC value for each sample was calculated by using a linear regression equation between the Net AUC (of Trolox concentrations) and the Trolox concentrations. Thus, ORAC values were expressed as micromole Trolox equivalents (TE) per gram (µmol TE/g). The Net AUC was obtained by subtracting the AUC of the blank from the AUC of Trolox or the AUC of the sample (BOT/fractions).

4.2.6. Data analysis

The data are shown as the mean of the replicates with the standard deviations. One-way analysis of variance (ANOVA) and Tukey's test for pairwise comparison at 5% of significance were used to detect significant differences between the antioxidant capacity of the BOT oil and its fractions using R software. In addition, a multiple factor analysis (MFA) was performed on polar and non-polar data (two tables) to describe and contrast the chemical composition profile of the BOT oil and its fractions obtained by GC-MS; this analysis was performed using XLSTAT software.

4.3. Results

4.3.1. The vacuum fractionation

After the fractional distillation process of BOT oil, four fractions were obtained, F1, F2, F3 and F4. Their yields related to the initial EO carried were 15.2, 34.0, 20.4 and 29.0 %, respectively. F1 resulted to be the most volatile fraction while F4 was the least volatile one (remaining fraction in the boiling flask).

4.3.2. Chemical composition of the fractions

The full chemical composition of the four fractions derived from the BOT oil is shown in Table 4-S1 (Supplementary material). Regarding the chemical composition profile of the BOT oil, this was previously reported by Ambrosio et al. (2019). Overall, the identification of the chemical composition of each fraction and the BOT oil by both columns was quite alike, as is shown in the individual factor map of the MFA (Fig. 4.1a), where the polar and non-polar identification dots were located very close to each other. Furthermore, a very good representation of the chemical composition data was achieved, since the first dimension of the MFA explained 81.71% and the second dimension explained 12.73% of the total variance. As observed in this plot (Fig. 4.1a), the three first fractions, F1, F2 and F3, were practically clustered together, suggesting that these fractions had a very close chemical composition profile. However, they differed from the BOT oil in the second dimension. For these three fractions and the BOT oil, limonene was detected as the major compound; however, fractions were remarkably more associated with this compound in the two dimensions of the MFA than in the BOT oil, which had that positive association only with limonene in the first dimension (Fig. 4.1b).Thus, indicating that F1, F2 and F3 presented higher relative of limonene (ranging from 86.98/87.36 to 91.83/93.2 %) compared to the BOT oil (78.65%/79.38%) (Table 4.1).

Nevertheless, F4 presented a fully different chemical profile when compared to the other three fractions (Fig. 4.1a), and it was the fraction that presented a very low relative amount of limonene, 0.96/0.61 % (Table 4.1). F4 was positively associated with a high amount of several compounds, and these are shown in the first and second quadrants of the MFA (Fig. 4.1b). Interestingly, minor compounds such as carvone, *trans*-carveol-, *cis*-carveol, *trans*-p-Mentha-2,8-dien-1-ol and *cis*-p-Mentha-2,8-dien-1-ol (Fig. 4.1b) were detected in this fraction and they were present at much higher amount compared to F1, F2 and F3 (Table 4.1). In addition, others minor 26 and 15 compounds were detected in F4 by non-polar and polar columns, respectively, which were present exclusively in this fraction (Fig. 4.1b, underlined names).

4.3.3. Antibacterial activity and Antioxidant capacity

Additionally, the biological properties of the BOT oil and its fractions were evaluated. Initially, their antibacterial activity was proved, which is shown in Table 4.1. Our results revealed that F4 was the only fraction that presented an antibacterial effect on the tested bacteria in contrast to the other three fractions (F1, F2, F3). MIC and MBC values showed

that F4 exerted a stronger antibacterial activity on *E. coli* U21 than on the beneficial bacterium *L. rhamnosus*, as observed by the lower MIC/MBC values for *E. coli* U21, which were four times lower than the MIC/MBC for *L. rhamnosus*. Thus, meaning that F4 had a

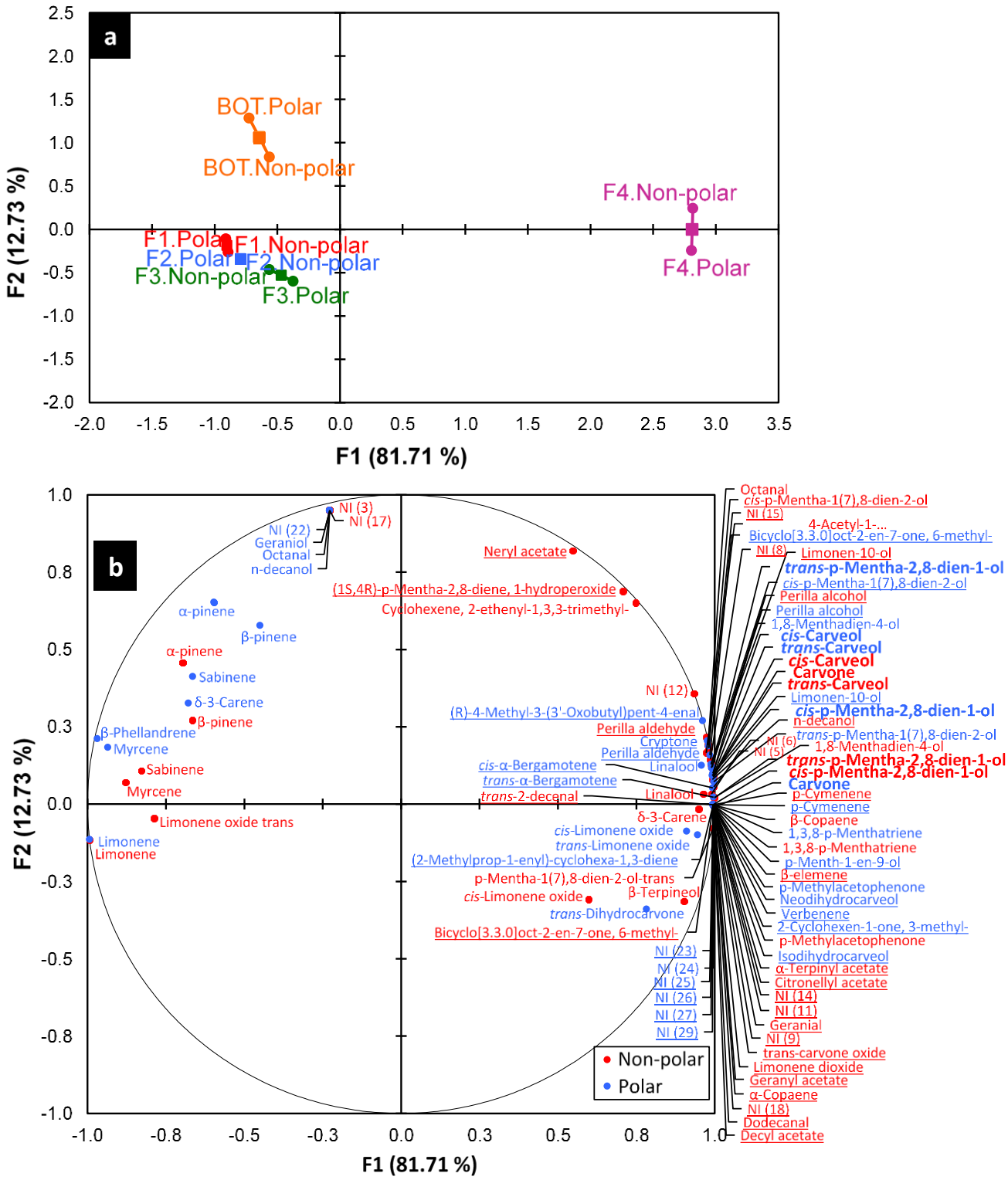

Figure 4.1. Multiple factor analysis (MFA) of the chemical composition profile of Brazilian orange terpenes EO and its fractions. The individual factor map of the overall chemical composition profiles by polar and non-polar identification (a) and biplot of the detailed chemical composition profile (b).

selective antibacterial performance. These results indicated that F4 would be the part of the BOT oil responsible for the observed selective antibacterial activity of the whole BOT oil. However, compared to F4, the whole BOT oil exhibited a higher selective antibacterial activity, as the BOT oil inhibited or killed bacteria at lower concentrations than F4. This suggests the possibility that the resulting antibacterial activity of the BOT oil could be a consequence of the synergism between F4 and the other three fractions.

Table 4.1. Antibacterial activity of BOT oil and its fractions

EO/Fractions	Antibacterial parameter (mg/mL)	Bacterial strain	
		E. coli U21	*L. rhamnosus* ATCC 7469
F1	MIC	-	-
	MBC	-	-
F2	MIC	-	-
	MBC	-	-
F3	MIC	-	-
	MBC	-	-
F4	MIC	3.70	14.80
	MBC	3.70	14.80
BOT*	MIC	1.85	3.70
	MBC	1.85	7.40

*Values determined in the study by (Ambrosio et al., 2019b)

Subsequently, the BOT oil and its fractions were subjected to screening for their possible antioxidant activity by the ORAC method, as it is shown in Table 4.2. Results showed that F4 exhibited the highest radical scavenging capacity (ORAC value was 844.51±20.20 μmol Trolox/g), however it was not found to be significantly different from the whole BOT oil. In contrast to the other fractions (F1, F2 and F3), F4 displayed a significant 1.8, 2.5 and 3.9-times higher antioxidant activity, respectively. Moreover, compared to the positive controls, α-Tocoferol and BHT, F4 displayed a significant 2.1 and 2.7-times higher antioxidant activity, respectively. Thus, these findings indicate that F4 would be the component of the BOT oil responsible for the resulting antioxidant capacity of the whole BOT oil, as well as for the antibacterial activity already observed.

4.3.4. Relationship between chemical composition and biological activities

Our results on the biological activity showed that F4 was the part of the BOT oil that presented either the unique or higher antibacterial or antioxidant activities. This would indicate that possibly the minor compounds of BOT oil detected in high amount in F4, such as: carvone, *cis*-carveol, *trans*-carveol, *cis*-p-Mentha-2,8-dien-1-ol and *trans*-p-Mentha-2,8-dien-1-ol would directly influence the selective antibacterial activity and the antioxidant capacity exhibited by BOT oil. Thus, this suggests that the presence of minor compounds

could be involved in conferring the biological properties of the BOT oil, instead of this role being attributed exclusively to its major compound, limonene. However, the synergistic interaction between limonene and the minor compounds inside of the BOT oil could also be a determinant for the resulting antibacterial and antioxidant properties of the BOT oil.

Table 4.2. Antioxidant capacity of the BOT oil and its fractions according to the ORAC assay

EO/Fractions	Antioxidant capacity µmol Trolox/ g of pure sample
F1	477.64±35.45^{b}
F2	343.24±26.57^{c}
F3	211.69±14.73^{d}
F4	850.67±16.04^{a}
BOT	785.14±79.35^{a}
α-Tocoferol	339.21±14.55^{c}
BHT	312.16±11.02^{c}

* Values are means ± Standard Deviation (SD) of triplicate determinations.
a-d: Mean values within a column having different superscripts are significantly by the least significant difference Tukey test ($p < 0.05$).

4.4. Discussion

Biological properties of citrus EOs have been widely investigated over the last 50 years, focusing on their application in food, cosmetic and pharmaceutical industries. The recent trend of searching for natural sources of antimicrobials for the food-producing animal sector, such as pig production, has led to considering EOs as a promising alternative to dietary supplementation of antibiotics. However, as a potential alternative to antibiotic growth promoters, it has been highlighted that EOs should comply with a selectivity between pathogenic and beneficial bacteria resident in the animal gut, as a beneficial modulatory effect on pig gut microbiota, in order to improve animal performance (Li et al., 2012b; Ouwehand et al., 2010; Si et al., 2006). Interestingly, after an evaluation of 28 EOs obtained from different plants, citrus EOs stood out for presenting a selective antimicrobial activity in the *in vitro* study by Ambrosio et al. (2017). Complementary and more detailed information about the selectivity of citrus EOS was supplied by (Ambrosio et al. (2019b) when they observed that citrus EOs were shown to cause higher disturbances on the normal growth kinetics of enterotoxigenic *E. coli* isolated from pig gut than on *Lactobacillus sp.* In our study, a citrus EO, BOT, which presented this selective antimicrobial spectrum, was fractionated under vacuum distillation and our results showed that the BOT oil comprised four fractions with different chemical composition profiles that directly influenced its biological activities.

The fractional distillation process could separate BOT oil into four fractions: F1, F2, F3 and F4 (Bottom fraction) using a collection interval between fractions of 10 min. Overall, the relative amount of the compounds present in each obtained fraction varied. Nonetheless, the whole BOT oil was characterized by having limonene as major compound (78.65/79.38%) (Ambrosio et al., 2019b). The three first fractions followed this pattern in higher purity (86.95-93.20%), while the F4 fraction had a very low amount of this compound (0.96/0.61). These results were in line with a previous study by Silvestre et al. (2016), in which fractional distillation of a green mandarin (*Citrus deliciosa* Tenore) EO allowed for the separation of this EO in 6 fractions removed at the top of the column, and one bottom fraction, using a collection interval of 10 min. The six first fractions as well as the whole mandarin EO were characterized by having limonene as major compound (41.8-75.9%) while the bottom fraction was found to have a lower limonene content (20.1%). Similarly, fractional distillation of a concentrated orange EO left a top fraction rich in limonene and a bottom fraction rich in valencene, with a very low limonene content (0.7-1.9%) (Beneti et al., 2011). Limonene concentration in our bottom fraction (F4) was quite similar to what was found in this previous study.

Moreover, minor compounds of BOT oil such as carvone, *cis*-carveol, *trans*-carveol, *cis*-p-Mentha-2,8-dien-1-ol and *trans*-p-Mentha-2,8-dien-1-ol were detected at relatively higher amount in F4 than in F1, F2 and F3 and even than the whole BOT oil. F4 exhibited the greatest biological activities among the fractions and had the lowest limonene content. Therefore, this observation suggests that those compounds would be those mainly responsible for the biological activities exhibited by F4 and consequently by the whole BOT oil. Nonetheless, the stronger biological activities showed by the whole BOT oil also suggest that the synergism between major and minor compounds would determine the biological activities of the citrus EO, BOT, and would be not exclusively determined by its major compound. Bakkali et al. (2008) have indicated that the biological activity of the major compound of an EO is modulated by the minor compounds it contains, forming a synergistic consortium to confer the total biological activities of the EO. For instance, antibacterial activity results showed that fractions rich in limonene (the major compound) did not exhibit any antibacterial effect on the tested bacteria, but F4 in fact presented a selective antibacterial activity, although lower than that of the BOT oil. Because the BOT oil is the combination of the four fractions, it is likely that the higher antibacterial activity of the whole BOT is conferred by the minor compounds in F4, potentiated by their synergistic interaction with limonene inside the BOT oil. This proves that limonene alone wouldn´t be exclusively involved in conferring the selective antibacterial activity of this citrus EO.

Already, the ineffectiveness of limonene alone on several pathogenic bacteria, including *E. coli*, and beneficial bacteria such as *Lactobacillus* sp. has been reported by

Fancello et al. (2016). Although, some studies have reported some weak antibacterial activity for limonene on *E. coli* and other pathogenic bacteria (Aggarwal et al., 2002; Dorman and Deans, 2000; Guo et al., 2018). Nonetheless, on the contrary, a study by Ouwehand et al. (2010) has shown that limonene stimulates the growth of *Lactobacillus* sp. instead of promoting its inhibition or killing it. Thus, it may be inferred that limonene in the BOT oil would be more related to the lower or non-activity, or even the stimulating activity, of beneficial bacteria, contributing in this way to the selective antibacterial activity of this citrus EO, whereas leaving the function of fighting pathogenic bacteria, such as *E. coli*, to the minor compounds, such as those found in F4.

Studies have indicated that the antibacterial effect of citrus EOs on pathogenic bacteria is more related to minor compounds contained in these EOs. These would be mostly oxygenated monoterpenes, which exhibit stronger antibacterial activity than hydrocarbon monoterpenes such as limonene (Espina et al., 2011; Settanni et al., 2012). The presence of oxygenated monoterpenes such as carvone, *trans*-carveol, *cis*-p-mentha-2,8-dien-1-ol, *cis*-limonene oxide and (Z)-patchenol, in higher relative amounts in mandarin EOs than in other EOs has been suggested as responsible for antibacterial activity of this EO. (Espina et al., 2011). Indeed, those three first compounds have been detected at remarkably relative amount in F4 and this supports the reasoning that they would be responsible for conferring the antibacterial activity of BOT oil on pathogenic bacteria, such as *E. coli*. Reinforcing this, in previous studies, the wide spectrum activity of carvone alone was observed on several pathogenic bacteria, including several *E. coli* strains (Aggarwal et al., 2002; Friedman et al., 2002; Oumzil et al., 2002). Moreover, carveol alone has also been a strong antimicrobial compound on gram-positive and gram-negative pathogenic bacteria, such as *E. coli,* inhibiting and killing it at 0.06 and 0.25 mg/mL, respectively (Guimarães et al., 2019). In addition, p-menthadienols compounds in a remarkable relative amount, such as *cis*-p-mentha-2,8-dien-1-ol (4.6-9.7%) and *trans*-p-mentha-2,8-dien-1-ol (16.3-22.1%), have been indicated as the responsible compounds for the high antimicrobial activity exhibited by the EOs that contain them (Bassolé et al., 2011; Jirovetz et al., 2007). The relative content of these two compounds detected in F4 was close to what was found in the previous study. Thus, this confirms the antibacterial role of the five oxygenated monoterpenes detected in BOT as the main responsible compounds for the antibacterial activity observed for F4 and therefore of the citrus EO, BOT, on pathogenic bacteria.

Additionally, the other compounds, those exclusively detected in F4, also would have a contributive role in the antibacterial activity such as geranial (Dorman and Deans, 2000; Settanni et al., 2012), perrilla aldehyde (Kim et al., 1995), geranyl acetate (Dorman and Deans, 2000; Fancello et al., 2016), *cis*-p-mentha-1(7),8-dien-2-ol (Jirovetz et al., 2007), *cis*-limonene oxide (Espina et al., 2011), citronellyl acetate and neryl acetate (Fancello et al.,

2016). Therefore, the selectivity towards pathogenic bacteria rather than beneficial bacteria by the BOT oil is likely conferred mainly by carvone, *cis*-carveol, *trans*-carveol, *cis*-p-Mentha-2,8-dien-1-ol and *trans*-p-Mentha-2,8-dien-1-ol in consortia with limonene. The probably mechanism of the selective antibacterial action of BOT oil, induced by the presence of those compounds would be related to causing higher disturbances of the permeability and integrity of the cytoplasmatic membrane of *E. coli* than *L. rhamnosus*, with the associated higher release of essential cellular constituents (Ambrosio et al., 2019a). Nevertheless, it is possible that there are other ways whereby those compounds could determine the mode of action of BOT oil on pathogenic bacteria (Nazzaro et al., 2013).

Similarly, F4 exhibited the greatest free radical scavenging capacity compared to the other three fractions, and this was comparable to the capacity exhibited by the whole BOT oil. It has been observed that increasing concentrations of particular compounds with high antioxidant capacities in a fraction obtained from an EO increases the antioxidant power in that fraction, compared to the antioxidant activity of the EO (Rostro-Alanis et al., 2019). This could explain the higher antioxidant capacity of F4 (even though this was not found to be significant). The five major compounds observed in F4 were concentrated, depending on the compound, from 3.2 to 12.1 times higher in comparison to their concentration in the whole BOT oil. These are likely also the responsible compounds for the antioxidant capacity of the BOT oil. This finding agreed with a previous observation by Rostro-Alanis et al. (2019), in which the bottom fraction that concentrated some compounds such as carvacrol, trans-caryophyllene and α-humulene at levels 5.1, 3.9 and 5.4 times higher, respectively, exhibited a higher antioxidant capacity than the whole oregano EO.

The antioxidant capacity of citrus EOs has been greatly reported and has been attributed mostly to phenolic compounds. However, although monoterpene hydrocarbons and oxygenated monoterpenes comprise the chemical composition of EOs, oxygenated monoterpenes usually more strongly characterize EOs (Ruberto and Baratta, 2000; Yi et al., 2018). Normally a scarce, if any, antioxidant activity is attributed to monoterpene hydrocarbons (Ruberto and Baratta, 2000), such limonene. For instance, limonene, detected as the most abundant compound present in EOs from Valencia orange, Ponkan and Eureka lemon, was found to display a poor antioxidant performance (Lu et al., 2019). Thus, this would be the case of the fractions of BOT oil rich in limonene, which exhibited lower antioxidant activity than F4.

Conversely, oxygenated monoterpenes were the most present class of compounds in the F4 profile. This chemical class harbors many examples of different functional groups including phenols, alcohols, aldehydes, ethers, esters, ketones, etc., with several variants (Ruberto and Baratta, 2000). The antioxidant activity of many of these compounds has already reported. For instance, alcohol compounds such as carveol and perrilla alcohol,

ketones such as carvone, and aldehydes such as perrilla aldehyde, have been found to be highly active antioxidant compounds (Ruberto and Baratta, 2000) and have been detected either at higher concentrations or exclusively in F4. Moreover, another alcohol compound, geranial (citral), was identified as the highest active antioxidant compound in mandarin EO. Esters such as citronellyl acetate, geranyl acetate and neryl acetate detected in that EO, individually, showed some antioxidant capacity as well, even more so than limonene (Yi et al., 2018). These compounds were also exclusively detected in F4. In addition, ethers such as cis-limonene oxide and trans-limonene oxide have also exhibited moderate antioxidant activity (Ruberto and Baratta, 2000). These two last compounds were also present in F4 at slightly higher amount than in F1, F2 and F3. Therefore, the presence of all these mentioned compounds in F4 could explain the highest antioxidant performance by F4 and thus would strongly influence the resulting antioxidant activity of the whole BOT oil. A possible synergistic effect between EO compounds on the antioxidant activity could determine the antioxidant mechanism of the whole EO (Lu et al., 2019). Hence, dietary supplementation with EOs, such as citrus EOs, could assist the control of biological damages caused by free radical production in the organism, thus offering health benefits against the oxidative stress in food-producing animals and also in products derived from these animals by preventing or retarding the oxidative activity, for instance, in raw meat (Giannenas et al., 2013).

The antioxidant mechanisms of EOs is based on both their ability to donate a hydrogen or an electron to free radicals and their ability to delocalize unpaired electrons within the aromatic structure, thus neutralizing free radicals and protecting other biological molecules against oxidation (Giannenas et al., 2013; Simitzis, 2017). Due to their antioxidant effect, EOs also affect lipid metabolism in animal tissues by enhancing the antioxidative enzymes activity (superoxide dismutase, catalase and glutathione peroxidase) and by inhibiting the formation of reactive oxygen species and off-flavors derived from the oxidation of polyunsaturated fatty acids (Simitzis, 2017). The beneficial effect produced by the antioxidant capacity of EOs on pigs fed a diet supplemented with EOs has already been observed (Li et al., 2012a; Xu et al., 2018; Zeng et al., 2015).

Results of this present study showed that certain compounds could be more involved in conferring the biological activities of the citrus EO, BOT, such as its selective antibacterial activity and its antioxidant potential.

4.5. Conclusions

Overall, BOT oil fractions differed in terms of their chemical composition profile and their biological activity. The three fractions richest in limonene exhibited lower antioxidant activity compared to the fraction poorest in limonene. The fraction poorest in limonene but richest in

other compounds like carvone, *cis*-arveol, *trans*-carveol, *cis*-p-Mentha-2,8-dien-1-ol and *trans*-p-Mentha-2,8-dien-1-ol showed selective antibacterial activity between pathogenic and beneficial bacteria as well as the highest antioxidant capacity, suggesting that these compounds play an important role in the biological activities observed for this fraction and consequently for the whole BOT oil, as opposed to the major compound, limonene, having this function exclusively. Therefore, this highlights the importance of the consortium between minor and major compounds for relevant biological activities of whole EOs. These findings, considered together, represent important information on the bioactivities of compounds associated with a citrus EO, and its future possible application as an antimicrobial/antioxidant agent for food-producing animals, such as pigs.

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Supplementary Table

Table 4-S1. Chemical composition of Brazilian orange terpenes' fractions: F1, F2, F3 and F4*.

Compounds[1]	LRI_C[2]		LRI_L[3]		F1 %[6]		F2 %[6]		F3 %[6]		F4 %[6]	
	NP[4]	P[5]	NP	P	NP	P	NP	P	NP	P	NP	P
NI (1)	922	-	-	-	0.05	-	-	-	-	-	-	-
α-Pinene	936	1019	-	1025.4	0.50	0.62	0.28	0.32	0.06	-	-	-
Verbenene	-	1211	-	1123.8	-	-	-	-	-	-	-	0.51
NI (2)	953	-	-	-	0.04	-	-	-	-	-	-	-
Sabinene	975	1118	976	1122	0.48	0.54	0.36	0.38	0.14	-	-	-
β-Pinene	981	1105	980	1100	0.14	0.15	0.10	-	-	-	-	-
Myrcene	989	1158	991	1160.2	1.17	1.59	1.05	1.36	0.46	0.83	-	-
Octanal	1003	-	1002.8	-	0.10	-	0.11	-	0.10	-	0.57	-
2-Cyclohexen-1-one, 3-methyl-	-	1598	-	1579	-	-	-	-	-	-	-	0.15
δ-3-Carene	1015	1146	1011	1146.8	0.15	0.16	0.15	0.14	0.08	-	0.43	-
β-Phellandrene	-	1208	-	1209.3	-	0.10	-	0.09	-	0.07	-	-
NI (3)	1028	-	-	-	0.37	-	0.28	-	0.29	-	-	-
Limonene	1037	1201	1031	1198.2	91.83	92.60	91.93	93.20	86.95	87.36	0.79	0.61
p-Cymenene	1092	1430	1087.9	1437.5	-	-	-	-	-	-	0.69	0.61
Linalool	1099	1538	1098	1543.3	0.19	0.19	0.24	0.22	0.32	0.37	0.54	0.74
1,3,8-p-Menthatriene	1116	1390	1111	1411	-	-	-	-	-	-	0.40	0.27
p-Mentha-2,8-dien-1-ol-trans	1125	1622	1123	1639	0.51	0.53	0.59	0.54	0.94	1.28	6.97	10.11
4-Acetyl-1-methylcyclohexene	1133	-	1137	-	-	-	0.07	-	-	-	0.53	-
cis-Limonene oxide	1137	1446	1134	1450.5	1.55	1.28	1.86	1.52	3.21	2.79	3.00	4.07
p-Mentha-2,8-dien-1-ol-cis	1141	1664	1138	1652.1	-	0.37	-	0.37	-	0.86	8.72	8.90
trans-Limonene oxide	1141	1458	1139	1461.6	1.07	0.60	1.30	0.72	2.23	1.48	-	2.57
NI (4)	1150	-	-	-	-	-	0.04	-	-	-	-	-
β-Terpineol	1153	-	1157	-	-	-	0.05	-	0.15	-	0.29	-
Bicyclo[3.3.0]oct-2-en-7-one, 6-methyl-	1172	1694	-	-	-	-	-	-	-	-	1.00	0.76
Cryptone	-	1673	-	1674.8	-	-	-	-	-	-	-	0.64
1,8-Menthadien-4-ol	1183	1680	1189	1681	0.07	-	0.08	-	0.18	0.29	1.66	3.06
p-Methylacetophenone	1189	1765	1182.7	1765	-	-	-	-	-	-	0.46	0.37
trans-p-Mentha-1(7),8-dien-2-ol	1191	1789	1180.5	1791	0.07	-	0.08	-	0.18	0.21	1.72	2.15
NI (5)	1201	-	-	-	0.20	-	0.22	-	0.56	-	1.71	-

trans-Dihydrocarvone	-	1609	-	1623.1	-	-	-	-	-	0.17	-	0.21
NI (6)	1204	-	1205.4	-	0.25	-	0.27	-	0.69	-	3.31	-
NI (7)	1210	-	-	-	-	-	-	-	0.09	-	-	-
Isodihydrocarveol	-	1774	-	1800	-	-	-	-	-	-	-	0.28
trans-Carveol	1222	1828	1217	1836.3	0.39	0.36	0.33	0.28	0.98	1.12	12.93	17.00
cis-p-Mentha-1(7),8-dien-2-ol	1233	1880	1233	1894.9	-	-	-	-	-	-	0.76	1.19
cis-Carveol	1236	1858	1229	1854.4	0.21	0.16	0.13	0.12	0.51	0.48	4.71	7.39
Carvone	1249	1731	1243	1733.6	0.35	0.34	0.28	0.26	0.94	1.22	10.95	16.45
trans-2-decenal	1263	-	1263.4	-	-	-	-	-	-	-	0.34	-
Geranial	1271	-	1271	-	-	-	-	-	-	-	0.50	-
n-Decanol	1274	-	1272.1	-	-	-	-	-	-	-	1.31	-
trans-carvone oxide	1279	-	1277	-	-	-	-	-	-	-	0.64	-
Perilla aldehyde	1281	1781	1273.4	1793.9	-	-	-	-	-	-	1.06	1.30
NI (8)	1289	-	-	-	-	-	-	-	-	-	3.02	-
Limonen-10-ol	1294	1985	1239	1979	-	-	-	-	-	-	1.08	1.79
NI (9)	1300	-	-	-	-	-	-	-	-	-	0.67	-
Perilla alcohol	1303	1994	1296.3	2006.6	-	-	-	-	-	-	2.15	1.55
NI (10)	1308	-		-	0.09	-	-	-	0.23	-	1.88	-
Limonene dioxide	1311	-	1294	-	-	-	-	-	-	-	0.53	-
(1S,4R)-p-Mentha-2,8-diene, 1-hydroperoxide	1322	-	-	-	-	-	-	-	0.18	-	1.65	-
NI (11)	1327	-	-	-	-	-	-	-	-	-	0.56	-
NI (12)	1335	-	-	-	-	-	-	-	0.13	-	2.47	-
NI (13)	1340	-	-	-	-	-	-	-	-	-	0.47	-
NI (14)	1344	-	-	-	-	-	-	-	-	-	0.75	-
Citronellyl acetate	1349	-	1352.4	-	-	-	-	-	-	-	1.57	-
α-Terpinyl acetate	1357	-	1347	-	-	-	-	-	-	-	0.89	-
Neryl acetate	1360	-	1362.9	-	-	-	-	-	0.12	-	1.30	-
NI (15)	1367	-	-	-	-	-	-	-	-	-	1.46	-
NI (16)	1372	-	-	-	-	-	-	-	-	-	0.44	-
Geranyl acetate	1375	-	1381	-	-	-	-	-	-	-	1.48	-
NI (17)	1377	-	-	-	-	-	-	-	0.07	-	-	-
α-Copaene	1386	-	1377	-	-	-	-	-	-	-	0.78	-
NI (18)	1393	-	-	-	-	-	-	-	-	-	0.97	-
β-elemene	1396	-	1390.4	-	-	-	-	-	-	-	0.82	-
Dodecanal	1409	-	1408.1	-	-	-	-	-	-	-	1.16	-

Decyl acetate	1416	-	1407.1	-	-	-	-	-	-	-	0.66	-
cis-α-Bergamotene	-	1552	-	1559.1	-	0.26	-	0.30	-	0.72	-	2.62
trans-α-Bergamotene	-	1559	-	1575.7	-	0.15	-	0.16	-	0.48	-	1.98
NI (19)	1418	-	-	-	-	-	-	-	-	-	0.38	-
β-Copaene	1447	-	1433.1	-	-	-	-	-	-	-	6.84	-
NI (20)	1667	-	-	-	0.21	-	-	-	-	-	-	-
NI (21)	1701	-	-	-	-	-	0.20	-	0.19	-	-	-
(2-methylprop-1-enyl)-cyclohexa-1,3-diene	-	1245	-	-	-	-	-	-	-	-	-	0.37
NI (22)	-	1633	-	-	-	-	-	-	-	-	-	0.30
Neodihydrocarveol	-	1717	-	1710	-	-	-	-	-	-	-	0.32
NI (23)	-	1727	-	-	-	-	-	-	-	-	-	0.55
NI (24)	-	1746	-	-	-	-	-	-	-	0.26	-	3.30
NI (25)	-	1839	-	-	-	-	-	-	-	-	-	2.65
NI (26)	-	1888	-	-	-	-	-	-	-	-	-	0.64
NI (27)	-	1919	-	-	-	-	-	-	-	-	-	0.81
p-Menth-1-en-9-ol	-	1927	-	1933	-	-	-	-	-	-	-	0.56
NI (28)	-	1946	-	-	-	-	-	-	-	-	-	1.99
NI (29)	-	1966	-	-	-	-	-	-	-	-	-	0.36
NI (30)	-	1973	-	-	-	-	-	-	-	-	-	0.49
NI (31)	-	1978	-	-	-	-	-	-	-	-	-	0.36
					100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

[1] Identification by GC/MS using non-polar and polar columns
[2] LRI_C: Linear retention index (Calculated)
[3] LRI_L: Linear retention index (Literature)
[4] NP: non-polar column DB-5MS
[5] P: polar column DB-WAX
[6] Relative amounts of the identified compounds based on the area of each peak in the chromatogram.
NI: Not identified compound

5. MICROENCAPSULATION ENHANCE THE *IN VITRO* ANTIBACTERIAL ACTIVITY OF A CITRUS ESSENTIAL OIL [4]

Abstract

Essential oils (EOs) have become a promising alternative to antibiotic use in animal breeding because of their biological properties. Citrus EOs, a by-product of citrus processing industries, could be feasible alternatives due to their huge production worldwide. Encapsulation techniques, such as microencapsulation, could allow for EO application in animal feed, preserving EO viability. This study analyzes the use of food grade polymers for microencapsulation of a commercial citrus EO, Brazilian orange terpenes, in a modified starch-chitosan matrix through spray-drying. Initially, physical properties, encapsulation efficiency and morphology of the microcapsules were investigated, as well as the antimicrobial property of the microencapsulate BOT (MBOT) powder on an enterotoxigenic *E. coli* U21 isolated from pig gut and on *Lactobacillus rhamnosus*. Results showed that microparticles had a slight irregular, spherical shape with a mean diameter of 2.77 μm and a high polydispersity. The moisture content of the MBOT powder was 5.39%, the water activity was 0.40 and the bulk density was 0.42 g/mL. Encapsulation efficiency reached 61.17%. In addition, the antibacterial activity results showed that MBOT exerted a higher on *E. coli* U21 and a lower activity on *L. rhamnosus*, thus presenting a selective antibacterial activity between the pathogenic and beneficial bacteria. Moreover, the MBOT exerted a higher selective antibacterial activity than the raw BOT, meaning that the antibacterial performance of the raw BOT was enhanced by encapsulation. This is probably because wall material besides protects the raw BOT against volatilization; it could have interacted in synergism with the raw BOT enhancing the antibacterial effect of the MBOT powder. Therefore, the modified starch-chitosan matrix could result advantageous to encapsulate BOT and turn feasible its application in animal feed.

Keywords: Modified starch-chitosan matrix; Spray-drying; *E. coli*,; *Lactobacillus*; Pig feed

5.1. Introduction

Essential oils (EO) are naturally volatile bioactive compounds derived from plants, which have turned into a promising alternative to antibiotic use in food-producing animals, such as pigs (Franz et al., 2010). This as a result of the banning of in-feed antibiotics as growth promoters for food-producing animals since 2006 by the European Community Regulation (EC) No. 1831/2003 (EU, 2003). The restriction was established as a measure in response to global concern regarding the growing emergency and propagation of antibiotic-resistant bacteria. Recently, EOs have gained increased attention, particularly, in the pig production sector, as EOs can improve pig performance as effectively as antibiotics (Vanrolleghem et al., 2019). The primary mode of action through which EOs can promote growth involves the

[4] Chapter adapted from:
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promotion of gut health and the function of young pigs during weaning, a critical stage of pig life (Gong et al., 2014). This mainly consists of the establishment of a healthy gut microbiota to fight pathogen colonization and gut disorders (Gong et al., 2014) as result of the antimicrobial activity of EOs complemented with other EO activities.

An interesting feature of EOs in contrast to an antibiotic, has to do with the fact that EOs are a mixture of several compounds, with every single compound having its own biological properties. Thus, EOs are of a complex nature and composition with diverse effects (Zeng et al., 2015b), such as multiple targeting antibacterial effect and with no bacterial resistance reported to date (Gong et al., 2014). A viable alternative to antibiotics in pig production should be safe for the public, financially effective and ecofriendly (Omonijo et al., 2018). Among the types of EOs, citrus EOs stand out as a potential alternative that meets these criteria, as they are common by-products of citrus processing industries and there is a large production of these EOs worldwide. Interestingly, citrus EOs have been found to present antibacterial properties against a range of pathogenic bacteria and they have been generally recognized as safe (GRAS) (Fisher and Phillips, 2008). Therefore, citrus EOs may be an alternative of natural antimicrobials to fulfil the needs of pig producing sector.

Nevertheless, due to the volatile nature of EOs, a method to effectively and practically apply EOs in pig feed and deliver the EO into the lower pig gut involves providing a enteric protection by means of encapsulation techniques, such as microencapsulation (Omonijo et al., 2018). This technique helps to enhance the oxidative stability, thermostability and shelf-life of EOs, preserving their biological and functional properties and ensuring the EOs targeted delivery from the feed to the gut of the animals (Bakry et al., 2016; Stevanović et al., 2018). In addition, microencapsulation facilitates EOs mixability, masks the EO odor and allows for combining with other feed ingredients (Stevanović et al., 2018). Therefore, the aim of this research was to study the microencapsulation of a commercial citrus EO, Brazilian Orange Terpenes (BOT), in a modified starch-chitosan matrix using spray-drying method, and to evaluate the physical properties, morphology and antimicrobial activity of BOT microcapsules on bacteria of occurrence in the pig gut.

5.2. Material and methods

5.2.1. Essential oil supply

A commercial citrus EO was used in this study, Brazilian orange terpenes (BOT), which is a by-product from orange processing and was supplied by a factory from São Paulo State, Brazil. Once the sample was received, it was kept in amber bottles under refrigeration (4°C) until use. Then, this oil was used as a core material for microencapsulation.

5.2.2. Microencapsulation

Preparation of emulsion

The emulsion consisted of an aqueous phase and an oil phase. The aqueous phase, which was the wall material, was comprised of a blend of a chitosan solution (2.27% w/v) and a modified starch solution (40%w/v) (Hi-cap, National Starch & Chemical Pty., Ltd., NSW, Australia). Each solution was prepared under constant stirring a day before emulsification and then kept overnight at room temperature to guarantee the full saturation of the polymers. The oil phase consisted of the BOT oil. Thus, to prepare the emulsion, the BOT oil was gradually added to the wall material solution containing Tween 80 (1% in relation to the total weight of the emulsion) while stirring at 15500 rpm for 5 minutes using a rotor-stator blender (Ultra-Turrax IKA T18 basic, Germany). The emulsion was prepared at the total solid content of 23g/100g, composed of modified starch 76.8 wt%, chitosan 3.2 wt% and BOT oil 20 wt%. Thus, the weight ratio of BOT oil to wall material was 1:4. This emulsion was used as the feed liquid for spray-dryer to the production of the microencapsulated powder.

Spray drying

The production of the microcapsules was carried out by drying the emulsion in a Büchi mini spray dryer B290 (Büchi, Flawil, Switzerland). Drying was performed in the concurrent phase flow at the following processing conditions: inlet air temperature = 140°C, outlet air temperature = 78±°2C, feed sample rate = 7 g/min, and sample temperature = 25°C. The dry product was then collected in a cyclone receiver; after the experiment had been completed, the material was removed from the walls and drying chamber. Once the microencapsulated powder containing the BOT oil was collected, it was stored in an opaque-hermetic container at 25°C until further analysis. Three independent drying cycles were performed.

5.2.3. Characterization and physical properties of microcapsules

Bulk density, moisture content and water activity (a_w)

Bulk density was determined by following the approach of (Mehyar et al., 2014), with slight modifications. Three grams of the microcapsule powder were loosely placed in a 10mL-graduated cylinder. The cylinder was vibrated, shaken, and gently tapped on a flat

surface until it reached a constant volume. The final volume was recorded, and the bulk density was calculated by dividing the sample weight by its volume.

The moisture content was measured using a moisture analyzer (MX-50, A&D Company, Tokyo, Japan) at 105 °C by drying the microcapsule powder (until moisture variation was <0.01%/min) and the water activity (a_w) was measured using a water activity meter (AquaLab 4TEV, Decagon Devices Inc., Pullman, USA) at 25.00 ± 0.50 °C.

Mean diameter and size distribution

The mean diameter and the size distribution of the microcapsules were obtained by light scattering using an LV 950-V2 instrument (Horiba, Kyoto, Japan). Samples from the microencapsulate powder were previously dispersed in absolute ethanol, then dispersed samples were added to the LV950-V2 sample bath containing the appropriate dispersion medium (absolute ethanol) until achieving transmittance levels suitable to perform the measurements. The mean particle size was expressed as the volume mean diameter ($D_{4,3}$), and the polydispersity was given by the span index, which was calculated according to Equation (3). The measurements were performed in triplicate.

$$Span = \frac{D_{90} - D_{10}}{D_{50}}$$

Where D_{10}, D_{50} and D_{90} correspond to the diameters relative to 10, 50 and 90% of the accumulated size distribution.

Encapsulation Efficiency analysis

Encapsulation efficiency (EE) of the EO was determined as the amount of core material, BOT oil, encapsulated inside of the powder particles. EE was determined based on the total oil content and the surface oil content following the Eq. (1):

$$\%\, EE = \frac{\%\, Total\ oil - \%\, Surface\ oil}{\%\, Initial\ load\ oil} \times 100 \quad (1)$$

Where, total oil refers to the entrapped oil (encapsulated) (Mehyar et al., 2014) and the surface oil. The surface oil is related to the unencapsulated oil present on the surface of microparticles, and the initial load oil is the amount of oil added to the emulsion.

Total oil

The total BOT oil content of the microcapsules was determined following the procedure described by (Mehyar et al., 2014), with slight modifications. Thus, total oil was determined by distilling 10 g of microencapsulated powder suspended in 100 mL of distilled water for 3 h in a Clevenger-type apparatus. The BOT was collected in the trap; its volume was measured and multiplied by a density factor of 0.9 g/mL to calculate the weight of oil recovered from the sample. Total oil was given by the proportion of the weight of oil recovered relative to the weight of distilled powder (%). The assay was performed in three independent replicates.

Surface oil

The surface oil (non-encapsulated oil fraction) was determined according to (Fernandes et al., 2013) with some modifications. Five grams of the encapsulated powder were weighed in a cone of filter paper (Whatman No. 40), which was prepared manually. The cone was placed in a tared funnel coupled to a 250 mL glass bottle. The microcapsules were washed five times using 10 mL of petroleum ether at room temperature, for a total of 50 mL of petroleum ether. After filtration through the filter paper, the solvent was evaporated from the glass bottle, and the extracted oil, which was considered the surface oil, was determined gravimetrically.

Because an amount of volatiles are lost during drying, another parameter to evaluate the drying process is a determination of the oil retention (Li et al., 2013). Thus, the proportion of total oil in the powder after spray drying in relation to the initial oil loaded indicates the remaining oil in the powder, and was calculated using Eq. (2) (Fernandes et al., 2013).

$$\%\ Oil\ retention = \frac{\%\ Total\ oil}{\%\ Initial\ oil\ load} \times 100 \quad (2)$$

Morphology and microstructure

Visualization of the surface morphology of the BOT microcapsules was performed using scanning electron microscopy (SEM). A thin layer of the powder was fixed on a 1 cm diameter glass surface of, then sputter coated with a 30-nm gold layer and observed in a scanning electronic microscope operating at an acceleration voltage of 20 kV (LEO 435 VP, Leo Electron Microscopy Ltd., Cambridge, England).

5.2.4. Antibacterial activity

Bacterial strains

An ETEC strain and a *Lactobacillus* species were evaluated in this study as models of pathogenic and beneficial bacteria, respectively, that occur in the pig gut. The ETEC strain, *E. coli* U21 (K88+/LT+/STb+/F18+/Sta+), was isolated from pig gut and provided by The Swine Heath Laboratory of the Department of Preventive Veterinary Medicine and the Animal Health-School of Veterinary Medicine and Animal Science at the University of São Paulo, Brazil. *Lactobacillus rhamnosus* ATCC 7469 was purchased from American Type Culture Collection (ATCC). The ETEC strain was cultivated in Tryptic Soy Agar-Difco (TSA) at 37 ºC for 18-20 h and *L. rhamnosus* was grown in MRS agar (Man, Rogosa and Sharpe agar) at 30 °C for 48 h. After activation, the bacteria were sub-cultured in Brain-Heart Infusion (BHI) broth or MRS, both supplemented with 15% v/v of glycerol. After incubation, they were stored at -20 ºC until their use.

Determination of Minimal Inhibitory Concentration (MIC)

The determination of the MIC of the microencapsulated BOT (MBOT) was performed by microdilution assay in a 96-well microplate following the standard protocol M07-A9 from the Clinical and Laboratory Standards Institute (CLSI, 2012), with some modifications. For the assay, the standard inoculum was prepared in sterile saline solution (0.85% w/v) from living colonies of the selected bacteria above, contained in plates of either TSA agar (*E. coli*) or MRS agar (*L. rhamnosus*.) at the optical density equivalent to the 0.5 McFarland Standard (0.08-0.13 at 625 nm). Subsequently, the standard inoculum was diluted at 1:100 to obtain an inoculum of 10^6 CFU/mL (final inoculum). The stock solution of microcapsules was prepared at 112 mg of MBOT/mL with MH or MRS broth. From the stock solution, two-fold serial dilutions were made in a range from 112 to 1.75 mg of MBOT/mL along the Y-axis of the microplate. Twenty microliters from the final inoculum were added to each well containing 180 µL of several MBOT concentrations, being the final volume in each well of 200 µL and bacterial population of approximately 10^5 CFU/mL. The following controls were used: growth control (180 µL of MH or MRS broth + 20 µL of inoculum); growth control containing wall material (180 µL of MH or MRS broth with wall material (modified starch/chitosan) + 20 µL of inoculum), growth control containing wall material-Tween 80 material (180 µL of MH or MRS broth with wall material-Tween 80 + 20 µL of inoculum). Finally, microplates were incubated at 37ºC for 24 h for *E. coli* and at 30°C for 36 h for *Lactobacillus* spp. The MIC was established as the lowest MBOT concentration that inhibited visible bacterial growth supported by resazurin testing at the end of the incubation period. For the resazurin test, 25 µL of resazurin (R7017; Sigma-Aldrich) solution at 0.0135% m/v were used per well. Thus,

after visual inspection, the presence of viable cells was evidenced through a change in the resazurin color from blue resazurin to pink resorufin (Pereira et al., 2014) after 1h of further incubation. Assays were carried out in triplicate in three independent replicates.

Minimal Bactericidal Concentration (MBC)

The determination of MBC was performed from microplate wells containing MBOT concentrations where there was no visible bacterial growth. An aliquot of 100 µL was taken from each well and seeded in MH or MRS agar. Then, plates were incubated for 24 h at 37ºC for *E. coli* U21 and for 48 h for *L. rhamnosus*. The MBC was defined as the lowest concentration of MBOT able to cause total bacterial death, represented by the visible absence of colonies on the agar plates.

5.2.5. Data analysis

The data are shown as the mean of the replicates with the standard deviations.

5.3. Results and discussion

5.3.1. Physical properties and morphology of microcapsules

The physical characteristics of BOT microcapsules is shown in Table 5. 1. The results showed that BOT microparticles reached mean diameters (d4,3) of 2.77 ± 0.03 µm. A previous study showed that microencapsulation of an orange EO with maltodextrin and whey protein as wall material, also via spray-drying, provided a powder with a particle mean diameter ranging from 10.21 to 12.44 µm at 30% of solid content and 15% of EO in the emulsion (Carmona et al., 2013). Similarly, microencapsulation of a commercial orange EO with chitosan alone (1:2) (Li et al., 2013) or with modified starch alone (1:4) (Rojas-Moreno et al., 2018) as wall materials provided powders with mean particle sizes of ~20 µm in each case. Moreover, microcapsules of lime EO (20%) having as wall material (80%) a blend of arabic gum/maltodextrin was reported as presenting a mean particle size ranging from 21.6 to 38.5 µm depending on the operating conditions (temperature) during drying (Bringas-Lantigua et al., 2012). In comparison to these previous studies, the mean size dimeter of BOT in modified starch-chitosan matrix microcapsules was ~10 times smaller. The diameter of microparticles by spray-drying may depend on several factors such as the characteristics and proportion of the wall material, emulsion properties (viscosity and total solid content), drying conditions (temperature) and type of atomizer used (Bringas-Lantigua et al., 2012;

Carmona et al., 2013; Fernandes et al., 2014; Rojas-Moreno et al., 2018). Particle size strongly influences the appearance, flowability, and dispersibility of a microcapsule powder (Reineccius, 2004). In this study, smaller particle sizes of BOT microcapsules could be advantageous to achieve adequate dispersion and homogenization of the powder in the animal feedstuff. However, the role of spray-dried products particle size regarding flavor retention is still being discussed (Reineccius, 2004).

In addition, BOT microparticle powders showed a high polydispersity - that is, a high heterogeneity of particle sizes as indicated by the scatter value (span index) equal to 1.12±0.01 (Table 5.1). This was also supported by Fig. 5.1, in which a certain heterogeneity of BOT particles in the powder was observed. However, previous studies reported higher scatter values for a microencapsulated oregano EO (with maltodextrin/modified starch/Arabic gum) and lavender EO (with maltodextrin/Acacia gum), which ranged from 1.79 to 2.59, and 1.76 to 2.1, respectively, indicating homogeneity of particles in both powders (Alvarenga Botrel et al., 2012; Burhan et al., 2019). Likewise, a span index of 2.13 indicated that the spray-dried rosemary EO-Arabic gum powder also presented a homogeneous particle distribution (Fernandes et al., 2013).

Table 5.1. Physical characterization of BOT microcapsules*.

Parameters	d4,3 (μm)	Span	Bulk density (g/mL)	Moisture content (g/100 g powder)	a_w[1]
MBOT	2.77±0.03	1.12±0.01	0.42±0.01	5.39±0.24	0.40±0.01

* Values are means ± Standard Deviation (SD) of triplicate determinations.
[1] a_w = water activity

It is also important to know the density characteristics of the microparticles, since this can be useful to predict the volume occupied by the final powder product within packaging or during storage (Campelo et al., 2018). In our study, the bulk density of the MBOT powder was 0.42 ± 0.01 g/mL, which was close to the values previously reported for a microencapsulated cardamom EO with whey protein/guar gum (0.49 g/mL) or whey protein/guar gum/carrageenan (0.41 g/mL) (Mehyar et al., 2014). However, it a lower bulk density has been reported for a microencapsulated lime EO with whey protein concentrate (Campelo et al., 2018) and a microencapsulated rosemary EO with modified starch (Fernandes et al., 2014), with values of 0.22 ± 0.04 and 0.25 ± 0.01 g/mL, respectively. A higher bulk density product translates to more convenient storage due to smaller container requirements, compared to a low-density product (Sun et al., 2019). Moreover, high bulk density values, as observed in our study, would indicate an absence of air in the powder, which means more stability to protect against oxidation during storage (Sun et al., 2019).

Bulk density can be influenced by other physical characteristics of the microcapsule powder such as the particle size (d4,3) the degree of polydispersity and the moisture content (Mehyar et al., 2014).

Furthermore, the moisture content and a_w of the MBOT powder was 5.39±0.24 % and 0.40±0.01, respectively. This was similar to results reported for an orange EO microencapsulated with native rice starch or modified rice starch, which had a moisture content ranging from 4.70±0.01 to 5.97±0.03 %. (Márquez-Gómez et al., 2018). Similarly, it was reported that the moisture content for a microencapsulated lemon EO in Arabic gum-maltodextrin matrix ranged from 5.15 to 7.10 %, depending on the temperatures applied during drying (Bringas-Lantigua et al., 2012). However, limonene alone, the major compound in citrus EOs, spray-dried encapsulated with modified starch (Hi-cap), whey protein concentrate or maltodextrin was reported as presenting lower moisture contents (from 1.2 to 2.7 %) (Jafari et al., 2007). Certainly, a moisture content of ~5% in a powder product is considered an optimal level to ensure best preservation of the powder (Márquez-Gómez et al., 2018) by reducing risk of microbial contamination and oil oxidation (Sun et al., 2019). Thus, the moisture content and the low a_w of the MBOT could be convenient to ensure its stability and property retention during the storage of the animal feedstuff (once added to it).

Regarding the encapsulation efficiency of BOT, this was 61.17±8.51 % (Table 5.2). This was a comparable encapsulation efficiency to what was reported for a spray-dried encapsulated orange EO with modified starch (1:4 EO:wall), which ranged from 61.72 ± 0.17 to 73.21 ± 0.33%, depending on the operating drying temperatures (Rojas-Moreno et al., 2018). However, slightly higher encapsulation efficiency was reported for an orange EO when encapsulated with a native rice starch alone or in combination with the modified rice starch (in a ratio 15:85 EO:wall material), with values of 73.13 and 73.33%, respectively (Márquez-Gómez et al., 2018). In addition to the encapsulation efficiency, evaluating the oil retention capacity of an encapsulation formulation is also important, since it indicates the proportion of the remaining EO (total oil) in the powder after drying with respect to the initial oil added. The oil retention in the MBOT powder was 65.79±6.83 %. Previous studies reported that microencapsulation of a commercial orange EO with maltodextrin/whey protein concentrate (Carmona et al., 2013) or modified starch (Rojas-Moreno et al., 2018) led to slightly higher oil retention percentages in the powders, ranging from 73.71 to 79.41 %, and from 74.55 to 77.66 %, respectively. It has been reported that the retention of an orange EO is markedly increased when the ratio of EO to wall material is decreased. For instance, when chitosan is used alone as a wall material to encapsulate an orange EO (Li et al., 2013). An optimal EO:wall material ratio is suggested as being one of the most important factors for the retention of an orange EO in a powder product (Li et al., 2013; Rojas-Moreno et al., 2018).

Table 5.2. Encapsulation efficiency of BOT oil in modified starch-chitosan matrix microcapsules*.

	Microcapsule component (g/100 g powder)			% Oil retention	%EE[1]
	Total oil	Surface oil	Entrapped oil		
MBOT	13.16±1.37	092±0.47	12.23±1.70	65.79±6.83	61.17±8.51

* Values are means ± Standard Deviation (SD) of triplicate determinations.
[1] %EE = Encapsulation efficiency

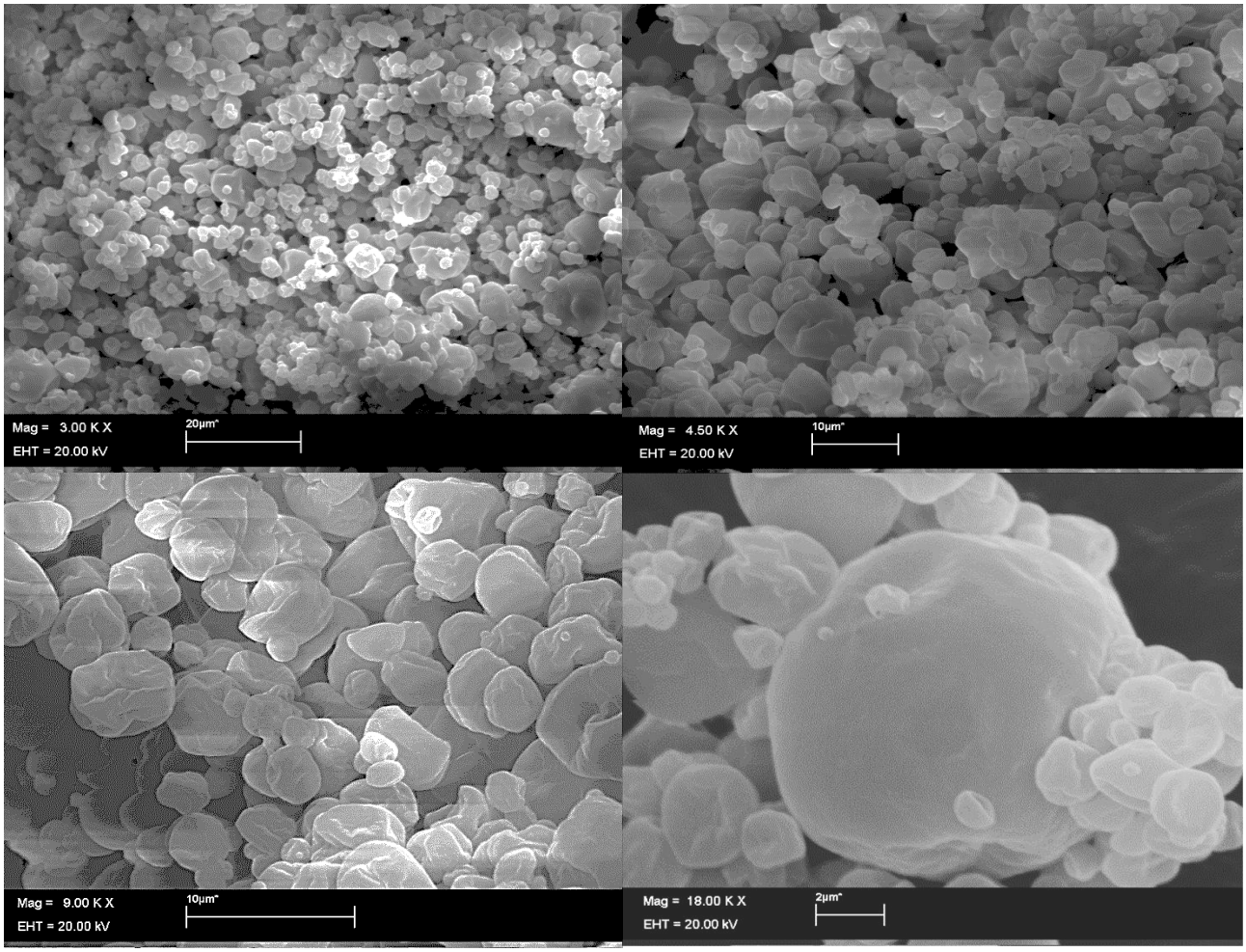

Fig. 5.1. Scanning electronic microscopy (SEM) images of BOT oil in modified starch-chitosan matrix microcapsules. a:3000x, b: 4.5.000x, b: 9.000x, c: 18.000x.

Finally, observation of the morphology of BOT microcapsules obtained by spray drying is shown in Fig.5.1. Most of the microcapsules presented slight irregular spherical shapes, with some having shrunk or experienced denting. No holes, pores or cracks were observed on the surface of the microcapsules, neither were any breakdowns of the full microcapsules observed. This indicated high stability of the MBOT. Structures with these characteristics were previously observed for microcapsules of an oregano EO in a β-cyclodextrin matrix or for microcapsules of an orange EO in a rice starch matrix (Arana-Sánchez et al., 2010; Márquez-Gómez et al., 2018). However, microencapsulating an orange EO with chitosan in a

ratio 1:1 EO:wall yielded well-defined, slightly shrunk spherical microcapsules(Li et al., 2013). Additionally, size variations were observed between the BOT microcapsules and some agglomerates. It was indicated that this may be a consequence of partial flocculation of the feeding emulsion, resulting in particles of different sizes which agglomerate after spray-drying (Sun et al., 2019).

5.3.2. Antibacterial activity

Additionally, the biological properties of the MBOT powder, specifically its antibacterial activity, were proved. Our results reveled that the MBOT exerted a stronger antibacterial activity on *E. coli* U21 than on the beneficial bacterium *L. rhamnosus*, as observed by the lower MIC values to *E. coli* U21 (Table 5.3). Thus, meaning that the MBOT maintained the selective antibacterial performance initially observed for the raw BOT (Table 5.3).

Table 5.3. Antimicrobial activity of BOT oil in modified starch-chitosan matrix microcapsules.

Treatments	Antibacterial parameter	*E. coli* U21		*L. rhamnosus*	
		mg microcapsules /mL	Raw oil content (mg/mL)	mg microcapsules /mL	Raw oil content (mg/mL)
Modified starch-chitosan	MIC	28.00	-	28.00	-
	MBC	28.00	-	28.00	-
Modified starch-chitosan + BOT	MIC	3.50	0.46	14.00	1.85
	MBC	7.00	0.93	14.00	1.85
Raw BOT	MIC		1.85	-	3.70
	MBC		1.85	-	7.40

However, compared to the raw BOT, the MBOT exerted a higher antibacterial activity. This suggests that possibly the antibacterial efficiency of the raw BOT was enhanced by microencapsulation, since a lower amount of the raw BOT in the microcapsules was needed to kill or inhibit bacteria, specially, the pathogenic one (*E. coli* U21). This enhanced antibacterial activity of the MBOT could be related to the EO protection against rapid volatilization by encapsulation, retaining the viability of the EO and its properties. Nevertheless, it was observed that the wall material alone had a certain antibacterial effect (although not selective) on the tested targets, which would indicate that the wall material and the raw BOT would have interacted in synergism, the antibacterial activity of the final MBOT powder. Proper protection can reduce the required effective dosage of EOs in pig feed and reduce the cost of pig production (Omonijo et al., 2018). The synergism between BOT and wall material would be advantageous in terms of cost-effectiveness. In addition, for successful targeted delivery of carriers (wall material) in feed to the lower gut, the process

conditions include slow digestion of the carrier matrix. The digestion rate depends on the type of compounds and their structural ordering (Stevanović et al., 2018). Polymers-based particles, such as the modified starch and chitosan, could slow digestion and facilitate delivery of the BOT oil into the lower pig gut. Our results were in line with a previous study by (Arana-Sánchez et al., 2010), which reported that three different microencapsulated oregano EOs, with β-cyclodextrin, were more effective than the raw EOs in inhibiting and killing bacteria, such as *E. coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The authors have hypothesized that this improved *in vitro* antibacterial activity of the microencapsulate powders could be associated with another positive effect of encapsulation, that is, the enhanced solubility of the EO in water by the wall material serving as a carrier. Thus, improving the EO stabilization and bioavailability in a liquid media culture (broth) (Arana-Sánchez et al., 2010).

The improved solubility of an EO by encapsulation would yield beneficial results since it is intended to have an effect of the EO in the pig gut through solubilization of the MBOT powder in the gut chyme during digestion of the feedstuff. Conversely, other studies have reported that EOs or EO compounds, when microencapsulated, have exerted the same antibacterial activity as the raw EOs/EO compounds on *E. coli* without testing the effect of the wall materials used alone; such as microencapsulated lemongrass EO (MIC = 22.23 mg/mL) (Leimann et al., 2009) and microencapsulated carvacrol (MIC = 0.25 mg/mL) (Sun et al., 2019). Already, some commercial products consisting of microencapsulated EOs or EO compounds have presented a higher antibacterial activity on *E. coli* than on *Lactobacillus* sp. of the pig gut microbiota in *in vivo* studies (Ahmed et al., 2013; Li et al., 2012; Zeng et al., 2015a). The selective antibacterial effect of our formulation - a commercial orange EO in modified starch-chitosan matrix microcapsules - on the pig gut microbiota, still needs to be elucidated in further experiments for its application in pig feed.

5.4. Conclusions

The present study investigated the microencapsulation of a commercial orange EO, Brazilian orange terpenes, into a modified starch-chitosan matrix by spray-drying, which was successfully performed, aiming its potential application in pig feed. Results showed that microencapsulation of BOT oil was achieved with significant BOT loading and encapsulation efficiency, indicating that the processing conditions were adequate to trap the BOT oil. The microparticle morphology, size and polydispersity were typical of spray-drying products. The MBOT presented a selective antibacterial activity between *E. coli* and *L. rhamnosus*, exhibiting a remarkably lower effect on the beneficial bacterium. Interestingly, the MBOT showed a higher antibacterial activity than the raw BOT, meaning that the antibacterial

performance of the raw BOT was enhanced by encapsulation. The wall material, in addition to protecting the BOT against volatilization, could have interacted in synergism with the raw BOT, favoriting the antibacterial effect of the BOT microencapsulate powder. Therefore, the enveloping modified starch-chitosan matrix could result advantageous to encapsulate EO and turn feasible the application of the EO in the animal feed. Of course, further research is needed to elucidate the effect of the MBOT in the pig gut microbiota.

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6. EFFECT OF A MICROENCAPSULATED CITRUS ESSENTIAL OIL ON *IN VITRO* FERMENTATION KINETICS OF PIG GUT MICROBIOTA[5]

Abstract

Essential oils (EOs) have emerged as a potential alternative to antibiotic use in pig breeding due to their antimicrobial properties. Citrus EOs, a common by-product of orange juice industry, can be financially an interesting alternative due to their huge offer in the global market. Thus, the effect of a microencapsulated citrus EO, Brazilian Orange Terpenes (BOT), on pig gut microbiota was evaluated by means of an *in vitro* fermentation model simulating different sections of the pig gut (stomach, ileum and colon). Treatments consisted in: BOT in its raw form (1.85 and 3.70 mg/mL), microencapsulated BOT (MBOT, at 3.50 and 7.00 mg/mL), colistin (2 µg/mL) and a control. Initially, results showed that the raw BOT and MBOT altered in a similar way the total bacterial kinetics in stomach only from 18h of fermentation and no metabolites production in terms of short-chain fatty acids (SCFAs) were detected at this fermentation stage. In ileum and colon fermentations, the raw BOT and the MBOT shifted ileal and colonic microbiota in a way that altered their total bacteria kinetics, reduced their diversity and altered their composition ($p < 0.05$) as evidenced by the significant reduction of certain bacterial taxa. However, more remarkable effects were found for the MBOT, indicating its higher antimicrobial effects compared to the raw BOT. This would indicate that probably the antibacterial efficiency of the raw BOT was enhanced by microencapsulation. Furthermore, the MBOT stimulated lactate production in ileum fermentation and greatly stimulated the overall SCFAs production in colon fermentation. Therefore, these results would indicate that besides the shifts on ileum and colonic microbiota by the delivered EO (BOT), the wall material of microcapsules (chitosan/modified starch) could have worked as an additional carbon source with prebiotic functioning, specifically, for colonic bacteria since it would have stimulated their growth and metabolic activity (SCFAs).

Keywords: Citrus essential oil;Microencapsulation; Pig gut microbiota; SCFA; ETEC

6.1. Introduction

The interest of using phytogenic feed additives in animal production, such as essential oils (EOs), has been growing during the last 20 years. The reason is because antibiotic use as feed additive to improve growth in farm animals was fully banned in 2006 by the European Union (EC 1334/2003, 2003), due to the concern of its use being related with the increase of antibiotic resistance in human bacteria. In pig production, antibiotics have contributed to improve the productivity of this sector by controlling enteric diseases in young pigs (Burch et al., 2008). EOs are plant extracts constituted by a mix about 20-60 aromatic-

[5] Chapter adapted from:
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volatile compounds (Bakkali et al., 2008; Burt, 2004). These compounds confer to EOs several biological properties, whereby have turned them a potential alternative to using antibiotics in animal feed such as pig production. Specifically, EOs for their antimicrobial, anti-inflammatory and antioxidant properties could exert beneficial effects on the pig gut ecosystem (Omonijo et al., 2018), which would impact positively on the pig performance. An important modulation of the gut microbiota of pigs by the antimicrobial effect of EOs, that is, suppressing pathogenic bacteria without compromising beneficial commensal bacteria would be desirable.

Earlier *in vitro* studies with pure cultures of pig gut bacteria have proved the efficiency of EOs or EO compounds in inhibiting pathogenic bacteria while not affecting noticeably beneficial bacteria (Ambrosio et al., 2017; Ouwehand et al., 2010; Si et al., 2006). Interestingly, EOs from citrus plant have been found for presenting this feature in the *in vitro* study (Ambrosio et al., 2017). Citrus EOs, commonly are a vital by-product of the citrus processing industries. These oils are the most produced EOs around the world, being the orange EO the most produced one (Barbieri and Borsotto, 2018; Iwabuchi et al., 2010). It has been estimated that the worldwide orange EO production reached 54.9 mil tons in 2018, being Brazil the major producer country, with a representativity of 52.7% of the global production (United Nations, 2019). Thus, due to the selective antimicrobial activity, the vast availability in the global market and the safety recognition to be used as feed additive (Fisher and Phillips, 2008), the application of citrus EOs in pig feed could turn feasible.

Nonetheless, the effectiveness of an EO on bacteria species may vary from pure cultures to that from mixed populations, as in the case of bacteria that are found in the pig gut environment. *In vitro* gut fermentation models are advantageous alternative assays that enable the stable cultivation of a complete intestinal microbiota and allow to investigate the effects on the composition and functionally of the gut microbiota in defined experimental conditions (Payne et al., 2012). For practical, cost, and ethical reasons and for the good reproducibility between experiments, *in vitro* gut fermentation models result to be an excellent alternative to *in vivo* assays (Gresse et al., 2017).

A few studies have proved that citrus peel EOs, alone or in combination with other EOs, can be potential natural growth promoters in broiler chickens, since positive effects on gut microbiota and microbial metabolites, and gut morphology were found (Erhan et al., 2017; Hong et al., 2012). Similarly, in-feed citrus peel extracts have led to an enhancement on the growth performance (Ebrahimi et al., 2014) and on the proximal intestinal morphology of broiler chickens too (Akbarian et al., 2013). Mostly, isolated EO compounds such as thymol, carvacrol and cinnamaldehyde have been widely proved for having a modulatory effect on pig gut microbiota in *in vivo* studies (Li et al., 2012b, 2018). Besides this effect, EOs or EO compounds can enhance the production of digestive secretions and nutrient

absorption, reduce the pathogenic stress, exert antioxidant activity and reinforce the pig immune system (Zeng et al., 2015). In addition, EOs/EO compounds may affect the organoleptic properties of the feedstuff, enhancing its flavor and palatability, which would impact positively on the feed intake, especially in pigs (Franz et al., 2010; van der Aar et al., 2017).

For these reasons, an important aspect that should be considered when it is intended to add EOs on the feedstuff, is the EOs volatile nature, since if an EO oil is added in their raw form, loss of its effectiveness due to volatilization can occur during the feedstuff storage. Moreover, it is necessary to consider an effective mode to deliver the EO inside to the pig gut. Thus, encapsulation techniques such as microencapsulation could assist to solve volatilization problems, preservation of EO properties and delivery of EOs to the pig gut (Omonijo et al., 2018). Therefore, this study aimed at investigating the effect of a microencapsulated commercial citrus EO, Brazilian Orange Terpenes (BOT), on pig gut microbiota by means of an *in vitro* fermentation model simulating several sections of the pig gut.

6.2. Material and methods

6.2.1. Essential oils supply

A commercial citrus EOs was used in this study, Brazilian orange terpenes (BOT), which was a by-product from orange juice production and was supplied by a factory from São Paulo State, Brazil. Once the sample was received, it was kept in amber bottles under refrigeration (4°C) until use. Then, this oil was used as a core material for microencapsulation by spray-dryer using as wall material a mixture of chitosan and modified starch. The amount of BOT oil used was 1:4 (w/w) relative to the mass of the wall material. The total BOT oil content in the microcapsules was 13.2%.

6.2.2. Preparation of EO/antibiotic solutions

Stock solutions of the raw BOT oil were prepared at 10.3 and 20.6% v/v (92.5 and 185 mg/mL respectively) with sterile distilled water using Tween 80 (T09799RA, Exôdo científica-Brazil) as emulsifier. Regarding the microencapsulated BOT (MBOT), stock solutions were prepared at 178.7 and 357 mg/mL with sterile distilled water. Colistin sulphate stock solution (C4461; Sigma-Aldrich) was prepared at 0.1 mg/mL with sterile distilled water too.

6.2.3.*In vitro* fermentation assay

The *in vitro* fermentation assay was carried out under simulated prevailing conditions of stomach, ileum and colon of pigs. Specific media for each condition were used in combination with the BOT oil, raw and microencapsulated (MBOT), in order to evaluate the effect of this citrus oil on the fermentation kinetics and metabolite production of pig gut microbiota.

Media preparation

For mimicking *in vitro,* the prevailing conditions of stomach, ileum and colon of pigs, three different media were prepared following the protocols reported in previous studies and they are summarized in Table 6-S1. Stomach media was prepared according to Beumer et al. (1992) with slight modifications. In brief, once the basal solution for the stomach simulation was prepared, it was boiled, cooled and the pH of the media was adjusted to pH=3 with HCl. Then, 47.5 mL of this basal solution were dispensed into serum bottles (capacity of 120 mL) and were sealed with rubber stoppers and aluminum crimp seals. Once the media was cooled, 2.5 mL of the enzyme solution (previously filter-sterilized, Table 6-S1) was added to the bottles using a syringe under sterile conditions.

Regarding the ileum media, it was prepared following the recipe by Blake et al. (2003) with slight modifications (Table S1). In brief, once the ileum-basal solution was prepared, it was boiled, cooled by flushing N_2/CO_2 through it and the pH was adjusted to pH = 6. Then, 50 mL of this basal solution were dispensed into serum bottles, which were sealed with rubber stoppers and aluminum caps. Next, a stream of N_2/CO_2 was flowed into the bottles for removing the O_2, bottles were autoclaved and cooled. Afterwards, filter-sterilized vitamin and trace element solutions were added to the bottles (0.05 and 0.10 mL respectively), using a syringe and under sterile conditions.

The colon media was prepared according the recipe by Williams et al. (2005) with slight modifications (Table 6-S1). In brief, the colon-basal solution was prepared, boiled, cooled by flushing N_2/CO_2 (80:20 v/v) through it, the pH was adjusted (pH = 6.5-7and 46.4 mL of this basal solution were distributed into serum bottles. Then, N_2/CO_2 was flowed into the bottles for removing the O_2 and bottles were sealed with rubber stoppers and aluminum caps. Next, bottles were autoclaved, cooled, and 0.6 mL of vitamin/phosphate solution (filter-sterilized), 2.4 mL of bicarbonate solution and 0.6 mL of reducing agent were added to the bottles using a syringe under sterile conditions too. Set-up bottles with the corresponding media were kept under refrigeration (4°C) until use. Media were prepared in advance.

Inoculum preparation

For this study, stomach, ileum and colon contents were collected from commercial pigs in a slaughterhouse located at the province of Gelderland, the Netherlands. Immediately after the pigs were slaughtered, contents from each pig gut location were collected under anaerobic conditions, placing the gut content in sterile serum bottles previously flushed with N_2/CO_2 (80:20 v/v). After the bottles were sealed with a rubber stopper and aluminum crimps seals, a sterile needle accoupled to a filter (0.2 µm) was inserted into the rubber stopper and bottles were placed into an anaerobic jar containing oxygen-removing sachets anaerobic gas generating sachets (Thermo Scientific™ Oxoid AnaeroGen). The collected samples were rapidly transferred to the laboratory and used when it was as fresh as possible. Once in the laboratory, the material from each gut location was weighted and samples from 3 pigs were pooled for each location inside of an anaerobic tent. Stomach, ileum and colon inoculum were prepared from each of the generated pool with a pre-warming step (39 ◦C), anaerobic (N_2/CO_2) and sterile saline solution (0.9% v/v NaCl) in a ratio of 1:1, 1:10 and 1:10 (w/w), respectively. Inoculums were prepared right before the experiment began to be used as fresh as possible.

In vitro fermentation procedure

The *in vitro* fermentation experiment was carried out at the experimental conditions indicated in Table 6.1. In total six treatments were performed as follow: Control (no addition of EO/antibiotic), the raw BOT oil at 1.85 mg/mL and 3.70 mg/mL, the MBOT oil at 3.5 mg/mL (≈ 0.463 mg of raw BOT/mL) and 7.0 mg/mL (≈ 0.93mg of raw BOT/mL) and colistin treatment at 2.0 µg/mL. Colistin was tested at that concentration because the MIC for this antibiotic on *E. coli* has been previously reported as ≤ 2.0 µg/mL (CLSI, 2012; EUCAST, 2019). In addition, according to JECFA (2016) the average daily intake of colistin for livestock animals was stablished on the basis of the colistin MIC_{50} 1 µg/mL of colistin for *E. coli*. For this experiment the serum bottles containing the corresponding stomach, ileum and colon media were taken off from refrigeration, warmed at 39°C and then 1 mL of each raw BOT, MBOT or colistin stock solutions were added to the bottles of each media using a syringe in order to reach the working EO/antibiotic concentrations. Bottles were stirred for homogenizing the EO/antibiotic solutions with the media. Subsequently, to start the fermentation assay, 1.5 mL of the fresh inoculum from each gut location were added to the bottles containing the respective media with the EO/antibiotic treatments using a syringe. Once, bottles were fully set-up, they were stirred again for fully homogenization and finally

brought to incubation at 39°C for 72h. Three replicates per media and treatment were performed in this experiment. During the incubation period, samples were taken at 0h, 12h, 18h, 24h, 48h and 72h of fermentation.

Table 6.1. *In vitro* batch incubation conditions simulating the pig gastric, ileal and colonic fermentation.

	Stomach simulation	Ileum simulation	Colon simulation
Inoculum (mL)*	1.5	1.5	1.5
Medium and Headspace	Aerobic (free air)	Anaerobic (N_2/CO_2, 80:20 v/v)	Anaerobic (N_2/CO_2 80:20 v/v)
pH	3	6	6.5 - 7
Temperature (°C)	39	39	39
Duration	72 h	72 h	72 h
Treatments mg/mL			
- Control	0	0	0
- BOT	1.85, 3.70	1.85, 3.70	1.85, 3.70
- MBOT	3.5, 7.0	3.5, 7.0	3.5, 7.0
- Colistin	0.002	0.002	0.002

*Once added the inoculum to the corresponding media, immediately, was taken an aliquot, which was the sample at time 0 h and was called as “Starpoint” of the fermentation.

DNA extraction, PCR amplification, and sequencing

Total bacterial DNA was isolated from the pellet of 1.5 mL stomach, ileum and colon fermentation samples. The pellets were obtained by centrifugation at 10 000 rpm for 10 min. The DNA isolation protocol comprised the following steps: the pellet was resuspended in 650 µL Stool Transport and Recovery (STAR) buffer (Roche Diagnostics Nederland BV, Almere, the Netherlands) in a tube containing zirconia-silica beads (0,1 mm) (Sigma) and glass beads (2.5 mm), then the tubes were homogenized 3 times at 5.5 m/S for 60s in a bead-beater (Berlin Technologies, CNIM, Montigny-le-Bretonneux, France). The homogenized samples were incubated at 95°C for 15 min and centrifuged at 4°C for 5 min (10 000 rpm). Supernatants were taken and the DNA was isolated using the Maxwell 16 Instrument (Promega, Leiden, the Netherlands) as described by van Gastelen et al. (2017). Amplification of the V5–6 hypervariable region of the bacterial 16S rRNA gene was performed using universal primers (784 f: 5′-[AG]GGATTAGATACCC-3′, 1064 r: 5′-CGAC[AG][AG]CCATGCA[ACGT]CACCT-3′) containing unique barcodes for each sample. Amplification by PCR reaction was performed in triplicate, using an initial denaturation at 98°C for 30s followed by 25 cycles of denaturation at 98°C for 10 s, annealing at 42°C for 10 s, elongation at 72°C for 10 s, and a final step of 72°C for 7min. PCR products were run in an electrophoresis chamber on a 1.3% agarose gel at 135 V to confirm the successful

amplification. The replicates of the PCR products for each sample were pooled and purified using magnetic beads CleanPCR kit (Clean NA, GC Biotech B.V., The Netherlands) according to the manufacturer's instructions and eluted in 35 µL of nuclease-free water (Qiagen). The DNA concentration was determined by Qubit BR dsDNA kit using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Purified amplicons were pooled at 200 ng per sample per library preparation and sent for Illumina HiSeq at GATC. The quantity of the libraries was checked by a Qubit 2.0 Fluorometer before to send for sequencing.

Quantitative real-time PCR

Quantitative real-time PCR was performed using published primer sets (Table 6-S2). SYBR green qPCR assays were performed with the DNA samples using an iCycler iQ real-time detection system (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands). All qPCR analyses were carried out in triplicate with a reaction volume of 10 µL. The reaction mixture contained iQ SYBR green mix, each primer at 10 µM (final concentration) and 2.5 µL of the sample DNA or PCR grade water. The amplification program comprised of an initial denaturation at 95ºC for 3 min followed by 39 cycles of 95°C for 15 s, 60 or 52°C for 30 s, and 72°C for 30 s. Following amplification, melting curves were obtained by slow heating at 0.5°C/s from 65 to 95°C. Standard curves were generated with 10-fold serial dilutions of the 16S rRNA genes amplified from of each targeted strain: Total bacteria and *E. coli*.

Analysis of metabolites production

Short chain fatty acids (SCFA) production was analyzed by high-performance liquid chromatography (HPLC). For this, 1.5 mL from stomach, ileum or colon fermentation samples were taken and centrifuged at 10 000 rpm for 10 min. Then, the supernatant was stored at -20°C until HPLC analysis. A SCFA standard curve from a stock solution (100 mM) containing lactate, formate, acetate, propionate, isobutyrate and butyrate was prepared. Crotonate was used as the internal standard. Vials contained sample supernatants/standard curve and crotonate in a ratio 4:1 (v/v). SCFA production was measured with a LC 2030C HPLC (Shimadzu, Den Bosch, the Netherlands) equipped with a column Metacarb 67h of 300×6.5 mm (Agilent, Amstelveen, the Netherlands) for the separation of organic acids compounds. The injection volume was 20 µL. The column working temperature was 45°C, the mobile phase was sulphuric acid 0.01 N at a flow of 1.0 mL/min. Metabolites were detected by a refractive index detector coupled (RID-20A, Shimadzu, Den Bosch, The Netherlands).

6.2.4. Bioinformatics and statistical analysis

The 16S rRNA gene sequencing data was analyzed using an in-house bioinformatics pipeline NG-Tax (Ramiro-Garcia et al., 2016) version 2.0 (Poncheewin et al., 2019). Operational taxonomic units (OTU) were defined using an open reference approach, and taxonomy was assigned to those OTU using a SILVA 16S rRNA gene reference database version 128. Two different in-house mock communities (Ramiro-Garcia et al., 2016) were also sequenced as controls in order to check the sequencing quality. The mocks were compared with their theoretical compositions by performing Pearson correlations using QUIIME. The phylogenetic diversity (PD) was calculated using the picante R package version 1.7.0 and a One Way Anova test with Tukey's post-hoc test were performed in order to check for significant differences between treatments. Next, principal coordinates analysis (PCoA) based on weighted Unifrac distances was performed on the microbial community composition of all samples using the microbiome R package version 1.7.2. To evaluate the effect of treatments and fermentation time on the microbial community, multivariate analysis of variance (PERMANOVA), using Adonis test with 999 permutations was performed using vegan R package version 2.5.3. The abundance of microbial taxa was expressed as percentage (relative abundance) of the total 16s rRNA gene sequences. To evaluate the effect of the treatments, a One-way Anova model and a Tukey's post-hoc test for pairwise comparison were used to detect significant differences using R software (version 3.6.1). The confidence level for all the analysis was 0.05.

6.3. Results

6.3.1. Total bacteria kinetics of pig gut microbiol fermentation

The total bacterial kinetics based on the 16S rRNA gene copies is shown in Fig. 6.1. It was observed that BOT, either raw or microencapsulated (MBOT) altered the number of total bacteria in stomach, ileum and colon fermentations. Overall, colistin did not alter the total bacteria population for any of the three fermentation stages compared to the control. The number of total bacteria in stomach was unaltered by BOT and MBOT until 18h after the start of the fermentation process, but both treatments reduced the number of total bacteria at later times in a similar manner, compared to the control. Regarding ileal fermentation, the raw BOT at 1.85 mg/mL and the MBOT (3.5 and 7.0 mg/mL) had a slight reductive effect on total bacteria population until 24h of fermentation. Nonetheless, the higher concentration of the raw BOT oil (3.70 mg/mL) caused a bacteriostatic effect until 12h of fermentation. In

colon fermentation, the raw BOT inhibited total bacteria population while the MBOT increased it in contrast to the control.

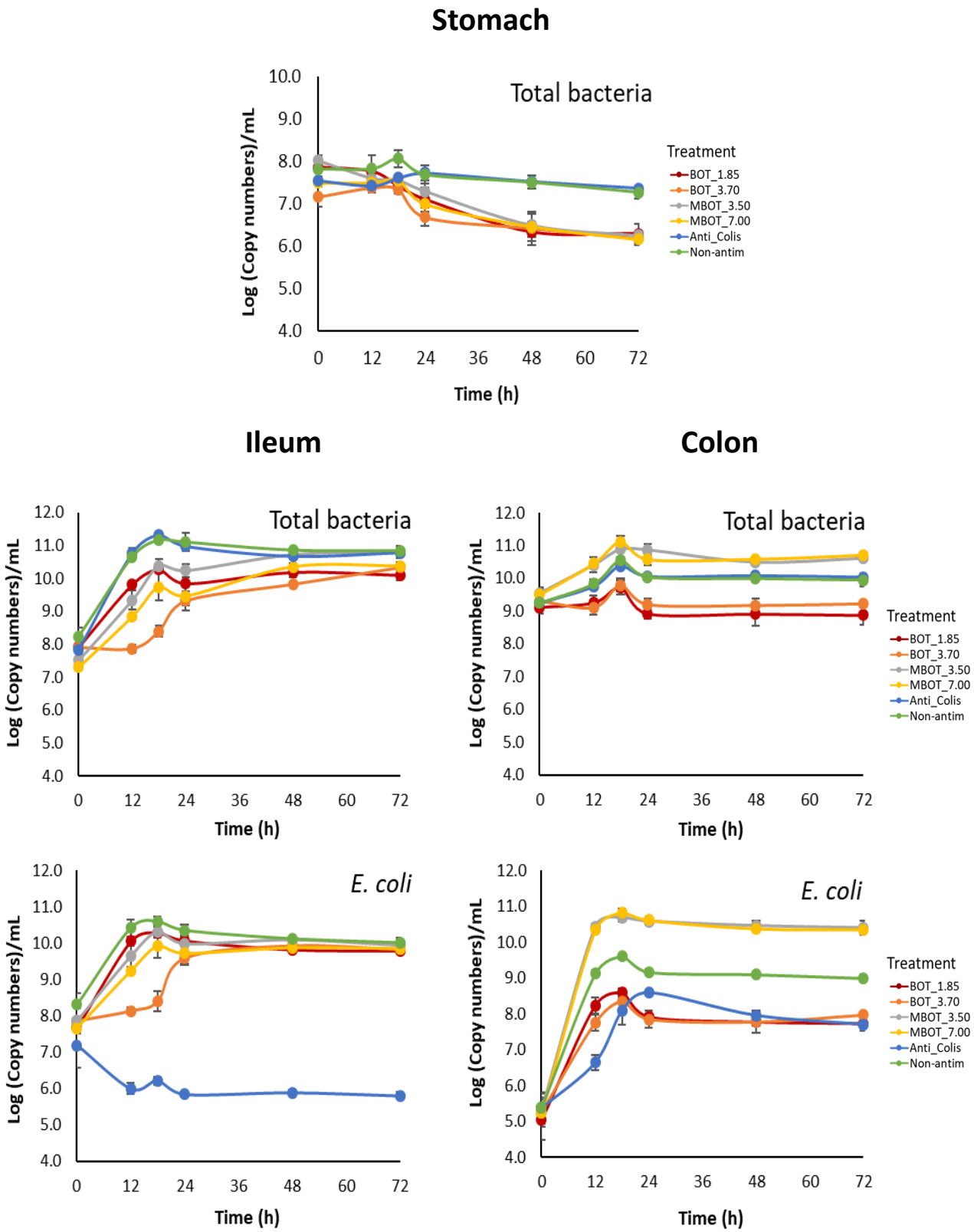

Figure 6.1. Total bacteria and *E. coli* kinetics based on the 16S rRNA gene copy numbers (Log_{10}[copies]/mL) by effect of the raw BOT, microencapsulated BOT (BOT) and colistin on pig simulated stomach, ileum, and colon fermentations.

6.3.2. Diversity and composition of ileal and colon microbial fermentation

Firstly, the phylogenetic diversity (PD) of the bacterial communities in the two gut fermentation stages, ileum and colon, as effect of the raw BOT oil and the MBOT was calculated (Fig. 6.2). Overall, during the whole fermentation term, the MBOT (either at 3.70 or 7.00 mg/Kg) compared to the raw BOT reduced significantly the PD of the ileal microbial fermentation in a same manner than colistin ($p<0.05$). However, time of fermentation (12, 24 and 72h) influenced progressive changes on the PD by effect of the raw BOT, MBOT and colistin when compared to the control (Fig. 6.2A). Regarding the colonic microbial fermentation, the raw BOT and the MBOT reduced significantly the PD in contrast to the control and colistin treatments (Fig. 6.2B), but the MBOT caused a higher reduction of the PD. Colistin did not change the PD compared to the control during the whole fermentation term.

Subsequently, it was explored the variations on the microbial community composition and the degree of similarity between them at OTU level as effect of the raw BOT oil, the MBOT and colistin on both gut fermentation stages, ileal and colonic were explored (Fig. 6.3A and 6.3B). Principal coordinate analysis (PCoA), based on weighed UniFrac distances matrices, revealed that the raw BOT, the MBOT and colistin significantly changed in different manner the bacterial composition in both, ileal and colonic fermentations, as supported by Permanova and Adonis test (Table 6.2). In addition, the bacterial composition was significantly altered by the time of fermentation within each treatment, since a clear clustering by the time effect was also observed in the PCoA plot for both, ileal and colonic fermentations (p-value's Adonis test < 0.05) (Fig. 6.3). These results suggested that the diversity of the bacterial community composition of ileal and colonic microbial fermentations were significantly changed by the raw BOT and MBOT compared to the control and regard to colistin treatment.

Table 6.2. Effects of experimental factor on *in vitro* fermentation pig microbiota. Adonis based on Weighted UniFrac distances

	Factor	Ileum	Colon
Permanova and Adonis test	Treatment	$R^2 = 0.50$ $p = 0.001$	$R^2 = 0.78$ $p = 0.001$
	Time point	$R^2 = 0.25$ $p = 0.001$	$R^2 = 0.09$ $p = 0.001$
	Treatment×Time point	$R^2 = 0.23$ $p = 0.001$	$R^2 = 0.08$ $p = 0.001$

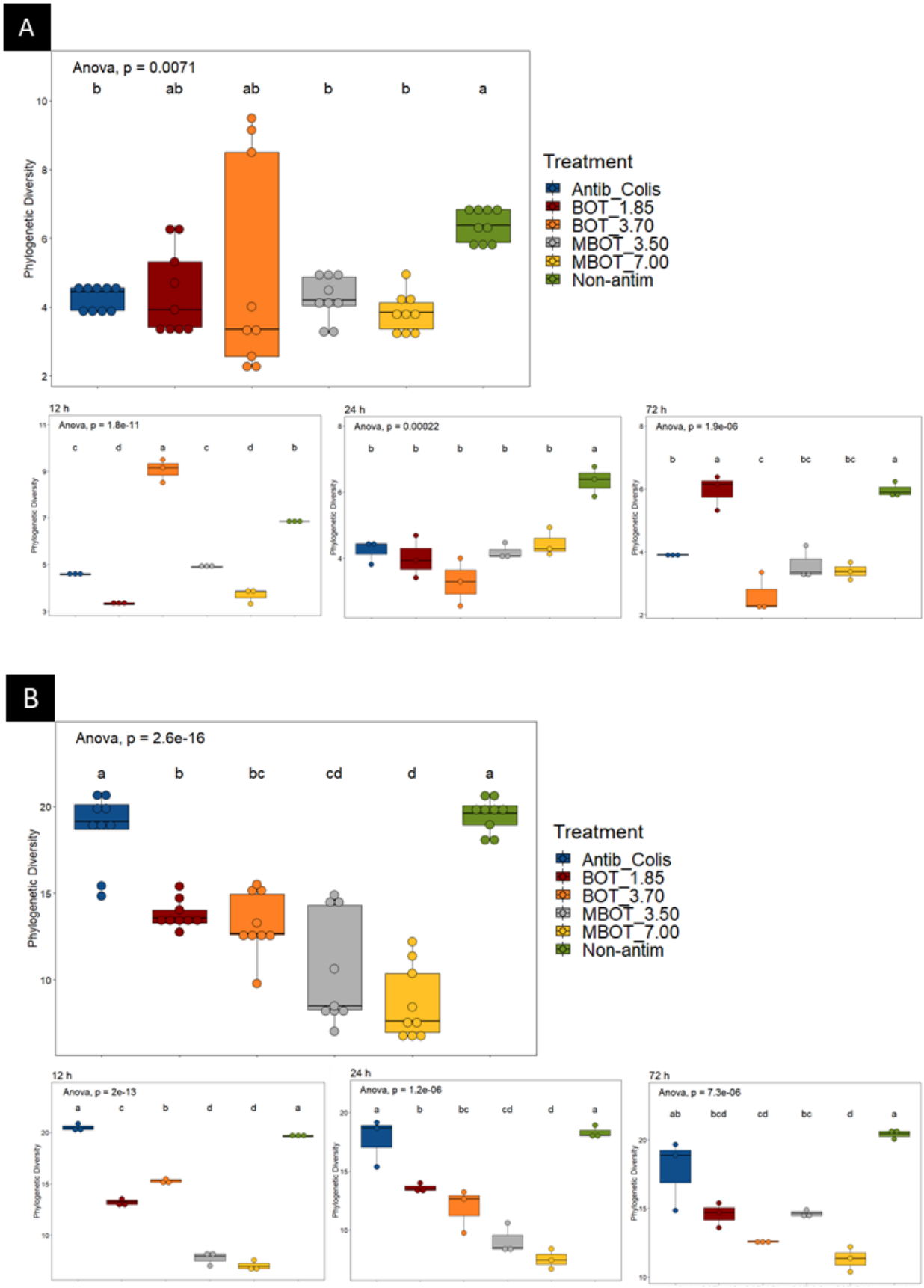

Figure 6.2. Changes in the phylogenetic diversity of (A) ileum and (B) colon microbial fermentations by effect of the raw BOT, microencapsulated BOT (MBOT) and colistin.

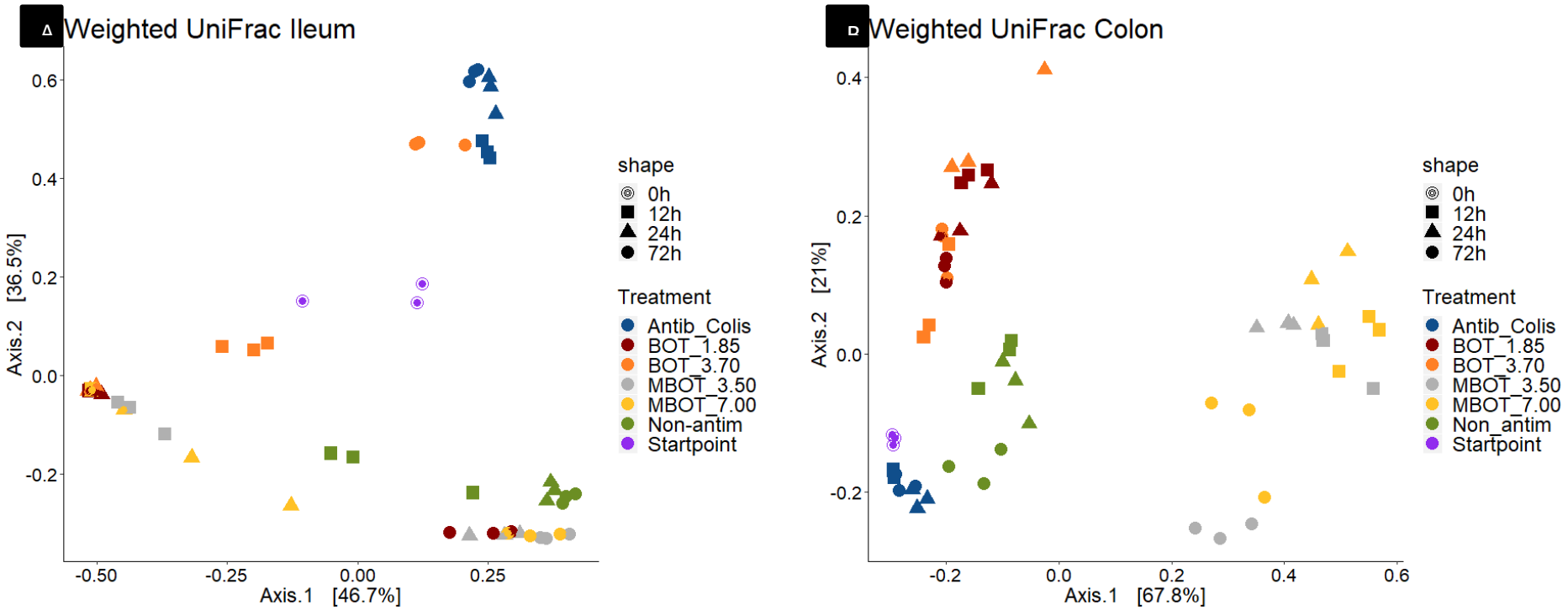

Figure 6.3. PCoA plots of the microbial composition of pig ileum (A) and colon (B) fermentations by effect of the raw BOT, microencapsulated BOT (MBOT) and colistin treatments and by effect of the time of fermentation. “Starpoint” of fermentation (bacterial composition at 0h).

Taxonomical distribution

Additionally, the taxonomic distribution of the bacteria in ileal and colonic microbial fermentations was explored. Based on the relative abundance, dynamic variations of main phyla and genera by effect of the citrus EO treatment during the whole ileal and colonic fermentations were observed (Fig. 6-S1).

For the ileal fermentation, we evaluated the changes at the top-3 phyla at the end of the fermentation (72 h), as shown in Table 6.3. Among these, *Firmicutes* was the most abundance phylum in the control treatment (92%), this was significantly reduced by the raw BOT at 3.70 mg/mL and colistin, while both treatments increased greatly *Actinobacteria* abundance. Conversely, the MBOT fully reduced *Actinobacteria*. Moreover, the raw BOT increased *Proteobacteria* abundance while colistin fully reduced it. Subsequently, at genus level, the variations on the top-5 genera in the ileal microbial fermentation were evaluated (Fig. 6.4, Table 6.3) and results showed that the raw BOT, MBOT and colistin significantly altered their abundance ($p < 0.05$) (Figure 6.4). *Bifidobacterium* was greatly increased by the raw BOT at 3.70 mg/mL (78%) and colistin (87%), while both treatments reduced the abundance of *Lactobacillus*. The raw BOT and MBOT fully reduced *Clostridium sensu stricto 1* and *Streptococcus* abundances while that colistin increased *Clostridium sensu stricto 1* compared to the control. The raw BOT compared to MBOT and the control, slightly increased *Escherichia_Shigella* abundance while colistin efficiently fully reduced it. Specifically, results of the estimation of *E. coli* absolute abundance (Fig. 6.1) showed that only colistin treatment reduced *E. coli* in the ileal fermentation.

Table 6.3. Relative abundance* of phyla and genera after 72h of ileal microbiota fermentation as effect of BOT oil treatment, raw and microencapsulated.

Treatment	Antib_Colis	BOT_1.85	BOT_3.70	MBOT_3.50	MBOT_7.00	Non-antim
Phylum						
Firmicutes	0.22±0.09^{b}	0.82±0.06^{a}	0.02±0.0.4^{c}	0.94±0.03^{a}	0.91±0.05^{a}	0.92±0.00^{a}
Proteobacteria	0.00±0.00^{c}	0.18±0.06^{a}	0.11±0.0.4^{ab}	0.06±0.03^{bc}	0.09±0.05^{abc}	0.04±0.00^{bc}
Actinobacteria	0.78±0.09^{a}	0.00±0.00^{b}	0.87±0.0^{a}	0.00±0.00^{b}	0.00±0.00^{b}	0.04±0.02^{b}
Other	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Genus						
Bifidobacterium	0.78±0.09^{a}	0.00±0.00^{b}	0.87±0.01^{a}	0.00±0.00^{b}	0.00±0.00^{b}	0.04±0.02^{b}
Clostridium_sensu_stricto_1	0.20±0.08^{a}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.01±0.00^{b}
Escherichia_Shigella	0.00±0.00^{c}	0.17±0.05^{a}	0.11±0.04^{ab}	0.06±0.03^{bc}	0.09±0.05^{abc}	0.04±0.01^{bc}
Lactobacillus	0.00±0.00^{c}	0.81±0.06^{b}	0.02±0.04^{c}	0.94±0.03^{a}	0.91±0.05^{ab}	0.87±0.03^{ab}
Streptococcus	0.02±0.01^{ab}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.03±0.02^{a}
Others	0.00±0.00	0.01±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

*Values are means ± Standard Deviation (SD) of triplicate determinations

One-way ANOVA test with Tukey's post hoc test was used. Results within a row with different superscript letters indicate significant difference ($p < 0.05$).

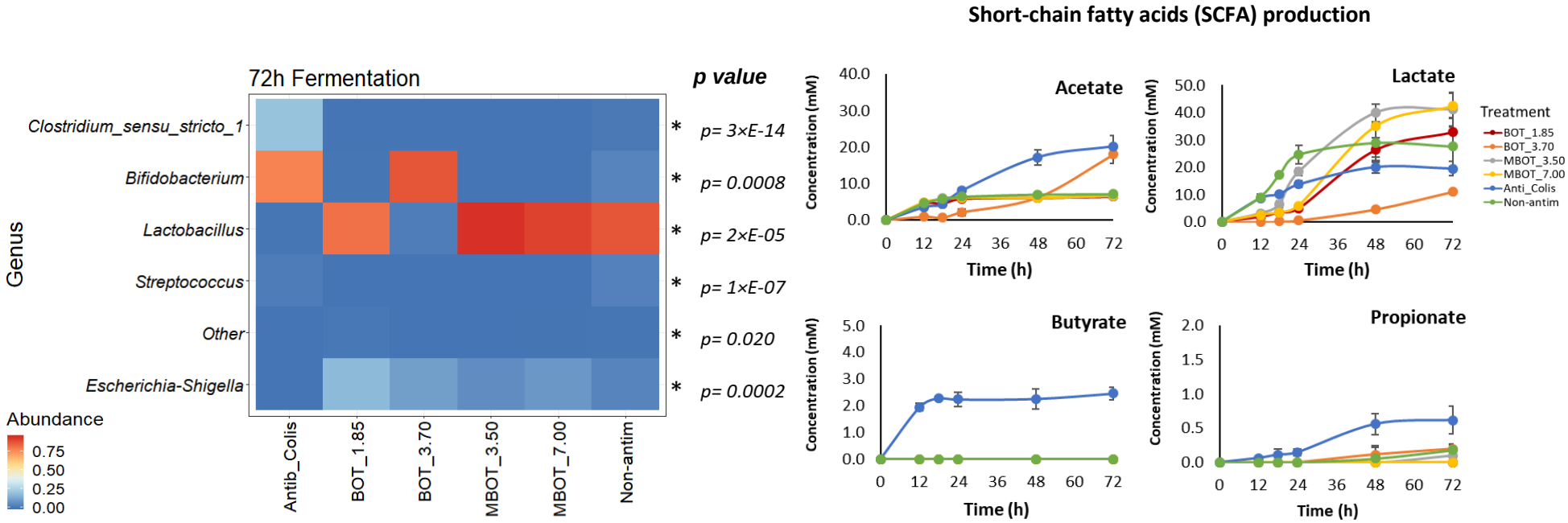

Figure 6.4. Heat map of the relative abundance of the top-5 genus present in ileum microbial fermentation (in the left) and short chain fatty acids (SCFA) production (in the right) by effect of the raw BOT, microencapsulated BOT (MBOT) and colistin after 72 h of fermentation. p-values were calculated by a one-way Anova model and asterisks (*) indicate significant differences ($p<0.05$) on the relative abundance by effect of the treatments. Vertical bars indicate the standard deviation.

Regarding colonic microbial fermentation, significant changes in the relative abundance of bacteria at phylum and genus level by effect of the BOT and MBOT treatments at the end of the fermentation (72 h) were observed as shown in Table 6.4. Firstly, at phylum level, the variations on relative abundance of the top-7 phyla were evaluated. Depending on the concentration tested, the raw BOT and the MBOT fully reduced *Bacteroidetes, Euryarchaeota* and *Synergistetes* compared to the control treatment. In addition, the MBOT significantly reduced *Firmicutes* while highly increased *Proteobacteria* abundance. The raw BOT increased *Fusobacteria* abundance but colistin fully reduced this phylum in contrast to the control. Inversely, colistin efficiently increased *Firmicutes* (to 95%) whereas reduced *Proteobacteria* compared to the control. Furthermore, at genus level, the variations on the relative abundance of the top-19 genera by effect of the BOT treatments is shown in the Fig. 6.5 and Table 6.4. The raw BOT and the MBOT depending on the concentration tested fully reduced *Clostridium sensu stricto 13*, *Terrisporobacter, Ruminiclostridium 9*, *Christensenellaceae R7 group, Ruminococcaceae UCG 002* and *Ruminococcaceae uncultured genus* abundances. Additionally, the MBOT significantly reduced *Clostridium sensu stricto 1, Lactobacillus, Syntrophococcus, Subdoligranulum* and *Turicibacter* abundances, while the raw BOT increased or kept the same abundances of these genera in contrast to colistin and the control. Inversely, the MBOT significantly increased the abundance of *Streptococcus* and *Ruminiclostridium_5* genera whereas the raw BOT increased noticeable *Fusobacterium* and *Peptostreptococcaceae Unknown Genus* (even though this was found not significant). In addition, the raw BOT efficiently decreased *Escherichia_Shigella* abundance (although this was found not signicant in contrast to the control) while colistin fully reduced it. Specifically, the raw BOT was as same efficient as colistin to decrease *E. coli* abundance (Fig 6.1). However, the MBOT increased *Escherichia_Shigella* and *E. coli*, even at an upper level than the control. Therefore, at genus level it was more evident that the MBOT exerted higher antibacterial activity than the raw BOT on colonic bacteria as evidenced by the significant reduction of several bacterial taxa. However, the increase in the abundance of some colonic bacteria by the MBOT would indicate that, besides the alterations by the delivered EO, the microcapsules (starch modified and chitosan) could have worked as an additional carbon source to promote the growth of these bacteria.

6.4. Short chain fatty acids (SCFAs) production

The production of fermentation end-products such as SCFAs was evaluated for the stomach, ileal and colonic microbial fermentations. As expected, no SCFAs production was detected in the stomach fermentation, since at this gut stage mainly enzymatic activity

Table 6.4. Relative abundance of phyla and genera after 72h of colon microbiota fermentation as effect of BOT oil treatment, raw and microencapsulated.

Treatment	Antib_Colis	BOT_1.85	BOT_3.70	MBOT_3.50	MBOT_7.00	Non-antim
Phylum						
Actinobacteria	0.01±0.01^{a}	0.02±0.01^{a}	0.02±0.01^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.01±0.01^{a}
Bacteroidetes	0.01±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.03±0.01^{a}	0.00±0.00^{b}	0.02±0.01^{ab}
Euryarchaeota	0.0±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.02±0.01^{a}
Firmicutes	0.95±0.02^{a}	0.79±0.02^{b}	0.76±0.05^{b}	0.42±0.06^{c}	0.39±0.04^{c}	0.78±0.04^{b}
Fusobacteria	0.00±0.00^{b}	0.16±0.02^{a}	0.20±0.06^{a}	0.02±0.01^{b}	0.01±0.00^{b}	0.03±0.01^{b}
Proteobacteria	0.01±0.00^{c}	0.03±0.00^{bc}	0.03±0.01^{bc}	0.53±0.08^{a}	0.60±0.04^{a}	0.13±0.05^{b}
Synergistetes	0.01±0.01^{a}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.01±0.00^{ab}
Others	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Genus						
Asaccharospora	0.05±0.09^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
Sharpea	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.05±0.06^{a}	0.00±0.00^{a}
Christensenellaceae_R7_group	0.07±0.03^{a}	0.00±0.00^{b}	0.00±0.00^{b}	0.01±0.01^{b}	0.00±0.00^{b}	0.06±0.02^{a}
Clostridium_sensu_stricto_1	0.07±0.02^{b}	0.16±0.00^{a}	0.18±0.02^{a}	0.02±0.01^{c}	0.01±0.00^{c}	0.07±0.01^{b}
Clostridium_sensu_stricto_13	0.03±0.00^{a}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.04±0.01^{a}
Escherichia_Shigella	0.00±0.00^{c}	0.03±0.00^{bc}	0.02±0.01^{bc}	0.51±0.07^{a}	0.59±0.04^{a}	0.12±0.05^{b}
Terrisporobacter	0.04±0.03^{a}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.01±0.00^{ab}
Fusobacterium	0.00±0.00^{b}	0.16±0.02^{a}	0.20±0.06^{a}	0.02±0.01^{b}	0.01±0.00^{b}	0.03±0.01^{b}
Lactobacillus	0.12±0.05^{b}	0.22±0.03^{a}	0.21±0.05^{a}	0.02±0.00^{c}	0.01±0.00^{c}	0.12±0.01^{b}
Mogibacterium	0.01±0.00^{a}	0.04±0.04^{a}	0.01±0.00^{a}	0.05±0.01^{a}	0.02±0.00^{a}	0.05±0.01^{a}
Others	0.24±0.05	0.11±0.01	0.10±0.01	0.16±0.01	0.10±0.02	0.25±0.03
Peptostreptococcaceae_Unknown_Genus	0.17±0.15^{a}	0.15±0.01^{a}	0.17±0.02^{a}	0.02±0.00^{a}	0.01±0.00^{a}	0.08±0.01^{a}
Ruminiclostridium_5	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.09±0.03^{a}	0.02±0.04^{a}	0.00±0.00^{b}
Ruminiclostridium_9	0.03±0.02^{a}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.04±0.00^{a}
Ruminococcaceae_UCG_002	0.08±0.03^{a}	0.00±0.00^{c}	0.00±0.00^{c}	0.02±0.01^{bc}	0.00±0.00^{c}	0.05±0.01^{ab}
Ruminococcaceae_uncultured_genus	0.02±0.01^{a}	0.00±0.00^{c}	0.00±0.00^{c}	0.01±0.00^{b}	0.00±0.00^{bc}	0.03±0.00^{a}
Streptococcus	0.004±0.00^{b}	0.02±0.00^{b}	0.02±0.00^{b}	0.05±0.01^{b}	0.16±0.06^{a}	0.004±0.00^{b}
Subdoligranulum	0.02±0.01^{a}	0.02±0.01^{a}	0.02±0.00^{a}	0.001±0.00^{b}	0.00±0.00^{b}	0.02±0.00^{a}
Syntrophococcus	0.01±0.01^{b}	0.03±0.00^{a}	0.03±0.00^{a}	0.002±0.00^{c}	0.0004±0.00^{c}	0.01±0.00^{b}
Turicibacter	0.03±0.01^{b}	0.05±0.01^{a}	0.05±0.01^{a}	0.01±0.00^{c}	0.00±0.00^{c}	0.02±0.00^{b}

*Values are means ± Standard Deviation (SD) of triplicate determinations

One-way ANOVA test with Tukey's post hoc test was used. Results within a row with different superscript letters indicate significant difference ($p < 0.05$).

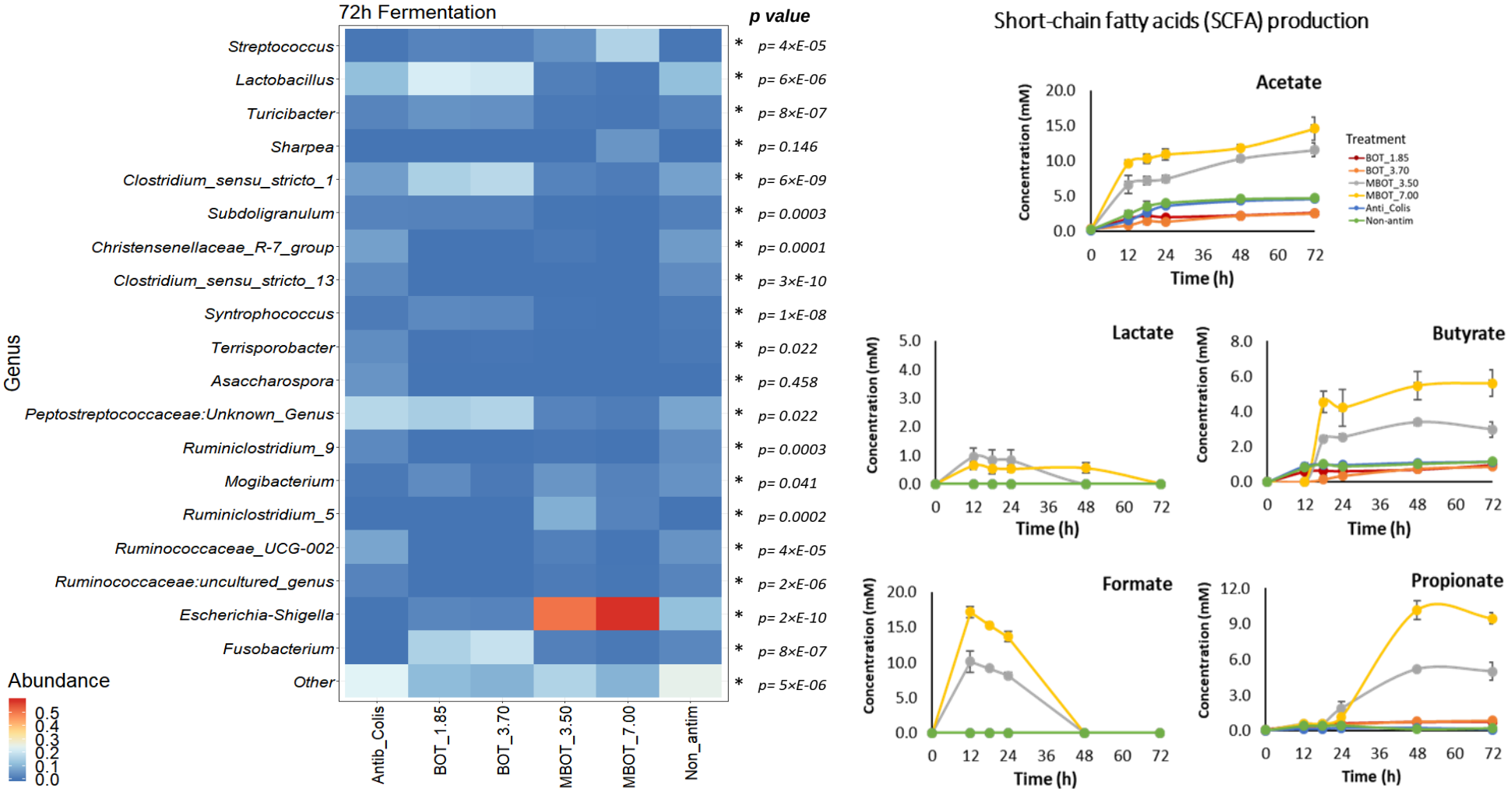

Figure 6.5. Heat map of the relative abundance of the top-19 genus present in colon microbial fermentation (in the left) and short chain fatty acids (SCFA) production (in the right) by effect of the raw BOT, microencapsulated BOT (MBOT) and colistin after 72 h of fermentation. p-values were calculated by a one-way Anova model and asterisks (*) indicate significant differences ($p<0.05$) on the relative abundance by effect of the treatments. Vertical bars indicate the standard deviation.

(pepsin cleaving proteins) occurs instead of microbial activity, thus no microbial metabolites are produced. The production of SCFA for the ileal microbial fermentation is shown in Figure 6.4. It was observed that acetate and lactate were the major SCFA produced. Lactate production was increased by the MBOT, which could be associated with the metabolic activity of *Lactobacillus,* whose abundance was slightly increased, although this increase was not significant. The raw BOT only at 3.70 mg/mL increased acetate production but decreased lactate production. Conversely, colistin stimulated acetate, butyrate and propionate productions while decreased lactate. Regard colonic bacterial fermentation, overall the MBOT raised the SCFAs production (acetate, butyrate and propionate) compared to the raw BOT, colistin and control treatments (Fig. 6.5), indicating that the metabolic activity of colonic bacteria was stimulated by the MBOT. This high production of SCFAs could correspond with the increased abundance and activity of some colonic bacteria such as *Streptococcus* and *Ruminiclostridium_5* in the colonic fermentation. Thus, these results would support the possible prebiotic functions of the wall material of microcapsules for colonic bacteria since besides to stimulate their growth would have stimulated their metabolic activity as well.

6.5. Discussion

Nowadays, EOs have turned a potential alternative to replace antibiotics as a feed additive to promote growth in livestock animals. Specifically, EOs have gained interest in pig production sector for their positive impacts on the growth performance and pig welfare (Bento et al., 2013; Franz et al., 2010). Through a meta-analysis it was revealed that EOs enhance the feed conversion rate (FCR) and keep the same average daily gain (ADG) of weaning piglets compared to antibiotics (Vanrolleghem et al., 2019). This growth-promoting effect by in-feed EOs has been suggested to be strongly related with beneficial effects of EOs on the pig gut ecosystem, such as modulation of the gut microbiota (Li et al., 2012b; Xu et al., 2018). Pig gut is characterized by having a large number of microorganisms, which play important roles in the pig health and well-being (Canibe et al., 2019). Thus, EOs for their antimicrobial activity can provoke changes on the pig gut microbiota. For this reason, a proper way of applying an EO in animal feed should be assisted by encapsulation techniques, such as microencapsulation. Besides being an efficient way to deliver the EO into the pig gut, microencapsulation can also prevent or reduce volatilization of the EO thus extending the EO shelf life in the animal feedstuff (Bento et al., 2013).

6.5.1. Total bacteria kinetics

In this study, we observed that the citrus oil, BOT, raw and microencapsulated, modified the pig gut microbiota in a simulated gut fermentation trial. Initially, shifts on the total number of bacteria by the effect of the raw BOT and MBOT in contrast to colistin and the control were found. In stomach simulation, the MBOT and the raw BOT did not alter total number of bacteria until 18h of fermentation, but both treatments reduced it afterwards in a similar manner. The lack of alterations on the total number of bacterial in stomach in a period shorter than 18h could be considered favorable since it is not intended to have any release of the EO and thus an antimicrobial effect on the stomach bacterial population during the gastric transit of the feedstuff, which is shorter than 18h for weaned piglets (Snoeck et al., 2004). In comparison to our results, previously, some pure EO compounds such as carvacrol and thymol (0.5 mg/mL) were reported for significantly reducing the number of total bacteria of a pig gastric simulation after 3h of fermentation (Michiels et al., 2009). But, in an *in vivo* study, the administration of thymol (at 0.5 or 2 mg/g), either raw or microencapsulated, did not change the total number of bacteria in stomach digest of weaned piglets (Michiels et al., 2010).

In ileal simulation, basically our results showed a slight bacteriostatic effect on the total bacteria population until 12h of fermentation by only the raw BOT (3.7 mg/mL), but afterwards the raw BOT and MBOT did not alter it. Inversely, the MBOT raised total bacteria of the colonic microbial fermentation in contrast to the raw BOT. On the contrary, several commercial EO products such microencapsulated oregano EO (10%) (Cheng et al., 2018; Zou et al., 2016), carvacrol (0.15 mg/g of feed) (Gutiérrez et al., 2010) or encapsulated thymol + cinnamaldehyde (18%) (Li et al., 2012a; Zeng et al., 2015) were found for not altering the total bacteria population in jejunum, ileum or colon of weaned piglets.

6.5.2. Diversity and Microbial Community Composition

The MBOT and the raw BOT significantly affected the diversity and composition of ileal and colonic microbial fermentations. The MBOT compared to the raw BOT reduced the ileal diversity, as much as colistin. However, none influence of EO supplementation, carvacrol, on ileal microbial composition has been observed in piglets (Gutiérrez et al., 2010). Regarding the colonic fermentation, both the raw BOT and the MBOT reduced the diversity, but the MBOT has a stronger effect. Conversely, it was reported that the administration of 2% of an commercial orange EO and limonene, the major compounds in citrus EOs, increased the diversity of caecal and colonic microbiota of mice (Wang et al., 2019). Nonetheless, a supplementation with a blend of pure EO compounds, carvacrol + thymol, did not affected

the colonic microbial diversity and richness of weaned piglets (Li et al., 2018). Additionally, a clear significant effect of the raw BOT, the MBOT and colistin on the bacterial community compositions of pig ileal and colonic simulated fermentations has been observed in our study ($p<0.05$), where each of the antimicrobial treatments altered the composition in a different manner when compared to the control. However, the administration of a commercial orange EO (2%) did not alter the colonic bacterial composition of mice (Wang et al., 2019). Conversely, compositional changes on colonic microbiota of weaned piglets by thymol and carvacrol supplementation was previously reported by Li et al. (2018).

The abundance of certain bacterial taxa were variable by effect of the raw BOT, the MBOT and colistin in ileum and colon fermentations. At phylum level, in ileum fermentation, the MBOT only caused a full reduction of *Actinobacteria*. However, the raw BOT only at 3.70 mg/mL and colistin reduced *Firmicutes* while increased *Actinobacteria. Proteobacteria* was increased by the raw BOT while colistin fully reduced it. Conversely to these results, dietary supplementation (0.12 µg/mL) with an encapsulated commercial EO product (carvacrol + thymol) has led an increase of *Firmicutes* and a decrease of *Cyanobacteria* and *Proteobacteria* abundances in chicken ileum microbiota (Yin et al., 2017). But, the administration of carvacrol alone (0.15 mg/g) was found to have an influence increasing *Actinobacteria* in ileum of weaned piglets with the concomitant decrease of *Bacteroidetes* and *Clostridium* cluster XIVa (Gutiérrez et al., 2010).

At genus level, it was observed that the raw BOT and the MBOT exerted a strong antibacterial activity on *Clostridium sensu stricto 1* and *Streptococcus* genera, which were fully reduced in contrast to the control and colistin. In addition, the MBOT fully reduced *Bifidobacterium* too. These results were in-line with previous findings by Gutiérrez et al. (2010), in which carvacrol administration was found for decreasing species from the genera *Streptococcus* and *Clostridium* cluster IV in ileum of piglets. Previously, an reductive effect on *Bifidobacterium* (although not fully such as in our study) in ileum of weaned piglets after administration of a encapsulated EO product containing orange, oregano, anis and chicory EOs (2%) was reported by Kroismayr et al. (2008). Furthermore, the MBOT did not alter *Lactobacillus* and *Escherichia-shigella* abundances, but the raw BOT on the contrary slightly raised *Escherichia-shigella*. In contrast to the EO treatments, colistin reduced *Lactobacillus* while efficiently fully reduced *Escherichia-shigella* abundance, and specifically caused a great reduction of *E. coli* in the ileal fermentation. No alterations on *Lactobacillus* abundance in pig small intestine (proximal, distal or ileum) after in-feed several EO compounds has been previously reported by (Kroismayr et al., 2008; Michiels et al., 2010). However, the supplementation with a microencapsulated oregano EO led to *E. coli* reduction without altering the *Lactobacillus* count in ileum microbiota of growing-finishing pigs (Cheng et al., 2018). It has been suggested as a positive modulatory effect of the gut microbiota of weaned

piglets by EO supplementation an increase in the ratio *Lactobacillus:Enterobacteria* in ileum (Manzanilla et al., 2004). However, a reserve effect was observed for the raw BOT and MBOT in our *in vitro* trial.

Regarding colon fermentation, at phylum level it was observed that the raw BOT and the MBOT fully reduced *Bacteroidetes*, *Euryarchaeota* and *Synergistetes* in a same manner. Nevertheless, additionally, the MBOT decreased *Firmicutes* while increased *Proteobacteria*. Colistin increased *Firmicutes*, decreased *Proteobacteria* and fully reduced *Fusobacteria*. Our results on the MBOT were in line with the effect at phylum level found for limonene administration (2%), which mainly reduced *Firmicutes* abundance in colon of mice (Wang et al., 2019). On the contrary, supplementation with thymol + carvacrol led to an increase in *Firminutes* abundance while reduced *Bacteroidetes* in colon of weaned piglets (Li et al., 2018).

At genus level, it was more obvious the strong antibacterial activity of the raw BOT and the MBOT on the colonic bacteria. But, overall it was observed that the MBOT exerted more remarkable effects compared to the raw BOT. Depending on the tested concentration, the raw BOT and the MBOT caused the full reduction of six of the 19 most abundant genera (see results section) compared to the control and colistin. However, additionally, the MBOT caused a significant reduction of *Clostridium sensu stricto 1, Lactobacillus, Syntrophococcus, Subdoligranulum* and *Turicibacter*, while the raw BOT increased them and *Fusobacterium*. In addition, the MBOT tended to increase *Streptococcus* and *Ruminiclostridium_5* abundances. Particularly, an increase in *Streptococcus* abundance in pig colon by the administration of an EO product (carvacrol + thymol) was suggested as an indicator of an improved intestinal health (Li et al., 2018). It has been indicated that the increase of other beneficial bacteria such as *Ruminococcus*, *Lactobacillus* and *Megasphaera* with the simultaneous reduction of *Enterobacteriaceae* in colon by thymol and carvacrol supplementation would be associated with low diarrhea incidence and the enhanced pig performance (Li et al., 2018). On the contrary to our results, the administration of an orange EO and isolated compounds normally present in this kind of EO, limonene and linalool, were found to increase beneficial bacteria such as *Lactobacillus*, *Parabacteroides* and *Barnesiella* in colon of mice, being a positive regulation of the intestinal microflora (Wang et al., 2019).

Furthermore, our results showed that only the raw BOT efficiently decreased *Escherichia_Shigella* abundance, while colistin fully reduced it. Both treatments also diminished specifically *E. coli* in a same manner. Contrariwise, the MBOT raised *Escherichia_Shigella* and *E. coli* at an upper level than the control. Several studies have reported that for instance supplementation with several EO compounds (thymol + carvacrol/thymol + cinnamaldehyde) effectively reduce *E. coli* in colon, rectum or faeces of weaned piglets with the simultaneous increase of *Lactobacillus* as same as antibiotics

treatments (Li et al., 2012b, 2012a; Xu et al., 2018; Zeng et al., 2015). Likewise, it has been reported that a commercial product having an orange, oregano, anise and chicory EOs was effective to decrease *E. coli* and *Salmonella* and increase *Lactobacillus* and *Bacillus* in piglet faeces (Ahmed et al., 2013). Similarly, oregano EO supplementation was found to promote the integrity of the intestinal barrier, probably by mean of modulation of the gut microbiota related to *E. coli* reduction in jejunum, ileum and colon of growing-finishing pigs, and inactivation of inflammation signaling pathways (Zou et al., 2016). The modulation of the small intestinal microbiota by feeding herbal extract or EOs could lead indirectly improvements on the digestive capacity of the small intestine, consequently impacting on the pig growth performance (Costa et al., 2013). In addition, EOs could improve the small intestinal morphology and the antioxidative capacity, being other mods of action of EOs to contribute with the enhancement of pig performance (Cheng et al., 2018; Franz et al., 2010).

6.5.3. SCFAs production

The composition of the gut microbial communities and their metabolite production have an effect on the health and subsequently on the nutritional status of the host (Bento et al., 2013). The activity of the intestinal microbiota can be indicated by their fermentation end-products, such as short chain fatty acids (SCFAs), which derive from the digestion and fermentation of carbohydrates and serve as source of additional energy for the host (den Besten et al., 2013; Tungland, 2018).

Besides the shifts on the gut microbiota composition, EOs can also alter the metabolism of the intestinal microbiota, such as the SCFAs production. In ileum microbial fermentation, the MBOT caused a slight stimulation of lactate production, associated probably with the activity of *Lactobacillus*. Conversely, the raw BOT (3.7 mg/mL) reduced lactate and increased acetate, which would be related to its effect on the abundance and metabolism of *Lactobacillus*, *Bifidobacterium* and *Escherichia-shigella*. Colistin compared to the EO treatments, greatly stimulated SCFAs production. Inversely to these results, supplementation with an EO product or avilamycin were shown to not altering the SCFAs production (acetate, lactate, butyrate, propionate, capric acid and valeric acid) by the ileal microbiota of weaned piglets, although a significant increase of *Bifidobacterium* by the EO treatment was observed (Kroismayr et al., 2008). Nonetheless, microencapsulated thymol (0.5 or 2.0 mg/g of feed) was reported for changing the activity of the distal small intestine microbiota of piglets evidenced by a decrease of acetate and lactate productions without clear evidence of alterations on coliforms, *Lactobacillus* or *Streptococcus* counts (Michiels et al., 2010). Likewise, administration of thymol + cinnamaldehyde was found for altering ileal microbiota

metabolism of broiler chickens by reducing propionate and increasing acetate and butyrate in ileum content (Cao et al., 2010).

In colon fermentation, our results have shown that the metabolic activity of colonic bacteria was modified mainly by the MBOT. Overall, the MBOT greatly raised the SCFAs production in the colonic fermentation. Most fermentation of feed takes place in the cecum and large intestine (den Besten et al., 2013) and SCFAs are the major fermentation end-products in those gut locations. SCFAs are mainly produced in colon from dietary fibers, which are not digested in the small intestine (Hamer et al., 2007). Increased SCFAs production in colon fermentation by the MBOT could indicate that the wall material of microcapsules (modified starch and chitosan) could have worked as prebiotic or as an extra carbon source, which besides to have stimulated the growth of some colonic bacteria, would have stimulated their metabolic activity.

The higher production of butyrate by the MBOT would be an advantageous effect, since butyrate is an important colon metabolite and energy source for epithelial cells and also influence several cellular functions affecting colonic health, such as inhibition of inflammation, reinforce colonic defense barrier and decrease oxidative stress (Hamer et al., 2007). *Firmicutes* are recognized as the main butyrate producers (den Besten et al., 2013), thus the increase of *Streptococcus* and *Ruminiclostridium_5* by the MBOT would have influenced the higher butyrate production.

Acetate and propionate were also greatly increased by the MBOT. An increase in acetate would be beneficial since acetate is as important as butyrate for energy supply to colonic epithelial cells. Moreover, acetate is one of the started units for butyrate and propionate production by colonic bacteria (den Besten et al., 2013). In addition, propionate has also potential health-promoting effects comprising anti-lipogenic, cholesterol-lowering and anti-inflammatory activity (Hosseini et al., 2011). Acetate and propionate are mainly produced by *Bacteroidetes* phylum (den Besten et al., 2013), but other phyla can also produce these SCFAs (Fernández et al., 2016). The higher acetate and propionate production could probably be associated with the metabolic activity of *Escherichia-shigella* (*Proteobacteria*), *Streptococcus* and *Ruminiclostridium_5* (*Firmicutes*) in this present study, whose abundances were increased by the MBOT.

Stimulation of SCFAs production by in-feed an EO product to weaned piglets may be directly related with the enrichment of functions involved in carbohydrates metabolism of colonic bacteria, specifically, those related to propanoate metabolism and butanoate metabolism (Li et al., 2018). Compared to our results, the supplementation with an encapsulated EO product constituted by a blend of carvacrol, cinnamaldehyde and capsicum oleoresin was found for modifying the SCFAs production in colon of piglets, in a way that increased the proportion of acetate but reduced butyrate and valerate (Manzanilla et al.,

2004). Nevertheless, on the contrary to our results, , the administration of an orange EO previously led to a significant reduction of the SFCAs content in colon of mice, decreasing acetate and butyrate productions (Wang et al., 2019). In a similar way, a commercial EO product having a blend of orange, oregano, anis and chicory EOs was found for decreasing acetate production as much as avilamycin in the colon of weaned piglets, which would have been associated with the decrease of *Bifidobacterium* and *Clostridium* counts (Kroismayr et al., 2008).

6.5.4. General outlook

Our results have shown that the MBOT and the raw BOT due to their antimicrobial activity, altered the diversity, composition and metabolic activity of the ileal and colonic microbiota in an *in vitro* fermentation trial, compared to colistin and control treatments. Remarkably, higher antimicrobial effects were found for the MBOT compared to the raw BOT, as evidenced by the reduction of bacterial taxa in ileum and colon fermentations. This would indicate that the antibacterial efficiency of the raw BOT was enhanced by microencapsulation since the concentrations of raw BOT into the microcapsules were lower than the concentrations tested in the treatment with the raw BOT, approximately fourth times lower. However, the impact of the effects observed for the MBOT in this *in vitro* fermentation trial, on the pig gut heath and pig performance still need to be elucidated in futures studies.

To date, the outcomes on the modulation of the pig gut microbiota by the antimicrobial effect of EOs are variable and specifics depending on the evaluated EO or EO compound. An important aspect also to be pointed out is that most of the EO products evaluated as a potential alternative to antibiotics in pig production sector cited at the literature and commercially available, consist of isolated single EO compounds, standing out thymol and carvacrol as the major applied. Nevertheless, the application of an isolated EO compound instead of a whole EO could result in the selection of bacteria resistant to it in a short-term. Gut bacteria easily could develop a mechanism to repeal the effect of an EO compound as in the case of an antibiotic, which consists of an isolated single compound too. In fact, antibacterial resistance to carvacrol has begun to be reported (Berdejo et al., 2019). Thus, it would be more recommendable the use of a whole EO since it is rich in a large number of different compounds which may exert different mechanisms to inactivate bacteria, turning so more difficult to bacteria develop easily a mechanism to resist the activity of a whole EO.

6.6. Conclusions

Our results showed on the basis of an *in vitro* set-up, that the microencapsulated citrus EO, BOT, shifted the pig gut microbiota in a way that more remarkably reduced its diversity

and altered its composition as evidenced by a significant reductions of bacterial taxa in ileum and colon fermentations in contrast to the application of BOT in a raw form. Probably, these higher effects by the MBOT is a result of the enhanced antibacterial activity of the raw BOT by microencapsulation. Furthermore, besides of the shifts on ileum and colonic microbiota by the delivered EO (BOT), the wall material of microcapsules (chitosan/modified starch) could have worked as an additional carbon source with prebiotic functionality, specifically, for colonic bacteria since it would have stimulated their growth and metabolic activity (SCFAs production). In addition, from a practical standpoint, the use of a citrus EO, assisted by microencapsulation, would be an attractive and interesting alternative as antimicrobial additive in pig breeding. However, the impact of the effects on modulation of the gut microbiota and its metabolic activity observed for the microencapsulated citrus EO, BOT, in this *in vitro* trial on the pig heath and pig performance still need to be elucidated in further studies.

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Supplementary material

Tables

Table 6-S1. Composition of media used for simulating *in vitro* stomach, ileal and colonic conditions

Pig gut stage simulation	Ingredients/quantity	Pre-flushing	Sterilization
Stomach	*Basal solution* (for 1L of distilled water) Proteose-peptone 8.3 g, D-glucose 3.5 g, NaCl 2.05 g, KH_2PO_4 0.6 g, $CaCl_2$ 0.11 g, KCl 0.37 g. 1 mL of L-cystein HCl solution (0.5g/L)	After boiling, to cool flushing N_2/CO_2***	Autoclaving
	Enzyme solution (for 1L of distilled water) Porcine bile 0.05 g, lysozyme 0.1 g, pepsin 13.3 mg	N_2/CO_2	Filtering (0.2 µm)
Ileum	*Basal solution* (for 1L of distilled water) Xylan 0.6 g, pectin 0.6 g, amylopectin 0.6 g, starch 5.0 g, casein 3.0 g, pancreatic digest of peptone from casein 3.0 g, K_2HPO_4 2.0 g, $NaHCO_3$ 0.2 g, NaCl 4.5g, $MgSO_4 \cdot 7H_2O$ 0.5g, $CaCl_2 \cdot 2H_2O$ 0.45 g, $FeSO_4 \cdot 7H_2O$ 0.005 g, haemin 0.01 g, bile salts 0.05 g, antifoam A 0.5 mL, tween 80 2.0 mL. 1mL of resazurin solution* 1 mL of L-cystein HCl solution (0.5g/L)	After boiling, to cool flushing N_2/CO_2	Autoclaving
	Vitamin (for 1L of distilled water) Menadione 1.0 mg, biotin 2.0 mg, pantothenate 10mg, nicotinamide 5 mg, vitamin B12 0.5 mg, thiamine 4.0 mg and paraminobenzoic acid 5.0 mg.	N_2/CO_2	Filtering (0.2 µm)
	Trace mineral solution (for 1L of distilled water) EDTA 500 mg, $FeSO_4 \cdot 7H_2O$ 200 mg, $ZnSO_4 \cdot 7H_2O$ 10 mg, $MnCl_2 \cdot 4H_2O$ 3.0 mg, H_3BO_3 30 mg, $CoCl_2 \cdot 6H_2O$ 20 mg, $CuCl_2 \cdot 2H_2O$ 1.0 mg, $NiCl_2 \cdot 6H_2O$ 2.0 mg, $NaMoO_4 \cdot 2H_2O$ 3.0 mg.	N_2/CO_2	Filtering (0.2 µm)
Colon	*Basal solution* (per 1L of distilled water) KCl 0.6 g, NaCl 0.6 g, $CaCl_2 \cdot 2H_2O$ 0.2 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, pipes buffer 1.5 g, NH_4Cl 0.54 g, trypticase 1.0 g, trace mineral solution 10 mL, Haemin** Solution 10 mL, and fatty acid solution 10 mL. 1mL of resazurin solution (0.2 g resazurin per 200 mL distilled water)	After boiling, to cool flushing N_2/CO_2	Autoclaving
	Trace Mineral Solution (per 1L of 0.02M HCl): $MnCl_2 \cdot 4H_2O$ 0.025 g, $FeSO_4 \cdot 7H_2O$ 0.020 g, $ZnCl_2$ 0.025 g, $CuCl \cdot 2H_2O$ 0.025 g, $CoCl_2 \cdot 6H_2O$ 0.050 g, SeO_2 0.050 g, $NiCl_2 \cdot 6H_2O$ 0.250 g, $Na_2MoO_4 \cdot 2H_2O$ 0.250 g, $NaVO_3$ 0.0314 g and H_3BO_3 0.250 g.	N_2/CO_2	Filtering (0.2 µm)
	Fatty Acid Solution (per 1L of 0.2M NaOH): Acetic acid 6.85 mL, propionic 3.0 mL, butyric 1.84 mL, *iso*-butyric 0.47 mL, 2-methyl-butyric 0.55 mL, valeric 0.55 mL and *iso*-valeric acid 0.55 mL.	N_2/CO_2	Filtering (0.2 µm)

	The reducing agent (1L of boiled distilled water) Na2S·9H2O 20.5 g, cysteine HCl 20.5g. Prepared into a fume cupboard due to the inhalation of toxic fumes.	N_2	Autoclaving
	The bicarbonate solution (1L of boiled distilled) Na2CO3 (sodium carbonate anhydrous) 82 g	Before/after autoclaving, and prior use flush with CO_2	Autoclaving
	The vitamin/phosphate solution (per 1L of KH_2PO_4 solution (54.7 g/L)) Biotin 0.0204 g, folic acid 0.0205 g, calcium dpantothenate 0.1640 g, nicotinamide 0.1640 g, riboflavin 0.1640 g, thiamin HCl 0.1640 g, pyridoxine HCl 0.1640 g, *para*-amino benzoic acid 0.0204 g, cyanocobalamin (vitamin B12) 0.0205 g.	N_2/CO_2	Filtering (0.2 µm)

* Prepared according to (Blake et al., 2003): resazurin solution = 0.2 g resazurin per 200 mL distilled water
** Prepared according to (Williams et al., 2005): Haemin solution = 0.1 g Haemin dissolved in small amount 0.05M NaOH and made up to 1L of boiled distilled water with CO_2 flushing through it.
*** N_2 + CO_2 at the ratio 80:20 v/v

Table 6-S2. Primers used for real-time PCR

Primer	Orientation	Primer sequence (5' to 3')	Annealing temperature (°C)	Reference
Total bacteria	Forward	GTGSTGCAYGGYYGTCGTCA	52	(Heinritz et al., 2018)
	Reverse	ACGTCRTCCMCNCCTTCCTC		
Escherichia coli	Forward	CATGCCGCGTGTATGAAGAA	60	(He et al., 2017)
	Reverse	CGGGTAACGTCAATGAGCAAA		

Figures

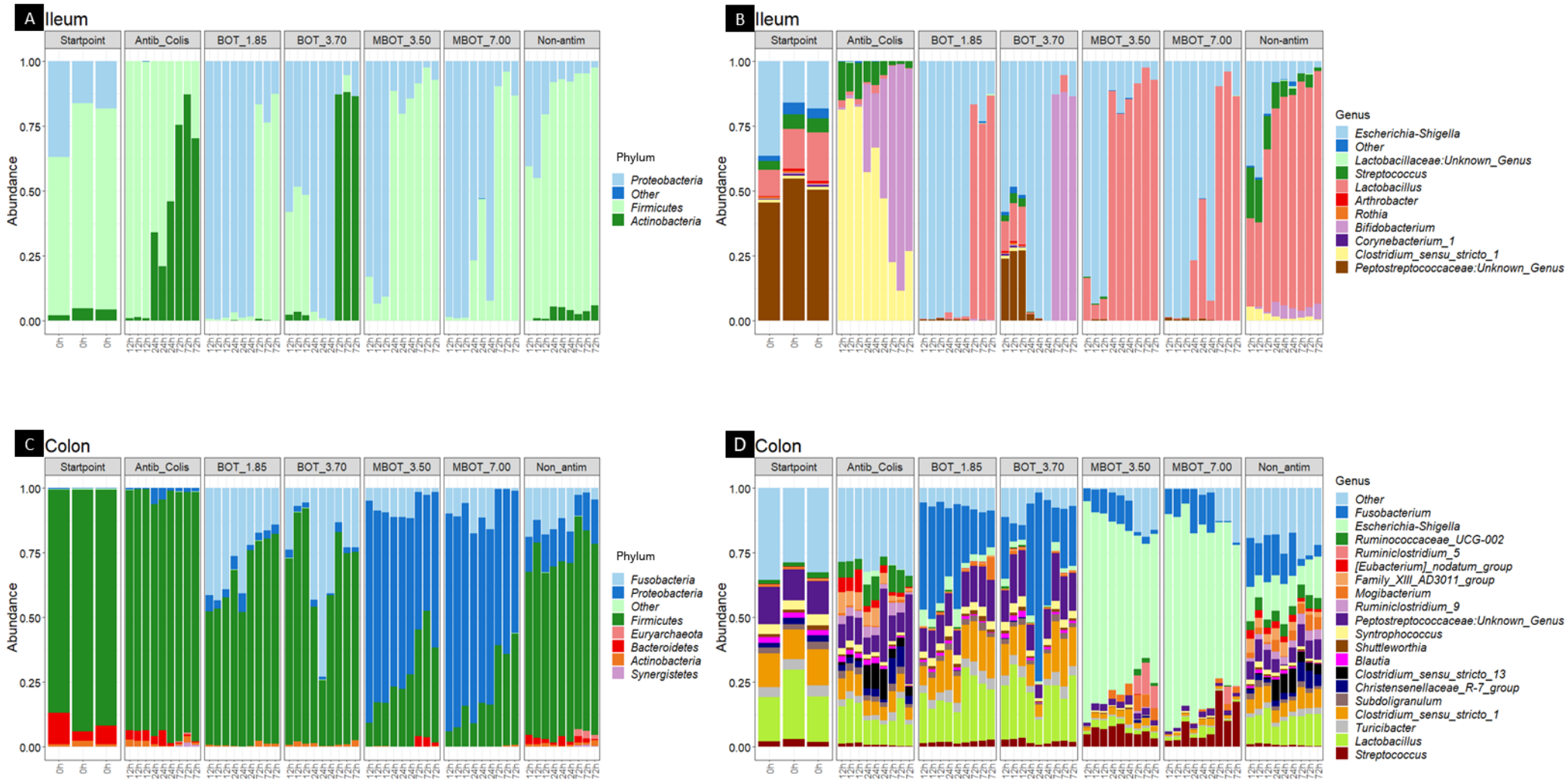

Figure 6-S1. Changes on the taxonomical distribution of ileum and colon microbial fermentations by the effect of the raw BOT, microencapsulated BOT (MBOT) and colistin at phylum (A and C) and genus (B and D) levels. “Starpoint” of fermentation (taxonomical distribution of bacteria at 0 h).

7. GENERAL DISCUSSION AND FUTURE OUTLOOKS

In the framework of this research, the pleasant orange smell accompanied all the stages of this thesis, even when pig' feces were needed to set-up a stage of the art in vitro fermentation. This thesis summarizes a series of studies performed with the aim to provide new insights into the antimicrobial effect of citrus essential oils (EO) related to their chemical composition, targeting their potential application as an alternative to antibiotics use in the feed of food-producing animals, such as pigs. This in view that in the last two decades have seen an increased tendency in the use of aromatic herbs and EOs as feed additives in animal nutrition due to the banning of antibiotics for this purpose within the European Community since 2006 (Franz et al., 2010). The antibacterial activity of citrus EOs initially was reported in the 60's on food spoilage bacteria (Subba, Soumithri & Rao, 1967), but subsequently their antimicrobial effect on gram-positive and gram-negative pathogenic bacteria has been proved in several studies (Fisher and Phillips, 2008; Friedly et al., 2009; O'Bryan et al., 2008). However, the antimicrobial effectiveness of citrus EOs against enteropathogenic bacteria affecting food-producing animals has not been largely investigated. Thus, in the course of researches conducted in this thesis we discuss finding and observations on the differential antibacterial effect of citrus EOs on pathogenic and beneficial bacteria of occurrence in the pig gut.

This thesis followed a workflow, in which firstly six commercial citrus EOs were screened by their antibacterial effect on enterotoxigenic *E. coli* (ETEC) strains isolated from pig gut and on *Lactobacillus* species. (**Chapter 2**). A potential selective antibacterial activity by these EOs was observed, that was, a stronger antibacterial activity on ETECs than on *Lactobacillus* sp, altering more remarkable the normal growth kinetics of ETECs compared to *Lactobacillus* sp. Minor compounds present in the evaluated citrus EOs (*cis*-limonene oxide, *trans*-carveol, carvone, *trans*-limonene oxide, *cis*-p-Mentha-2,8-dien-1-ol, *trans*-p-Mentha-2,8-dien-1-ol, perrilla alcohol, *cis*-carveol, *cis*-p-Mentha-1(7),8-dien-2-ol and 1,8-menthadien-4-ol) were inferred to be involved in driving this feature of antimicrobial spectrum instead of citrus EOs' major compound, limonene, doing this exclusively. Selectivity towards pathogenic bacteria rather than beneficial bacteria is not new in the search for antimicrobial substances aiming an effect on the gut microbiota of farm animals, such as pigs. This has been indicated as a desirable feature of the antimicrobial spectrum by EOs as a potential alternative to antibiotics used as growth promoters in pig feed (Gresse et al., 2017; Si et al., 2006). Thus, in this chapter, we have observed that the evaluated citrus EOs fulfilled this feature.

Subsequently, insights on the mechanism underlying the selective antibacterial activity of a citrus EO was provided in **Chapter 3**. Higher disturbances on the cytoplasmatic membrane permeability and integrity, with the concomitant higher release of essential

cellular constituents (proteins and reducing sugars) of ETEC cells than *Lactobacillus* sp. cells was found as the probable mechanism whereby a citrus EO exert a selective antibacterial activity. In addition, sequential exposure to a citrus EO (3 times) at subinhibitory concentrations was conducted following the fact that gut bacteria of pigs frequently undergo a sequential exposure to the antimicrobial effect through the constant ingest of the feedstuff contained the antimicrobial (Ambrosio et al., 2018). Sequential exposure led to a higher reduction of the bacteria viability compared to a single exposure, exerting the sequential exposure higher reduction of ETEC than *Lactobacillus* sp. viability. To our knowledge, this was the first study that reports the mechanism behind the selective antibacterial effect of a citrus EO on bacteria of occurrence in the pig gut. Moreover, this chapter also heightened the differential effect on bacteria viability between sequential and single exposures to a citrus EO. Also, the effectiveness of EO sub-MICs in a sequential exposure. Most of the *in vitro* studies have determined the effectiveness of an antimicrobial, such EOs, based on a single exposure results (Ebani et al., 2018; Michiels et al., 2009). Thus, when testing *in vitro*, the effectiveness of an EO or other antimicrobials, either on single isolated bacteria or mixed bacteria populations (such gut microbiota) should take in consideration this differential effect and test the effectiveness in a sequential exposure assay if the aim is to use EOs or other antimicrobial substances as feed additive. Of course, in *in* vivo studies already this condition is reflected. In addition, sequential exposure results have suggested that the use of sub-MICs could be sufficient to be dosage in the pig feed when these *in vitro* results are translated to *in vivo* studies.

Next in **Chapter 4**, insights of the citrus EO' components involved in conferring the selective antibacterial activity as well as the antioxidant capacity exhibited by a citrus EO was provided. This was attained through the study of citrus EO fractions obtained by fractional distillation. Minor compounds belonging the class of oxygenated monoterpenes: carvone, *cis*-carveol, *trans*-carveol, *cis*-p-Mentha-2,8-dien-1-ol and *trans*-p-Mentha-2,8-dien-1-ol were stand out as the main compounds influencing those biological activities, instead of the major compound, limonene, playing this role exclusively. These compounds were initially inferred within the group of detected minor compounds that would be related with the exhibited selective antibacterial activity by a group of several citrus EOs in **Chapter 2**. Thus, confirming the initial findings in **Chapter 2**. However, a synergistic interaction between major and minor compounds looks to be a determinant for the resulting antibacterial and antioxidant activities of a citrus EO (**This chapter**). The characteristic and biological properties of an EO is driven by the compounds present in the EO and their synergistic interaction inside to the EO, since it is probably that minor compounds may modulate the activity of the major EO compound (Bakkali et al., 2008). Nonetheless, most of the studies to date have tested formulation of EO products destined to pig feed on the use of isolated major

EO compounds from several EOs (Bento et al., 2013; P. Li et al., 2012; S. Y. Li et al., 2012; Li et al., 2018; Xu et al., 2018; Zeng et al., 2015), probably, because the biological properties of EOs has been widely attributed to their major compounds since they have shown biological activities when tested separately (Bassolé and Juliani, 2012). Moreover, another reason would be related to the fact that the demanded amount of an antimicrobial compound by the animal production sector is considerable huge, and to fulfill this demand is more viable industrially the synthesis or isolation of individual EO compounds. To date, the great demand of some EO compounds for several bulk applications is insufficient to supply the demand and the harvest from natural sources is in some cases supplemented by chemical synthesis (Jongedijk et al., 2016; Lange, 2015). However, the use of isolated single EOs compounds could bring a similar problem that to date we are facing with the case of antibiotics. Conversely, a whole EO rather than an isolated single EO compound use is a way for an arsenal of different compounds fights pathogenic bacteria, each at the more different mechanism of action on bacteria (Radulovic et al., 2013). Thus, easy bacterial resistance development in short-term could be controlled using whole EOs.

In addition, an aspect to consider when the aim is to apply EOs in the pig feed is the fact that EOs need to be protected against volatilization and from the impact of the environmental conditions in order to preserve their proprieties. Additionally, a way for an efficient site-targeting delivery of the EO within the pig gut need to be considered. Encapsulation can resolve both aspects (Omonijo et al., 2018). Thus, in the **Chapter 5** a modified starch-chitosan matrix as wall material allowed the efficiently microencapsulation of a citrus EO (encapsulation efficiency, 61.17%). The higher selective antibacterial activity exhibited by the microencapsulated citrus EO than the raw one was an indicative result of the enhanced antibacterial performance of the raw citrus EO by encapsulation. Probably, due to the synergistic interaction between the raw citrus EO and the wall material, which would have favorited the antimicrobial performance of the microencapsulated powder product. The contributive role of the wall material on the antimicrobial properties of an encapsulated product would be a positive aspect since it would reduce the rate of the EO inclusion in the encapsulation formulation. This reduction would be advantageous in terms of cost-effectiveness for the pig industry, as EOs' potential alternative to antibiotics. Proper protection by microencapsulation, for instance, will avoid that the majority of EOs be lost during the feedstuff processing, storage and delivery to the lower intestine of pigs where most pathogen' colonization occurs (Lillehoj et al., 2018; Omonijo et al., 2018).

Finally, in **Chapter 6** the effect of the microencapsulated citrus EO (in **Charter 4)** on pig gut microbiota by means of a stage of art *in vitro* fermentation was evaluated. Compared to the raw citrus EO, the microencapsulated one more remarkable shifted ileal and colonic microbiota in a way that altered their total bacteria kinetics, reduced their diversity and

altered their composition as evidenced by the reduction of certain bacterial taxa. The higher antimicrobial effects exhibited by the microencapsulated citrus EO would be a result of the enhanced antibacterial effect of the raw citrus EO by microencapsulation, as previously observed in **Chapter 5**, thus reaffirming that observation. In addition, the stimulation of short chain fatty acids (SCFAs) production mainly in colon fermentation by the microencapsulated citrus EO indicated that probably the wall material of microcapsules (modified starch-chitosan matrix) could have served as an additional carbon source with prebiotics functioning, specially, for colonic bacteria. Several *in vitro* or *in vivo* studies have showed that plant extracts and EOs act along the animal digestive tract modulating the microbiota in a way that beneficially affect the ecosystem of the gut microflora by controlling pathogens (Franz et al., 2010; Lillehoj et al., 2018). That effect has been related to the increase of *Lactobacillus* group and decrease of *E. coli* or total coliforms (Ahmed et al., 2013; P. Li et al., 2012; S. Y. Li et al., 2012; Xu et al., 2018; Zou et al., 2016). This applies especially to that critical phase of the pigs' development, post-weaning, in which they are more susceptible (Franz et al., 2010). Conversely to this, the microencapsulated citrus EO failed in controlling the growth of *Escherichia-shigella* and *E. coli* in ileum and colon fermentations compared to the antibiotic treatment colistin. Nonetheless, *Lactobacillus* growth was stimulated in ileum fermentation by the microencapsulated citrus EO. The raw citrus EO controlled *E. coli* in colon fermentation as much as colistin and increased *Lactobacillus* abundance. Substances with antibiotic activity, such EOs, display multiple modes of action and wide spectrum of activity over the gastrointestinal microbiota (Lillehoj et al., 2018). It is still challenged to predict what microbial changes are responsible for promoting pig performance by in-feed plant extract or EOs (Franz et al., 2010; Lillehoj et al., 2018). The impact of the effects on modulation of the pig gut microbiota by the microencapsulated citrus EO, observed in this chapter, on the pig heath and performance still need to be elucidated in further studies.

Conclusions

EOs have been investigated as alternative to antibiotics in animal production, such pig production with more emphasis since the prohibition of antibiotics as enhancer of the animal performance since 2003 when the regulation by the European Union was stablished to be applied from 2006 (EU, 2003). Basically, for the active antimicrobial compounds that EOs harbors, EOs have turned an important promising antibiotic alternative. In this thesis, the focus has been to study citrus EOs, maybe the most promising type of EOs as source of antimicrobial agents, for their application as such in pig production for two reasons: a) the huge worldwide production of citrus EOs, as they are the main by-product of the citrus processing industry, which from a practical standpoint their implementation in short term

could turn financially viable; and b) because of the potential selective antimicrobial activity of citrus EOs between pathogenic and beneficial bacteria of occurrence in the pig gut showed by our *in vitro* results.

The unraveling of the citrus EOs' selective antimicrobial spectra that this thesis has left involves the facts of citrus EOs causing higher disturbances on the growth kinetics of the pathogenic *E. coli* than *Lactobacillus* sp. (**Chapter 2**), probably by causing higher disturbances on the permeability and integrity of the cytoplasmatic membrane, provoking consequently higher release of cellular constituents of the pathogenic *E. coli* than the beneficial *Lactobacillus* sp. (**Chapter 3**). The characterization of the chemical composition of citrus EOs allowed to infer that minor compounds present in these EOs would be involved in conferring their selective antibacterial activity, these would be mainly oxygenated monoterpenes such as carvone, *cis*-carveol, *trans*-carveol, *cis*-p-Mentha-2,8-dien-1-ol and *trans*-p-Mentha-2,8-dien-1-ol (**Chapter 4**). In addition, the antibacterial activity of a microencapsulated citrus EO (**Chapter 5**) on pig gut microbiota was observed (in an *in vitro* fermentation set-up), in a way that reduced its diversity and altered its composition (**Chapter 6**). However, the unraveling of citrus EOs effects in a future *in vivo* study is needed.

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