TARSILA RODRIGUES ARRUDA

ANTIMICROBIAL AND ANTIOXIDANT ACTIVE PACKAGING INCORPORATED WITH HOP (*Humulus lupulus* L.) EXTRACT: DEVELOPMENT, CHARACTERIZATION AND APPLICATION IN MEAT PRODUCT

Thesis submitted to the Food Science and Technology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

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Co-advisers: Allan Robledo Fialho e Moraes
Patrícia Campos Bernardes

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ABSTRACT

ARRUDA, Tarsila Rodrigues, D.Sc., Universidade Federal de Viçosa, August, 2024. **Antimicrobial and antioxidant active packaging incorporated with hop (*Humulus lupulus* L.) extract: development, characterization and application in meat product**. Adviser: Nilda de Fátima Ferreira Soares. Co-advisers: Allan Robledo Fialho e Moraes and Patrícia Campos Bernardes.

The search for alternatives to fossil-based plastics has grown abruptly in recent years, leading to the investigation of biopolymers as possible substitutes and, in special, polylactic acid (PLA) has shown good prospects in many industrial areas. In parallel, active packaging using biopolymers has gained interest as an ally to the food sector, potentially decreasing food losses. In this sense, the present study aimed to develop and characterize active antimicrobial and antioxidant food packaging based on PLA and natural bioactives from hop (*Humulus lupulus* L.). To provide a better understanding of the natural bioactives and their technological aspects, including complexation with cyclodextrins, prospects in active packaging development boosted to elaborate a comprehensive review in Chapter Article 1. Chapter Article 2, a commercial β-acids-rich hop extract was directly incorporated in different concentrations (0.1-5% w/w) into PLA-based sheets. Mechanical and thermal findings indicated that possibly the different fractions of the extract logged into distinct sites in the polymeric matrix, with the potassium salts of β-acids located majorly inside the PLA sheets and the propylene glycol on the outer surface. Sheets added with 5% of hop β-acids significantly improved the inhibitory effects towards non-sporulated Gram-positive bacteria (*Listeria monocytogenes* and *Staphylococcus aureus*). The developed sheets were then tested concerning their biodegradability in soil, with Chapter Article 3 verifying the effects of the hop extract incorporation into the polymeric matrix. The results revealed that hop β-acids incorporation into the polymeric matrix did not reduce the degradation process but limited the bulk hydrolysis, probably due to the lower diffusion of water molecules among the polymer chains. Thus, in the active sheets, the degradation occurred mainly through surface erosion. The study conducted in Chapter Article 4, on the other hand, investigated the inclusion complexation of the commercial β-acids rich hop extract with two distinct cyclodextrins, β-cyclodextrin (βCD) and 2-hydroxypropyl-β-cyclodextrin (HPβCD) and the evaluation of the obtained inclusion complexes (IC). The inclusion complexation enhanced β-acids thermal and oxidative stability and both IC presented great application prospects. Despite the lower stability evidenced by βCD-based IC in

comparison with the IC prepared with HPβCD, possibly due to a higher content of β-acids on the molecule's outer face, it exhibited faster release into meat products simulant solution, along with a higher antibacterial activity. Finally, the study presented in Chapter Article 5 investigated the individual incorporation (20% w/w) of the IC previously studied into PLA films. IC-added films were evaluated concerning their performance (mechanical, thermal, and morphological features), along with their *in vitro* (antioxidant, antimicrobial, quorum quenching, and antibiofilm properties) and *in situ* (test with a bologna real food system) bioactivity. This work confirmed the potential of inclusion complexation with cyclodextrins as carriers for hop β-acids and application in active packaging development. *In situ* results corroborated with the *in vitro* findings and indicated a possible extension of the lag phase in the natural microbiota of bologna and for *L. monocytogenes* intentionally inoculated. Overall, the studies herein presented are a good asset for the biopolymeric active packaging development, also reinforcing the potentiality of hop β-acids' application.

Keywords: Sustainability; Biopolymers; Natural bioactives; Cyclodextrins; Quorum sensing; Biofilm; Food preservation.

RESUMO

ARRUDA, Tarsila Rodrigues, D.Sc., Universidade Federal de Viçosa, agosto de 2024. **Embalagem ativa antimicrobiana e antioxidante incorporada com extrato de lúpulo (*Humulus lupulus* L.): desenvolvimento, caracterização e aplicação em matríz cárnea**. Orientador: Nilda de Fátima Ferreira Soares. Coorientadores: Allan Robledo Fialho e Moraes e Patrícia Campos Bernardes.

Nos últimos anos, a busca por alternativas aos plásticos derivados de componentes fósseis cresceu rapidamente. Isso impulsionou a investigação de biopolímeros como possíveis substitutos, destacando-se o poli(ácido lático) (PLA) por sua boa aplicabilidade em várias áreas industriais. Paralelamente, embalagens ativas com biopolímeros têm despertado interesse como aliadas no setor alimentício, com potencial para reduzir as perdas de alimentos. Neste sentido, o presente estudo teve como objetivo desenvolver e caracterizar embalagens alimentícias ativas, antimicrobianas e antioxidantes, produzidas à base de PLA, incorporadas com bioativos naturais do lúpulo (*Humulus lupulus* L.). Para uma melhor compreensão dos bioativos naturais, no capítulo Artigo 1, foi abordado uma revisão abrangente sobre os aspectos tecnológicos do extrato de lúpulo, incluindo sua complexação com ciclodextrinas e suas perspectivas no desenvolvimento de embalagens ativas. No capítulo Artigo 2, um extrato comercial de lúpulo rico em β-ácidos foi incorporado diretamente em diferentes concentrações (0,1-5% m/m) em folhas de PLA. As análises mecânicas e térmicas sugeriram que as diferentes frações do extrato se alojaram em locais distintos na matriz polimérica, com os sais de potássio de β-ácidos localizando-se principalmente no interior das folhas de PLA e o propileno glicol na superfície externa. Folhas com 5% de β-ácidos de lúpulo mostraram um aumento significativo nos efeitos inibitórios contra bactérias Gram-positivas não esporuladas (*Listeria monocytogenes* e *Staphylococcus aureus*). As folhas desenvolvidas foram então testadas quanto à sua biodegradabilidade em solo. No capítulo Artigo 3, verificou-se que a presença de β-ácidos de lúpulo não reduziu a taxa de degradação do material, mas limitou o processo interno de hidrólise, provavelmente devido à menor difusão de moléculas de água entre as cadeias poliméricas. Assim, nas folhas ativas, a degradação ocorreu principalmente por erosão superficial. No estudo apresentado no capítulo Artigo 4, foi investigado a complexação por inclusão do extrato de lúpulo rico em β-ácidos com β-ciclodextrina (βCD) ou 2-hidroxipropil-β-ciclodextrina (HPβCD), e os complexos de inclusão (CI) obtidos foram caracterizados em função das propriedades térmicas e de liberação do agente ativo. A complexação melhorou a

estabilidade térmica e oxidativa dos β-ácidos, evidenciando boas perspectivas de aplicação. Apesar da menor estabilidade do CI à base de βCD comparado ao CI com HPβCD, possivelmente devido ao maior teor de β-ácidos na face externa do complexo, este CI exibiu liberação mais rápida em solução simulante de produtos cárneos e maior atividade antibacteriana. Finalmente, no capítulo Artigo 5, foi investigada a incorporação individual (20% m/m) de ambos CI em filmes de PLA. Os filmes com CI foram avaliados quanto ao desempenho mecânico, térmico e morfológico, bem como sua bioatividade *in vitro* (antioxidante, antimicrobiana, "quorum quenching" e antibiofilme) e *in situ* (teste com sistema alimentício real de bologna). O trabalho confirmou o potencial da complexação com ciclodextrinas como transportadoras de β-ácidos de lúpulo para aplicação em embalagens ativas. Os resultados *in situ* corroboraram com os *in vitro*, indicando possível extensão da fase *lag* da microbiota natural da mortadela e da bactéria *L. monocytogenes* inoculada. No geral, os estudos aqui apresentados reforçam o potencial de aplicação dos β-ácidos de lúpulo no desenvolvimento de embalagens ativas biopoliméricas.

Palavras-chave: Sustentabilidade; Biopolímeros; Bioativos naturais; Ciclodextrinas; *Quorum sensing*; Biofilme; Conservação de alimentos.

SUMMARY

1. GENERAL INTRODUCTION

In aerospatiale, food packaging, textile, biomedical, automobilist, and many more industries, plastics are everywhere. Still conventionally produced by petroleum-sourced polymers, plastics are versatile and considerably cheap materials widely employed in diverse areas. However, these conventional plastics have generated deep discussions concerning their source and incorrect discard, as they represent not only an environmental problem but also constitutes a public health issue ("On the plastics crisis", 2023). Therefore, several attempts have been made in recent years to circumvent the negative impacts of the incorrect discard of fossil-based plastics, including the enhancement in politics towards the usage of recyclable materials and the development of novel biopolymers (James et al., 2024).

Polylactic acid (PLA) is a good example of a biopolymeric material, as it is produced from renewable sources (i.e., through the fermentation of sugars to lactic acid, followed by polymerization) and has proven biodegradability. Compostable under industrial conditions, PLA is one of the highest commercially employed bioplastics. Furthermore, it presents similar properties to conventional plastics, such as polyethylene and polystyrene, and has a lower carbon footprint compared to petroleum-based materials (Ferreira et al., 2024).

In the same context, the interest in biopolymers and their applicability in the consumer market has increased the attention to their usage as active packaging. The incorporation of active substances, e.g., antimicrobials and antioxidant compounds, into biopolymers can be an interesting approach to replace conventional plastics in food packaging and a potential ally in food preservation. In special, the usage of natural bioactives goes in accordance with the current consumers' demand for replacement/decreased usage of synthetic additives in food production (Siddiqui et al., 2023).

However, the linking of all these individual features still has some drawbacks. For example, during the active packaging development, the interactions between the active components and the polymer/food matrix must be highly considered as they will dictate the release kinetics during the food's shelf life. Furthermore, bioactive natural sources, including essential oils and vegetal extracts, are usually prone to degradation under the conditions employed in plastic production (i.e., extrusion) also presenting pronounced sensorial aspects, which can interfere with the food's characteristics and lead to lower consumers' acceptance. Thus, strategies have been studied in this sense, to help improve the stability of natural bioactives, decrease their sensorial impacts within the food product, and promote a more controlled release during

the food's shelf life. It is the case of the nanotechnology approaches, as in the inclusion complexes with cyclodextrins (CD) (Puebla-Duarte et al., 2023).

CD are cyclic oligosaccharides that can form host:guest inclusion complexes (IC) with a wide range of molecules. Especially for hydrophobic components, the increasing in their solubility in water and stability can broaden the guest applicability range, including in active packaging manufacturing. Besides the inclusion complexes' features, the CD can also improve material features, such as mechanical strength and stability of biodegradable plastics (Szente and Fenyvesi, 2018).

From this perspective, the present work proposed the investigation of distinct materials in an attempt to develop sustainable plastics with bioactive properties for application as active food packaging. Chapter Article 1 brought a comprehensive literature review of the usage of natural bioactive in active packaging and how the inclusion complexes can be a potential ally in the path; Chapter Article 2 evaluated the direct incorporation of different concentrations of a commercial β-acid rich hop extract into PLA-based sheets that afterward was characterized. Moreover, the degradability of the materials in the soil was assessed in Chapter Article 3. Chapter Article 4 managed to study the inclusion complexes with two cyclodextrins (β-cyclodextrin and 2-hydroxypropyl-β-cyclodextrin) and the commercial β-acid rich hop extract, and the impacts on β-acids' functionality. Finally, in Chapter Article 5, the tested IC were individually applied in PLA-based films and were evaluated the effects of such incorporation in both performance and bioactivity *in vitro*, with studies of antioxidant and antimicrobial activities, as well as the quorum quenching and antibiofilm properties of IC-added films; and *in situ*, with films being tested as active packaging for a ready-to-eat meat product (bologna).

1.2. References

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2. ARTICLE 1: NATURAL BIOACTIVES IN PERSPECTIVE: THE FUTURE OF ACTIVE PACKAGING BASED ON ESSENTIAL OILS AND PLANT EXTRACTS THEMSELVES AND THOSE COMPLEXED BY CYCLODEXTRINS

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2.1. Introduction

Packaging systems favor maintaining food quality, integrity, and safety, also avoiding the scope of contamination that can arise during all food supply chains (Kalpana et al., 2019). Besides its function as a barrier, the package creates an interface between food and consumers, giving essential information and being responsible for the product's first impression.

New technologies have been developed to use active/intelligent food packaging in the last few years. Intelligent tools (i.e., indicators and sensors) and active films can be mentioned as examples of this current trend. For example, active packages involve the deliberated inclusion of subsidiary constituents in or on either the packaging material or the package headspace to enhance the performance of the package system (Robertson, 2013). These packages emerge as a constantly evolving technique that, by the direct interaction between the food product and bioactive substances intentionally incorporated in the package, can preserve the food quality, meanwhile can extend its shelf life for a more extended period, when compared to passive packaging (Bhargava et al., 2020).

Moreover, consumers' demand for natural additives is increasing more than ever. Responding to this, numerous plant extracts (PEx), essential oils (EOs), and their derivatives have been studied by their potential use for active food packaging (Bajić et al., 2019a). The incorporation of these natural substances in films can achieve distinct proposals, such as antimicrobial (Andrade et al., 2020; de Oliveira et al., 2020), antioxidant (Cao & Song, 2020; Jiang et al., 2020), or even repellent activity (Lee et al., 2020).

A promising source of bioactive compounds in the food industry is agro-food by-products. A large volume of leaves, branches, peels, roots, stems, and seeds are discarded as industrial waste. This biomass is usually under valorized and considered a problem worldwide due to its environmental impacts (Dedousi et al., 2017). The use of these potential active compounds for active/intelligent packaging consists not only in an environmentally friendly

and natural activity but also as functional and cost-effective new packaging technologies (Chiralt et al., 2020).

However, some aspects can put barriers to applying natural bioactive substances in food packaging systems, such as the relative instability (i.e., thermal, oxidative) of these compounds during the manufacturing of the polymers or storage of the final product. Also, considering the undesirable sensorial aspects of EOs and some PEx (e.g., intense aroma and/or flavor), there are specific situations in which sensory changes in the food are not well accepted. Thus, using these substances is not the best alternative, as they could mischaracterize the product (Gu & Liu, 2020; Huang et al., 2019a).

Taking that into account, to increase the stability of the active compounds, complexes with cyclodextrins (CDs) have been studied as an excellent alternative for the development of active films (da Rocha Neto et al., 2019; Kalogeropoulos et al., 2009; Lu & Liu, 2020; Marques et al., 2019).

CDs are cyclic oligosaccharides from enzymatic degradation of starch, formed by (α-1,4)-linked 6-12 D-glucopyranose units. The most common natural CDs have a hydrophilic outer surface and a hydrophobic central cavity, which allows them to capture or encapsulate other molecules (Di Cagno, 2017; Jansook et al., 2018). They are gaining attention to the food packaging industry due to their tasteless, colorless, odorless, non-caloric, non-carcinogenic, biodegradable, and non-toxic characteristics (Liu et al., 2022).

In addition, it is known that complexation with these substances can effectively improve the properties of the guests' molecules, such as stability, solubility, chemical availability, and thus increase its bioavailability (Gu & Liu, 2020; Jansook et al., 2018).

The ability to help mask undesirable flavors and the improvement of stability of bioactive molecules are fascinating characteristics, especially considering the food industry. To present biological activity, molecules need to reach the acting site without losing integrity and cross the bacterial cell membrane. Therefore, they need to be protected to preserve bioactive molecules' structural integrity. The CDs possess the capability of delivering them to the main targets without losing any bioactivity, especially considering aqueous medium (Munin & Edwards-Lévy, 2011; Pinho et al., 2014; Zarandona et al., 2020). Furthermore, using bioactive compounds in the form of inclusion complexes with CDs reduces potentially adverse changes in the product's sensorial properties, contrary to the direct exposure of the bioactive molecules to the food components (Pinho et al., 2014).

With the current industrial interest in natural sources of bioactivity and the versatility of CDs, there is a clear need for a fresh review covering CDs as carriers of natural compounds for

active food packaging. For this reason, the purpose of the present contribution is to provide a brief overview of the most up-to-date research combining the ideas of natural antimicrobial/antioxidant substances (which can be obtained from conventional sources or food by-products) and the use of CDs and modified CDs as their potential carriers. We pay particular attention to the aspects that must be considered during the employment of natural substances for food contact, the advantages, drawbacks, and how CDs can be a good bet for contributing to the expansion of active packaging to an industrial scale. Moreover, the current and prospects of active packaging which must be addressed are discussed. Finally, this review is expected to fill the gap about the potential use of natural bioactive compounds in active food packaging and inspire future research directions for CDs in the food packaging industry.

2.2. Essential oils and plant extracts as sources of natural compounds for food packaging

Many natural substances from plants have demonstrated excellent anti-inflammatory, antiviral, anticarcinogenic, antimicrobial, and/or antioxidant properties (Essien et al., 2020). Considering food application, these compounds' antioxidant and antimicrobial activities have great importance since allowing the development of food preservatives with a natural profile. EOs and PEx are common forms of presentations of a mixture of natural active substances. They have gained a lot of industrial attention (e.g., food and pharmaceutical industry) in the last years.

EOs are organic substances and can be obtained from plant materials such as flowers, buds, leaves, seeds, bark, and stems (Aidi Wannes et al., 2010; Hill et al., 2013). Meanwhile, PEx result from the considered primary step in obtaining the crude mixtures of natural compounds (Sabo & Knezevic, 2019). The composition and quality of EOs and PEx can be affected by numerous factors inherent to the plant, such as variety, geographical origin, development stage, climate conditions, the season of growth, ripening stage, and harvest (Karacabey et al., 2013; Sabo & Knezevic, 2019).

The composition profile of these natural substances delimits their application as food preservatives. Knowing their action mechanism is essential since different compounds at distinct concentrations can be found in EOs and PEx. This aspect strongly influences the choice of the natural substances, depending on each food matrix and their characteristics (e.g., composition, fat content, main target microorganisms, oxidation profile, etc.).

2.3. Bioactive mechanisms of EOs and PEx

2.3.1. Antimicrobial properties

The presence of spoilage microorganisms can produce a series of changes in the nutritional and sensory properties of the foods. This, associated with many diseases caused by foodborne pathogens that represent a public health threat, instigates additional hurdles to the microbial growth in food (Hassan & Cutter, 2020; Pina-Pérez & Ferrús Pérez, 2018; Saggiorato et al., 2012).

The mechanisms of action of the EOs and PEx are directly associated with the chemical structure of its constituents. Despite the variability in the composition of these fractions extracted from plants, EOs usually have a more pronounced antibacterial activity when compared to PEx, mainly due to the presence of hydrophobic compounds (Değirmenci & Erkurt, 2020).

It is essential to mention that the antimicrobial properties of EOs are intrinsically related to their chemical composition, type, and concentration (Ju et al., 2019). The main compounds associated with the antimicrobial activity of EOs are described as aldehydes, phenols, and oxygenated terpenoids (Ju et al., 2019; Khaneghah et al., 2018).

The hydrophobicity allows them to interact with the bacterial cellular membrane, changing its permeability and allowing the outflow of ions and other cell contents. When a substantial loss of cell contents is achieved or occurs the loss of vital ions and molecules, the microbial cell dies (Khaneghah et al., 2018). Besides its activity against the bacterial cellular membrane, EOs constituents may have other simultaneous routes of action, such as cell wall injury, mitochondrial perturbation, ribosome dysfunction, DNA damage, and interference at *quorum sensing* factors, which can lead to decreasing at swarming motility, biofilm formation and proteolytic activity (Ju et al., 2019).

As mentioned, the chemical composition of PEx depends primordially on the plant characteristics and the extraction process. However, the primary substances associated with the antimicrobial activity of natural extracts are the phenolic compounds (Sabo & Knezevic, 2019). Catechin, epicatechin, epigallocatechin, carvacrol, thymol, and eugenol are examples of this class of chemicals (Mir et al., 2018; Peng et al., 2013). The antimicrobial potential of phenols is determined by the availability of hydroxyl groups and conjugated double bonds in the reactive groups (Ultee et al., 2002).

Polyphenols' primary mechanism of action is associated with the interaction with the microbial cell components, resulting in conformational changes on the phospholipid bilayer.

Hence, fluidization of membrane lipids occurs and membrane expansion, resulting in its destabilization and the leak of intracellular components (Ultee et al., 2002).

Antimicrobial activity of EOs (e.g., obtained from cinnamon, orange, hop, oregano, clove, garlic, etc.) and PEx (e.g., extracted from hops, propolis, grapefruit, moringa, ginger, rosemary, etc.) had been described against a broad spectrum of microorganisms, including Gram-positive and Gram-negative bacteria, yeasts, and molds (Balaguer et al., 2013; Bassanetti et al., 2017; He et al., 2020; Hussain et al., 2008; Iturriaga et al., 2012; Leite et al., 2013; Nionelli et al., 2018).

One of the limitations of using EOs and PEx as antimicrobials is the lack of information about the precise mechanism of action, despite the increasing number of studies dealing with it. Further investigations must be accomplished to combat bacterial pathogens and their resistance. The interest in the antimicrobial potential of EOs and PEx goes beyond conventional concerns about the growth of microorganisms, also covering mechanisms virulence, motility, sporulation, conjugation, and biofilm formation, primarily focusing on *quorum quenching* activity (Cáceres et al., 2020; Camele et al., 2019; Moradi et al., 2020; Rodriguez-Garcia et al., 2014; Sabo & Knezevic, 2019).

Furthermore, studies about antimicrobial activity of natural sources of bioactive compounds, such as EOs and PEx, are increasingly needed due to their multi-component composition, which turns more difficult for bacteria to develop resistance than to antibiotics that are often composed of only a single molecular entity (Cowan, 1999; Kotzekidou et al., 2008; Salma et al., 2016).

2.3.2. Antioxidant properties

Oxidation is one of the most important processes involved in food degradation (Viuda-Martos et al., 2011). It occurs during the food supply chain and can affect nutritional and sensory properties (rancid odors and flavors). Nevertheless, lipid oxidation may lead to potentially toxic compounds (Kubow, 1992).

Nowadays, the interest in natural antioxidants has considerably increased for use in many products, including foods and their packages, aiming to replace synthetic antioxidants, which are being restricted due to their genotoxicity (Sasaki et al., 2002), environmental toxicity (Sarmah et al., 2020) and low acceptance by consumers (Pokorný, 2007).

EOs and PEx are excellent sources of natural antioxidants. Therefore, the antioxidant mechanism of each fraction will strongly depend on its chemical composition. The antioxidant activity of EOs is associated with biologically active compounds, such as terpenoids and

phenolic acids, for example, phenylpropanoids (Alves-silva et al., 2013; Thokchom et al., 2020). In general, these compounds act as antioxidants by donating hydrogen to highly reactive compounds, thereby preventing further radical formation (Iversen, 1999).

Similarly, PEx are complex systems that contain bioactive components in different concentrations (Mir et al., 2018). As mentioned before, the main active agents present in PEx are usually phenolic compounds. The antioxidant properties of polyphenols are associated with their capability to act as reducing agents, hydrogen donating antioxidants, and singlet oxygen quenchers. In some cases, metal chelation properties have been proposed (Rice-Evans et al., 1996). The content of flavonoids in PEx was also responsible for their antioxidant potential (Mir et al., 2018).

Contrary to antimicrobial activity, some studies demonstrated that, for the same plant, PEx exhibited more pronounced antioxidant activity than EOs (Abu-Orabi et al., 2020; Ahmed et al., 2019; Aidi Wannes et al., 2010). This may occur due to the phenolic composition of extracts, which differs from EOs'. PEx usually present higher amounts of phenolic compounds when compared with EOs, obtained from the same fraction of the same plant (Değirmenci & Erkurt, 2020). In conventional extraction with solvents, the content of polyphenols and flavonoids in PEx depends on the solvent polarity, among other factors inherent to the plant.

Reports in literature describe organic solvents and their aqueous formulation as the most frequently used to extract phenolic components (Değirmenci & Erkurt, 2020; Sun et al., 2015; Trabelsi et al., 2014). For example, ethanol has been known as an excellent solvent for polyphenol extraction, being safe for human consumption. On the other hand, methanol has been generally associated with the extraction of lower molecular weight polyphenols (Alara et al., 2020; Do et al., 2014). However, a recent study emphasized that the literature evidenced that there is no solvent generally acceptable as the best for extraction of polyphenols since a significant influence that detects the solubility of solutes in solvents is the structure of the solute which refers to phenolic compounds in this context (Alara et al., 2021).

Ahmed et al. (2019) observed a high correlation between antioxidant activity and total phenolic contents of basil extracts. On the contrary, they found a low correlation between antioxidant activity and total phenolic contents of basil EOs.

Considering the bioactive properties of EOs and PEx, antioxidant and antimicrobial activities can be mentioned as most targeted for developing food packaging. The incorporation of antioxidant and/or antimicrobial agents into polymeric films can help to extend the shelf-life of perishable food by protecting against oxidation, vitamin losses, enzymatic browning, and microbial growth (Mir et al., 2018).

2.4. Current application of EOs and PEx in active food packaging

An innovative example of extending food shelf-life is active packaging (Hassan & Cutter, 2020). In this type of packing, the use of natural sources of bioactive compounds has attracted more and more attention. Mainly, these packages are composed of polymer matrix added with additives, including different kinds of EOs and PEx. Among other benefits, EOs and PEx can provide antioxidant properties and/or antimicrobial protection against food pathogens and spoilage microorganisms to packaged food.

The extraction of natural active substances is conducted primarily within *in natura* materials (i.e., root, stem, leaf, alabastrum, flower, and fruit) and requires specific procedures. Natural agents are relatively safe and easy to obtain, being incorporated into films or coated directly to the food (e.g., active edible coating) (Huang et al., 2019a). Since the development of biodegradable packaging seems to be one of the most exciting strategies shortly (i.e., in substitution to the ones with fossil origin), the exploration of natural compounds as a source of bioactivity adds an even more promising vision (Mir et al., 2018). However, it is essential to point out that these compounds can be toxic to humans (Bernhoft, 2010; Di Nunzio et al., 2017; Vilas-Boas et al., 2021). Since the migration of the incorporated compounds is one of the target points observed during active packaging development, the concentration of the natural must be adjusted concerning their migration rate (i.e., toxic compounds cannot be incorporated into food packaging).

The visual aspects of a biodegradable film without (Figure 1A) and with (Figure 1B) the incorporation of a natural extract are presented in Figure 1. The differences in the appearance of the films can be attributed to the incorporation of the natural extract. For example, the lower crystallinity attributed to the active film prepared by the casting method with the extract added can be responsible for its less hazy opaque color. Furthermore, since it presents polyethylene glycol in its constitution, it can act as a plasticizer inducing changes in the crystallization behavior. For example, a study conducted by Łopusiewicz et al. (2018) found that the opacity of modified PLA/melanin films was lower than the pure PLA film, possibly attributed to the color and the content of the melanin particles.

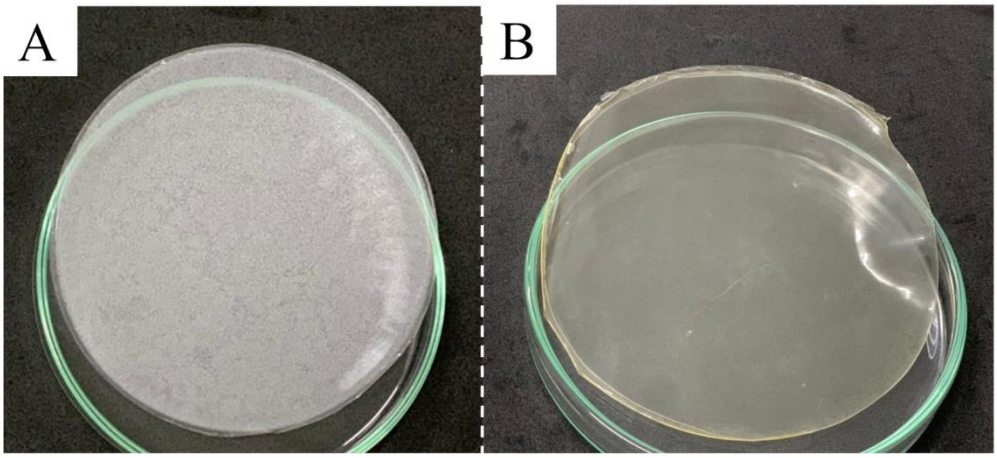

Figure 1. Visual aspects of biodegradable films based on poly(acid lactic): A. Control; B. incorporated with hop β-acids natural extract.

In terms of EOs, some have been widely studied for their application in packaging by their bioactive profile, such as those from clove, lemon, rosemary, grape seed, garlic, cumin seed, straw fruit, cinnamon, and patchouli (Ju et al., 2019). PEx have also been strongly targeted for food packaging development, a source of bioactivity. Green tea extract, grape seed extract, grapefruit seed extract, mint/pomegranate peel extract, sea buckthorn leaves extract, pine bark extract, thyme extract, oak extract, brown algae extract, and hop extracts are some examples of PEx that were already studied considering their incorporation in active films (Bajić et al., 2019a; Brobbey et al., 2017; Mir et al., 2018; Talón et al., 2017). An update in EOs/PEx and their effect on food packaging is summarized in Table 1.

Table 1. Essential oils and plant extracts with their respective main effects on active packaging.

Sources	Major bioactive	Base materials	Effect on food packaging	References
Essential oils				
Anise essential oil	(E)-cinnamaldehyde and (E)-anethole	Polyethylene terephthalate (PET) layer in polypropylene (PP) film	Insect-repellent activity	(Lee et al., 2020)
Lemon, Thyme, and Cinnamon essential oils	Limonene, thyme phenol, cinnamic aldehyde	Chitosan	Antibacterial activity	(Peng & Li, 2014)
Combination of essential oils components	Thymol and carvacrol	Low-density polyethylene (LDPE)	Antimicrobial activity	(Krepker et al., 2017)
Oregano essential oil	Phenolic compounds (carvacrol and thymol)	PP and PET	Antimicrobial activity	(Otero et al., 2014)

Orange essential oil	D-Limonene (major compound), α-pinene, Linalool, α-phellandrene, octanal, and decanal	Poly (butylene adipate-co-terephthalate) (PBAT)	Antimicrobial activity	(Andrade et al., 2020)
Savory essential oil	Carvacrol	Lemon waste powder/cellulose nanofiber	Antibacterial activity	(Soofi et al., 2021)
Ginger and grape seed essential oils	Volatile compounds, polyphenols, and others	LDPE polymers	Antioxidant properties	(Wrona et al., 2021)
Clove essential oil	Limonene, eugenol and β-caryophyllene	Soy protein isolate-montmorillonite (reinforcement)	Antimicrobial activity	(Echeverría et al., 2018)
Plant extracts				
Grapefruit seed extract	Flavonoids, citric acid, ascorbic acid, tocopherol, limonoid, polyphenols	Agar/alginate/collagen blend and hydrogel films	Antimicrobial activity	(Wang & Rhim, 2015)

	Flavonoids, citric acid, ascorbic acid, tocopherol, limonoid, polyphenols Limonene	Carrageenan films	Antimicrobial activity Antimicrobial properties	(Kanmani & Rhim, 2014)
	Flavonoids, citric acid, ascorbic acid, tocopherol, limonoid, polyphenols	PBAT	Antimicrobial activity Antimicrobial properties	(Andrade et al., 2020)
Thyme extract	Polyphenols	Chitosan and starch	Antioxidant activity	(Talón et al., 2017)
Hop extract (ethanolic)	hop resins (e.g., α- and β-acids), aroma components, and polyphenols	carboxymethyl cellulose, oxidized potato starch, soy protein isolate, and gelatin	Antioxidant activity	(Kowalczyk & Biendl, 2016)
Hop extract (supercritical CO_2)	α-acids and β-acids as the main bioactive compounds, and also xanthohumol and polar components are found in traces	Chitosan	Antibacterial activity	(Bajić et al., 2019b)

Amaranthus leaf extract	Phenolic compounds, betacyanin	Polyvinyl alcohol (PVA) and gelatin	Antioxidant, antibacterial and intelligent (color change according to spoiled samples) properties	(Kanatt, 2020)
Neem leaves extract	Carotenoids, phenolic compounds, flavonoids, triterpenoids, ketones, valavinoids, saponins, glycosides, steroids, and tetra-triterpenoids azadirachtin	Seaweed-based biopolymer	Antimicrobial properties	(Kumar et al., 2019, 2020)

In antimicrobial packaging, antimicrobial agents must migrate from the packaging material to the food, even if in some cases they can only act at the food surface (where most of the contamination occurs) and exert the active effect straightly on target microorganisms. The antimicrobial activity strongly depends on the migration properties of the biologically active molecule. In package systems, the bioactive molecules exist within a film material that contains many micropores (i.e., spaces at the polymer matrix that can enable the accommodation of the active substances). Over time, they gradually diffuse through the micropores to the film's surface, where they contact the food (Marinello et al., 2019; Xing et al., 2016). The antimicrobial profile must be extended during the stipulated storage period, and, in this concern, the release rate of antimicrobials from the package into the food must be monitored.

Therefore, if the migration rate of an antimicrobial compound is faster than the growth rate of the target microorganisms, the antimicrobial compound will be depleted before the end of the required period (storage time). Once the antimicrobial molecule is depleted, the packaging system will lose its antimicrobial activity, and microorganisms will grow. On the other hand, if the release rate of the antimicrobial compound is too slow to control the growth of microorganisms, they will rapidly grow to contaminate/spoil the food before the antimicrobial agent is released.

Similarly, the antioxidant properties are strongly related to the release rate of the bioactive compounds. Therefore, maintaining the critical concentration of antioxidant agents at the food surface is necessary to inhibit oxidative changes. However, when added to polymer matrices, two mechanisms can be mentioned as responsible for bioactive compounds' antioxidant properties in food packaging: specific antioxidant action by diffusion of bioactive compounds from the coated package, acting directly to food components (Atarés & Chiralt, 2016), or act as an oxygen barrier, in the packaging itself, due to its oxygen scavenger capability (Bonilla et al., 2013).

There are numerous polymers indeed for food contact. The incorporation of bioactive compounds not only affects the food itself but modifies the characteristics of packing material and can enhance its shelf life and durability (Mir et al., 2018). Active films usually present modifications at their physicochemical, mechanical, barrier, antimicrobial, and antioxidant properties when compared to conventional films (i.e., without the addition of bioactive substances) (Alizadeh-Sani et al., 2020; Bajić et al., 2019a, 2019b; Norajit et al., 2010).

Amaranthus is recognized by its high amount of betalains, which determine the plant foliage color (red, orange, green). *Amaranthus tricolor* L. has been legally accepted as a food ingredient. The incorporation of *Amaranthus* leaf extract into films not only allows the

obtention of an intelligent tool with natural pH-sensitive dyes but also can produce an active package (i.e., PVA/gelatin films) with antimicrobial and antioxidant properties capable of being applied for shelf life extension of perishable food commodities, such as fish and meat (Kanatt, 2020).

Neem (*Azadirachta indica*) leaves, another source of bioactivity for films development, can produce ethanolic extracts with antimicrobial activity. Seaweed-based films are a sustainable alternative for food packaging. Incorporating neem leaf extracts provides antimicrobial activity, especially against Gram-positive bacteria (e.g., *Bacillus subtilis* and *Staphylococcus aureus*), and improves the film mechanical properties and water vapor permeability. In this context, gamma-irradiated seaweed-neem biodegradable films (2.5 kGy, 5% w/w neem leaves extract) presented enhanced performance: higher hydrophobicity, smoother surface, and improved mechanical, barrier, biodegradability, and antimicrobial properties (Kumar et al., 2019, 2020).

An interesting point about active packages, but very little addressed, is the development of insect-repellent films for food packaging. Some insects represent a true enemy of packaged food. For example, the Indian meal moth *Plodia interpunctella* is one of the most notorious insect pests worldwide stored products. Their larvae can penetrate food packaging using their mandibula, a real problem for cereal, legumes, bread, confectionery, noodles, powdered formula, and other processed foods, including pet food (Jia et al 2018). Polyethylene terephthalate (PET) incorporated with star anise EO proved to be a potential alternative as insect-repellent for bread packaging. Furthermore, the films were tested for production on a pilot scale, and the results showed applicability to a natural food system (Lee et al., 2020).

The combination of different natural compounds for active package development is another technique that can be performed (Wrona et al., 2015). A synergistic effect can be observed (Krepker et al., 2017). For example, plant EOs (lemon, thyme, cinnamon) or even their constituents (carvacrol, thymol) used in combination might provide a new formulation option for developing antimicrobial packaging (Krepker et al., 2017; Peng & Li, 2014).

Grapefruit seed extract (GSE) is a commercially available substance obtained from the seeds, pulps, and peels of grapefruit (*Citrus paradise Macf. Rutaceae*). Its biological activity is well recognized, and GSE is applied in several conditions by its antibacterial, antifungal, antiviral, antiparasitic, anticancer, antioxidant, and antifeedant properties (Riahi et al., 2021). Carrageenan (Kanmani & Rhim, 2014), agar/alginate/collagen blend, and hydrogel-based films (Wang & Rhim, 2015) are some examples of package materials incorporated with GSE that exhibited promising results for inhibition of foodborne pathogens.

Hops (*Humulus lupulus* L.) are used worldwide as a raw material in brewing. Soft resins (α- and β-acids), xanthohumol, terpenes, and phenolic compounds are some compounds in hop cones that exhibit attractive active potentials (Hrncic et al., 2019). Recently, the bioactivity of its rich composition has been explored for different applications, including active packaging (Bajić et al., 2019a, 2019b; Kowalczyk & Biendl, 2016). However, much remains to be investigated regarding the use of hops and their substances in the development of active packaging.

To develop an active film against a Gram-negative foodborne pathogen, Andrade et al. (2020) studied the incorporation of orange EO in poly(butylene adipate-co-terephthalate) (PBAT). The results evidenced the efficacy of the natural active substances against *Escherichia coli*. Despite the decrease in tensile properties associated with an increase of orange EO in the film, the authors mentioned that the films presented sufficient strength for its usage as packaging.

In parallel, Otero et al. (2014) also studied the activity of packaging films coated with oregano (*Origanum vulgare*) EO against Gram-negative bacteria in comparison with a food preservative ethyl lauroyl arginate HCl (LAE). The active polypropylene (PP) and PET films coated with oregano EO and LAE demonstrated antimicrobial effects against two *E. coli* O157:H7 strains. However, when tested in a food model (Zamorano cheese), the effectiveness was slightly reduced due to the interaction of active substances/polymer matrix/food. Interestingly, at concentrations tested (up to 8%), orange EO did not exhibit a significative sensorial effect on cheese packaged in PET films, different from what was observed for PP/EO active films, where the panelists evidenced sensorial discrepancies with the control sample, even under 6% of orange EO. These results highlight the importance of studies on polymer/natural active substance interactions.

Among antimicrobial, antioxidant properties are also important for active food packaging. Indeed, some natural sources have both potentials. Soy protein isolate-montmorillonite (MMT) films incorporated clove EO were able to decrease microbial growth (spoilage microorganisms) and lipid autoxidation when applied for tuna fillets packaging (17 days of storage at 2 °C) (Echeverría et al., 2018). Mango kernel ethanolic extracts (Maryam Adilah et al., 2018) and aqueous thyme extracts (Talón et al., 2017) are other sources of bioactive compounds that can be used in hydrophilic food packaging (protein-based and chitosan-based films, respectively).

However, many aspects must be considered to guarantee the desired effects, including the polymer used, the source of bioactive compounds and their composition, the desired

activity, and, in many cases, the food to be packaged and its characteristics. For example, natural substances as a source of bioactivity take a plus, the sensory attributes. The sensory aspects of EOs/PEx or their active compounds can, in specific situations, perform a positive interpretation for consumers by its harmony with the food packaged.

Some bioactive compounds, in addition to their antimicrobial/antioxidant aspects, also are characterized as important non-hazardous GRAS flavors (e.g., carvacrol, citral, limonene, vanillin), and their incorporation in polymer matrices can allow the obtention of active flavored packaging, such as citric and spiced (Costa et al., 2018; Khezerlou et al., 2019). Colorant properties are another aspect that natural sources can provide and improve the sensory characteristic of products (Jafarzadeh et al., 2021) or can control photo-oxidation by reducing light transmission (Moraczewski et al., 2020). For example, a study conducted by Lopes et al. (2021) evidenced that the judges highly valued the golden color of potato phenolic present on active film during the sensory evaluation of smoked fish fillets. This is a strand of research that can be further explored in studies involving the use of natural compounds for active food packages.

However, in other cases, the sensory establishment of the natural substances incorporated into films can negatively affect consumer perception of packaged food products. This and other possible entrapments of the use of natural bioactive compounds are discussed, along with an attractive alternative application in section 2.6.

A market report provided by Grand View Research Company (San Francisco, California, USA) stated that, in 2024, packaging market revenue in the United States would reach $6 billion for active packaging (Grand View Research, 2016). Undoubtedly, developing novel packaging systems is severely related to research carried out in these areas. Furthermore, as active packaging has gained attention in food preservation scenarios, the utilization of natural bioactive compounds has also been arising as a matrix force to this technology. This behavior is mainly triggered by consumers' profiles, increasingly demanding and adopting healthier consumption habits.

On the other hand, the regulatory aspects around active packaging remain still vague. This is one of the major impediments that still prevent a broader market penetration of these materials. For example, in Europe, Regulation 450/2009/EC established the rules for using such materials in food contact applications, responsible for increasing interest in active packaging (European Comission, 2009). This regulation also brought other safety guidelines. For example, it stated that nanotechnology could not be used without further evaluation, even when direct contact with packaged food is not considered because of a functional barrier.

Moreover, in Europe, all active packaging systems that intentionally release substances into the food must comply with the legislation for direct food additives (Regulation 1333/2008/EC); that is, the released substance must be listed on the country's Positive List of Additives, and its use must characterize a technological need (European Parliament and the Council of the European Union, 2008).

Recently, in 2021, the *European Food Safety Authority (EFSA)* announced an administrative guide that describes how to submit applications for approval of materials to be "used in components of active and intelligent materials and articles intended to come into contact with food" (EFSA, 2021).

2.4.1. Application of nano-EOs and PEx in active food packaging

Nanotechnology has mainly been studied to improve the application of natural bioactive compounds in active food packaging (Yildirim et al., 2018). In the last decades, aiming to overcome the limitations presented by EOs and PEx (e.g., poor solubility, high instability, and degradability), scientific researchers have focused on the development of nanostructures able to improve the efficiency of natural compounds as preservative agents (Alfei et al., 2020).

It has been demonstrated that the nanoencapsulation (i.e., incorporation or dispersion of active components in the form of small vesicles) through particles with a nanometric diameter (1 to 100 nm) as delivering carriers of bioactive natural compounds leads to a remarkable improvement of important properties (Figure 2), such as the increase of their solubility and stability, as well as to a decrease of their inactivation rate (Pal et al., 2019). Therefore, this technology offers a better performance of natural bioactive compounds as preservative agents during active food packages development.

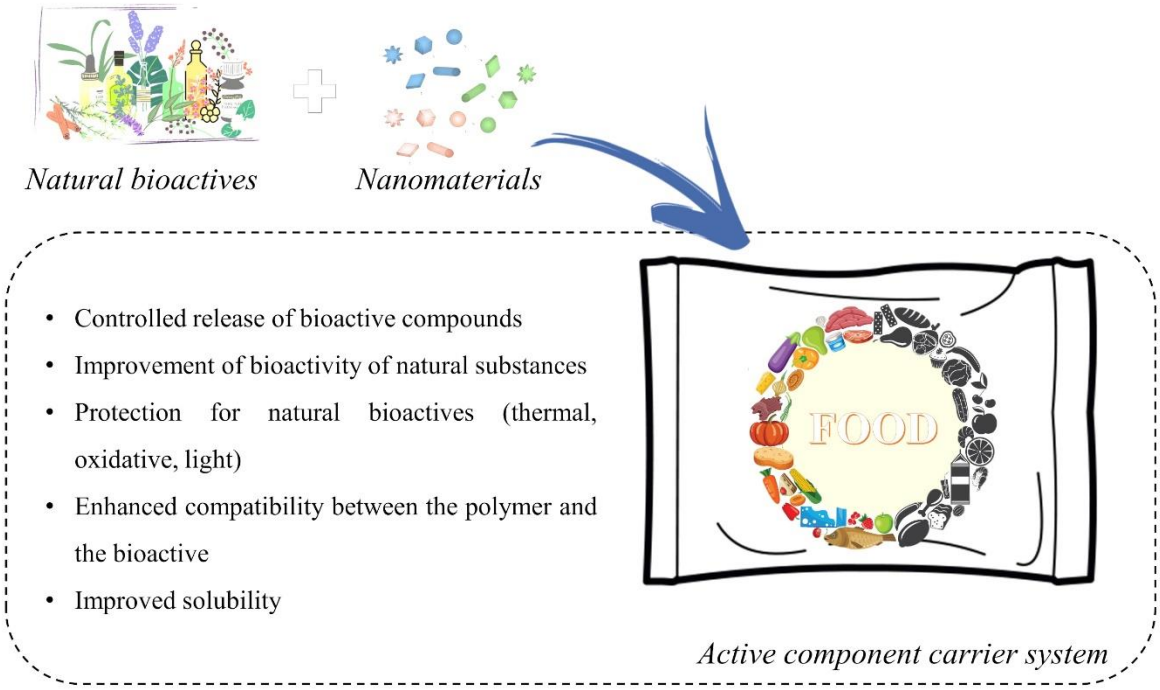

Figure 2. Nanotechnology in active packaging systems based on natural bioactive compounds.

Nanostructures can incorporate polymer matrices into two categories: bioactive nanoparticles are nanosized materials with intrinsic preservatives properties. In contrast, bio-activated nanoparticles are nanomaterials enriched with active synthetic or natural chemical substances or enzymes (Alfei et al., 2020). Here, we will approach this last category with the respective incorporation of EOs and PEx.

Various substances can be applied as wall materials for nanoencapsulation of bioactive compounds. Among them, carbohydrates (chitosan, pectin, alginates, maltodextrin, cellulose derivatives, modified starch, carrageenan, etc.), proteins (whey protein, sodium caseinate, gelatin, soy proteins, zein, casein, etc.), fat, and waxes (lecithin, bee wax, hydrogenated vegetable oils, etc.), and some polymers (polyglycolides, polylactides, polyethylene glycol, polycaprolactone, polyvinyl alcohol, etc.) are primarily used (Pateiro et al., 2021). The process of choosing the wall material depends mainly on the natural substance characteristics (e.g., hydrophobicity, active sites) and the polymer/food properties, which directly influence the chemical and structural compatibility.

A study conducted by Kamkar et al. (2021) evaluated the application of nanocomposite active packaging based on chitosan biopolymer loaded with nano-liposomal garlic essential oil to preserve refrigerated chicken breast fillet. The nanoliposomes presented considerable stability and preserved the packaged products more when added to chitosan films.

Compared to control samples (packaged in sterile polyethylene bag), the fillets packaged with active films showed lower total volatile nitrogen, peroxide value, thiobarbituric acid- reactive substances, and also microbial count, including total viable count, coliforms, *Staphylococcus aureus,* and psychrotrophic bacteria, due to the antioxidant and antimicrobial properties of garlic EO. Therefore, it is essential to mention that, in addition to the improved bioactive properties, the nanoliposomes influenced the physical characteristics of the films by improving their thickness, water-solubility, elongation at break, some microstructural properties, and antioxidant activity (in comparison to garlic EO in free form) (Kamkar et al., 2021).

Another study developed an active film based on nano-formulated cinnamon EO. The whey protein isolate-based film was incorporated with chitosan nanofiber and cinnamon essential oil. Both additives were emulsified, and nanostructured lipid carriers form. The nano structuration of the cinnamon EO improved its stability and antimicrobial properties against Gram-negative (*E. coli* and *P. aeruginosa*) and Gram-positive (*S. aureus*) bacteria. The enhanced antibacterial properties of cinnamon EO-loaded non-lipid carriers compared to the emulsified EO may be due to the gradual and sustained release of cinnamon EO through the nanoparticles during the incubation time in the film samples (Mohammadi et al., 2020).

In addition, there are numerous techniques for nanoencapsulation of bioactive compounds, such as coacervation, nanoprecipitation, spray-drying, emulsification-solvent evaporation, ionic gelation, and electrochemical-based technologies (Pateiro et al., 2021).

Recently, several studies highlighted the efficiency of electrospinning and electrospraying over the considered traditional encapsulation techniques (Aytac et al., 2017; Celebioglu et al., 2018b; Lamarra et al., 2020; Min et al., 2021; Rostamabadi et al., 2020). Furthermore, this technique is characterized by the absence of heat, an important advantage for preserving thermally unstable bioactive substances, such as EOs and PEx. Thus, electrospinning achieves high encapsulation efficiency values, ensuring the stability of compounds upon processing and storage (Lamarra et al., 2020).

A current review described the backgrounds involving the development of nanoparticles based on polysaccharides for entrapment of hydrophobic compounds, such as EO, pointing to chitosan as the most widely used polymer in electro-spinning/electrospraying technologies for this purpose (Dierings de Souza et al., 2021).

Nanofibers are another effective strategy for encapsulating bioactive compounds. These nanosized fibers have excellent prospects, especially considering their application in food packaging. Electrospinning has been used as an efficient processing method for stabilizing active compounds due to electrospun fibers' high encapsulation efficiency and stability (Aytac

et al., 2017, 2018; Celebioglu et al., 2018a; Wu et al., 2020). Consequently, many structural and functional advantages of the electrospun nanofibers have led to greater attention on the research and development of the active packaging coating/material through an electrospinning process (Altan & Çayır, 2020; Shahbazi et al., 2021)

For example, carboxymethyl cellulose-gelatin nanofibers produced via electrospinning successfully encapsulated *Mentha longifolia* L. EO (Shahbazi et al., 2021). These nanofibers were used for the formation of nanofibrous films with interesting properties. The active films presented lower water vapor permeability than neat carboxymethyl cellulose-gelatin films. Moreover, they exhibited improved microbial population and chemical property of peeled prawns when the nanofibers were added in concentrations over 1%. Antioxidant properties were also found for the active films. When incorporated with 2% of bioactive nanofibers, the films promoted the highest sensory scores in terms of odor, color, texture, taste, and total acceptance throughout the study period (14 days).

In another study, Altan & Çayır (2020) investigated the production of zein ultrafine fibers plasticized with tributyl citrate (produced via electrospinning) used for encapsulation of carvacrol and consecutively film formation. The authors evidenced that electrospun fibers with homogeneous fiber distribution (without beads) and the highest encapsulation efficiency were obtained from the electrospinning of the solution containing 36% zein concentration and 7% carvacrol. Moreover, carvacrol-loaded electrospun fibers may be applied as active coatings to extend the shelf life of food products, especially considering their low water vapor permeability playing a pivotal role in preserving packed food products that are sensitive to changes in the surrounding environment.

Nano-emulsion methods can also be applied to incorporate natural bioactive compounds in food packaging. For example, oil-in-water nano-emulsion has been widely utilized to improve hydrophobic substances' dispersion in the hydrophilic phase, promoting the compatibility between hydrophobic substances and water-soluble matrices. This can be greatly useful especially considering EOs, since their direct incorporation into the film-forming matrix may present a poor fusion between the matrix and bioactive components, resulting in the poor performances of the active films (Sun et al., 2021).

In this context, nano-emulsion films can be obtained from hazelnut meal protein and clove EO, exhibiting improved antioxidant and antimicrobial properties (Gul et al., 2018). Another example is functional gelatin-based composite films incorporating oil-in-water lavender EO nano-emulsions, which presented strong antibacterial properties against Gram-

positive (*S. aureus* and *L. monocytogenes*) and Gram-negative (*E. coli*) bacteria, in addition to excellent antioxidant activities (Sun et al., 2021).

Nanotechnology is an interesting tool for developing active coatings for food products. Nano-chitosan coatings incorporating nano-encapsulated cumin (*Cuminum cyminum* L.) EO was successfully applied to sardine fillet preservation. The active coatings exhibited improved antioxidant and antimicrobial properties during the evaluated storage time (16 days). Moreover, the highest sensory properties were obtained during the experimental period for the coating incorporated with nano-encapsulated EO. This is due to the reduction of diffusion rate and thus increased antimicrobial and antioxidant activities caused by the encapsulation process. Consequently, sensory perception can be improved (Homayonpour et al., 2021).

A similar study, described by Zhang et al. (2020), investigated the development of active coatings based on chitosan-gelatin containing nano-encapsulated tarragon EO to preserve pork slices. The results suggested that the active coating could significantly inhibit the quality deterioration of pork slices (during 16 days of storage). Among the advantages of nano-encapsulation, this technique contributed to the sustained release of tarragon EO and enhanced the antioxidant, antibacterial and sensory properties.

Many studies around nano-EOs and PEx have raised concerns about these materials' safety and environmental impacts. These nanomaterials' overall risk and safety to human health have not been completely understood due to the lack of studies in this field. However, the commonly applied nanomaterials are biocompatible and included at low dosages in the composites (Rangaraj et al., 2021). Even though the migration of these substances from the packaging to the food product must be considered (Dimitrijevic et al., 2015). Nanomaterials that proved to migrate in insignificant levels or not to migrate are approved (EFSA, 2012).

As well, as their bioactivity in food matrices is mainly improved by the sizeable surface area, the particles within the nanometer range may behave differently within the human body and have different biological fates (Mahato et al., 2021). This implies that issues concerning their toxicity may be different based on their petite sizes (Munro et al., 2009); thus, the GRAS decisions must be made considering this (Sharma et al., 2020). Despite the specificity of each nanomaterial developed, further studies must be conducted on risk assessment and proper regulation, considering prospects of active packaging and their placement in the market.

In this context of nanotechnology as an interesting alternative to the development of active packaging based on natural compounds emerges the use of CDs with their inclusion complexation phenomenon being able to deliver structures in nanoscale. The CDs as essential tools to carrying natural substances will be approached in Section 2.6.

2.5. By-products in food industry/processing and active packaging application

Considerable amounts of waste are generated (30-50% of total food weight) during food processing. Alternative and novel materials, described as by-products, are derived from underused food products, renewable resources, and the valorization of agro-industrial and marine wastes (Chiralt et al., 2020). Seeds, pomace, pulp, peel, husk, and unused flash are examples of these by-products, which are cheap sources of bioactive compounds, nutraceuticals, and fibers suitable for various applications (Jafarzadeh et al., 2020).

Some research has demonstrated the great potential of these materials as great sources of important components, such as phenolic compounds (i.e., flavonoids, polyphenols, and tannins) (Sá et al., 2020; Sganzerla et al., 2020; Zhao & Saldaña, 2019). Since by-products could have the same, or even more, bioactive substances than the frequently used industrial parts (Ayala-Zavala et al., 2013), the commercial application of these materials to recover their bioactivity seems promising. In addition, the employment of food by-products can assure efficiency and process sustainability.

Polyphenols and other interesting compounds obtained from by-products may contribute to the antioxidant and antimicrobial potential of the packaging system. Films incorporated with these substances can also be presented as eco-friendly and sustainable materials. Also, value addition and valorization of food wastes would make this a cost-effective alternative (Bhargava et al., 2020). Furthermore, recent research studies have employed bioactive compounds from food by-products incorporated in package materials (Table 2). Aiming at their application in the food industry (i.e., food matrices and packaging), by-products have their potentially active substances extracted by different processes, especially using organic solvents. Therefore, the main application form of these materials is as ‘by-product extracts’ (Chiralt et al., 2020).

Table 2. Potential applications of by-products in active food packaging.

By-products sources	Methods of extraction	Matrix	Bioactive compounds	Properties	References
Neem leaves extract	Extraction with 95% ethanol (v/v)	Seaweed biopolymer + glycerol	Carotenoids, phenolic compounds, flavonoids, triterpenoids, ketones, valavinoids, saponins, glycosides, steroids, and tetra-triterpenoids azadirachtin in	Antimicrobial activity against Gram-positive bacteria: *S. aureus* and *B. subtillis;* No effect against Gram-negative bacteria *E. coli*	(Kumar et al., 2019, 2020)
Turmeric dye residue extract	Soxhlet extraction with solvents (ethanol/ isopropanol, 1:1)	Turmeric flour + sorbitol	Curcumin and curcuminoids (demethoxycurcumin and bisdemethoxycurcumin)	Antioxidant activity	(Maniglia et al., 2014)
Red pitaya peel extract	Extraction with 30% ethanol (v/v)	Starch/polyvinyl alcohol + glycerol	Betalains	Antioxidant activity and antimicrobial effects against Gram-negative (*E. coli* and *Salmonella*) and Gram-positive (*S.*	(Qin et al., 2020)

				aureus and *L. monocytogenes*) foodborne pathogens.	
Pomegranate peel extract	Extraction with 80% ethanol (v/v) solution containing 1% of hydrochloric acid (v/v)	κ-carrageenan + glycerol	Polyphenols (phenolic acids, flavonoids, and tannins)	Antioxidant activity and antimicrobial activity against *E. coli* and *Salmonella* (Gram-negative) and *S. aureus* and *L. monocytogenes* (Gram-positive); Films were more effective against Gram-negative bacteria.	(Liu et al., 2020)
Blueberry pomace	Separation of the pomace by filtration followed by freeze-drying process	Cassava starch + sorbitol	Aromatic compounds	Protection against UV radiation	(Luchese et al., 2018)

Feijoa peel flour	-	*Pinhão* starch + citric pectin + glycerol	Phenolic compounds, flavonoids, carotenoids, alkaloids	Antioxidant activity and antimicrobial activity against *E. coli, S.* Typhimurium, and *Pseudomonas aeruginosa;* The active film seemed an excellent alternative for maintaining the quality of apples during storage	(Sganzerla et al., 2020)
Mango kernel extracts	Extraction with absolute ethanol (99,9% purity)	Protein isolate + fish gelatin + glycerol (soy lectin was used as emulsifier)	Gallic acid, ellagic acid, ferulic acid, cinnamic acids, tannins, vanillin, coumarin, and mangiferin	Antioxidant properties	(Maryam Adilah et al., 2018)
	Soxhlet extraction with methanol	Mango kernel starch (added with mango kernel fat and plasticizers)	Gallic acid, ellagic acid, ferulic acid, cinnamic acids, tannins, vanillin, coumarin, and mangiferin	Primary antioxidant properties and UV-absorbing effects	(Melo et al., 2019)

Cashew tree pruning residue	Acetosolv pulping (92.8 vol% acetic acid and 0.7 wt% hydrochloric acid in distilled water) followed by fractionation in ethanol	Bacterial cellulose produced from cashew apple juice and added with lignin and cellulose nanocrystals (both from cashew tree pruning fiber)	Lignin	UV- absorbing and antioxidant properties; Active films did not exhibit antimicrobial activity against Gram-negative (*E. coli*) and Gram-positive bacteria (S.*aureus*) as well as yeasts (*S. cerevisiae*), at conditions tested	(Sá et al., 2020)
Sunflower hull extracts	Extraction with 80% aqueous methanol (1:10) (hull:solvent)[a]	Potato starch + glycerol	Phenolic compounds, with chlorogenic acid the main compounds, followed by coumaric and ferulic acid derivates, mono-caffeoylquinic and dicaffeoylquinic acid derivates)	Antioxidant capacity; No antimicrobial activity was detected against *E. coli* and *L. innocua* at conditions evaluated[b]	(Menzel et al., 2019)
Grape seed extracts	Extraction with 60% methanol	Pea starch + glycerol	Phenolic compounds, phenolic acids, flavonoids	Antioxidant activity and bacteriostatic effect,	(Corrales et al., 2009)

				with reduced bacterial growth (*Brochothrix thermosphacta*) in pork loins under refrigerated storage	
Tomato by-products (peels, seeds, and small amounts of pulp) extract	Ultrasound-assisted extraction using 98% ethanol (v/v)	*PVA + chitosan* *PVA+Itaconic acid*	Carotenoids and phenolics compounds	PVA/chitosan films exhibited antibacterial activity toward *S. aureus*, *P. aeruginosa,* and *S. enterica* Enteritidis, with higher effect against Gram-negative bacteria; PVA/Itaconic acid films presented some antibacterial activity against *P. aeruginosa*	(Szabo et al., 2020)
Potato peel extract	Soxhlet extraction with n-hexane	Potato starch + glycerol	Phenolic compounds	Antioxidant activity with the potential to pack smoked fish fillets	(Lopes et al., 2021)

Red apple pomace extract	Extraction with 80% (v/v) ethanol solution with 0.5% (v/v) of HCl	Chitosan + TiO_2 nanoparticles	Phenolic compounds and pigments (anthocyanins)	Potent antioxidant and antibacterial ability; pH-responsive color-changing properties	(Lan et al., 2021)
Rowanberry, blue-berried honeysuckle, and chokeberry pomace	Aqueous extraction	Fish gelatin + glycerol	Phenolic compounds (hydroxycinnamates in rowanberry and chokeberry, and anthocyanins in blue-berried honeysuckle extract)	Improved antioxidant activity (especially for blue-berried honeysuckle extract, 3-fold); antibacterial properties against *E. coli*, *P.fluorescens*, *S. aureus,* and *L. innocua*	(Staroszczyk et al., 2020)
Mulberry pomace extracts	Ultrasonic-assisted extraction with 75% (v/v) acidified ethanol solution	Psyllium seed gum	Phenolic compounds	pH-sensitive behavior (monitor of food spoilage) and improved antioxidant properties	(Zhang et al., 2021b)
Residual biomass of green coffee oil (press cake and sediment)	Extraction with 70% (v/v) ethanol	Carboxymethyl cellulose	Phenolic compounds (chlorogenic acids)	High UV absorption and antioxidant effects on fish oil	(Vidal et al., 2022)

Sea buckthorn (*Hippophaerhamnoides linn*) pomace extract	*	Esterified potato starch	Phenolic compounds	Antioxidant and antimicrobial properties (against spoilage bacteria), improving quality of beef jerky	(Guo et al., 2020)

- : Extracts obtained by a supplier.

*: Not mentioned in the study.

[a] Ethanol 80% was also tested, but MeOH extraction was shown to be fast and easy.

[b] Potential bacteriostatic action was observed around 40 mg/mL. Further analysis is required

The processing of blueberry juice generates a significant volume of waste that is usually discarded. However, this wasted material is rich in phenolic compounds, fibers, minerals, and vitamins that can perform biological activities in packaging systems or improve package mechanical properties. For example, blueberry pomace incorporated into cassava starch film improved the film performance and indicated beneficial effects to prevent food deterioration caused by UV radiation, suggesting the potential application of these by-products in active packaging (Luchese et al., 2018).

Pomegranate edible and no edible parts (including peels) contain abundant polyphenols, such as tannins, flavonoids, and phenolic acids. Comparing extracts obtained from pomegranate flesh and peel incorporated into the κ-carrageenan matrix, Liu et al. (2020) verified that due to different polyphenols composition, films produced with pomegranate by-products extract (i.e., peel) possessed higher UV light barrier, antioxidant and antimicrobial properties.

Turmeric residue obtained from turmeric dye extraction was used to obtain a film plasticized with glycerol (Maniglia et al., 2015). Turmeric films revealed the presence of curcuminoids (i.e., curcumin, demethoxycurcumin, and bisdemethoxycurcumin), despite the partial loss of these actives due to the film manufacturing process (i.e., high temperature). The study evidenced that shorter heating times during film production are needed to reduce curcuminoids loss. A similar work, conducted by Maniglia, Domingos, de Paula, & Tapia-Blácido (2014), demonstrated that edible films produced from the turmeric dye extraction residue (turmeric flour), containing sorbitol as a plasticizer, also exhibit antioxidant activity and could constitute an active packaging material.

Enhanced antioxidant and antimicrobial active properties also can be delivered by incorporating betalains from red pitaya (*Hylocereus polyrhizus*) peel into starch/PVA films (Qin et al., 2020).

Unlike other fruits, cashew commercialization is focused on its nuts, and the pseudofruit (cashew “apples”) jointly tree pruning fiber are considered by-products and mostly wasted. Films produced from bacterial cellulose (from cashew apple juicy) and added with lignin and cellulose nanocrystals (from cashew tree pruning fiber) were effective in providing antioxidant properties. However, no antimicrobial activity was demonstrated (Sá et al., 2020).

Feijoa (*Acca sellowiana*) waste by-products can also functionalize food packaging. In addition, bioactive compounds in feijoa peels can provide intensive antioxidant properties and antimicrobial activity against pathogenic bacteria. These results allowed the successful use of feijoa peels flour in developing active films for the postharvest conservation of apples (Sganzerla et al., 2020).

Furthermore, the meat industry also can be a great source of interesting by-products that can be applied to package development. For example, chicken feathers are recognized as reinforced material for food packaging (Baba & Ozmen, 2015). This poultry industry by-product can be utilized as a film base material after extraction of chicken feather protein and, along with gelatin, can act as a polymer matrix to incorporate clove oil that can be used as an active packaging for smoked salmon (Song et al., 2014).

Chitosan is a crustacean by-product that has also been used in active packaging due to its antimicrobial activity. Some studies have questioned its effectiveness in antimicrobial package development, attributing the bactericidal effect of chitosan solutions exclusively to its increased acidity (pH 5) (Foster & Butt, 2011). Meanwhile, the antimicrobial activity of chitosan has been described in active films, especially against Gram-positive bacteria (Szabo et al., 2020). However, the precise mechanism of chitosan antibacterial capacity is not fully elucidated.

A study conducted by Huang et al. (2019b) used chitosan (in combination with tannic acid) and cellulose acetate to fabricate nanofibrous mats using layer-by-layer self-assembly technology. The results indicated that the antibacterial activity of the mats revealed over 86% antibacterial activity against Gram-negative bacteria (*E. coli*) and up to 99% antibacterial activity against Gram-positive bacteria (*S. aureus*). These interesting antimicrobial properties were partially attributed to positively charged chitosan that can act as a biological adhesive due to the negatively charged lipopolysaccharide on the outer membrane of bacteria (Božič et al., 2012).

Therefore, considering the countless possible applications of by-products, these findings highlight the substantial potential of exploring food by-products in active packaging for the food industry.

2.6. Cyclodextrin: a potential carrier for bioactive compounds

Some setbacks can be observed when using natural compounds as active agents in food packaging. Many natural substances present pronounced sensory characteristics, which can negatively interfere with the properties of the packaged product. An example occurs with the addition of EOs, given many volatile compounds.

Natural bioactive substances (i.e., antioxidant, antimicrobial) may also present low stability to temperature, oxygen, and light factors. In addition, as mentioned in section 2.2, depending on the interaction between the active molecule and the food matrix/packaging

material, its release can attend to food needing to maintain its conservation during the storage period. In other cases, this release may be too slow or too fast, so it cannot keep up with the preservation of the packaged food.

Among the alternatives studied to overcome these negative aspects, CDs have excelled as natural substances carriers through natural compounds inclusion complex (IC) systems.

IC formation is described in recent studies as a specific nanoencapsulation system that relies on a model or proof-of-concept guest compounds (Cid-Samamed et al., 2022; Dehghani et al., 2020; Menezes et al., 2019; Sharma & Satapathy, 2021). The host-guest interaction required for IC obtention can only be made with a few components being used as a substrate 'host molecule' (e.g., CDs and lactoglobulins). The main substances employed as host molecules aiming at the IC development with bioactive substances are the CDs (Fuenmayor et al., 2021). However, as CDs' nanometric cavity accommodates a large spectrum of natural molecules, the ICs obtained are an interesting alternative to introduce the nanotechnological concept into active food packaging.

CDs are produced from a renewable natural material, starch, by a relatively simple enzymatic conversion and can act as vehicles to protect biologically active compounds (Szente & Fenyvesi, 2018). Given the structure of these cyclic oligosaccharides, with polarity contrasting between inside and outside, IC can be formed with different solid, liquid, and gaseous compounds as guest molecules (Astray et al., 2020b).

Studies demonstrated that CDs present a hydrophilic outer surface and a hydrophobic internal cavity. Thus, compounds that show low solubility in water are the perfect combination for the space inside the CD molecule (Carneiro et al., 2019; Jansook et al., 2018; Rezaei et al., 2019). CDs are already extensively used in cosmetics, food, and chemical industries, especially in pharmaceutical systems, to increase drugs' chemical stability, solubility, and bioavailability (dos Santos Lima et al., 2019).

Among the possible benefits attributed to the formation of IC between CDs and natural bioactive compounds aiming food package applications can be mentioned: the enhancement of solubility, stabilization against derivatizing agents (e.g., heat, visible or UV light and oxygen), control of volatility, and sublimation properties, attenuation of sensorial impacts, modulation of delivery rate of substances and physical isolation of compounds (Pinho et al., 2014).

The most common presentation form of these naturally occurring molecules, (i.e., obtained by enzymatic synthesis) is the α-, β-, and γ-CD (Figure 3), formed by six, seven or eight glucose units, and cavities with a diameter of 0.57, 0.78, and 0.95 nm, respectively

(Astray, Mejuto, & Simal-Gandara, 2020; Hădărugă, Bandur, David, & Hădărugă, 2019; Marques, 2010).

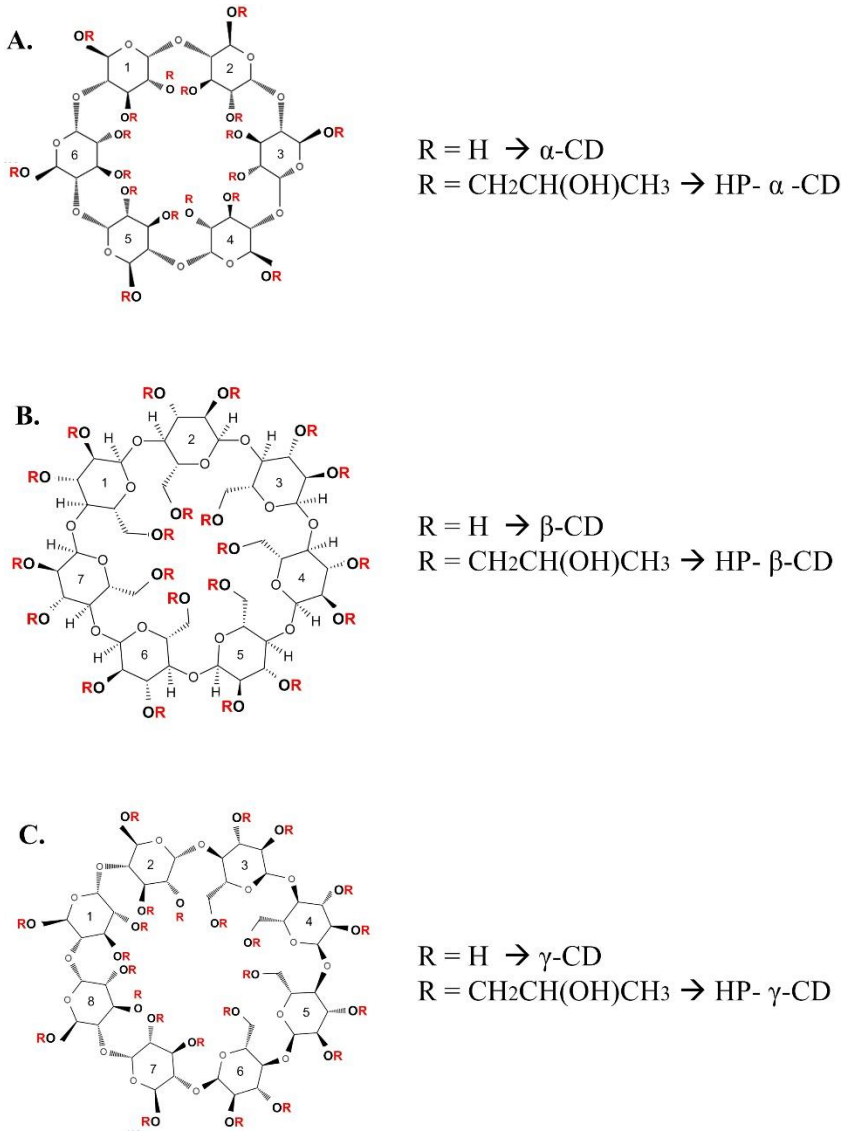

Figure 3. Chemical structure of main cyclodextrins (CDs): A. α-CD; B. β-CD; C. γ-CD.

The size of the CD cavity allows selectivity for the complexation of guest molecules, and the physical, chemical, and biological properties of these guest molecules may be drastically modified. For example, complex formation with CDs may lead to increased dissolution rate, membrane permeability, and bioavailability of nutraceuticals of low solubility (Kalogeropoulos et al., 2009). During IC formation (Figure 4), the most commonly used host:guest molar ratio is 1:1, but 2:1 or 1:2 (or even other ratios) have also been found, depending on the molecular structure of both molecules (Aytac et al., 2018; Szente & Fenyvesi, 2018; Yildiz et al., 2019; Yildiz et al., 2018).

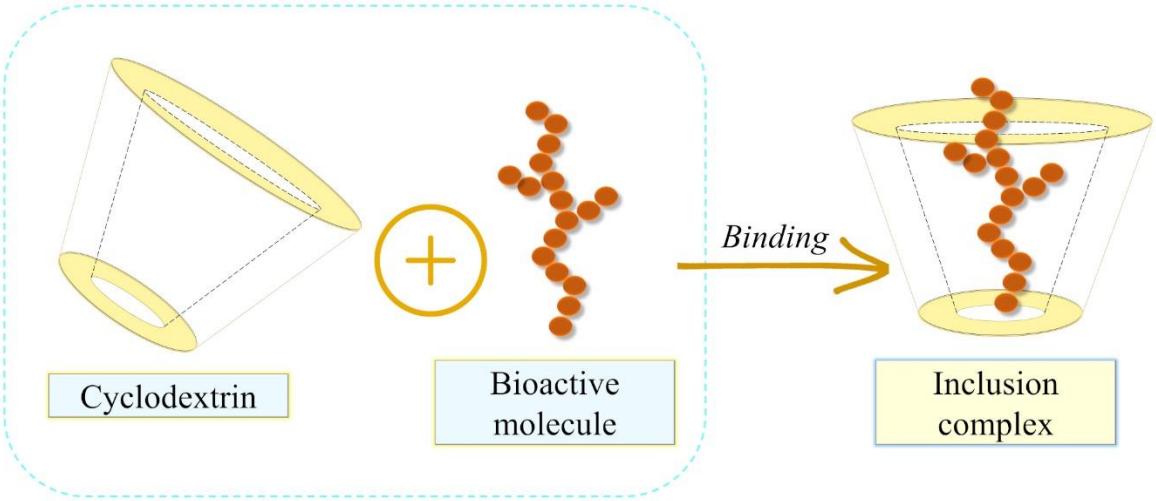

Figure 4. Representative scheme of inclusion complex formation.

Natural CDs have limited water solubility, limiting their applications as solubilizing complexing agents. Compared to other natural CDs, β-CD presents the lowest water solubility (approx. 18.5 mg/mL, at 25 ºC), followed by α-CD (145 mg/mL) and γ-CD (249 mg/mL) (Jansook et al., 2018). However, it is the most targeted for use in drugs and food due to its lowest price and its GRAS concept (Hădărugă et al., 2019). The food-grade β-CD has a price between US$ 5-6/kg (Astray et al., 2020a).

CDs structures can also be chemically modified, originating derivates with distinct and desired properties. For example, methylated, ethylated, hydroxypropylated, hydroxyethylated, acetylated, sulfoethyl, sulfopropyl, sulfobutyl-CDs, and polymeric β-CDs have been employed in the rational design of water-soluble or insoluble carrier systems (Marques, 2010).

The chemical modifications on CD molecules mainly focus on increasing the water solubility and bioavailability of biologically active compounds in the food and pharmaceutical fields. Despite that toxicological aspects and biodegradability of modified CDs are still unexploited, their non- or low-toxicity profile recognizes some. Yet, through an endothelial permeability assay, Monnaert et al. (2004) evidenced that native CD (α- > β- > γ-CD) are more toxic than CD derivatives (methylated and hydroxy propylated α-, β-, and γ-CDs), concerning their abilities to cross the blood-brain barrier. Contradictory to the generally presented in the literature, that shows that the permeability is strongly dependent on the nature and/or chemical modification of the CD, (Monnaert et al., 2004) could not find a structure/permeability

relationship. For instance, the water permeability is relatively invariable by methylation for α-CD, reduced to βCD, while increased for γ-CD.

Among the CD chemical derivatives, 2-hydroxypropyl-β-CD (HP-β-CD) is one of the most investigated as bioactive compounds carrier, mainly due to its high water solubility (>600 mg/mL) and bioavailability (Hădărugă et al., 2019). HP-β-CD is also considered a very effective and toxicologically benign derivative (Gould & Scott, 2005). Furthermore, highly soluble CD derivates can form stable complexes with the given guest (e.g., bioactive compound), being stable during storage but also susceptible to rapid decomposition in the biological media, contributing to the rapid absorption of a guest molecule and reducing toxicological effects of host molecule (i.e., interactions with cell membranes) (Astray et al., 2009).

Favorable interactions occur between the host and guest molecules during the IC formation, including electrostatic/coulomb forces, van der Waals interactions, hydrogen bonding, and hydrophobic effects. These interactions allow the obtention of more stable structures. However, their impact depends on various factors such as type of guest, the solvent used, etc. (Jansook et al., 2018).

Several methods can form ICs, and the selection of the process is based on the properties of the guest molecule, the nature of the CD chosen, and the cost involved (Marques, 2010). The most common methods are coprecipitation, kneading, grinding, solution, damp mixing and heating, slurry complexation, and neutralization (Pinho et al., 2014).

Besides the cavity size of CD molecules, other factors affect IC formation and stability. For example, the guest molecules' molecular structure and chain length, solvent composition, temperature, pH, and the chosen complexation methods must be considered (Hu et al., 2021).

The development of active food packaging with CD inclusion complexes is highly appealing nowadays, especially natural bioactive compounds as guest molecules. However, knowing CDs and their ICs main characteristics, it is essential to understand the central aspects of their application in active packaging development.

2.6.1. Cyclodextrin inclusion complexes in food packaging

CDs in the food industry are more pronounced than most people know. Around the world, there is available in markets a great amount of food with CDs included in its composition, such as chocolate, debittered juices, and soy products deodorized. Moreover, CDs' safety and reasonable price make them a promising tool for food technology (Fenyvesi et al., 2016).

In general, CDs are versatile molecules with multiple applications that can improve many segments (Fenyvesi et al., 2016; Jansook et al., 2018). More specifically, considering the food sector, CDs can be employed for research and development of new products (i.e., encapsulation of flavors; protection against oxidative degradation, heat-induced changes and light-induced decomposition; taste modifications and elimination of bitter and disgusting tastes and odors of foods; food preservation), nutritional purposes (i.e., cholesterol sequestrant) and intelligent food packaging (Astray et al., 2009; Marques, 2010). Some studies have already demonstrated IC potential applications directly in food matrices (Muñoz-Shugulí et al., 2021). On the main topic of this review, active packaging, CDs display an important role as a carrier of natural biologically active molecules.

CDs can be easily found for buying at great amounts aiming at the employment in industrial processes, even the chemically modified ones. Interestingly, in addition to pure CDs, there are IC between CDs and specific bioactive compounds (e.g., menthol, pinene, peppermint, citral, etc.) already available in the market.

Considering their specific employment in food packaging, CDs are a two-fold material. First, by reducing the residual organic volatile contaminants in packaging materials or acting as additive/second component (e.g., composite film, blends development) during film formation by incorporation of “empty” CDs; and secondly, as a carrier for bioactive compounds, by IC formation (e.g., active packaging; Figure 5) (Astray et al., 2009; Pinho et al., 2014). In addition, as an advantage to active food packages, CDs can act to reduce the diffusion kinetics, control the release of active compounds, and thus maintain food quality and safety (Astray et al., 2020b). A representative scheme of the functionality of CDs is presented in Figure 6.

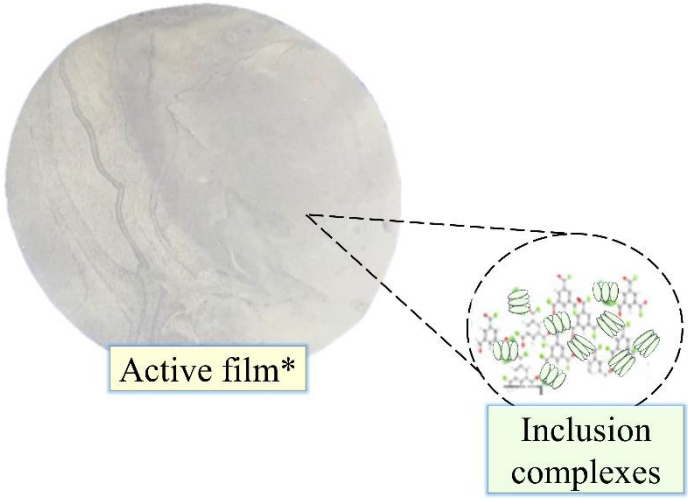

Figure 5. Scheme of inclusion complexes dispersed on active film. *Film produced by the authors: cellulose acetate added with HP-β-CD:hop β-acids.

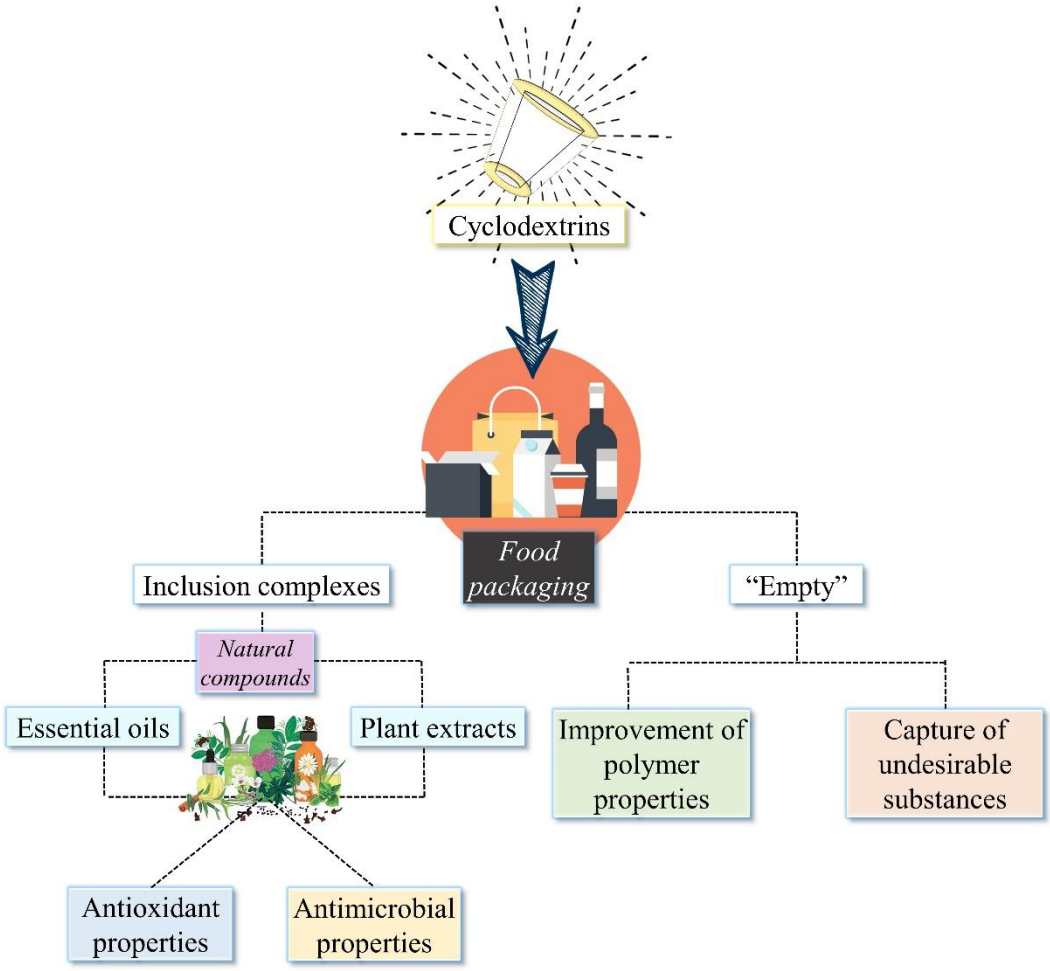

Figure 6. Graphical representation of the application of cyclodextrins in food packaging.

For food packaging development, taking the regulatory safety status into account, it is suggested that the eventual migration of most used CDs into the food from the packaging has no harmful effects on consumers (Szente & Fenyvesi, 2018). When ingested, CDs are not absorbed from the gastrointestinal tract due to their bulky and hydrophilic nature (Gidwani & Vyas, 2015).

The regulatory status of CDs in foods differs between countries (Astray et al., 2009). α-, β- and γ-CDs are registered in the Codex Alimentarius of the Joint FAO/WHO Expert Committee on Food Additives with the International Numbering System (INS) No. 457, 459 and 458, respectively, among the General Standard Food Additives (GFSA) (GSFA, 2019). In turn, β-CD is registered in the European Union as additive E-459 (Official Journal of the European Union L283/77, 2003). This status underwent revision in 2016 but did not lead to changes (Mortensen et al., 2016). Furthermore, since they are considered additives in Europe, their release in the food products must be accounted for in the product labeling (European Parliament and the Council of the European Union, 2022; Velásquez et al., 2021).

In the USA, CDs are considered GRAS by the FDA (list of food additives that are 'generally recognized as safe') and can be commercialized as such. In Europe is followed the recommendation of Joint FAO/WHO Expert Committee on Food Additives (JECFA) and, although α- and γ-CD can be consumed indefinitely (due to their favorable toxicological profiles), a maximum acceptable daily intake (IDA) of 0.5 mg/kg of body weight is pre-established for β-CD (Mortensen et al., 2016).

As for the derived molecules, some chemically modified CDs, such as hydroxypropyl-α-, β- and γ-CDs (HP-α-CD, HP-β-CD, HP-γ-CD, respectively) and randomly methylated α- (RAME-α-CD), β- (RAME-β-CD) and γ-CDs (RAME-γ-CD) are approved as pharmaceutical excipients; however, they have not yet been recognized as ingredients for use in foods (Fenyvesi et al., 2016).

Nevertheless, there are no specific regulations considering the inclusion complexes between CDs and bioactive compounds in food products. In such cases, the regulation of both ingredients, bioactive substance and CD, must be considered (Matencio et al., 2020).

On a large scale, packaging materials are conventionally produced at elevated temperatures (in general, between 100 ºC and 200 ºC, depending on polymer). Concerning the already discussed drawbacks of natural bioactive compounds, the high-temperature condition may lead to considerable loss through volatilization or thermal conversion (degradation, isomerization) of these active substances. This loss can be avoided if the bioactive component is mixed with the polymer in a complex form. Furthermore, another advantage is the better

homogeneity of the system because the majority of the active guests are not directly compatible with the polymer matrix (Szente & Fenyvesi, 2018).

The thermal stability provided by inclusion complexes formation with CDs has demonstrated excellent prospects for applying various natural bioactive compounds as active packaging components. For example, Celebioglu et al. (2018b) promoted the formation of inclusion complexes between modified CDs (HP-β-CD, HP-γ-CD, and methyl-β-CD 'M-β-CD') and thymol, an essential oil compound, at a 1:1 molar ratio. As a result, the enhancement of the thermal stability of the bioactive compound was successfully achieved. In addition, the volatility of thymol (a highly volatile substance) was effectively suppressed by the inclusion complexation. 88-100% of thymol maintained preserved in inclusion complexes.

A similar study conducted by Celebioglu et al. (2018a) obtained inclusion complexes from HP-β- CD, HP-γ-CD, and M-β-CD with eugenol (1:1 molar ratio), another essential oil component. Again, the compound's volatility was effectively suppressed (maintenance of 70-95% of the bioactive compound inside the inclusion complex). In addition, the thermal stability achieved by inclusion complexation allowed the preservation of the bioactive compound in temperatures up to 310 ºC. Meanwhile, the pure compound supports temperatures up to 200 ºC.

Both mentioned compounds are recognized for their antimicrobial and antioxidant activities. However, the improvement of their properties enables their application in active packaging development, considering the industrial processes. Various studies have already investigated the application of eugenol (Cheng et al., 2019a; Dammak & Sobral, 2019) and thymol (Lukic et al., 2020; Ramos et al., 2020) as active components for films development.

Nevertheless, the laboratory scale provides a limited perspective of industrial production. On a small scale, it is common to use solvent casting technique to film obtention, which allows the process to occur in relatively mild conditions (under 100 ºC). Such approaches do not represent the loss of bioactive compounds achieved on an industrial scale. When extrusion is employed to film obtention, the natural bioactive compounds are carefully added in the last minutes of the process to reduce possible losses by degradation or volatilization. Still, these substances can be lost in large amounts (~30% or over) (Ramos et al., 2020). Thus, inclusion complexation can be an interesting alternative to the industrial development of active films, especially concerning natural bioactive compounds. To illustrate, Figure 7 presents the visual aspects of active films incorporated with natural bioactive compounds in both forms (pure and complexed with CDs), obtained by solvent casting method.

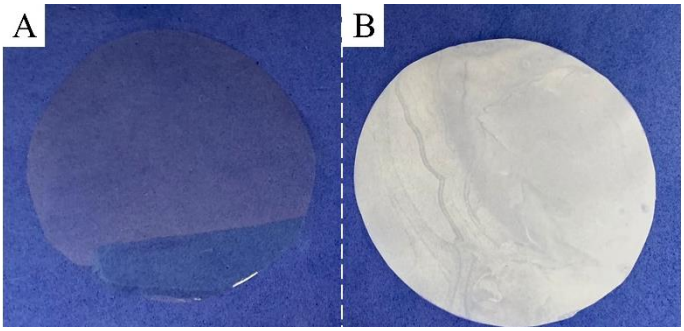

Figure 7. Cellulose acetate active films incorporated with hop bioactive compounds: A. pure hop extract; B. HP-β-CD:hop β-acids inclusion complexes.

Because of the mentioned thermal sensitivity of the compounds, the direct incorporation of natural bioactive compounds into packaging film using a melt extrusion method is considered not possible (Chen et al., 2019). However, some studies proved that inclusion complexes with CDs improve the thermal stability of natural substances, making them very useful as a biological control for additives. For example, ethylene-vinyl alcohol (EVOH) films incorporated with citral and trans-cinnamaldehyde inclusion complexes with β-CD (1:1 molar ratio) seemed an exciting approach to food preservation since it was responsible for extending the beef's shelf life about 4 days when stored at 4 ± 1 ºC.

An important detail about this study is that the films were made by extrusion processes (around 185 ºC), with the inclusion complexes being mixed directly with the polymer resin (1% w/w) (Chen et al., 2019). Thus, inclusion complexation can be an interesting alternative to the industrial development of active films, especially concerning natural bioactive compounds. In some cases, there is no other way that can be used to retain the bioactive compounds in the matrix.

Recently, another study investigated the development of antimicrobial films through the extrusion processes. The active component, in this case, was ferula (*Ferula asafetida*) ethanolic extract, which was complexed with β-CD in a molar ratio of 1:1. The complexation successfully enhanced the extract's thermal stability since the complexes were directly mixed (5% w/w) with low-density polyethylene (LDPE) resin on the extruder (200 ºC), and the natural bioactive compounds persisted on the active film. As a result, the antimicrobial efficiency of the films tested *in vitro* evidenced a significant potential against *Aspergillus niger* and *Saccharomyces cerevisiae* (Niazmand & Razavizadeh, 2021).

Previously, during the development of packaging for contact with food, it was tried as much as possible to control food-packaging interactions so that they did not happen. However,

considering the concept of “passive packaging,” these interactions were harmful to food quality since most implied undesirable sensory changes and migrations of toxic components from the packaging to the packaged food (Hotchkiss, 1997). On the contrary, active packaging uses purposeful interactions precisely to promote the quality of the packaged product.

In active packaging, the release rate of the bioactive compound must be highlighted. Some aspects interfere at the release rate of the IC guest active molecule: concentration of the bioactive substance in the polymer, its diffusion coefficient at the polymer, its affinity towards the CD, its partition coefficient between the polymer and CD, and between the polymer and the packaged item, and also on temperature and time (Joo et al., 2011).

When the bioactive molecule is incorporated into a polymer with high compatibility, for example, EO to a very hydrophobic polymer, its release will be prolonged, preventing the correct expected activity. ICs with CDs may help in this situation since, by its structural characteristic, CD creates hydrophilic micro-regions in the hydrophobic matrix, absorbing water, facilitating dissociation of the complex, and creating channels for migration of the active guest molecules (Plackett et al., 2006).

Various studies aim to investigate the impact of inclusion complex formation on the release kinetics of bioactive compounds during active films development. A recent review mentioned that the combination of natural compounds, such as EOs, with CDs is a promising strategy to enhance the application efficiency, and consequently the obtention of a sustainable release, in active films/coatings (Zhang et al., 2022).

In this context, a study developed chitosan films added with inclusion complexes for the controlled release of 2-phenyl ethanol (Zarandona et al., 2020). 2-phenyl ethanol is a natural compound with antimicrobial properties found in more than one hundred food products, including tea leaves, cocoa, and coffee (Etschmann et al., 2002). This bioactive is currently used to enhance flavor and odor (Yadav & Lawate, 2011). Thus, the idea of incorporation of this substance in active films seems promisor.

Adding 2-phenyl ethanol in inclusion complex form (using β-CD, in equimolar ratio) into chitosan film-forming solutions (at 10% w/w) led to chitosan films with improved properties. Especially considering the release properties, the results suggested that the active substance was evaporated during film formation since a maximum release of 8% was achieved when used in pure form. On the other hand, the natural bioactive films preserved the inclusion complex's bioactivity (Zarandona et al., 2020). These results suggest that the CD complexation helps ensure the release of bioactive compounds for a prolonged time, considering the protection offered and the interactions between the host molecule and polymer matrix.

Another study reaffirmed the sustained release promoted by incorporating natural substances as inclusion complexes (Zhou et al., 2020). The β-CD:cinnamaldehyde inclusion complexes (prepared at 1:1 molecular ratio) were crosslinked with a nonwovens polyethylene terephthalate (PET). The evaluation of the antibacterial pads produced indicated the success of incorporating cinnamaldehyde protected by CD. Antibacterial tests revealed that the pads could inhibit the growth of both Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria.

When applied with tray packaging, the antibacterial pads were used to preserve cold fresh pork by prolonging its shelf life effectively. Furthermore, the antibacterial pads added with inclusion complex could control the sustained release of cinnamaldehyde (a highly volatile natural PE), allowing long-term antibacterial effect, with great potential in active packaging to extend the shelf life of food products (including refrigerated food matrices) (Zhou et al., 2020).

The most significant release profile may be achieved when the package matrix is composed of a relatively hydrophobic polymer (Joo et al., 2011). On the other hand, the low compatibility of CD external surface (hydrophilic) with the less hydrophilic polymers results in disadvantages on stability and structure of packaging material, leading to problems involving reduced barrier to water and/or oxygen, elasticity, and thermal stability (Poverenov et al., 2013).

The trigger for the release of active compounds from IC is humidity. This characteristic is beneficial, especially for the packaging of high moisture food (Szente & Fenyvesi, 2018) and concerning spoilage aspects on food products. An explanation for this is the water absorption by CD inclusion complexes under higher humidity conditions, leading to some dissolving of CDs so that the complexed bioactive compounds are released faster (Agbenin & van Raij 2001). A study evidenced that CDs have changed their molecular structure when absorbing water, improving their solubility (Seo et al., 2010). Considering the specific case of fresh food, it is well-known that the spoilage process of these products is usually accompanied by increased humidity levels in the headspace of the packaging. Thus, active packaging incorporated with ICs could successfully fit in this case (Ayala-Zavala et al., 2008).

In this context, a study conducted by Yin et al. (2021) identified that, at the same temperature, a high-humidity environment (75%) accelerated the release of 1,8-cineole (1,3,3-Trimethyl-2-oxabicyclo[2.2.2]octane; a bioactive monoterpene commonly found in EOs) from an IC with HP-β-CD, and the amount of 1,8-cineole released at equilibrium (45%) was also higher than that of low humidity (0%). To illustrate this, at 23 ºC (near room temperature), the amount of active compound released in 75% humidity could reach 25%, but only 15% in 0% humidity.

The solubility of the natural bioactive compounds is another crucial aspect to discuss in inclusion complexation. As mentioned, naturally occurring bioactive compounds may present a hydrophobic profile (e.g., EOs components). This characteristic can be an issue considering the compatibility with more hydrophilic films, such as using novel biodegradable polymers (chitosan, methyl cellulose, carboxymethyl cellulose, etc.). This can be overtaken by the complexation process with modified CDs, such as hydroxypropylated CDs, promoting greater homogenization of the active compound on the polymer matrix and its target release. This property can also attract developing active coatings with fast orally dissolving characteristics. Furthermore, the higher solubility of natural substances may enhance their bioactive properties, as occurred to curcumin complexed with HP-γ-CD (Celebioglu & Uyar, 2020).

All aspects discussed in this review impact how ICs have become the target of studies involving their application in active packaging. Previous studies demonstrated the applicability of CD complexes as preservative agents. For example, an active film containing 0.1% iodine-β-CD inhibits putrefaction for 2 months at 20 ºC in fish paste or frozen seafood (Hara et al., 2000).

Nowadays, new approaches have been included for active packaging development. Just as there has been a more significant opening for biodegradable polymeric materials (e.g., chitosan, cellulose acetate, methylcellulose, polylactic acid), the choice of the bioactive agent has been directed to more natural sources. ICs formed by CD and PEx/EOs or their major compounds have entered the scene as potential sources of bioactivity (i.e., antioxidant, antimicrobial) in active food packaging (Table 3). Furthermore, this technology also positively impacts biodegradable (Liu et al., 2022; Velázquez-Contreras et al., 2022), contributing even more to the current packaging necessities and trends.

Table 3. Application of cyclodextrins (CD) as a carrier of natural bioactive compounds in food packaging

Cyclodextrin	Natural bioactivity source	Polymer	Activity	Reference
β-CD	Palmarosa and star anise essential oils	Polyethylene terephthalate (PET)	For both inclusion complexes, was observed less growth of *Penicillium expansum*, less weight loss and ethylene and CO_2 production, and less firmness loss of packaged apples, after 12 days at 23 °C (compared to control without essential oil)	(da Rocha Neto et al., 2019)
	Cinnamon essential oil	Poly(lactic acid) (PLA) nanofibers	Antimicrobial activity against *S. aureus* and *E. coli*; increasing of pork shelf life (about 5 days, at 25 ºC)	(Wen et al., 2016)

	Carvacrol	Sodium alginate	Films incorporated with ICs demonstrated improved water resistance, mechanical properties, light barrier property, and heat aging. The films also evidenced controlled release of the active compound and enhanced antifungal activity against *Trichoderma* sp. (isolated from postharvest white mushrooms stored at 4 °C). In addition, the active films alleviated oxidative damage and delayed senescence of the	(Cheng et al., 2019b)

		postharvest white mushrooms	
Citral and trans-cinnamaldehyde	Ethylene-vinyl alcohol (EVOH)	Reduction in the total bacterial count of packaged raw beef, and extension of its shelf life of about 4 days, at 4 ºC. IC demonstrated high thermal stability, enabling application in extrusion processes (185 ºC)	(Chen, Li, Ma, Mcdonald, & Wang, 2019)
Thymol and carvacrol	PLA	Antifungal activity, with *Alternaria alternata* inhibition after 10 days of incubation, at 25 ºC	(Friné et al., 2019)
2-phenyl ethanol	Chitosan	Controlled release of the antimicrobial compound; Retention superior to 90% of the active	(Zarandona et al., 2020)

		substance after the film preparation, while just 8% of 2-phenyl ethanol remained in the absence of complexation with β-CD, indicating higher antimicrobial activity	
Lemongrass oil	Polyvinyl alcohol (PVA)-Starch	Antioxidant activity and antimicrobial activity against *Shewanella putrefaciens*; Oxygen barrier improved	(Chen, Zong, Chen, & Xie, 2020)
α-tocopherol	Linear low-density polyethylene (LLDPE)	Antioxidant activity	(Koontz et al., 2010)
Gallic acid	Chitosan, gelatin, and their blends	Films incorporated with IC showed a higher release rate, being suitable to	(Rezaee et al., 2018)

		higher anti-oxidant demands and short-term protection.	
Carvacrol, *trans*-cinnamaldehyde, and eugenol essential oils	Chitosan	Antimicrobial activity against *E. coli*, *S. aureus* *Salmonella typhimurium*, and *L. monocytogenes;* decreased water vapor permeability of chitosan films	(Xiuxiu Sun et al., 2014)
Mustard essential oil	Sodium cellulose sulfate (NaCS)	Great antimicrobial activity against *E. coli*, *S. aureus*, and discreet activity against *Bacillus subtilis* and *Aspergillus niger*	(Chen & Liu, 2016)
Curcumin	Gelatin	Significant increase in antioxidant activity and the stability of this activity (due to a	(J. Wu et al., 2018)

			lower release rate of active compound) in food simulant (apple juice)	
	Trans-anethole	Gelatin	Antimicrobial activity was observed against Gram-negative (*E. coli, Pseudomonas aeruginosa*) and Gram-positive (*S. aureus, B. subtilis*) bacteria	(Ye et al., 2017)
	carvacrol and *trans*-cinnamaldehyde	Low-density polyethylene (LDPE)	Films with LDPE+βCD + active agent evidenced slow-release rates and, consequently, slow effectiveness against the fungi *Botrytis Cinerea*	(Canales et al., 2019)
2-O-methyl-β-CD	hexahydro-β-acid	Chitosan	Decrease in moisture content of the film, increase of water	(Lu et al., 2019)

			solubility and water permeability, improvement of antioxidant activity and antimicrobial activity against foodborne pathogens (Gram-positive and Gram-negative and fungi)	
Hydroxypropyl-β-CD	Carvacrol	Chitosan	Antimicrobial activity against *S. aureus* and *E. coli* after 20 days of storage (25 °C, 43% environmental RH)	(Higueras et al., 2015)
Triacetyl-β-CD	Allyl isothiocyanate	LDPE	High humidity conditions promoted more prolonged release of the active compound. The IC showed high thermal stability,	(Shin et al., 2019)

			enabling film formation through extrusion processes. The active film could be applied to food packaging aiming at antimicrobial purposes (e.g., high moisture food matrices)	
Hydroxypropyl-γ-CD	Resveratrol	Cellulose bilayer film (cellulose acetate + hydroxyethylcellulose)	Films with greater hydrophilicity, antimicrobial activity against *Campylobacter* strains, anti-q*uorum sensing* activity	(Â. Silva et al., 2016)
γ-Cyclodextrin	Thymol	Electrospun zein nanofibrous webs	Antibacterial activity against *S. aureus* and *E. coli* and reduce the bacterial count in raw beef stored up to 5 days at 4 °C	(Aytac et al., 2017)

A significant number of studies in literature evaluated β-CD as a host molecule before IC formation. EOs seemed to be a current suggestion to guest molecules due to their hydrophobic profile and strong bioactivity. For example, da Rocha Neto et al. (2019) mentioned that β-CD:palmarosa or star anise EOs (prepared at approximately 1.67 mL of EO/g of β-CD) demonstrated potential application in double-bottom package (PET). These PET active packages exhibited interesting antimicrobial effects against blue mold growth (at 23 ºC), thereby extending apple shelf life. The packages associated with antifungal properties reduced ethylene production, respiration rate, softening, pH, and increasing titratable acidity (da Rocha Neto et al., 2019).

Active packages can contribute to the development of novel technologies that may help to reduce postharvest losses caused by fungi, such as *Penicillium expansum* and *Trichoderma* sp., in countries with a lack of suitable cold chain to fresh products (Cheng et al., 2019b; da Rocha Neto et al., 2019). The fungi of the genus *Alternaria* are another agent related to postharvest diseases and economic losses. They also represent a potential risk to public health since they can produce a broad spectrum of mycotoxins and secondary metabolites. Active packages developed with β-CD:thymol or carvacrol IC (1:2 molar ratio) at 2.5% and 5% w/w, respectively, showed *Alternaria alternata* inhibition (after 10 days of incubation), evidencing an interesting potential use in the agro-food industry (Friné et al., 2019).

ICs can also be applied in packaging systems aiming to increase the shelf life of meat products, such as pork (Wen et al., 2016) and raw beef (Aytac et al., 2017; Chen et al., 2019), by inhibiting spoilage microbiota or even food pathogens (i.e., Gram-positive and Gram-negative bacteria).

In addition to antimicrobial properties, some studies also demonstrated the great potential of IC in antioxidant packages. For example, curcumin (Wu et al., 2018), α-tocopherol (Koontz et al., 2010), and gallic acid (Rezaee et al., 2018) are some examples of natural sources with antioxidant properties that can be applied to IC formation aiming at active food packaging development.

Figueroa-Lopez et al. (2020) investigated the development of active films by incorporating different inclusion complexes. Oregano EO (OEO) was complexed with two CDs (α-CD and γ-CD, at 80:20 w/w and 85:15 w/w host:guest weight ratios, respectively). Both ICs obtained resulted in homogeneous films when incorporated in poly (3-hydroxybutyrate-co-3-hydroxyvalerate) matrix (added with 15 wt % α-CD:OEO and 25 wt% γ-CD:OEO). Due to the greater encapsulation efficiency, the inclusion complex between γ-CD and OEO exhibited higher antimicrobial and antioxidant properties. Consequently, the films added with this

complex presented good performance (maintenance of bioactivity) up to 15 days due to the high protection offered by the inclusion complex system.

Another study developed cardboard boxes coated with CDs entrapped with an EOs mix (carvacrol:oregano:cinnamon, 70:10:20 wt%) for fresh tomato storage. This active box maintained the tomato firmness for 6 days at 8 ºC (short transport simulation). Furthermore, the decay rate was reduced from 9−15% to 2% after an abusive storage period (6 days/8 °C+12 days/25 °C) (Buendía−Moreno et al., 2020).

It is also important to mention that the inclusion complexation with CDs can also improve the antioxidant/antimicrobial effects of the natural compounds. For example, Serna-Escolano et al. (2019) reported that the antifungal effect of thymol and carvacrol complexed with HP–β-CD (1:1 molar ratio) against *Geotrichum citriaurantii* was higher than that presented on the free form. Das et al. (2019) also verified the enhanced antimicrobial (antibacterial and antifungal) activities of several M–β-CD:EOs complexes prepared at 2:1 molar ratio (lavender oil, lemon balm oil, peppermint oil, thyme oil, borneol, citral, linalool, menthol, and thymol) and their main components against *E. coli*, *S. aureus*, and *S. pombe* strains.

The shelf life of various food products could be successfully prolonged owing to the possibly enhanced bioactivity and the improved thermal stability, photostability, and as well as to the controlled release of natural bioactive compounds entrapped in CDs. Thus, the choice of CD (host molecule) and active source (guest molecule) must be taken carefully, considering the application (food matrix) and the interaction with the polymer. Several studies demonstrated CD-based films successfully applied to food preservation, including fruits and vegetables, fish, meat, milk, and juices (Liu et al., 2022). Although the current cost of most of the CDs is a negative aspect that prevents all the potential applications mentioned in the literature, production costs are continuously falling. Nevertheless, this technology's outstanding results and prospects assure that CDs will be more widely used in active packaging development shortly.

2.7. Future trends of active packaging

The incorporation of natural compounds as a source of bioactivity, by itself, is already a future trend for the development of food packaging (Wrona et al., 2021). However, to overcome the hurdles still associated with applying natural bioactive substances (e.g., essential oils), the use of protective substances as CDs has gained some attention and intents to an extent

even more. Likewise, biodegradable package materials are necessary to avoid further environmental pollution, which is another advantage of using CDs in the packaging (Szente & Fenyvesi, 2018).

More recently, CD-based research has focused on developing CD polymers such as CD nanosponges (Silva et al., 2019). These nanosponges are novel delivery systems for various bioactive substances. They consist of solid porous particles with nanocavities (internal or external) in their structure, which is responsible for their ability to entrap the guest molecules. Nanosponges can load high levels of compounds which is a significant difference compared to other nanocarriers. Furthermore, nanosponges enhance the solubility of hydrophobic and poorly water-soluble bioactive compounds and improve their controlled release (Tejashri et al., 2013)

CD nanosponges, food-grade nanocarriers, are produced from the reaction of CDs with a crosslinker (e.g., carbonyldiimidazole, pyromellitic anhydride, dimethyl carbonate, and diphenyl carbonate) in acidic or neutral forms (Rezaei et al., 2019). CD nanosponges can make inclusion and non-inclusion complexes with various types of hydrophilic or hydrophobic substances aiming its application in food packaging, for example, coriander EO (Cavalli et al., 2006; Silva et al., 2019) and cinnamon oil (Simionato et al., 2019).

Active films/edible coats incorporated with natural compounds intending to reduce or prevent *quorum sensing* mechanisms and biofilm production in food matrices are also being explored (Akyuz et al., 2018; Mohan et al., 2019). Another aspect that must be considered is the bacterial stress response associated with active antimicrobial packaging. Moreno, Atarés, Chiralt, Cruz-Romero, & Kerry (2018) observed partial recovery and growth of the bacteria in chicken breast fillets packaged in starch/gelatin films containing 10% N-α-lauroyl-l-arginine ethyl ester monohydrochloride, after 6 days of storage. The authors attributed this behavior to a possible response to stress induced by the antimicrobial compound.

Another novel alternative for developing food packaging systems is the combination of electrospinning and CD inclusion complexes. This combination has been described to the obtention of novel active food packaging materials with high active compound loading with a stable release (Vidal et al., 2021). Therefore, this innovative alternative can also be addressed to natural sources entrapment, aiming for food package development. Biodegradable polymers are also an environmentally friendly trend that, associated with active technologies, have excellent prospects to be explored in the following years, contributing to the sustainable development of the food industry.

2.8. Concluding remarks

Although CDs have been extensively investigated for their application in Food Science, the current interest in using these molecules as a tool for developing active food packaging has gained special attention. This is reinforced by including naturally occurring bioactive compounds as a source of antimicrobial/antioxidant properties. In addition, the thermal and oxidative stability of bioactive substances obtained from EOs, PEx, and food by-products can be successfully improved by inclusion complexation with CDs. This opens doors to the industrial manufactory of active package systems based on natural compounds.

Nanotechnology is being described as the key for many industrial segments, helping to develop novel technologies and upgrading other ones. The perspective given by this review is focused on the versatility of CDs, a promising contributor to the development of nanotechnology. Several research groups are discovering novel CDs with distinct properties, contributing to developing CD-based materials available for use in new applications. Surely this technology will gain space in the packaging industry scenario in the coming years (with the help of the gradual decline in the prices of this technology), extrapolating the status of the target on scientific research.

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3. ARTICLE 2: BEYOND BREWING: β-ACID RICH HOP EXTRACT IN THE DEVELOPMENT OF A MULTIFUNCTIONAL POLYLACTIC ACID-BASED FOOD PACKAGING

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3.1. Introduction

Packaging is an important step in the food industry. Its primary function is to ensure food safety by isolating food from the external environment and thus prolonging its shelf-life [1]. However, this traditional preservation method is the one that produces large quantities of urban solid wastes, with only a small percentage being recycled [2]. Traditional food packaging materials are mainly petroleum-based polymers. These materials cannot meet the demand for sustainable development in modern society due to the oil shortages along with serious environmental problems and even threaten human health due to their incorrect discard and slow degradation rates [3,4]. Furthermore, recycling also can be a complicated and expensive task, since most of the packages are constituted of a mixture of materials with different characteristics, thus compromising the recovery, selection, cleaning, and reprocessing of these traditional materials. Therefore, great effort has been put into escalating novel bio-based materials as potential substitutes for conventional sources [5,6].

Polylactic acid (PLA), a novel biodegradable biopolymer, is a linear aliphatic polyester produced 100% from renewable sources. It is obtained through bacterial fermentation of polysaccharides such as from sugar, corn, cane and potato, or through chemical synthesis [7,8]. Despite its drawbacks (e.g., high brittleness, limited flexibility, high hydrolysis rate, and low thermal resistance), PLA's advantages are highlighted due to its biocompatibility, biodegradability, degradation into non-toxic components, thermoplasticity, Generally Recognized As Safe (GRAS), and processability [7]. These features are responsible for consistent studies investigating its applicability as a food packaging material, especially concerning the development of active packaging [9–11].

The food industry has been claiming new technologies that help to improve food quality, and antimicrobial and/or antioxidant active packaging has emerged as a great candidate for combining protection and bioactivity [12]. Consumer demands for fresh-tasting, mildly pre-

served, and convenient foods, along with changing retailing practices have been intensely arising, thus, more sophisticated technologies have begun to emerge [13]. Conventional packaging, strongly based on an inert relationship with food, can no longer attend to the changes that have begun around the interaction between food and packaging. Interestingly, the addition of natural substances with antimicrobial and/or antioxidant properties into packaging material has demonstrated good prospects for the enhancement of food shelf-life [14,15]. It also accomplishes the consumer's preference for reducing/replacing synthetic chemical additives [16].

Hops' (*Humulus lupulus* L.) phytochemicals (e.g., α- and β-acids, xanthohumol, essential oils, and phenolic compounds) display a wide range of biological and pharmacological properties that expands the plant application beyond its traditional use: brewing [17–19]. α- and β-acids, known as hop bitter acids or soft resins (due to their solubility in hexane), are prenylated phloroglucinol derivates produced in hop lupulin glands (present in hop cones) and are characterized by their non-polar profile. These compounds are mainly found in the hop plant [18,20,21]. Among them, β-acids, also called lupulones, are GRAS components and have proved to exert remarkable antimicrobial activity against foodborne pathogens [19,22], and some antioxidant effects have already been reported [23,24]. Lupulone, colupulone, and adlupulone are the major compounds of this class of bitter acids (c.a. 10% of hop dry weight) [20].

Although there are many studies on hops, fewer comprise their potential for active packaging development. Furthermore, none have investigated hop β-acids as an antimicrobial/antioxidant additive to PLA-based active packaging. As the addition of active compounds into the polymer matrix does not necessarily lead to improved biological activity [25] and can significantly change the intrinsic properties of the polymer (e.g., permeability and mechanical properties), the incorporation effects of these bioactives on the polymer matrix should be investigated.

Thus, after adequately assessing the bioactive properties of the β-acids from the hop extract, this study aimed to characterize and evaluate the antimicrobial and antioxidant properties of PLA-based sheets added with increasing concentrations of β-acid-rich hop extracts (0%, 0.1%, 1%, 2.5%, and 5% w/w of potassium salts of β-acids, 'KBA'). This commercial extract has been widely used concerning its strong antimicrobial activities in the brewing or sugar industry (in some cases, it is also well-handled for animal food preservation). It comprises a viscous liquid product that allows safe handling and automated dosing, being also more stable than the pure β-acids. Furthermore, it comprehends a Food Grade/Kosher Product, enabling its application to food preservation such as through active packaging.

The structural, physical, and biological features of the active sheets were evaluated by Fourier-transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), thermogravimetric analysis (TGA), hydrophobicity evaluation, scanning electron microscopy (SEM), and ultraviolet spectrophotometry (UV–vis). Antimicrobial activities were tested against Gram-positive and Gram-negative bacteria. Additionally, changes in the morphology of Gram-positive bacteria caused by the antibacterial effects of active sheets were evaluated through atomic force microscopy (AFM).

3.2. Material and Methods

3.2.1. Material

β-acids rich hop extract (BetaBio45, Hopsteiner, Germany) was kindly donated by Wallerstein Indl e Coml Ltda (São Paulo, Brazil). It consists of a brownish solution and according to the manufacturer, it contains 45.0% ± 2.5% w/w potassium salts of β-acids, 22.5 ± 12.5% w/w food grade propylene glycol, 1.0-2.0% w/w hop oils with corresponding residual moisture. The pH value is 10.7 ± 1.0 and the density 1.07 ± 0.03 g/mL at 20 ºC. Hop β-acids present at the extract are mainly colupulone, lupulone and adlupulone, extracted through CO_2 extraction and quantified trough HPLC according to Analytica ICS 13 and EBC 7.7 and 7.8, as described by the manufacturer.

PLA Ingeo™ 4043D (95.7% L-lactide, density = 1.24 $g.cm^{-3}$) (Natureworks LLC, USA), chloroform P.A. (Vetec, Brazil) were used to plastic sheet preparation. Anhydrous ethanol (99.8% purity degree, Neon, Brazil), methanol (99.8% purity degree, Merck, Germany), DPPH (2,2-diphenyl-1-picrylhydrazyl) (Sigma Aldrich, USA), ABTS (2,2-azino-di-(3-ethylbenzothialozine-sulphonic acid) (Sigma Aldrich, USA), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Sigma-Aldrich, USA), lithium chloride (Dinâmica, Brazil), sodium chloride (Fmaia Bioquímica e Química, Ltds, Brazil) were also used during the experiment.

Listeria monocytogenes ATCC 15313, *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 11229, *Pseudomonas aeruginosa* ATCC 15442, and *Bacillus cereus* ATCC 14579 used in the antimicrobial analysis were obtained from the culture collection of the Laboratory of Food Packaging, Federal University of Viçosa.

3.2.2. β-acids rich hop extract assessment

3.2.2.1. Antioxidant activity

The antioxidant activity of KBA was determined *in vitro* using the radicals ABTS [26] and DPPH [27].

Prior to the antioxidant assays, the β-acids rich extract was diluted to achieve 45 ppm. First, the absorbances of the solutions containing the radicals were adjusted to 0.700 (±0.05) at 517 nm (DPPH: 1 mmol/L) and 734 nm (ABTS: 7 mmol/L ABTS + 2.45 mmol/L $K_2S_2O_8$, 1:1 v/v). For each assay, 3.5 mL of the respective radical solution was added to 0.5 mL of the diluted KBA-rich extract. The absorbance was measured using a spectrophotometer (model UV 1800, Shimadzu, Japan), after 6 min at 734 nm and 30 min at 517 nm for ABTS and DPPH analysis, respectively. Trolox was used as a standard antioxidant and its calibration curves were established (0–200 μmol/L). Results were expressed as μmol of Trolox equivalents per g.

3.2.2.2. Antimicrobial evaluation

The antimicrobial properties of the commercial extract (in terms of KBA) were evaluated against foodborne pathogenic bacteria: *S. aureus*, *L. monocytogenes*, *P. aeruginosa*, *E. coli* and *B. cereus*. For inoculum preparation, bacterial strains were activated twice in brain heart infusion broth (BHI, Kasvi, Italy), incubated at 37 ±2 ºC, for 24 h, streaked over plate count agar (PCA, Oxoid, England), and incubated again under the same conditions. Selected colonies were taken from the plate and suspended in 0.85% (w/v) saline solution until obtaining a visual suspension turbidity similar to 0.5 McFarland standard (approximately 10^8 CFU/mL) (Probac, Brazil).

The Minimum Inhibitory Concentration (MIC) of KBA was determined using the broth microdilution test in 96-well microplates as previously described by Arruda et al. [17]. 100 μL of BHI broth was added to the microplate wells. Then, 100 μL of a stock suspension of the extract in water (45,000 μg/mL of KBA) was added to the wells of the first row and serially diluted. 100 μL from the twice diluted bacterial suspensions (10^6 CFU/mL inoculum) were transferred to the wells, resulting in a final bacterial concentration of approximately 10^5 CFU/mL [28]. The tested concentrations of KBA ranged from 11,250 to 0.34 μg/mL. The microplates were incubated at 37 ± 2 ºC, for 24 h. MIC was defined as the lowest antimicrobial concentration capable of preventing visible growth. BHI broth and propylene glycol were used as control. Considering the Minimal Bactericidal Concentration (MBC) determination, aliquots of 100 μL from each well without visible growth (MIC), and the well immediately adjacent, were inoculated onto BHI agar (Himedia, India) and incubated at 37 ± 2 ºC, for 24 h. MBC was

defined as the lowest concentration of KBA (in µg/mL) required to kill a bacterium, evidenced by the absence of bacterial growth after inoculation [17]. MIC and MBC were also evaluated under refrigeration conditions (7 ºC/10 d) for *L. monocytogenes*.

Also, the agar diffusion method [29], with modifications, was performed to assess the bacterial susceptibility against KBA. Mueller-Hinton agar (Sigma-Aldrich, India) plates were inoculated with a swab dipped into a bacterial solution with a turbidity equivalent to the 0.5 McFarland standard. A 6 mm diameter hole was made in each inoculated plate center. A 5 µL aliquot of the KBA extract was added to the agar cavities. The antibiotics tetracycline (Inlab, Brazil) and ampicillin (Inlab, Brazil) were used as positive controls, and propylene glycol was used as a negative control. The plates were incubated at 37 ± 2 ºC, for 24 h, and *L. monocytogenes* was also investigated at 7 ºC/10 d incubation conditions. The inhibition zones were measured in millimeters. A higher inhibition zone diameter indicates a higher antimicrobial activity.

3.2.3. Preparation of PLA-based sheets

PLA-based sheets were obtained by *casting* method, from the dispersion of the PLA pellets in 200 mL of chloroform (1:10 w/v) under magnetic agitation (300 rpm, 12 h, 25 $\pm$ 2 ºC) [30,31]. Then, the β-acids rich hop extract was introduced to the dispersion so the final concentrations of KBA were 0.1% (PLA-BA0.1%), 1% (PLA-BA1%), 2.5% (PLA-BA2.5%), and 5% (PLA-BA5%) wt, regarding the polymer mass. The polymer dispersions were homogenized in Ultra-turrax (1 min, 4800 rpm, T25, IKA), degassed, and poured onto a glass plate (18 cm x 34 cm). KBA extract-free formulation served as control (PLA-0). The solvent of all the treatments was allowed to evaporate under a chemical hood at room temperature for 24 h [31–33]. The sheets were peeled from the trays and cut before the testing.

3.2.4. PLA-based sheets assessment

3.2.4.1. Fourier-Transform Infrared Spectroscopy (FTIR)

The FTIR spectra of the sheets were obtained using an FTIR spectrometer, model 660-IR (Varian Inc., CA, USA). Attenuated Total Reflectance (ATR) spectra of the samples were obtained in the 4000-700 cm^{-1} region, using 16 scans and 4 cm^{-1} resolution.

3.2.4.2. X-ray diffraction (XRD) analysis

The X-ray patterns of PLA-based sheets were measured by a D8 diffractometer (Bruker AXS, Germany), using Cu-Kα radiation (λ=0.1514 nm) in a 2θ range from 5° to 60° with a scanning rate of 0.05° s^{-1}.

3.2.4.3. Thermogravimetric analysis (TGA)

TGA of the PLA sheets was performed on a thermogravimetric analyzer (Model DTG-60H, Shimadzu, Japan), under a nitrogen atmosphere (50 mL/min). Approximately 3 mg of each sample was weighed and heated from 25 ºC to 600 ºC at a rate of 10 ºC/min.

3.2.4.4. Thickness and mechanical properties

The thickness (µm) of PLA-based sheets was evaluated in 10 different locations, randomly selected, with a digital micrometer (model 547–401, Mitutoyo, Japan). The final result was expressed as an average value [34].

The mechanical properties evaluation followed the ASTM D882-18 [35]. Sheets' samples were cut into 175 mm x 25 mm rectangular specimens and tested on a Universal Testing Machine (model 3367, Instron Corporation, USA) equipped with a 1 kN load cell. Five specimens of each treatment were tested on each repetition. The initial distance of grids' separation was 125 mm, and the rate of separation was 50 mm/min. Prior to the analysis, the test specimens were conditioned for 48 h at 25 ± 2 ºC and 50 ± 2% RH. These conditions were maintained during the mechanical tests. The tensile properties analyzed were ultimate tensile strength (UTS, in MPa), elongation at break (EB, in %), and modulus of elasticity (Young's modulus, YM, in MPa) [35].

3.2.4.5. Water vapor permeability (WVP)

To determine the WVP of the sheets, the gravimetrical methodology was used following the guidelines from the ASTM E96/E96M [36] standard method, with modifications. For this method, two saturated salt solutions were employed aiming to maintain differential RH values between the cups' inner and outer media, triggering the water permeation through the film into the capsules [37,38]. Salts were chosen accordingly to their respective RH values at room temperature (25 ºC) and the relative hydrophobic characteristic of PLA (i.e., in this case, a moderate RH difference may be required to trigger the water migration without affecting the reproducibility of the experiment).

The plastic sheets (Ø = 83 mm) were placed on poly(methyl methacrylate) cups containing a saturated solution of lithium chloride (12 ± 5% RH, at 25 ± 2 ºC) and sealed with paraffin. After assembly, the cups were placed in desiccators containing saturated sodium chloride (75 ± 5% RH, at 25 ± 2 °C), and the weight was monitored to provide at least ten data points during the first 24 h of analysis, and then, the cups were periodically weighted for 17

days. The gain of mass over time allowed the determination of the water vapor transmittance rate (WVTR) according to the following equation:

$$WVTR = m/(t \times A) \quad (1)$$

In which m/t corresponds to the slope of gain of mass (g) over time (h), and A is the permeation area (m²). Next, the WVP was obtained according to:

$$WVP = (WVTR \times X_T)/[P_S \times (RH_1 - RH_2)] \quad (2)$$

In which X_T is the sheet thickness, P_S is the saline water saturation pressure, RH_1 is the RH in the desiccator containing NaCl, and RH_2 is the RH on the cup containing LiCl. WVP was expressed as 10^{-10} g/(Pa·s·m).

3.2.4.6. Measurement of hydrophobicity

The surface hydrophobicity of PLA-based sheets was determined through the sessile drop contact angle technique, in a goniometer (Kruss Advance for Drop Shape Analyzers Version 1.7, Hamburg, Germany). Mili-Q water, formamide (Merck) and α-bromonaphtalene (Merck) drop (2 μL) were deposited on the sheet samples. All measurements were made on the "air side" of the plastic sheets. The degree of hydrophobicity of the treatments was assessed through the Van Oss approach, which is also known as the Lifshitz–van der Waals/acid-base approach [39]. The correlation between the liquid surface tension and the contact angle was used to determine the free energy of hydrophobicity (ΔG_{hydrop}).

3.2.4.7. Scanning electron microscopy (SEM)

The surface and the cross-sectional area of the plastic sheets were examined with a scanning electron Tabletop Microscope (model TM3000, Hitachi High-Technologies, Tokyo, Japan), using a secondary electron detector and operating under a low vacuum. Uncoated samples were attached to the stubs' surface with a double-sided carbon tape aid, and the accelerating voltage of 15 kV was used.

3.2.4.8. Optical parameters

Color values (CIE L*a*b) and the opacity of the PLA-based sheets were measured with a colorimeter ColorQuest XE (HunterLab, Reston, VA, EUA), working with D65 (average daylight) and 10° angle (field of view) [40]. The opacity value was automatically evaluated by the Universal Software V4.01 (Hunterlab Associates Laboratory), as a relation of the sample opacity on the black and the white standard. The L* (lightness, brightness), a* (redness/greenness), and b* (yellowness/blueness) color values were obtained using the CIELAB scale, also directly obtained by the mentioned software. The saturation index or chroma (C*) was calculated through the following equation [41]:

$$C^* = \sqrt{(a^*)^2 + (b^*)^2} \quad (3)$$

The light transmission (T%) of the sheets was also measured through a spectrophotometer (model UV 1800, Shimadzu, Japan), with wavelengths ranging from 200 to 700 nm, with a step size of 5 nm.

3.2.4.9. Release test

Migration properties of the active sheets were evaluated according to the methodology described by [42], with modifications.

Two kinds of standard food simulants were used: distilled water, as a non-acidic aqueous food simulant (pH ≥ 4.5); and 95% (v/v) ethanol solution, as a simulant for fatty foods [43–45]. Freshly prepared sheet samples from each treatment (6 cm x 2 cm) were weighed on an analytical balance and subsequently immersed, separately, in 50 mL of the simulating solution, under agitation (200 rpm, 25 ± 2 ºC). An aliquot of the sample solutions was taken at intervals at first 10 h (15 min, 30 min, 1 h, 2 h, 3h, 4 h, 5h, 6h, 7h, 8 h, 9h, 10 h) and daily on the subsequently 22 days to measure its absorbance at 354 nm, in a UV-Vis spectrophotometer (model UV1800, Shimadzu, Japan). After the absorbance reading, the aliquots were returned to the simulating solution. The accumulative release percentage was calculated by the ratio of the release determined by the calibration curve (based on KBA-extract sequentially diluted on the respective simulation solutions) and the theoretical total release.

Furthermore, Higuchi and Korsmeyer-Peppas models were applied to the data to estimate the release kinetics:

Higuchi model: $\frac{M_t}{M_\infty} = K_1 \times t^{\frac{1}{2}}$ (4)

Korsmeyer-Peppas model: $\frac{M_t}{M_\infty} = K_2 \times t^n$ (5)

In which $\frac{M_t}{M_\infty}$ is the release percentage of KBA at time t(h); K_1 and K_2 are the rate constants (a characteristic of the matrix related to the diffusion process); and n is the diffusion index (characteristic of the release mechanism).

3.2.4.10. Antioxidant activity of sheets

The antioxidant properties of the active PLA sheets were investigated through ABTS [26] and DPPH [27] radical cation-based assays, with modifications. These methodologies are usually proposed by several studies to evaluate the antioxidant activity of plastic materials [44,46,47]. Weighted samples of each treatment (1 cm²) were immersed in 10 mL of ethanol and stored under magnetic stirring (200 rpm, 25 ±2 ºC), in the dark, for 24 h. Subsequently, 0.5 mL extraction solution was added to 3.5 mL ethanol-free radical solution and the absorbance was measured at 734 nm after 6 min for the ABTS method and 517 nm after 30 min for the DPPH method, respectively. The analysis conditions were similar to the ones described in Section 2.2.1. The ABTS/DPPH radical-scavenging activity (%) was calculated as follows:

$$ABTS/DPPH(\%) = (A_{control} - A_{sample})/A_{control} \quad (6)$$

In which $A_{control}$ is the absorbance of the adjusted radical solution without samples, and A_{sample} corresponds to the absorbance of radical added with the extraction solution after the corresponding time [34].

3.2.4.11. Antimicrobial activity of sheets

The antimicrobial activity of the PLA-based sheets was investigated towards Gram-positive (*S. aureus*, *L. monocytogenes* and *B. cereus*) and Gram-negative (*P. aeruginosa*, *E. coli*) bacteria. The inoculum was prepared as mentioned in Section 2.2.2.

3.2.4.11.1. Agar diffusion test

Bacterial suspensions (c.a. 10^8 CFU/mL, Section 2.2.2) were individually spread onto solidified Mueller Hinton agar using a swab. Sheet samples were cut into disks (Ø = 10 mm) and placed at the center of the agar surface. The plates were incubated at 37 ºC/24 h. For *L. monocytogenes*, the antimicrobial activity was further investigated under refrigeration, at 7 ºC for 10 days. Discs of tetracycline 30 µg (Cecon, Brazil) and ampicillin 10 µg (Cecon, Brazil) were used as positive controls [17,48]. Inhibition zones were measured and presented in mm.

3.2.4.11.2. Direct contact and vapor phase analysis

The antimicrobial activity of the sheets was evaluated by direct contact through the broth dilution method [49]. 10 µL aliquots of the inoculum (c.a. 10^8 CFU/mL, Section 3.2.2.2) were individually transferred to tubes containing 10 mL of BHI broth, resulting in a final inoculum of approximately 1 x 10^5 CFU/mL. Then, sheet samples (2 cm²) were immersed in the inoculated tubes. To simulate food storage, the tubes were placed inside polyethylene/nylon bags. The incubation period was 37 ± 2 ºC, 24 h (*L. monocytogenes*, *S. aureus*, *E. coli*, *B. cereus* and *P. aeruginosa*) and at 7 ± 2ºC, 10 days (*L. monocytogenes*). The presence or absence of microbial growth was evaluated through visual turbidity.

Before vapor phase testing [50], Petri dishes containing solidified BHI agar were inoculated with bacterial suspensions (c.a. 10^8 CFU/mL, Section 3.2.2.2) with a swab aid. The PLA-bases sheets were cut into rectangular shapes (2 cm x 4 cm) and fixed onto the lids of the plates. The plates were incubated at 37 ± 2 ºC, for 24 h. The antimicrobial activity of *L. monocytogenes* was also evaluated at 7 ±2º C for 10 days. The presence or absence of microbial growth was evaluated after the respective incubation period.

3.2.4.11.3. Morphological evaluation

Possible morphological changes caused in cells by the action of hop β-acids present in PLA sheets were investigated through Atomic Force Microscopy (AFM), according to the methodology described by Salive et al. [51], with modifications. The analysis was conducted from the results of the dilution method that evaluated the antimicrobial activity by direct contact between sheets and bacteria, described in item 3.4.11.2. Respective tubes containing the sheets without KBA extract addition were used as controls. First, the sheets were removed, and the tubes were centrifugated at 5000 x g for 10 min (model 4K-15; Sigma, Germany). The supernatant was discarded and the cells were resuspended at 1 mL sterile milli-Q water.

For sample preparation, 1 mL was collected from the cell resuspensions, and the aliquots were centrifuged at 2000 x g for 10 min (SBC 140-115, Force Mini Select BioProducts; New York, USA). The supernatant was discarded, and then seven successive washes were performed with 1 mL of 0.85% (w/v) saline solution. After washing, samples were resuspended in sterile distilled water. Then, 10 µL aliquots of the samples were individually placed on sterile glass slides, (1 cm x 1 cm) and air-dried in a laminar flow hood for cell adhesion. The prepared slides were left in a desiccator containing silica-gel, at room temperature (25 ± 2 ºC) until AFM analysis. An atomic force microscope (NT-MDT, N Ntegra Prima; Moscow, Russia) in intermittent mode was used.

3.2.5. Statistical analysis

The experiment was carried out three times in a completely randomized design. The results were subjected to analysis of variance (ANOVA) with a confidence level of 95% ($p < 0.05$) followed by Tukey's posthoc test ($p < 0.05$). The software R was employed in the statistical analysis. All experiments were done in triplicates and the results were expressed as the mean ± standard deviation.

3.3. Results and Discussion

3.3.1. Antioxidant and antimicrobial properties of KBA

KBA is responsible for the biological properties of the commercial hop extract used in the present experiment. Therefore, its antioxidant and antimicrobial activities validation is particularly important to further investigate the antioxidant and antimicrobial active capacity of the PLA-based sheets containing KBA.

Previous studies have shown the free radical scavenging potential of hop β-acids [23,34]. The antioxidant activity of KBA present in the extract was 2192.21 ± 37.63 µmol Trolox eq/g and 2506.53 ± 46.60 µmol Trolox eq/g, for ABTS and DPPH radicals 'assays, respectively. The antioxidant properties of hop β-acids can be attributed to their double bonds and the presence of active hydroxyl groups [23,52]. Furthermore, six-member ring structures, such as hop β-acids, are associated with strong hydroxyl free radical scavenging properties [53].

Concerning KBA's antimicrobial properties, the values of inhibition zones, the MIC, and the MBC of KBA are presented in Table 1. No antibacterial activity was evidenced by propylene glycol at the concentrations tested in the present study. Hop bitter acids (i.e., α- and β-acids) have been recognized by lower antimicrobial activity against Gram-negative bacteria

[18], which was confirmed in the current investigation. The KBA extract did not exhibit inhibition zones against both tested Gram-negative bacteria (*P. aeruginosa* and *E. coli*); also, MIC and MBC were notably higher than those against Gram-positive bacteria (Table 1). The higher resistance of Gram-negative bacteria can be attributed to the lipopolysaccharide-containing outer membrane, which can imply the inhibition of KBA's diffusion and/or inactivation by serum phosphatides [54,55]. Similar results were found for *B. cereus* that, despite being a Gram-positive bacterium, is a sporulate microorganism, which can be responsible for its greater resistance to KBA.

Table 1. Antimicrobial activity of potassium salts of hop β-acids: inhibition zones, Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) against Gram-positive foodborne pathogens.

Bacteria	**Inhibition zone[a] (mm)**	**MIC (µg/mL)**	**MBC (µg/mL)**
S. aureus	43.42 ± 2.38 c	<0.34	0.69
L. monocytogenes	60.25 ± 8.74 b	<0.34	0.69
E. coli	0.00 ± 0.00 d	5,625	5,625
P. aeruginosa	0.00 ± 0.00 d	5,625	11,250
B. cereus	0.00 ± 0.00 d	5,625	11,250
L. monocytogenes (incubation at 7ºC)	79.25 ± 6.11 a	<0.34	0.69

[a]Means ± standard deviation (n=3); Means with a different letter (a, b, c, d, e) within columns differ significantly by Tukey's test ($p \leq 0.05$).

On the other hand, hop of KBA demonstrated great antibacterial potential against both *L. monocytogenes* and *S. aureus* Gram-positive microorganisms. A higher inhibition zone was verified against *L. monocytogenes*, and an even greater inhibitory effect was evidenced under refrigeration conditions ($p < 0.05$) (Table 1). Considering the MIC values of KBA, *S. aureus* and *L. monocytogenes* (at optimal and refrigeration conditions) did not present visual growth at the lowest concentration tested (0.34 µg/mL). For the MBC assay, the aliquots from 0.34 and

0.69 µg/mL wells were tested, and the lowest concentration was not sufficient for the bactericidal effect (MBC); however, cells exposed to 0.69 µg/mL of KBA did not grow at BHI agar.

3.3.2. ATR-FTIR analysis

In order to explore the interaction between the PLA matrix and KBA extract, the manufactured sheets were analyzed by FTIR (Fig. 1). There were some characteristic peaks from PLA polymer that were not affected by KBA extract incorporation, appearing in all PLA-based sheets, such as the sharp peak at 1746 cm^{-1} that can be assigned to the ester carbonyl stretching vibration (C=O).

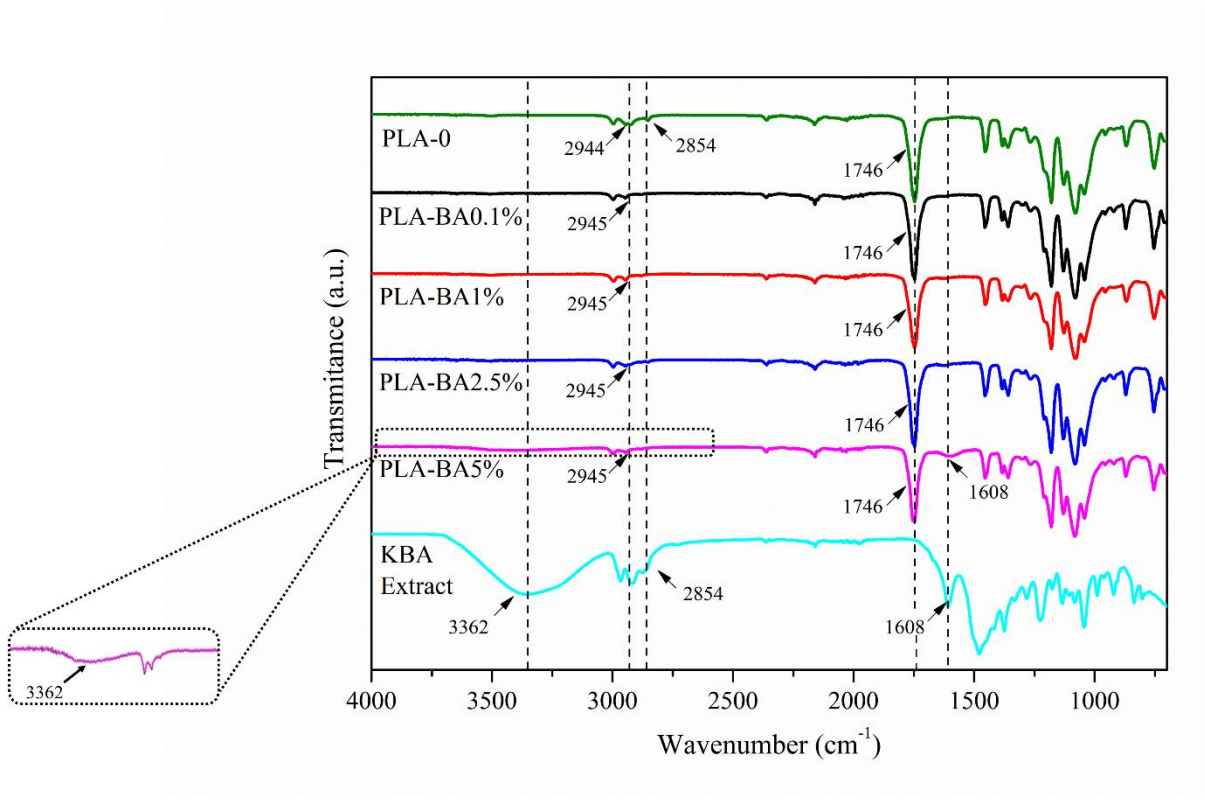

Fig. 1. ATR-FTIR spectra of the KBA-free (PLA-0) and KBA-loaded (0.1-5% w/w of KBA) PLA sheets.

Nevertheless, KBA extract incorporation induced some changes in FTIR spectra of KBA-added sheets, as occurred with the PLA characteristic double peak presented at 2944 cm^{-1} (i.e., attributed to the antisymmetric and CH_3 stretching) that dislocated, with the appearance of a peak at 2945 cm^{-1} on the active sheets (Fig. 1). A similar behavior was found by Tian et al. [56] when hop β-acids were incorporated into the chitosan film containing nano-silica. The

bands at 1383 cm^{-1} and 1861 cm^{-1} resulted from the -CH vending modes, while the 1085 cm^{-1} band corresponds to the C-O stretching vibration from ester units [57].

Moreover, the bands at 868 cm^{-1} and 756 cm^{-1} are conventionally associated with the C=C stretch in the amorphous and crystalline phases of PLA, respectively [58]. The addition of the active agent altered these bands' intensity related to the rearrangement of molecular order within the polymer matrix [59]. Another difference between PLA-0 and PLA sheets added with KBA extract is the absence of the band at 2854 cm^{-1}, associated with C-H bond stretches from the PLA matrix [60]. This can be attributed to the participation of hydrogen in intermolecular bonds with the KBA extract's components. A similar phenomenon has been suggested by Kowalczyk et al. [16] for hydrophilic films loaded with potassium salts of hop iso-α-acids.

Likewise, the characteristic band at 1608 cm^{-1} observed at the KBA extract spectrum is also discreetly seen only for PLA-BA5%. This band corresponds to the stretching of the C=O bond present at hop β-acids molecules [34], indicating the incorporation of KBA into the PLA sheet. For PLA sheets produced with lower concentrations, the band at 1608 cm^{-1} cannot be observed, probably due to the limit of the technique. Meanwhile, the broad peak at 3362 cm^{-1} is related to O–H groups stretching vibration in KBA extract. This peak cannot be found for PLA sheets incorporated with KBA. The exception is on the PLA-BA5% spectrum, in which there is a slightly appearing of this band (highlighted in Fig. 1), indicating that is due to the concentration of KBA present in the PLA-based sheet.

Others KBA concentrations are too low to evaluate the hydrogen bonding interaction performed at the inner structure of the sheet due to low penetration of IR through ATR associated with the considerable thickness of PLA sheets. At PLA-BA5%, some O-H may be induced by the interaction of KBA extract with the PLA-based sheets, also concerning the presence of propylene glycol. Therefore, when KBA was added to the polymer matrix, FTIR spectra discretely changed accordingly.

3.3.3. XRD

XRD was used to detect changes in the PLA structure due to the addition of KBA extract. Fig. 2 exhibits XRD diffraction patterns of PLA-BA0.1%, PLA-BA1%, PLA-BA2.5%, PLA-BA5%, and PLA-0. The diffractogram of the PLA-control sheet presented a broad background marked by two major characteristic peaks around 16.9º and 19.1º and also by other less prominent at approximately 22.4º and 9.4º, implying the semi-crystalline structure of the biopolymer. As the KBA extract was added to the sheets, the diffraction peak at 9.4º weakened

on XRD patterns, which is consistent with the decrease in crystallinity [45]. In a study conducted by Tian et al. [62] the addition of hop β-acids also promoted a decrease in chitosan-based films crystallinity, associated with changes in film structure and altering the position and number of diffraction peaks.

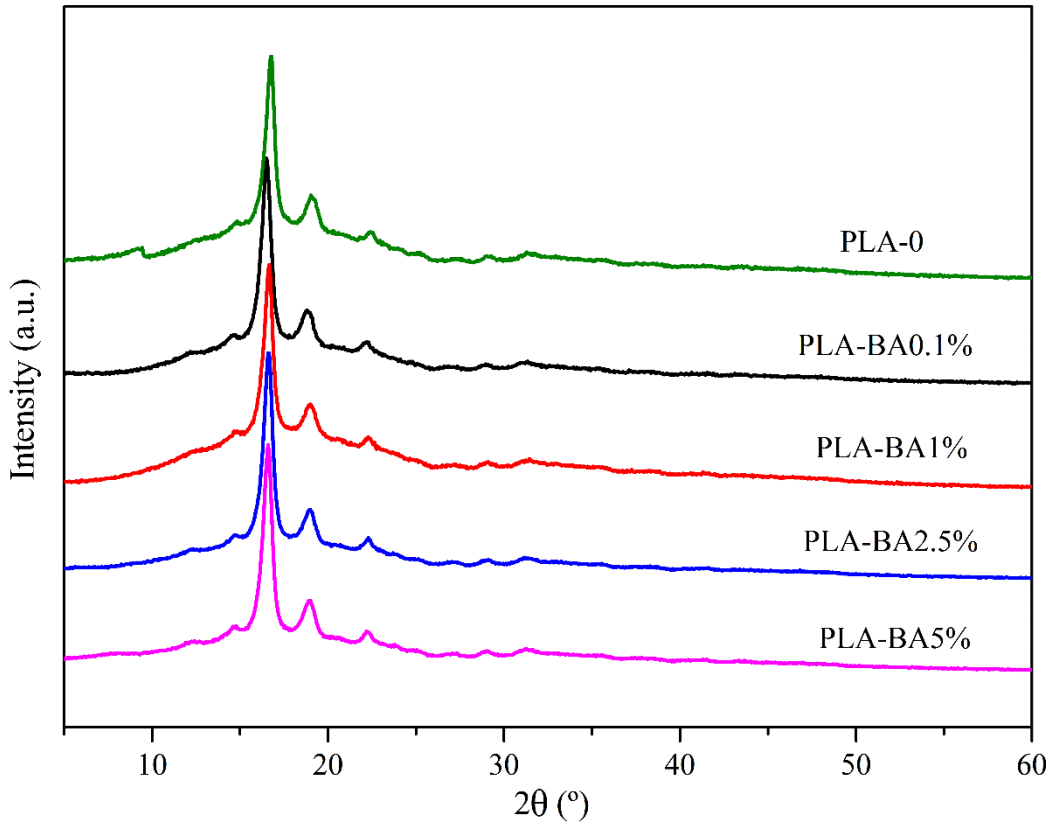

Fig. 2. XRD of PLA-based sheets.

Furthermore, compared with sheets composed of pure PLA, with the addition of KBA extract, all the characteristic peaks slightly shifted to a lower 2θ, indicating that the diffraction angle (θ) attenuated and the PLA crystalline interplanar spacing increased a bit. This can reinforce the possibility of changes in the crystal structure of PLA, probably attributed to the incorporation of extract components into the internal structure of PLA. Similar behavior was observed by Wang et al. [63] by adding cellulose nanocrystals into PLA films.

3.3.4. Thermal stability

Based on the data obtained in the TGA and DTG curve (Fig. 3), it is possible to observe the thermal stability of the material and the influence of the KBA extract on the degradation temperature compared to the pure PLA sheet.

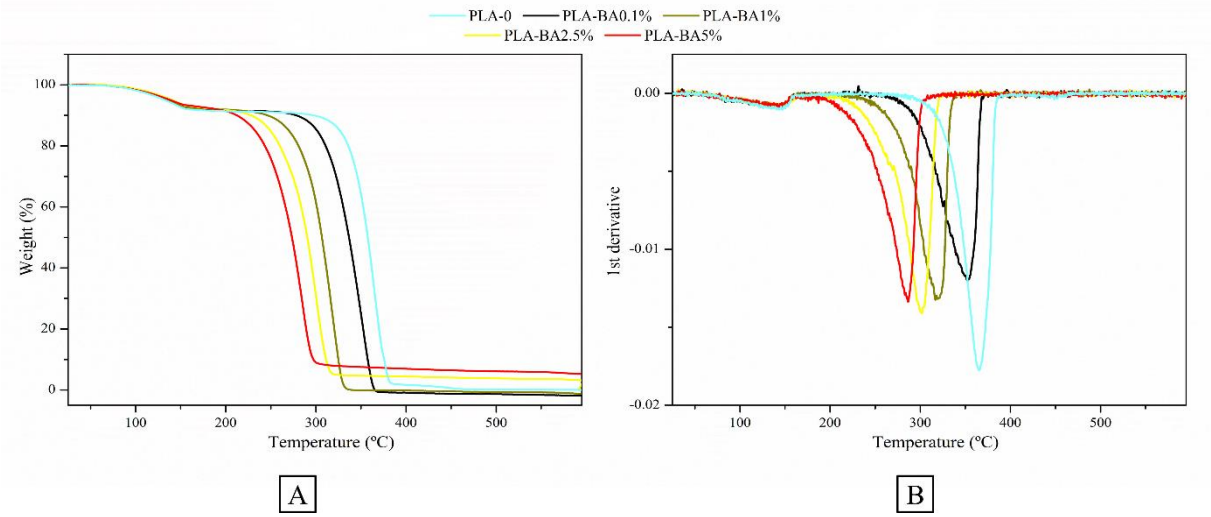

Fig. 3. TGA (A) and DTG (B) curves and of PLA-based sheets.

For PLA-based sheets, the general behavior was similar: the major mass loss occurred mainly in two steps (Fig. 3A). All of them presented a loss mass greater than 90%. At the derivative (DTG) curves (Fig. 3B) it is possible to distinguish two major peaks. The first peak is regular to all PLA-based sheets, around 85-155 ºC. It is possible to attribute this peak to the moisture present in the sample [64,65], which slightly decreased with the hop extract incorporation and can be a result of the hydrophobic structure of KBA molecules. Then, the second peak differentiated among the treatments: as the KBA extract was added, lower degradation temperatures occurred in the active sheets (Fig. 3A).

The temperature of maximum degradation for PLA-0, PLA-BA0.1%, PLA-BA1%, PLA-BA2.5%, and PLA-BA5% was 366 ºC, 353 ºC, 319.8 ºC, 302.7 ºC, and 287 ºC, respectively (Fig. 3B). The higher degradation temperature was reported by PLA-0, which presented an almost complete degradation occurring between 297 ºC and 383 ºC. PLA-BA0.1%, PLA-BA1%, PLA-BA2.5%, and PLA-BA5% presented the following degradation temperature intervals: 277-373 ºC, 236.5-338 ºC, 223-323 ºC, and 205-305 ºC, respectively. These results comprehend that the addition of the KBA extract into PLA sheets progressively reduced the thermal stability of the plastic material. Degradation of KBA extract components overlapped with PLA degradation.

These KBA-extract-dependent changes in the thermal degradation profile of PLA sheets can be attributed to the plasticizer effect of the added components. Propylene glycol naturally present in the hop extract has recognized plasticizing behavior in plastic materials production [66,67]. Moreover, KBA interaction with PLA matrix can weaken the interactions polymer-polymer and increase the polymeric chain mobility, as observed for some essential oils [59,68]. These findings reinforce the results discussed in the XRD analysis.

Villegas et al. [68] and Kazemi-Pasarvi et al. [69] also evidenced a higher decrease in the initial degradation temperature when PLA films were added with a higher thymol concentration. Although the data found in the present study evidenced lower thermal stability for PLA active sheets (compared with the control material), melt processing should not be affected, as degradation starts at higher temperatures than those used for standard PLA processing (e.g., extrusion, injection). In fact, the plasticizer effect attributed to KBA extract incorporation can improve the processability of the PLA sheets.

Currently, PLA is one of the most economically competitive alternatives to traditional highly used non-biodegradable polymers such as polypropylene and polyethylene terephthalate; and since it can be produced from annually renewable sources (e.g., sugar beets and corn starch), or other renewable biomass products and wastes, this biodegradable polymer represents an interesting sustainable alternative to petroleum-based plastics [70]. However, it presents some issues that still may limit its commercial applications, including the inherently high brittleness and low toughness. Therefore, the incorporation of a natural bioactive extract that also improves the polymer plasticization (i.e., providing higher flexibility and lower brittleness) can be a potential tool to enhance its processability and application as an active food packaging.

3.3.5. Thickness, mechanical properties, WVP and hydrophobicity of PLA sheets

The effects of incorporating KBA extract on thickness, mechanical properties, and WVP of PLA-based sheets are summarized in Table 2. The KBA extract incorporation into PLA sheets did not exert significant alterations in the thickness values ($p \geq 0.05$), which ranged from approximately 319.63 μm to 334.94 μm (Table 2). Minimal differences in thickness can be related to variations caused by the casting method and distribution of KBA extract.

Table 2. Thickness, mechanical properties (ultimate tensile strength, UTS; elongation at break, EB; and Young's modulus, YM), water vapor permeability (WVP) and free energy of hydrophobicity ($\Delta G_{Hydrophobicity}$) of PLA-based sheets incorporated with 0% (PLA-0), 0.1% (PLA-BA0.1%), 1% (PLA-BA1%), 2.5% (PLA-BA2.5%) and 5% (PLA-BA5%) of potassium salts of β-acids (KBA).

Treatment	Thickness (μm)	EB (%)	UTS (MPa)	YM (MPa)	WVP (10^{-10}g/Pa.s.m)	$\Delta G_{Hydrophobicity}$ (mJ/m²)
PLA-0	323.19 ± 12.37 ns	12.64 ± 1.11 c	23.42 ± 1.65 a	892.35 ± 86.85 ns	2.77 ± 0.22 ns	-64.21 ±8.72 a
PLA-BA0.1%	319.63 ± 7.42 ns	12.36 ± 1.30 c	20.92 ± 1.38 ab	876.36 ± 78.95 ns	2.22 ± 0.21 ns	-62.57 ± 6.42 a
PLA-BA1%	337.16 ± 2.34 ns	13.50 ± 0.83 bc	20.66 ± 1.08 ab	830.92 ± 37.10 ns	2.48 ± 0.12 ns	-63.90 ± 5.81 a
PLA-BA2.5%	332.03 ± 14.25 ns	14.26 ± 0.95 ab	20.91 ± 1.02 ab	848.19 ± 52.06 ns	2.49 ± 0.17 ns	-22.93 ± 7.78 b
PLA-BA5%	334.94 ± 17.29 ns	15.17 ± 2.17 a	19.33 ± 2.11 b	810.76 ± 119.23 ns	2.77 ± 0.41 ns	- 8.46 ± 3.25 b

Means ± standard deviation (n=10 for thickness, n=3 for WVP and $\Delta G_{Hydrophobicity}$, n=5 for mechanical properties); Means with a different letter (a, b, c, d, e) within columns differ significantly by Tukey's test ($p \leq 0.05$).

Concerning the mechanical properties, the YM did not differ statistically ($p \geq 0.05$), regardless of extract presence. However, some effects were observed for EB and UTS (Table 2). As KBA extract was incorporated into PLA sheets, EB gradually increased, mainly when KBA extract was higher than 2.5% ($p < 0.05$). Meanwhile, UTS decreased with KBA incorporation, highlighting the reduction of 17.4% from the PLA-0 control sheet ($p < 0.05$). These results corroborated those observed in previous analyses and can be related to the plasticizing effect of KBA extract (KBA and/or propylene glycol) on polymer matrix [71,72]. Possibly, the tensile strength reduction occurred due to the partial substitution of strong interactions between polymeric chains, by the plasticizer's action [71,73]. This behavior leads to decreased rigidity and improved extensibility and flexibility of the sheets (corroborated by the results from Sections 3.3.3 and 3.3.4).

When additives, including active components such as KBA extract, are incorporated into a polymer matrix, compatibility between the component and the matrix may happen. Therefore, the initial migration of the components occurs for amorphous regions (i.e., areas that presented lower density in the polymeric structure). As KBA extract concentration increases, all amorphous regions will be filled and, consequently, the substances will start to occupy the crystalline region, lowering tensile strength [74].

Furthermore, the addition of KBA extract did not exert significant changes ($p \geq 0.05$) in the WVP values of PLA sheets (Table 2). Despite surface hydrophobicity reduction for sheets added up from 2.5% of KBA ($p < 0.05$), all PLA-based sheets were maintained as hydrophobic materials. These results can be attributed to the distribution of KBA and propylene glycol, the major components in the hop extract into PLA sheets. Propylene glycol is fully miscible with chloroform (i.e., used for casting the sheets) and can be oriented on the surface of the PLA matrix during solvent evaporation. Meanwhile, KBA presents hydrophobic structures that can migrate through the polymer matrix (Fig. 4). Therefore, despite the sheets' surface hydrophobicity having decreased when the maximum hop extract concentration was added, and also the propylene glycol having a high affinity with water, the water vapor permeation did not differ from the control sheet due to the hydrophobic inner and the maintenance of material thickness. It is worth mentioning that this increase in the KBA added sheets' hydrophilicity can be an interesting advantage concerning eco-friendly worries, compared to the hydrophobic materials [75]. Further studies are suggested to verify the impact of KBA extract on the biodegradability of PLA sheets.

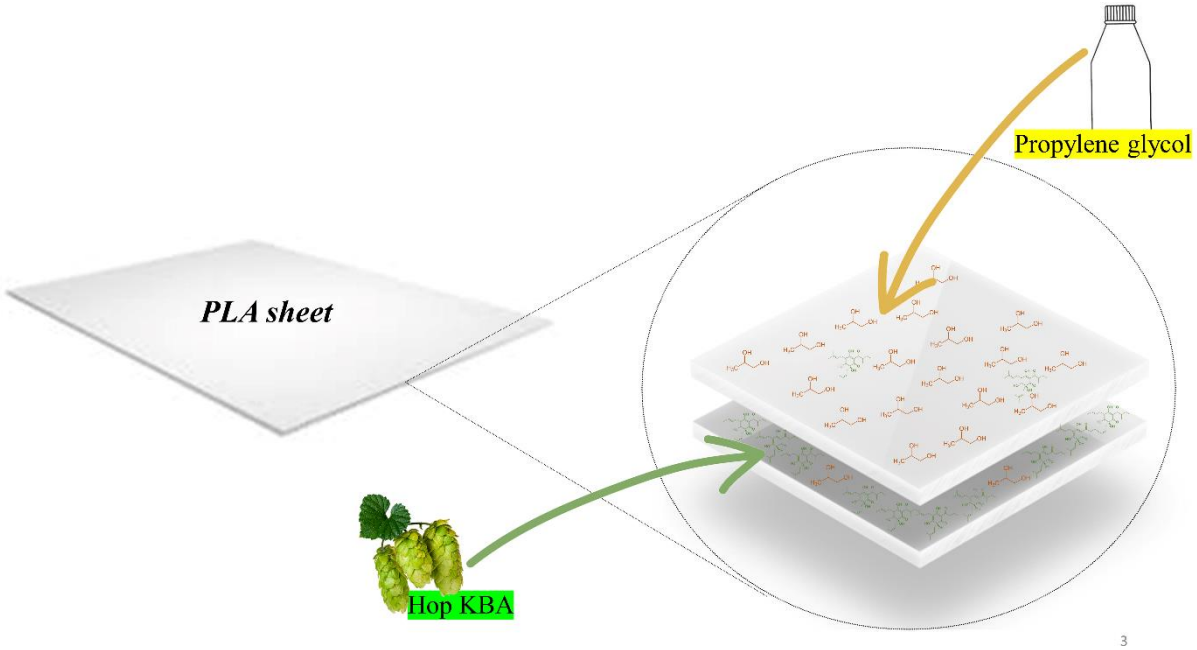

Fig. 4. Representative scheme of the localization of the KBA extract's components on the PLA-based sheets.

3.3.6. SEM

Microscopic characteristics of sheets were evaluated by SEM (Fig. 5). The surface microphotographs were obtained on the sheet-air interface (Fig. 5A). Despite not exerting a significative influence on the thickness of PLA sheets, the incorporation of the KBA extract resulted in major changes in sheets' microscopic appearance, exhibiting more irregular surfaces than the control plastic material. The homogeneity of the plastic materials' surfaces decreased as the content of KBA-rich hop extract increased (Fig. 5A).

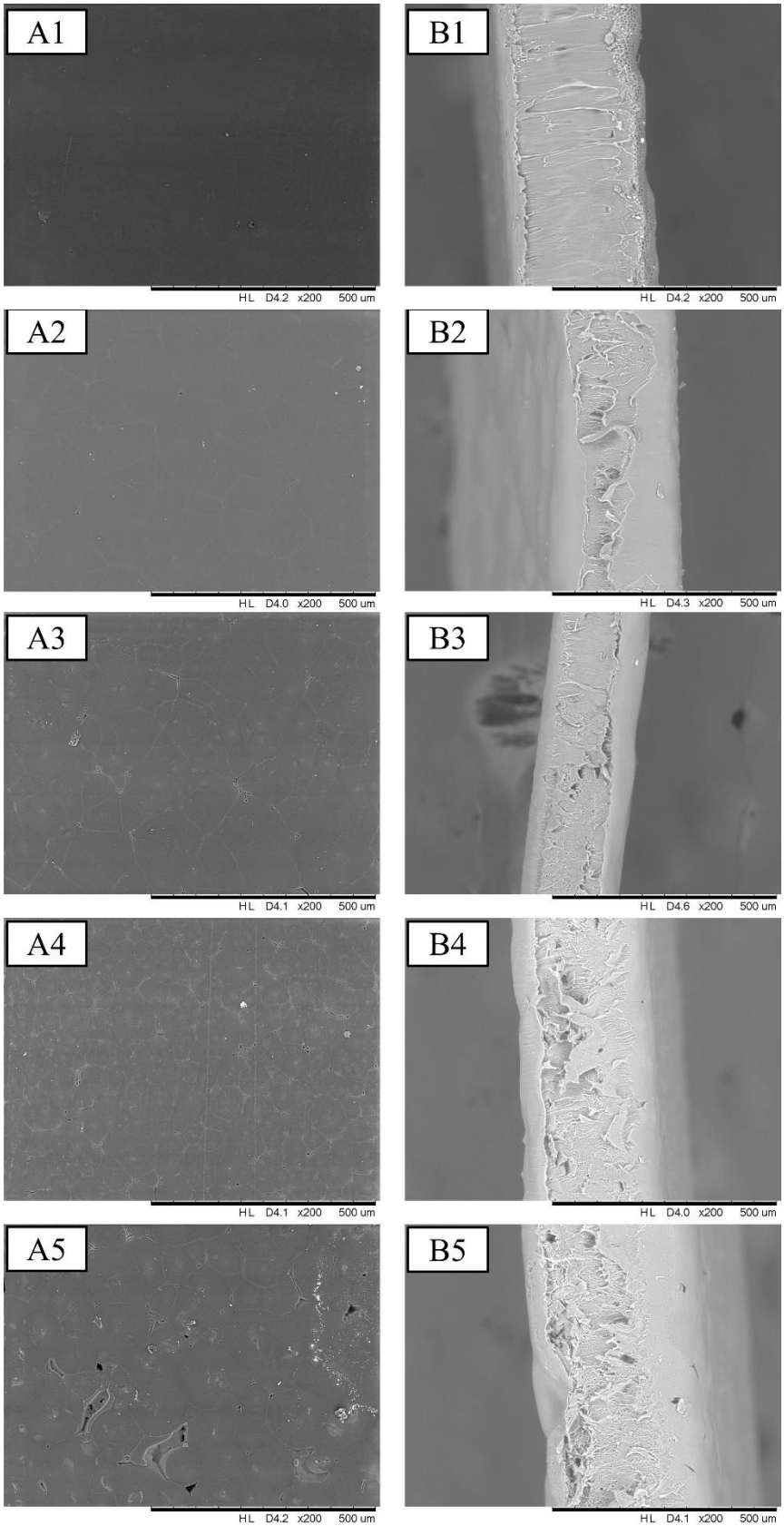

Fig. 5. SEM images of surface (A) and cross-section (B) of PLA sheets: PLA-BA0 (A1, B1), PLA-BA0.1% (A2, B2), PLA-BA1% (A3, B3), PLA-BA2.5% (A4, B4), and PLA-BA 5% (A5, B5).

Corroborating with the previous hypothesis of propylene glycol being located at sheets' surfaces (Section 3.3.5), accordingly with KBA extract incorporation, the surfaces became more heterogenous, covered with cracks (Fig. 5A). Similar behavior was observed by Orliac et al. [67] when studying different plasticizers on thermo-molded films made from sunflower proteins, which was due to the elimination of the plasticizer from the protein matrix to the film surface. Therefore, possibly, the propylene glycol from PLA sheets' surfaces may have increased the surface tension to break the sheets' structure during the drying process. As the solvent evaporated, propylene glycol separated from the PLA matrix, causing wrinkles [34]. Furthermore, as the extract concentration incremented, the surface tension may have increased and fractures enlarged, appearing crack joints and micropore formation, especially at PLA-BA5% (Fig. 5A-5).

Meanwhile, the cross-section images did not indicate the presence of globules or lumps and aggregates, which could be indicative of poor compatibility between KBA and PLA. Possible irregularities visible at the cross-section of PLA-0 are related to the cutting process of the samples. The porous layer observed in the control sheet (Fig. 5B-1) did not appear in cross-section images of KBA-added sheets. This laminar structure of the PLA matrix can be related to the sequential drying of the sheet (giving a different arrangement of the polymer chains) [76]. On the other hand, the possibility of propylene glycol being located at the sheets' surface may help in controlling solvent evaporation, which explains PLA-BA sheets did not present this bubbled layer (Fig. 5B).

At the inner location of PLA-based sheets added with higher amounts of KBA, it can be verified ruptures at the polymer structure, with clear plastic deformation of the material; thus, suggesting that KBA produces a plasticizing effect. As the concentration is lowered, this characteristic is seen with less evidence. Similar results were obtained by Villegas et al. [68] when adding thymol and cinnamaldehyde to PLA films.

3.3.7. Appearance and optical characterization

Optical properties are an indispensable factor for packaging materials [77]. PLA sheets were visually homogeneous with no brittle areas or bubbles and could be easily peeled from the casting plates; the sheets' side that was in contact with air during solvent evaporation presented itself as slightly wrinkled for all treatments. Color parameters can be observed in Table 3. L* values reduced and b* values increased with the incorporation of KBA extract ($p < 0.05$), indicating brightness reduction and yellowness increase of the sheets, respectively. It can be

attributed to the coloring effect of hop KBA which are recognized as yellow-brownish compounds [78]. Meanwhile, results for a* parameter indicated that the addition of KBA into PLA sheets promoted an improved greenish aspect up to 2.5% ($p < 0.05$), but not for PLA-KBA5%. (Table 3).

Table 3. Color parameters and opacity characterization of PLA-based sheets added with 0% (PLA-0), 0.1% (PLA-BA0.1%), 1% (PLA-BA1%), 2.5% (PLA-BA2.5%), and 5% (PLA-BA5%) of KBA.

Treatment	L*	a*	b*	C*	Opacity
PLA-0	64.43 ± 0.52 a	-2.56 ± 0.05 d	4.02 ± 0.19 d	7.93 ± 1.28 d	12.28 ± 0.32 c
PLA-BA0.1%	63.80 ± 1.01 ab	-3.17 ± 0.33 c	5.87 ± 0.54 d	6.67 ± 0.63 d	12.88 ± 1.32 c
PLA-BA1%	59.10 ± 0.47 b	-6.12 ± 0.14 b	12.44 ± 0.37 c	13.86 ± 0.39 c	13.66 ± 0.46 bc
PLA-BA2.5%	60.48 ± 2.26 b	-7.17 ± 0.01 a	17.67 ± 0.38 b	19.06 ± 0.36 b	15.52 ± 1.21 b
PLA-BA5%	59.54 ± 1.19 b	-3.01 ± 0.45 cd	30.20 ± 1.39 a	30.35 ± 1.34 a	23.05 ± 1.15 a

Means ± standard deviation (n=3); Means with a different letter (a, b, c, d, e) within columns differ significantly by Tukey's test ($p \leq 0.05$).

Moreover, C* values of PLA sheets increased with the KBA incorporation, indicating greater saturation of active sheets. Higher values of chroma represent an enhancement of color intensity, with colors more "alive and full", probably associated with the yellow-brownish color provided by the KBA extract [79]. Only PLA-BA0.1%, which corresponds to the sheet with lowest KBA incorporation, did not exhibit significant differences to control plastic ($p \geq 0.05$). Darker materials can be an advantage, especially concerning to packaging of light-sensitive foods [11]. A similar study conducted by Kowalczyk et al. [44] also verified the darker yellow-green appearance of gelatin/carboxymethyl cellulose films due to the incorporation of potassium salts of hop iso-α-acids extract. The appearance of the control and PLA-BA sheets can be visualized in Fig. 6A.

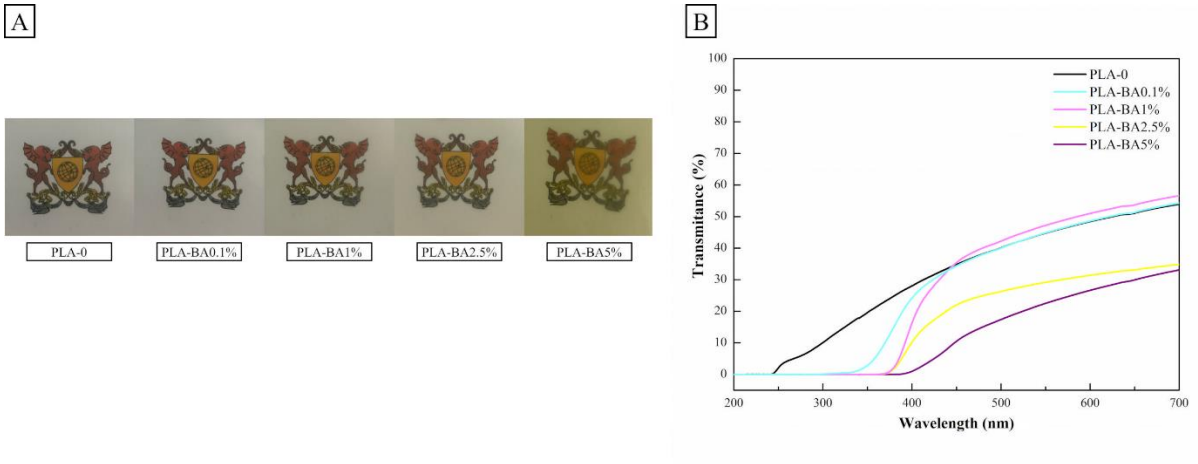

Fig. 6. Optical characterization of PLA sheets: (A) physical appearance; and (B) optical transmittance of PLA-based sheets added with different concentrations of KBA extract.

Opacity also increased with KBA extract addition (Table 3), presenting significant differences at higher concentrations (PLA-BA2.5% and PLA-BA5%) ($p < 0.05$). Opacity results can be attributed to both factors: color aspects of KBA and the possible presence of propylene glycol on sheets' surfaces as discussed in previous Sections. Xu et al., [80] also found higher opaque plastics within the incorporation of hop β-acids.

The light transmission properties of the sheets were determined at selected ultraviolet (UV) and visible wavelengths from 200 to 700 nm (Fig. 6B). It is important to state that despite quantification light absorbance/transmittance can be more complex in multicomponent systems, considering a PLA sheet composed by the polymer and the active extract, it is possible to assure the singular effect of the KBA extract itself by comparing the active sheets with the

control one. Therefore, it is a common methodology aiming at the investigation of the optical characterization of food packaging systems [81,82].

We observed that the light transmittance decreased with an increase in the content of KBA, with the light transmittance of PLA-BA5% sheets was lower than 1% up to 400 nm (UV region). Overviewing the UV region, all KBA-added sheets presented a decrease in light transmittance in comparison to the control sample. This is due to the ability of hop β-acids to strongly absorb UV rays [34,78]. Similar results were found in other packaging studies [45,62,83], conducted with the incorporation of hop β-acids.

However, in the visible light range, the transmittance of the PLA sheets increased with wavelength. The results obtained indicated that the plastics will not completely block light transmission in the visible light region (600 nm), but PLA-BA5% allowed a reduction of transmittance of over 20% in comparison to the control sheet. At 700 nm, PLA-BA1% presented a light transmittance similar to the one evidenced by the control sheet. Nevertheless, it presented a slightly higher visible light transmittance (starting at 445 nm) compared to PLA-0. Higher transmittance values were also found when PLA-poly(butylene adipate-co-terephthalate) films were incorporated with thyme (5-10% w/w) [84].

The oxidation process can be triggered by light incidence, including UV and visible light. Therefore, the ability to block UV and visible light can be a great advantage to packaging materials and an indispensable attribute of some packaged foods [85]. As PLA-BA sheets presented interesting UV-blocking properties, they can effectively resist UV light from damaging food or prevent the formation of free radicals [34]. For example, as many proteins have strong UV absorption around 280 nm [86], KBA-added sheets could be a great option to protect protein-rich food.

3.3.8. *In vitro* KBA release

The KBA *in vitro* release behaviors from PLA sheets to food simulants were assessed through UV–vis spectroscopy. The cumulative release (%) results concerning distilled water (aqueous food simulant) and 95% ethanol (fatty food simulant) are presented in Fig. 7A and 7B, respectively. KBA poorly migrated to water, achieving less than 2% (w/w) of cumulative release after 528h (22 days) for PLA-BA5%. Sheets with a lower concentration of KBA exhibited a decreased cumulative release at the end of the analysis, comprehending around 0.65% (w/w) (PLA-BA2.5%) and 0.39% (w/w) (PLA-BA1%). The sheet added with 0.1% did not evidence migration of KBA to the simulant solution (Fig. 7A). These preliminary results indicated that KBA-added PLA sheets are not quite suitable for aqueous non-acidic foods. Similar

results were found by Ordoñez et al. [87], which studied the release kinetics of ferulic and cinnamic acids from PLA thermo-processed films and verified that no quantitative release was detected into aqueous media.

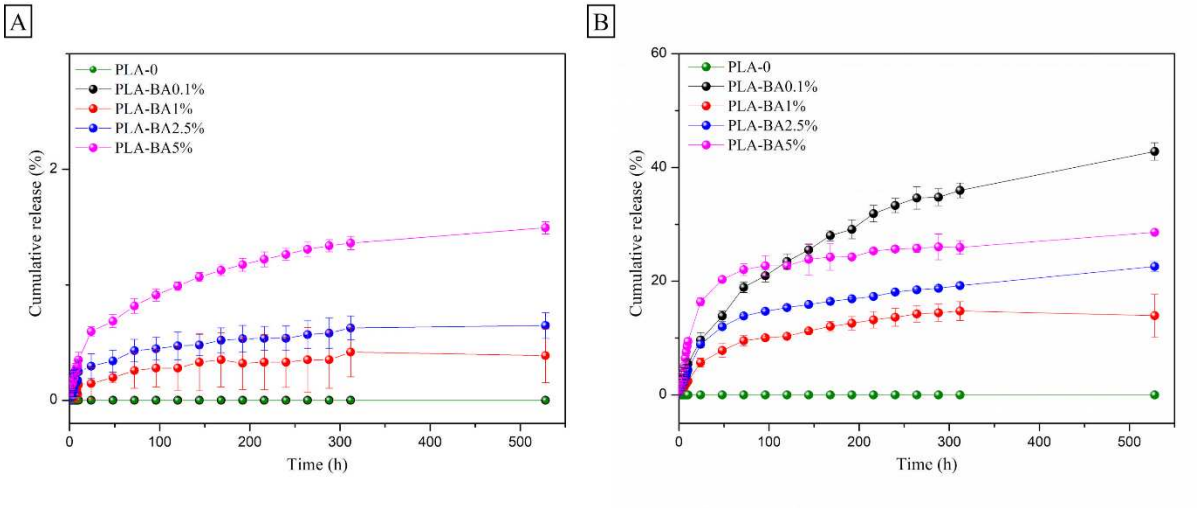

Fig. 7. Cumulative release (%) of KBA from PLA-based sheets on aqueous food simulant (A) and fatty food simulant (B).

Meanwhile, when 95% ethanol was used, higher cumulative releases were found, achieving up to 42.8% (w/w) at the end of the experiment (22 days), corresponding to PLA-BA0.1%. Secondly, PLA-BA5% presented 28.6% (w/w) of cumulative release. Sheets added with 1% and 2.5% of KBA exhibited, respectively, 22.6% and 13.95% of cumulative releases after 22 days. Improved migration was already expected to the fatty food simulant due to the hydrophobic nature of hop KBA [21]. Hence, these hydrophobic compounds can easily dissolve and are released in solvents with higher ethanol content [34]. Previous studies reported the connection between the higher ethanol concentrations on food simulants and PLA matrix being responsible for greater release behavior [87].

Greater release rates can be observed in the first 48h of incubation, followed by a steady upward trend. Interestingly, considering it can be observed that PLA-BA0.1% continued the release at high (but decreased from the start) rates after the initial burst; meanwhile, other PLA active sheets displayed a lower release rate, tending to equilibrium. The initial rapid release rate may be related to the release of KBA near the surface of the PLA sheets [62]. Concerning the preparation of the polymer sheets by the casting method, a fraction of dissolved bioactives displays the tendency to partial aggrouping near the sheet surface, which is called the 'skin layer' [88].

Higher differences in the concentration between the inner and outer environment act as a driving force for the release of KBA from the polymer structure. Then, the cumulative release rate shows a downward trend, which may be attributed to the release rate of the surface compounds being faster than the release of the KBA inside the polymer. After the continuous release of KBA, the concentration gradient (in x out) is close to zero, thus the driving force for outward diffusion disappeared, tending to reach the release equilibrium [62]. Therefore, considering these results, PLA-BA active sheets are interesting options for foods with high-fat content.

In order to further understand the release characteristics of the active ingredient into the food simulants, Higuchi and Korsmeyer-Peppas models were used (Fig. S1, Table 4). The Higuchi model is based on Fick's laws of diffusion, assuming that swelling and dissolution of the matrix are small or negligible and that the matrix has a square root of time dependence. Concerning the adjustment of the Higuchi model on the sheets for both food simulants, correlation coefficients were desirable ($0.9625 < R^2 < 0.9843$, for distilled water; and $0.9669 < R^2 < 0.9957$, for 95% ethanol).

Table 4. Parameters of Higuchi/Korsmeyer-Peppas model for aqueous food simulant (distilled water) and fatty food simulant (95% ethanol).

Treatment	Aqueous food simulant (distilled water)					Fatty food simulant (95% ethanol)				
	Higuchi		Korsmeyer-peppas			Higuchi		Korsmeyer-peppas		
	K_1	R^2	K_2	n	R^2	K_1	R^2	K_2	n	R^2
PLA-BA0.1%	-	-	-	-	-	2.0567	0.9957	2.2096	0.4867	0.9961
PLA-BA1%	0.0225	0.9625	0.0422	0.3831	0.9754	0.8449	0.9669	1.2881	0.4217	0.9764
PLA-BA2.5%	0.0367	0.9638	0.0909	0.3308	0.9839	1.1797	0.9710	1.9211	0.4094	0.9815
PLA-BA5%	0.0807	0.9843	0.1454	0.3904	0.9933	1.6949	0.9336	4.0933	0.3357	0.9636

Aiming to clarify these results, the Korsmeyer-Peppas model was also investigated ($R^2 \geq 0.96$). Previous studies have indicated that when $n < 0.5$, the diffusion behavior of the active ingredient into the sheet matrix follows a Fickian diffusion; when $0.5 < n < 1$, diffusion behavior follows a non-Fickian diffusion; and when $n \geq 1$, composite Case-II transport mechanism prevails [89]. Therefore, it can be stated that the release of KBA from PLA active-based sheets was based on the Fickian diffusion pattern (Table 4). To investigate the capacity of these sheets in real food systems (e.g., fatty food), from where compound migration could favor the relaxation of the polymer matrix and facilitate the delivery of KBA, is a great suggestion to further studies.

3.3.9. Bioactivity of PLA-based sheets

Conventional packaging must simply protect a product in the process of distribution, transport, and storage. Among the functions of these packages, the most basic one is containment, by simply avoiding product loss and preventing pollution during transport [90]. However, in addition to the current trend (and need) of substituting petrol-based plastics, traditional packaging cannot help with another concern: the reduction/total substitution of chemical additives in food products. On the other hand, active food packaging can be a revolutionary technology in this matter to the food industry, as it enables the incorporation of natural bioactives on the packaging material and can provide their controlled release through the product shelf life. Therefore, active packaging can not only meet sustainability concerns (as they can be produced with novel biobased materials), but also the consumers' demands for food consumption. In this context, KBA-added PLA sheets can be an interesting tool joining all these concepts from a raw material still little explored by the food industry: hops.

3.3.9.1 Antioxidant activity

Oxidative processes are one of the main causes of food waste. Therefore, packages containing antioxidants could be employed to inhibit the oxidation damage of fatty foods (i.e., more prone to oxidation), being a key approach for enhancing food quality and extending their shelf life.

Studies have shown that hop β-acids present some interesting antioxidant properties [17,91]. Therefore, the antioxidant capacity of the PLA sheets was investigated aiming to extend their application to food packaging. Fig. 8 presents the radical scavenging activity (through ABTS and DPPH free radicals) of the control sample and the KBA-containing sheets. Both

radicals' assays indicated that the antioxidant properties increased with the gradual incorporation of KBA into the polymer matrix, reinforcing the applicability of the developed active packaging at fatty food matrices.

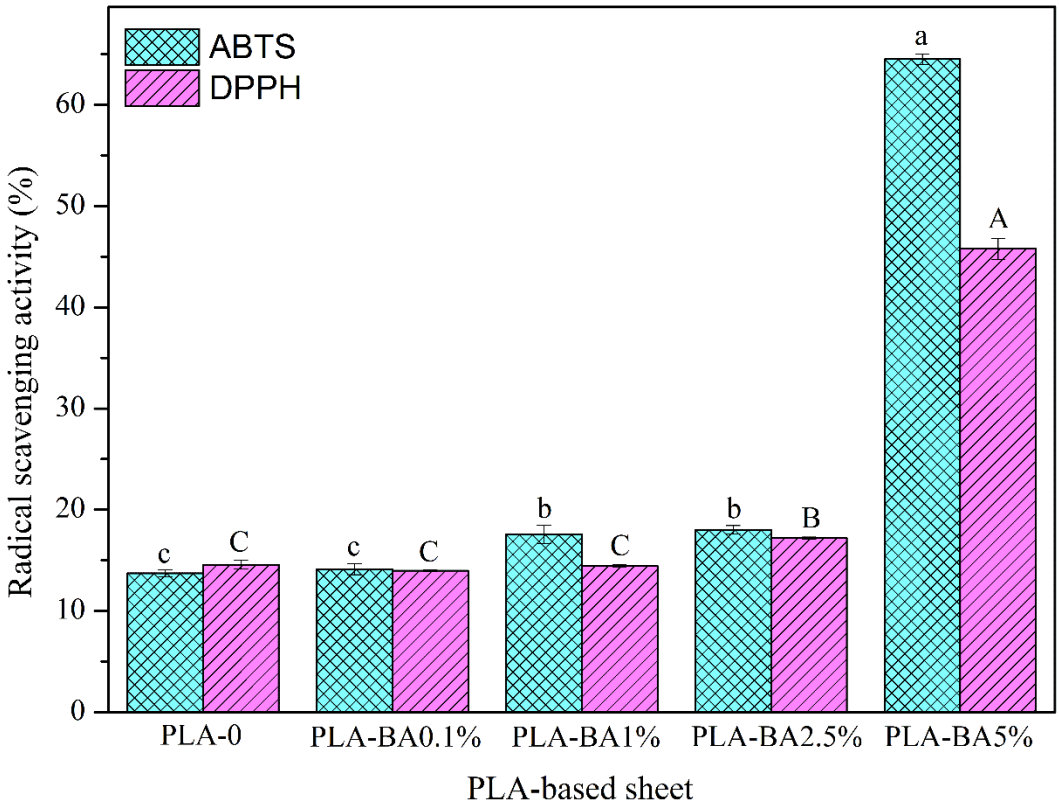

Fig. 8. ABTS and DPPH radical scavenging activity of PLA-0, PLA-0.1%, PLA-BA1%, PLA-BA2.5%, and PLA-BA5% sheets.

Concerning the ABTS scavenging activity, PLA-BA0.1% did not present a significant higher antioxidant capability than the control sample ($p \geq 0.05$). At the DPPH assay, sheets added with higher KBA concentrations (PLA-BA2.5% and PLA-BA5%) presented significant improvement in their scavenging activity ($p < 0.05$). PLA-BA5% exhibited significantly higher antioxidant capacity than the other studied sheets ($p < 0.05$). The explanation for these results may be the strong antioxidant capacity of hop β-acids [23], especially due to the presence of active hydroxyl groups in β-acids [52]. These findings are supported by previous studies that evaluated the antioxidant properties of hop β-acids-added films [34,45,46].

Despite the antioxidant properties of hop β-acids not being their most recognized characteristic, some studies in the literature already confirmed the antioxidant potential of hop β-

acids in food preservation, either through active packaging additive or direct incorporation. Tian et al. [45] reinforced the antioxidant properties of hop β-acids by testing chitosan-based films added with these bioactives (0.3%) in a food matrix highly susceptible to oxidative reactions: soybean oil. The results indicated a strong antioxidant activity shown by the obtained film, protecting soybean oil from oxygen and temperature interference. In another study, hop β-acids were applied along with another hop phytochemical (xanthohumol) to development of a nanoliposome. The lupulone-xanthohumol loaded nanoliposome successfully partially replaced nitrite in cooked beef-sausage: using 60 ppm nitrite beside 100 ppm nanoliposome effectively prevented microbial growth and lipid oxidation, while retaining the proper sensory properties of the tested food [92].

3.3.9.2. Antimicrobial properties

3.3.9.2.1. *In vitro* antibacterial evaluation of the active PLA sheets

Antimicrobial packaging has proved to be an outstanding technology for the maintenance of food quality, especially when the bioactive compounds are from natural sources [12]. The bacterial susceptibility to KBA-containing PLA sheets was verified by the agar diffusion test. Direct contact and vapor phase analysis (on liquid and solid media, respectively) were also performed. Therefore, PLA active sheets were tested on solid, liquid, and air media. All tests were performed against Gram-positive and Gram-negative foodborne pathogens.

Concerning the agar diffusion test, results were expressed as the size of the inhibition zones (Fig. 9). Inhibition zones were only found against *S. aureus* and *L. monocytogenes,* when tested with the sheet with higher KBA content (PLA-BA5%) (Table 5), corroborating with previous information indicating greater antibacterial activity of hop β-acids against Gram-positive bacteria [17,19]. PLA-BA2.5% presented a reduction in cell density around the sheets against these bacteria, however, did not completely inhibit bacteria growth with a clear inhibition zone formation (Fig. 9). These results are following those presented in Section 3.1, which revealed that for inhibition of Gram-negative or sporulate Gram-positive bacteria (*B. cereus*), much higher concentrations of KBA are needed.

Table 5. Mean diameter (mm) of inhibition zones for the PLA sheets towards pathogenic bacteria under optimum temperature (37 ºC) and refrigeration (7 ºC)

Bacteria*	Treatment					Tetracycline	Ampicillin
	PLA-0	PLA-BA0.1%	PLA-BA1%	PLA-BA2.5%	PLA-BA5%		
S. aureus	0.00 ± 0.00 aC	0.00 ± 0.00 aC	0.00 ± 0.00 aC	0.00 ± 0.00 aC	18.08 ± 0.52 abB	17.67 ± 2.50 cB	46.00 ± 1.90 bA
L. monocytogenes	0.00 ± 0.00 aC	0.00 ± 0.00 aC	0.00 ± 0.00 aC	0.00 ± 0.00 aC	20.51 ± 4.67 aB	38.50 ± 0.84 bA	40.58 ± 1.63 bA
E. coli	0.00 ± 0.00 aB	0.00 ± 0.00 aB	0.00 ± 0.00 aB	0.00 ± 0.00 aB	0.00 ± 0.00 cB	17.67 ± 5.68 cA	20.50 ± 2.66 cA
P. aeruginosa	0.00 ± 0.00 aB	0.00 ± 0.00 aB	0.00 ± 0.00 aB	0.00 ± 0.00 aB	0.00 ± 0.00 cB	8.50 ± 1.38 dA	0.00 ± 0.00 eB
B. cereus	0.00 ± 0.00 aC	0.00 ± 0.00 aC	0.00 ± 0.00 aC	0.00 ± 0.00 aC	0.00 ± 0.00 cC	22.42 ± 1.56 cA	10.50 ± 9.40 dB
***L. monocytogenes* (incubation at 7ºC)**	0.00 ± 0.00 aC	0.00 ± 0.00 aC	0.00 ± 0.00 aC	0.00 ± 0.00 aC	12.25 ± 0.75 bB	78.83 ± 2.32 aA	77.50 ± 3.02 aA

Means ± standard deviation (n=3); Means with a different letter (a, b, c, d, e) within columns differ significantly by Tukey's test ($p \leq 0.05$). Means with a different letter (A, B, C) within rows differ significantly by Tukey 's test ($p \leq 0.05$).

All five pathogens were tested under optimum temperature conditions (37 ºC); *L. monocytogenes*, a psychrotrophic bacteria, was also tested under refrigeration conditions (7 ºC).

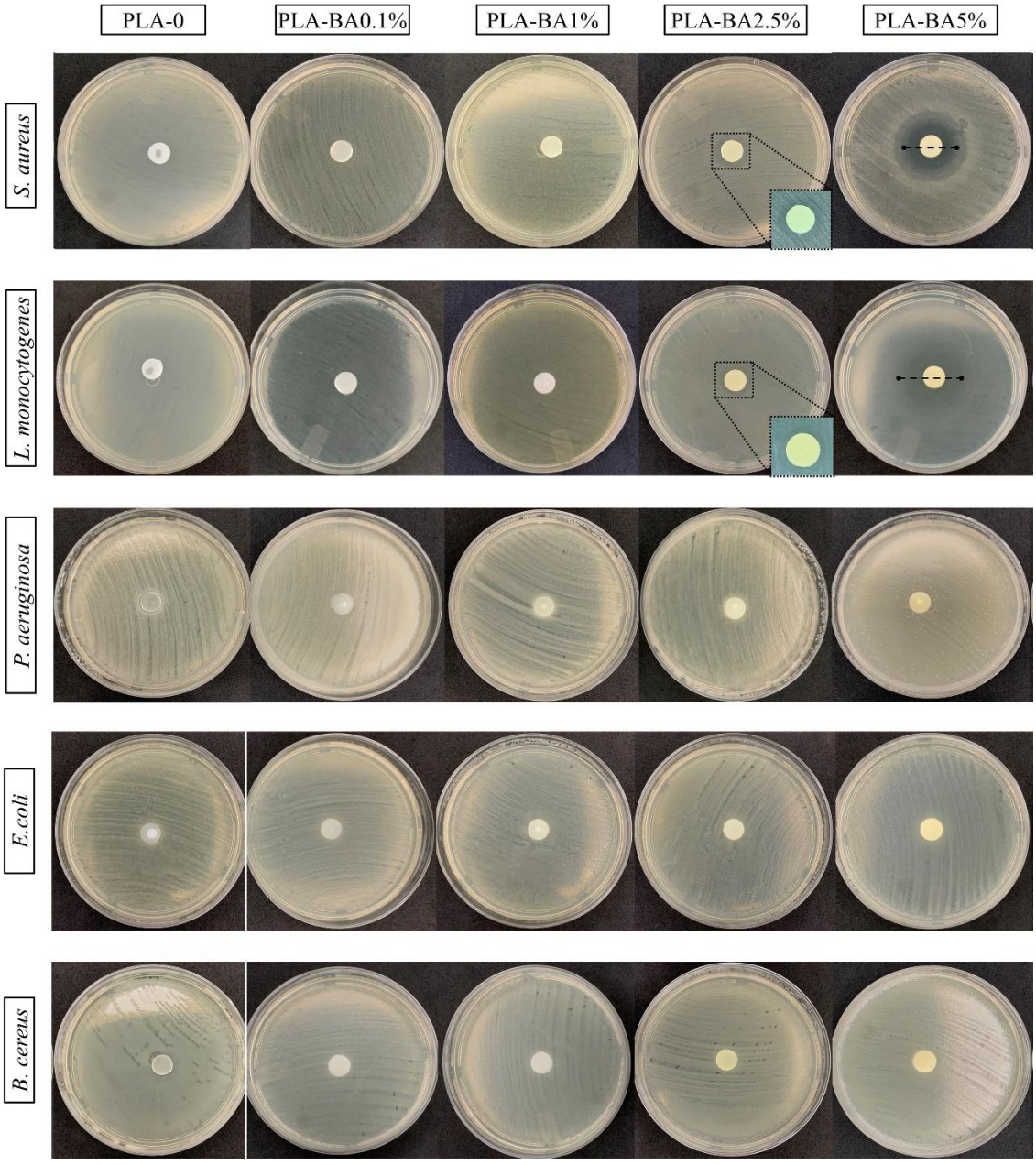

Fig. 9. Antibacterial activity of PLA sheets against Gram-positive and Gram-negative bacteria though zones of inhibition performs.

The inhibitory effect towards *S. aureus* and *L. monocytogenes* is presumably caused by the interference of the hop KBA with the bacterial cytoplasmic membrane, promoting its leakage. This process could result in the inhibition of the active transport of sugars and amino acids, and consequently in the inhibition of cellular respiration and synthesis of proteins, RNA, and DNA [55].

At 37 ºC for 24h, there was no difference between both Gram-positive bacteria ($p \geq 0.05$). However, incubation temperature seemed to be a significant factor, and the refrigeration

temperature restricted the diffusion of KBA with a lower inhibition zone found for *L. monocytogenes* incubated at 7 ºC for 10 d ($p < 0.05$). This difference can be attributed to the slow release of KBA from PLA sheets at lower temperatures [93]. Furthermore, as KBA presented low diffusion rates on aqueous systems, limited diffusion can be expected in the culture medium [87].

The antibacterial activity of PLA-based sheets was further evaluated by the direct contact and vapor phase diffusion methodologies, with results shown in Fig. S2 and S3, respectively. When in direct contact with the inoculated culture media (37 ºC/ 24 h), PLA-BA5% was able to inhibit the growth of both non-sporulate Gram-positive bacteria (*S. aureus* and *L. monocytogenes*) (Fig. S2). Similar results were observed for *L. monocytogenes* under refrigeration conditions (7 ºC/10 d). Tubes containing the sheets with lower KBA concentrations presented turbidity compared to the control (containing PLA-0), indicating no inhibitory effect on the tested bacteria. This antilisterial activity of hop β-acids is strongly corroborated by other studies in the literature [94–96].

The sheets were not able to inhibit bacteria growth through vapor phase analysis and KBA-added sheets presented a similar outcome to the control sheet at both temperatures (Fig. S3). These results imply that PLA active sheets are not suitable to promote inhibition on the headspace of packaging systems, requiring direct contact with the food. Hop α- and β-acids are part of the group of "nonvolatile hop resins" [97] which, in addition to the entrapment effect of the polymer matrix [12], can explain the low migration to the plate headspace. A study conducted by Marques et al. [50] found an interesting activity when performing the vapor phase test of cellulose acetate enriched with garlic essential oil (10% w/w) against *S. aureus* and *L. monocytogenes*. However, it is important to emphasize the volatile character of essential oils, which contribute to the effect on the air interface.

The antimicrobial properties of hop KBA, along with their GRAS concept, allow their application as an important tool in food preservation [18]. Some studies have already confirmed their potential for minimizing/preventing the growth of microorganisms in food matrices, especially in meat products [19,98]. Kramer et al. [19] employed a very similar β-acid rich hop extract to inhibit *L. monocytogenes* on pork bologna. In the mentioned study, at 0.4 g/kg of KBA extract, *L. monocytogenes* populations were reduced up to 3 orders of magnitude in comparison to bologna without the bioactive extract, while at a 0.8 g/kg concentration, KBA extract either prevented the growth of *L. monocytogenes* or resulted in at least 4 orders of magnitude lower final populations. Furthermore, at the tested conditions, no significant sensorial or color impacts were evidenced.

Another study investigated the application potential of these hop compounds on the shelf life of a meat product (i.e., pork), this time with the hop hexahydro-β-acids (a more hydrophilic derivative of hop β-acids produced by catalytic hydrogenation) incorporated into chitosan edible films (0.1-0.3% w/v) [99]. Compared to pork packaged in pure chitosan and polyethylene films (a traditional food package), chitosan-hexahydro-β-acids films prolonged the shelf life of fresh pork, due to a reduction in microbial proliferation, thiobarbituric values, pH, and total volatile base nitrogen contents during storage at 4 °C for 16 days. Therefore, these results indicated both antimicrobial and antioxidant prospects of the hop β-acids derivatives even in an *in situ* study. When the dosage of hexahydro-β-acids was increased from 0.1% to 0.3% (w/v), the freshness of pork was prolonged by 7−8 days.

3.3.9.2.2. Morphological aspects of bacterial cells on media with KBA-added sheet

From the results of direct contact analysis, aliquots were taken aiming to evaluate the morphological aspects of bacterial cells treated with PLA-based active sheets. Therefore, AFM analysis was conducted with *S. aureus* and *L. monocytogenes* cells in contact with PLA-0 and PLA-BA5%, incubated at optimum growth temperature (37 ºC/24 h). AFM results of bacteria are shown in Fig. 10. Cells from both bacteria treated with PLA-0 are full and intact with a smooth cell wall, demonstrating no visual impact of the control sheet, thus no antibacterial events towards the tested microorganisms (Fig. 10A1 and 10B1, for *S. aureus* and *L. monocytogenes*, respectively). Furthermore, after 24 h of incubation at optimum conditions, these cells started to multiplicate, with the Z ring visualized in the AFM images (indicated by blue arrows).

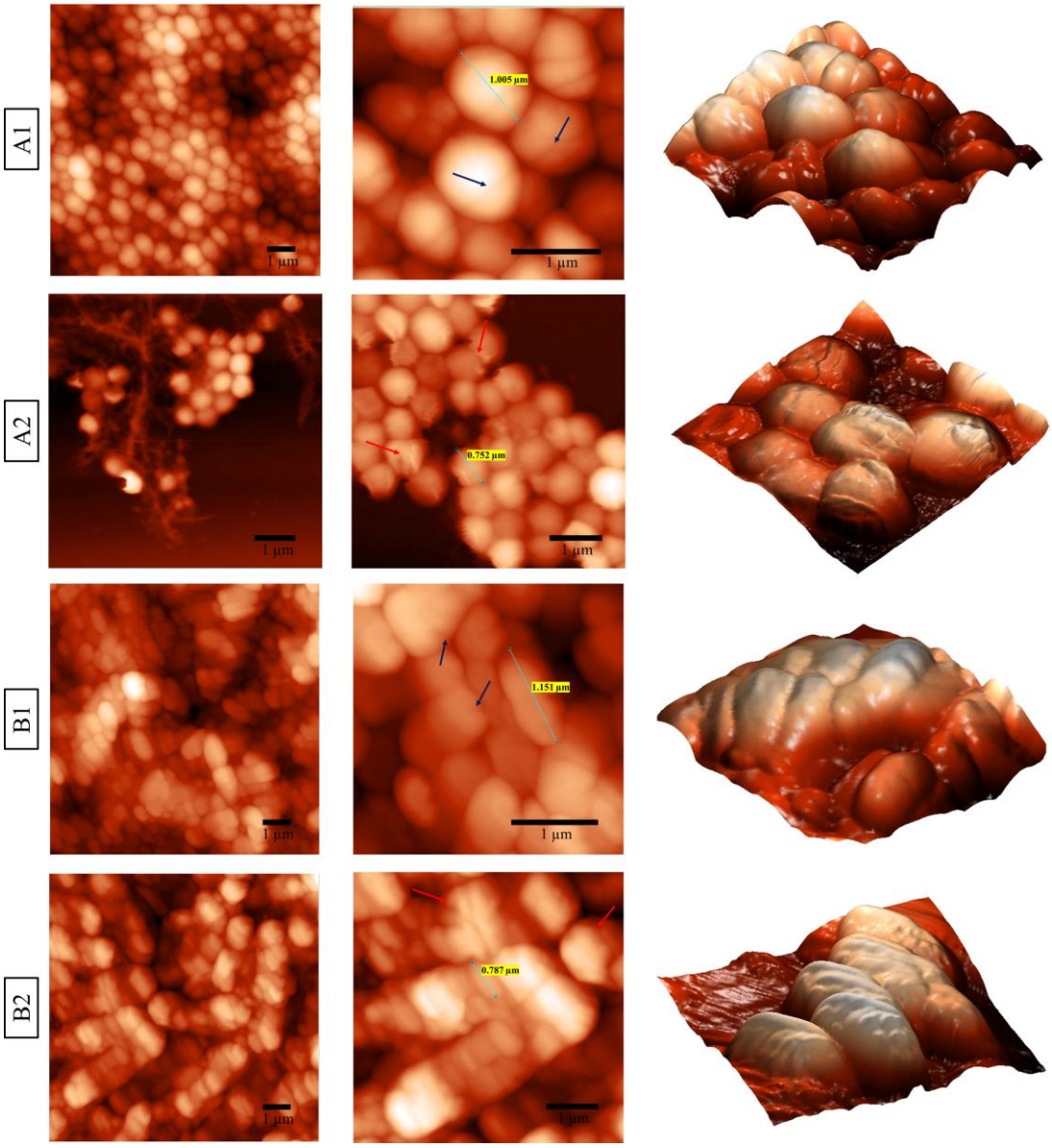

Fig. 10. AFM images of *S. aureus* (A) and *L. monocytogenes* (B): treated with PLA-0 (A1, B1) and treated with PLA-BA5% for 24 h (A2, B2).

On the other hand, after treatment with PLA-BA5%, cell morphology was greatly affected (Fig. 10A2 and 10B2, for *S. aureus* and *L. monocytogenes*, respectively). The morphology of bacterial cells treated with KBA-added PLA sheets shrank and some cells appeared seriously damaged, presenting deformities and leakage of cytoplasmic materials (red arrows). These abnormalities were found for both bacteria, but we can point out some particularities considering each one: observing the AFM images for *S. aureus* treated with PLA-BA5%, the broken cells were more evident, and was a clear appearance of some characteristic material presumably attributed to intracellular material; PLA-BA5%-treated *L. monocytogenes* pre-

sented a higher distribution of shrunken cells with notorious ruffled appearance to their membranes, and the cells appeared more accumulated and/or adhered. These results are consistent with data found for hop β-acids [62,100], which reinforces the effect of β-acids on the integrity and permeability of the cell membrane, leading to cell wall lysis, membrane instability, and separation of the cell membrane and cell wall.

Despite no differences observed in the inhibition zones of PLA-BA5% against both bacteria (Section 3.3.9.2.1), through the morphological evaluation it is assumed that the bactericidal effect was higher for *S. aureus*, in comparison with *L. monocytogenes* (i.e., higher cells cleavage). Previous studies already emphasized the discrepancies in the susceptibility to hop components between species of Gram-positive bacteria [17,98,101]. This can be justified by the composition of bacterial cell surfaces: the peptidoglycan structure of *L. monocytogenes* cell wall is similar to the one described for many Gram-negative bacteria (e.g., *E. coli*) [102] and presents a greater number of proteins attached, making it more hydrophilic [103]. Therefore, a higher staphylococcal susceptibility can be evidenced by the bactericidal mechanism of KBA diffused from PLA sheets. On *L. monocytogenes*, the effect was greatly bacteriostatic, preventing microorganism growth, and some bactericidal events were also found.

3.4. Conclusions

PLA biobased sheets were successfully prepared using KBA extract as an active additive. The results indicated that the KBA extract major constituents presented distinct behavior when added to PLA-based sheets: KBA molecule logged into the polymer matrix, meanwhile, propylene glycol predominantly stayed localized on the sheets' surface. PLA sheets' thickness was not altered by KBA extract incorporation; however, it exerted a plasticizer effect probably due to the accommodation of KBA molecules in the polymer matrix, corroborated by mechanical and thermal studies. Release results indicated that KBA-added polymers presented a great fit to fatty food, exhibiting a Fickian diffusion behavior. Furthermore, KBA-added PLA sheets presented significative bioactive properties, with reduced light transmittance, strong UV-blocking properties, and significantly enhanced antioxidant and antibacterial activities. PLA-KBA5% significantly improved the inhibitory effects towards non-sporulated Gram-positive bacteria. These results were confirmed through the morphological evaluation of the treated cells of *S. aureus* and *L. monocytogenes*, which indicated a direct influence on the cell membranes of both bacteria.

This study reinforces the applicability of hops' β-acids to other sectors of the food industry, such as the development of sustainable active packaging. The results obtained point toward the possibility of using KBA-loaded PLA sheets as an ally to food safety. Therefore, the present work lays the foundation for further needed investigations concerning the development of KBA/PLA sustainable active food packaging at a pilot/commercial scale, such as swelling degree investigation, stability, and toxicological studies, biodegradability test, release studies at different temperature and medium conditions, and more importantly, studies approaching the direct contact of the bioactive sheets within real food systems, especially in fatty foods, companied with a physicochemical and sensorial evaluation.

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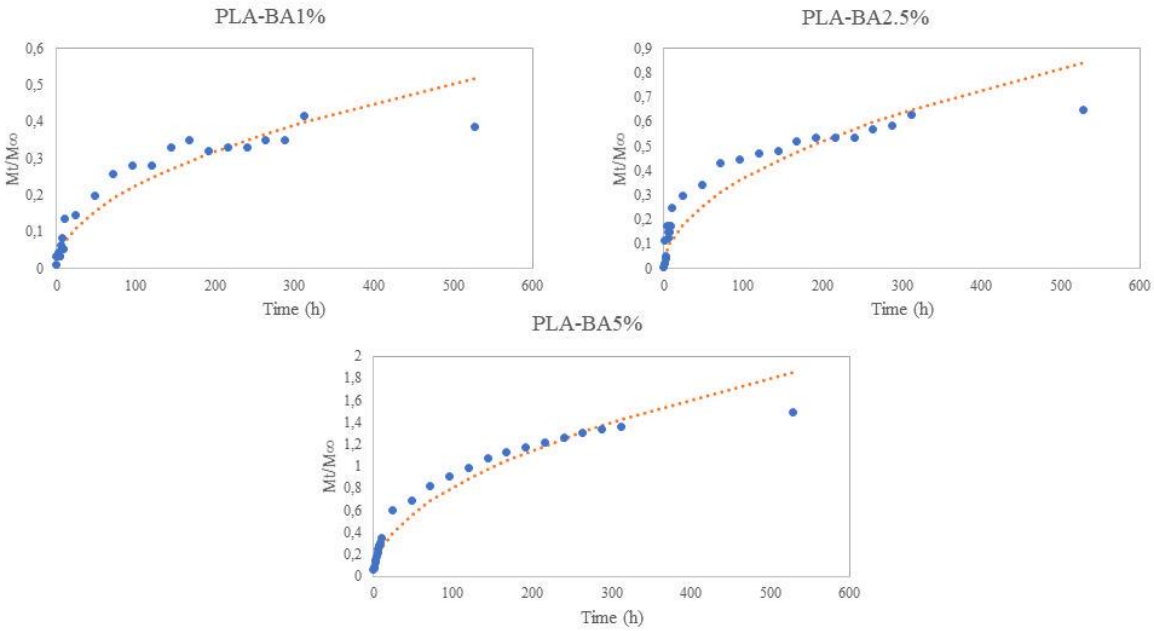
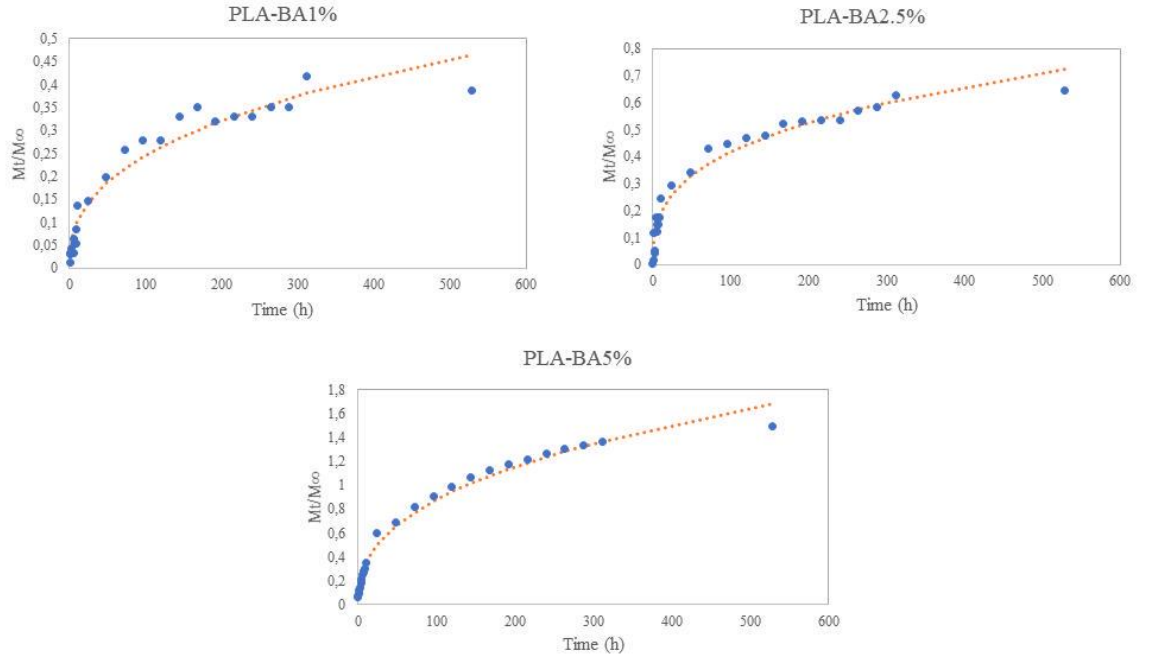
3.6. Supplementary Material

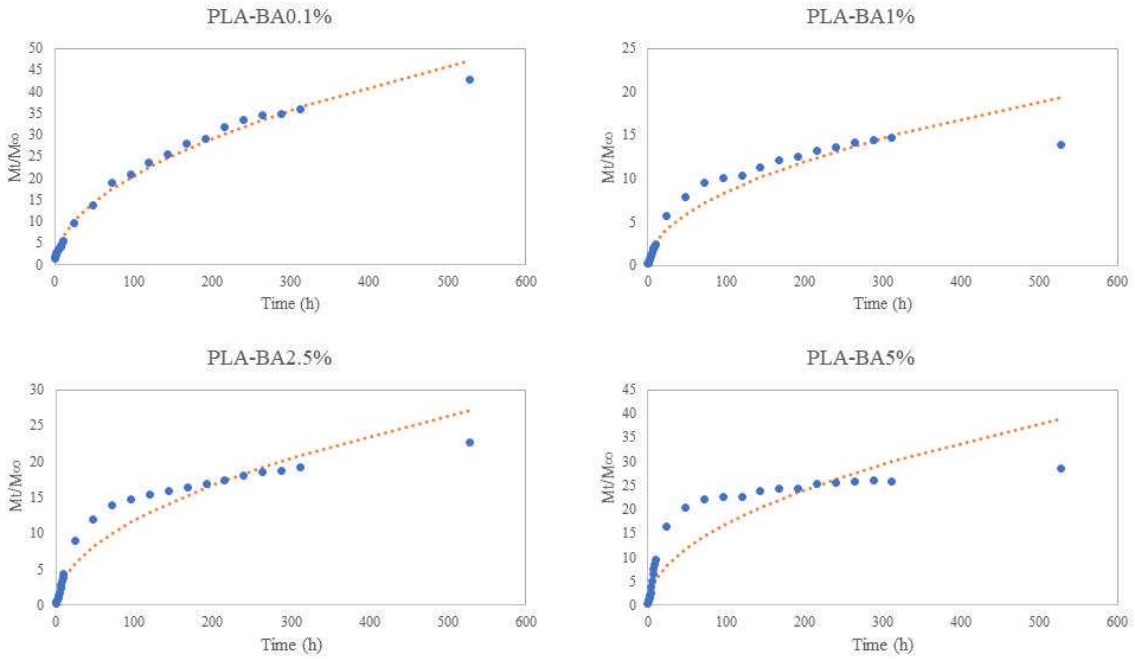
Kinetic release: Distilled water – Aqueous food simulant
Higuchi model:



Korsmeyer-Peppas model:



Kinetic release: 95% ethanol – Fatty food simulant
Higuchi model:



Korsmeyer-Peppas model:

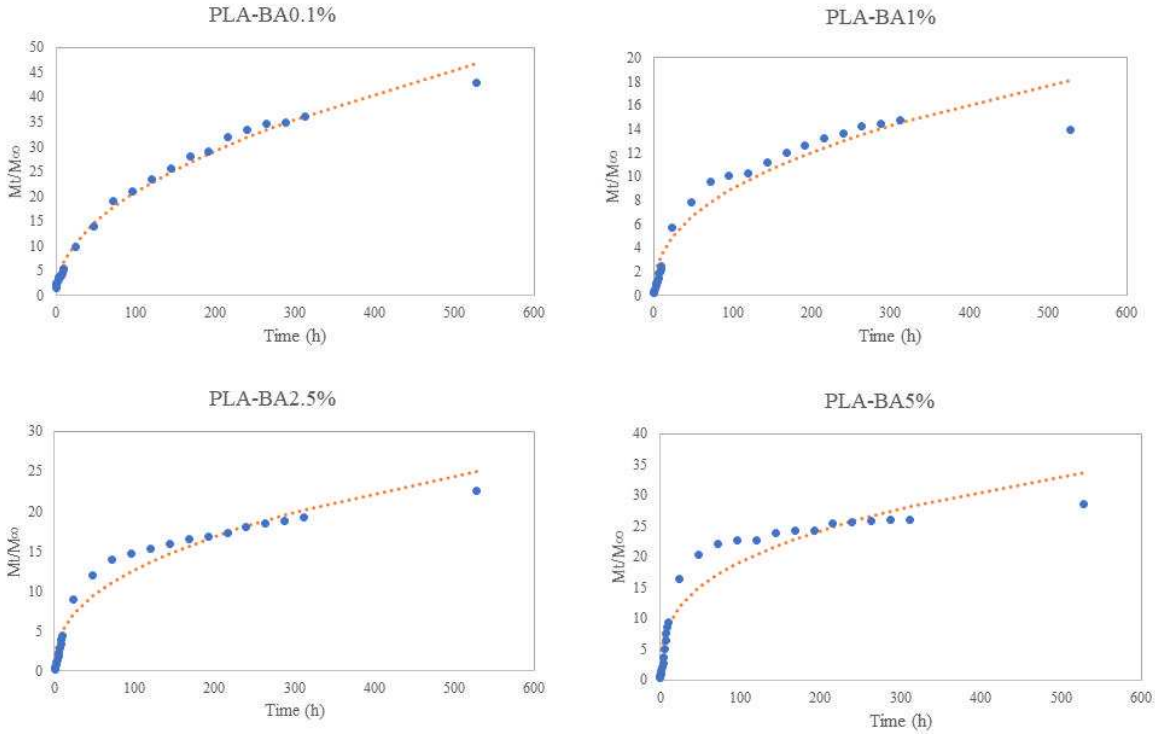


Fig. S1. Kinetic release potassium salts of β-acids from poly(lactic acid)-based films into aqueous (distilled water) and fatty (95% ethanol) food simulants.

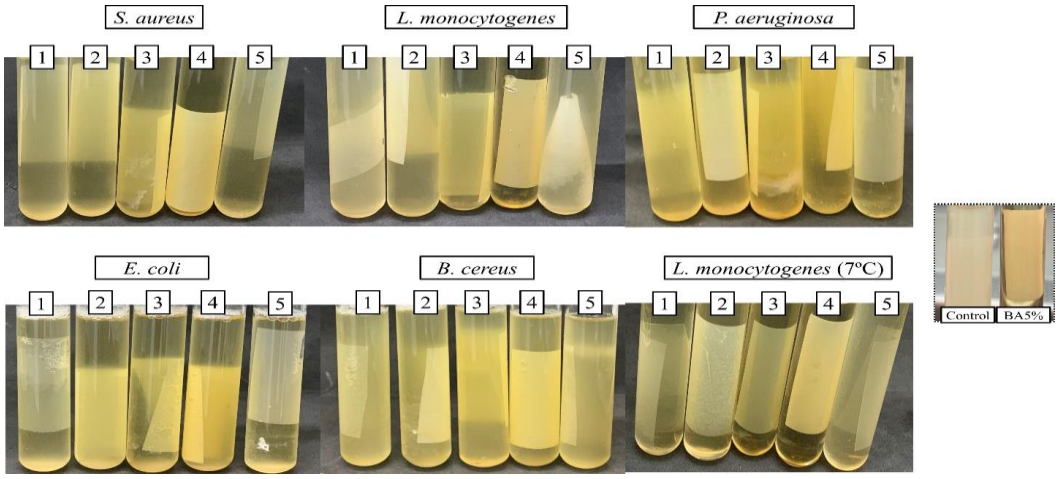


Fig. S2. Antibacterial properties of active PLA sheets with 0.1% (1), 1% (2), 2.5% (3) and 5% (4) of KBA and control sheet (5), testes through direct contact analysis against *S. aureus*, *L. monocytogenes*, *P. aeruginosa*, *E. coli* and *B. cereus* at 37 ºC/24 h. *L. monocytogenes* was also tested at 7 ºC/10 d.

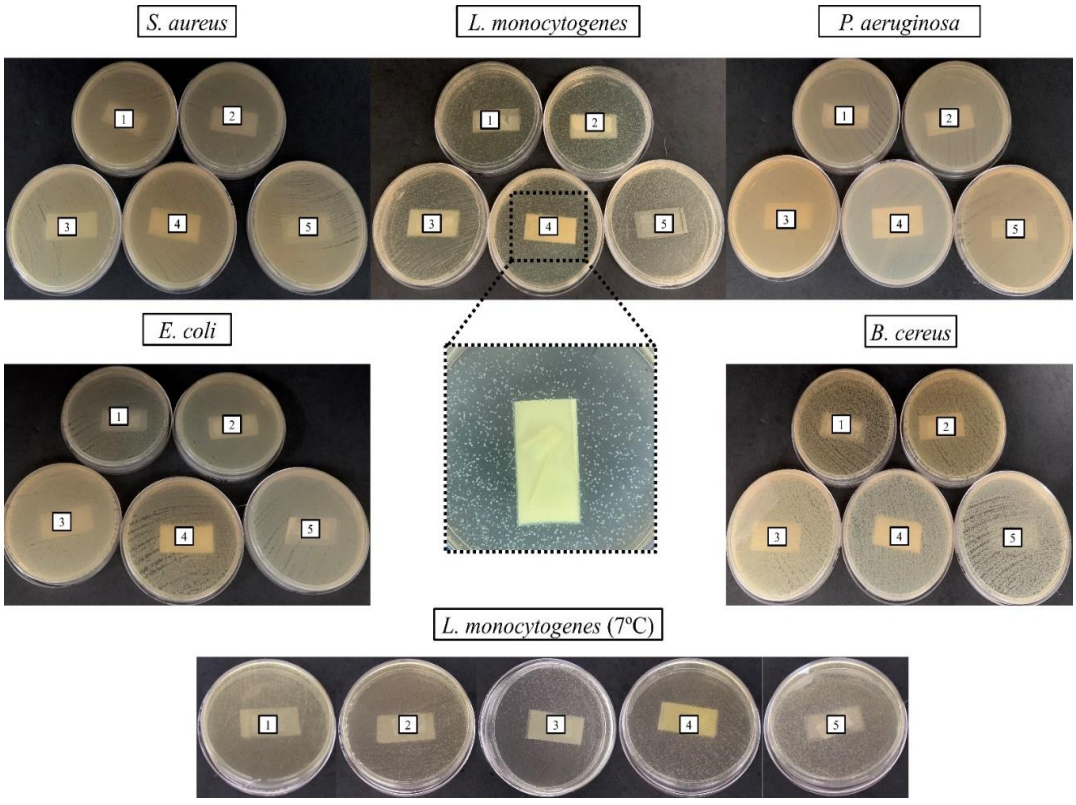


Fig. S3. Antibacterial properties of active PLA sheets with 0.1% (1), 1% (2), 2.5% (3) and 5% (4) and control sheet (5), testes through indirect contact analysis against *S. aureus*, *L. monocytogenes*, *P. aeruginosa*, *E. coli* and *B. cereus* at 37 ºC/24 h. *L. monocytogenes* was also tested at 7 ºC/10 d.

4. ARTICLE 3: EFFECT OF THE INCORPORATION OF β-ACID RICH HOP EXTRACT ON DEGRADATION IN SOIL OF POLYLACTIC ACID (PLA) SHEETS

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4.1. Introduction

Due to their versatility, durability, stability, and low cost, among other attributes, plastics have covered every sector of human needs, replacing other materials such as glass, wood, and metal [1]. However, the fact that they are still mainly produced from fossil origin and their incorrect discard have become outstanding topics surrounding plastic materials utilization: conventional plastics present a persistent nature and a slower rate of decomposition in natural settings [2].

Therefore, facing this environmental issue, several attempts to create alternatives to petroleum-derived plastics have been made in the last decades. Either these surrogating materials have to present a lower environmental impact in their production processes, or their residues can be treated and incorporated into nature without generating pollution. These so-called bioplastics consist of materials generally produced from renewable sources (e.g., plants, animals, or microorganisms) and can mean they are biodegradable according to international standards [3]. Among them, poly(lactic acid) (PLA) was the first polymer based on renewable raw materials commercialized at a large scale [4].

The degradation mechanism of PLA can be mainly described through two steps: (1) chemical hydrolysis of the ester bond of PLA, catalyzed by temperature (2) aerobic bacterial transformation of the fragmented residues into carbon dioxide and water in compostable atmosphere [4,5]. Despite being recognized as a compostable material, meeting the specifications of international standards, the literature evidenced that PLA disintegration is slow in soil under natural conditions, indicating that this polymer can remain in the soil for a considerable time [6,7]. In fact, PLA is hardly degradable at ambient temperature because it is resistant to microbial attack [8].

The rate of the aforementioned steps is influenced by both the biotic (i.e., microrganisms) and abiotic (temperature, moisture, oxygen content and soil properties, e.g., pH, macro-, meso- and microelements) conditions but also by the characteristics of the polymer [6], which

mainly include chemical structure, polymer chain, crystallinity, and the presence/type/quantity of additives. These last ones can migrate from the plastic or not be degraded, potentially posing toxicity risks to terrestrial and aquatic ecosystems [9].

Furthermore, the presence/type/quantity of additives in plastic materials can greatly affect the rate of plastic degradation. In this sense, it is important to point out the manufacturing of antimicrobial/antioxidant packages, characterized by the incorporation of active substances into/to the polymer matrix. It is, therefore, extremely important to assess the degradation of each case individually, especially considering that antimicrobials can affect the biotic degradation conditions.

In a previous work, active sheets were successfully manufactured through the incorporation of different concentrations of a β-acids rich hop extract (KBAE) into the PLA matrix [10]. Nevertheless, their degradation profile was not assessed. Therefore, the present study aims to investigate the degradation in soil of the PLA-based active sheets and verify if KBAE incorporation would interfere with the degradation of the elaborated plastic materials, especially considering the presence of the antimicrobial/antioxidant active agents (hop β-acids).

4.2. Material and methods

4.2.1. Material

PLA Ingeo™ 4043D (95.7 % L-lactide, density =1.24 g.cm^3) (Natureworks LLC, USA), and chloroform P.A. (Vetec, Brazil) were used for plastic sheet manufacture. As an active component, a β-acids rich hop extract (BetaBio45, Hopsteiner, Germany), kindly donated by Wallerstein Indl e Coml Ltda (São Paulo, Brazil), was used. The extract is a brownish solution and contains 45.0 % ± 2.5 % w/w potassium salts of β-acids, 22.5 % ±12.5 % w/w food grade propylene glycol, and 1.0–2.0 % w/w hop oils with corresponding residual moisture. According to the manufacturer, the hop β-acids present in the extract are mainly colupulone, lupulone and adlupulone, obtained through CO_2 extraction.

4.2.2. Methods

4.2.2.1. Sheets manufacture

PLA-based sheets were obtained by casting method, following the methodology previously described by Arruda et al. (2023) [10]. The dispersion of the PLA pellets was prepared in 200 mL of chloroform (1:10 w/v) under magnetic agitation (300 rpm, 12 h, 25 ± 2 ºC). Then, the β-acids rich hop extract was added to the dispersion until the final concentrations of KBAE

were 0.1% (KBAE-FREE.1%), 1 % (KBAE1%), 2.5 % (KBAE2.5 %), and 5 % (KBAE5%) wt, regarding the polymer mass. The polymer dispersions were homogenized in Ultra-turrax (1 min, 4800 rpm, T25, IKA), degassed, and poured onto a glass plate (18 cm × 34 cm). KBAE-free formulation served as control (KBAE-free). The solvent of all the treatments was allowed to evaporate under a chemical hood at room temperature for 24 h. The sheets were peeled from the trays and cut before the testing.

4.2.2.2. Soil burial test

The PLA sheets' degradation was studied by simulating the processes occurring under natural conditions, according to Rogovina et al. (2013) [11], with modifications. The samples (3 cm x 3 cm) were placed in plastic nets to facilitate their removal and buried in soil with neutral pH (ca. 7.0 ± 0.5) for a period of 180 days. The vessels containing the samples were exposed to ambient light but sheltered from rain and watered twice a week. Every 30 days, the samples were picked up from the soil and cleaned with a brush to remove the excess of soil attached to the samples. The degradation process was evaluated over time in terms of visual appearance (including alteration in the specimen size), changes in morphology (Scanning Electronic Microscopy - SEM), weight loss, thermal analysis (thermogravimetric analysis - TGA), molecular structure (Fourier Transform Infrared Spectroscopy – FTIR), and crystallinity (X-ray dispersion – XRD).

4.2.2.3. Sheets characterization over time

4.2.2.3.1. Visual analysis and size variation

After the cleaning step, the PLA sheets' appearance (color, aspect, and roughness) was subjectively assessed. Besides that, their size was measured (mm) with a ruler and compared to the initial mold. The final area (*Af*) was compared to the initial area (*Ai*) according to equation 1, allowing to determine the size reduction (SR%):

$$SR(\%) = \frac{Ai - Af}{Ai} x100\% \qquad \text{Eq. 1}$$

4.2.2.3.2. Mass loss

Mass loss (ML) of the sheets was evaluated overtime until the 180th day. After the cleaning step, the sheets were weighted on a digital scale (minimum 10 mg, e = 0,001, Bel Mark M214A, Italy). The mass loss (%) was determined according to the equation 2:

$$ML(\%) = \frac{mi-mf}{mi} x100\% \quad \text{Eq. 2}$$

In which m_i is the initial mass of the sample (day 0, before burial), and m_f is the mass of the sample in a given time [12].

4.2.2.3.4. Scanning Electron Microscopy (SEM)

Morphological changes (e.g., holes, tears and other defects) in sheet surfaces were monitored using micrographs obtained through a Scanning Electron Tabletop Microscope (TM3000, Hitachi High-Technologies, Japan) with a secondary electron detector, operating under low-vacuum and 15 kV of accelerating voltage. The uncoated samples (around 1 cm²) were attached to the stubs' surface with the aid of a double-sided carbon tape.

4.2.2.3.5. Thermogravimetric analysis (TGA)

Over the 180-day trial, TGA was conducted on a Shimadzu thermogravimetric analyzer, (Model DTG-60H, Japan). Approximately 5 mg of samples were weighed and heated from 25 to 600 ºC at a 10 ºC/min rate, under an inert atmosphere of nitrogen (50 mL/min).

4.2.2.3.6. Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR spectra of the sheets were obtained using an FTIR spectrometer, model 660-IR (Varian Inc., CA, USA), equipped with Attenuated Total Reflectance (ATR). FTIR-ATR spectra of the samples were obtained in the 4000–700 cm^{-1} region, using 16 scans and 4 cm^{-1} resolution.

4.2.2.3.7. X-ray diffraction (XRD)

The possible changes in samples crystallinity were assessed through X-ray diffractograms obtained in a BRUKER X-ray diffractometer (model D8 Discover, USA) equipped with an X-ray tube (Cu-Kα radiation, λ = 0.1514 nm), in a 2θ range from 5º to 40º with a scanning rate of 0.05º s^{-1}.

4.2.2.4. Statistical analysis

The software R was used for statistical analysis. Quantitative data were collected in triplicates and the results were expressed as the mean ± standard deviation. When appropriate,

the data obtained were submitted to analysis of variance (ANOVA) followed by Tukey's post hoc test or non-linear regression ($p < 0.05$).

4.3. Results and discussion

4.3.1. Visual appearance, microscopic changes, and mass loss of PLA sheets

PLA is a polymer widely studied as a more sustainable option to replace conventional plastics. Although it is considered a biodegradable material, the literature shows that its biodegradability behavior varies according to many factors: environmental conditions (soil composition, water, compost, temperature, light exposure, pH, etc.), exposure time, and the polymer own features (hydrophilicity/hydrophobicity, size, shape, molecular weight, polymer crystallinity, presence of additives, blends with other polymers, etc.) [8,9]. The main mechanisms involved in PLA degradation are hydrolytic, oxidative, microbial, enzymatic, and photodegradative, and, in environmental natural conditions, two or more mechanisms can occur simultaneously [6,8,13].

Photographs of PLA samples buried for 180 days in soil were taken and can be visualized in Figure 1. The incorporation of KBAE impacted the initial appearance of the PLA sheets, enhancing green and yellow tones as the extract concentration increased. After burial in soil, all samples became brittler and opaquer with time, especially the KBAE-added ones, regardless of the extract concentration. Additionally, the KBAE sheets seemed to be more fragile and brittle than the control. For example, after 90 days, the samples KBAE2.5% and KBAE5% were fragmented or had missing parts, making it difficult to remove the sheets from the soil. These findings match those observed in earlier studies, which also describe an increase in opacity and brittleness in PLA sheets when exposed to different degradation conditions [14]. This opaquer feature, or brightness reversion, of PLA samples is often related to the occurrence of hydrolytic degradation [5,15], which, in this case, could have happened due to the soil moisture and the interaction of water molecules and the PLA chain.

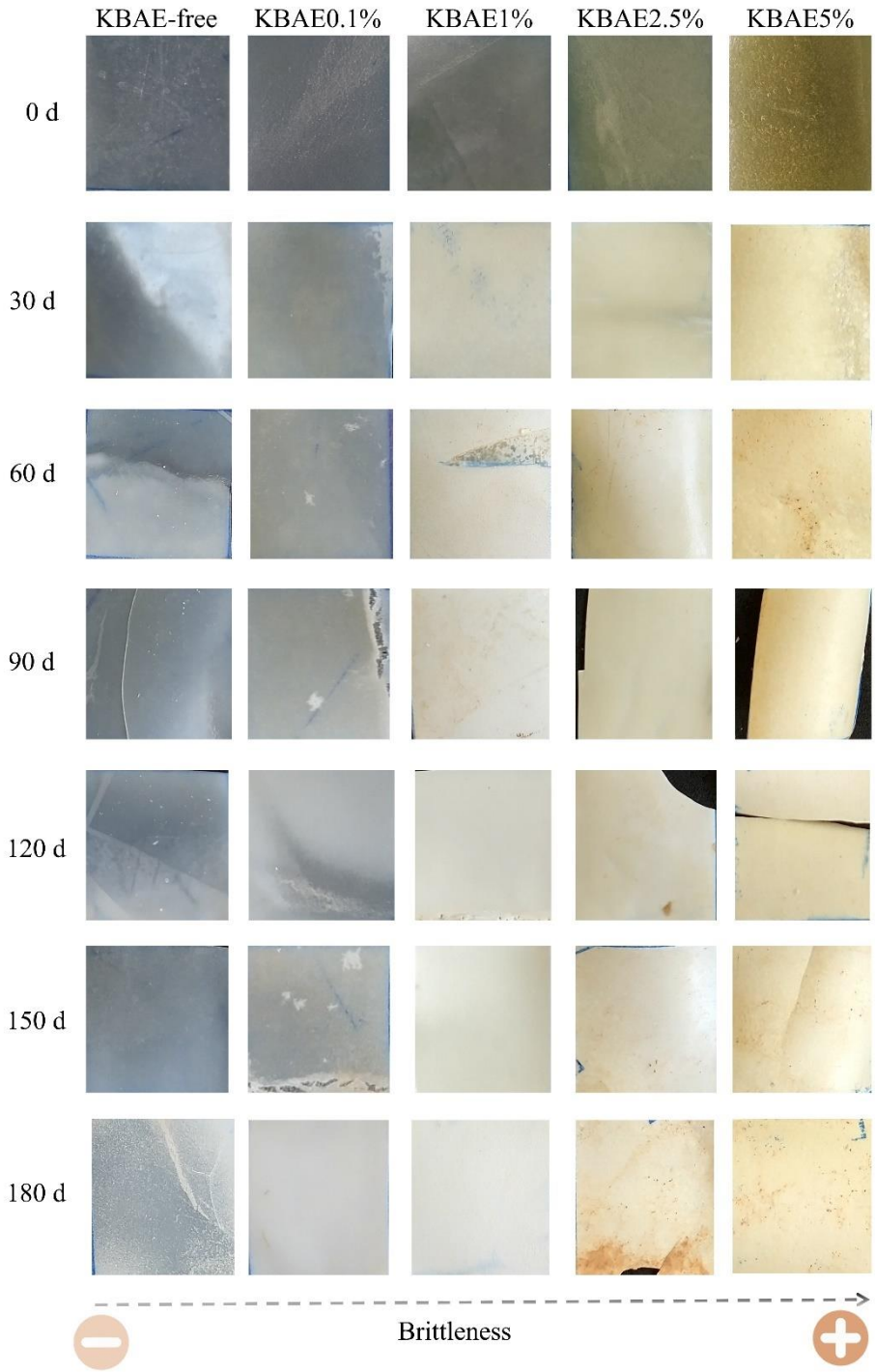

Figure 1. Photographs of PLA sheets incorporated with different concentrations of KBAE (0% to 5%) before and after burial in soil for up to 180 days.

Changes in the sheet structure were observed at the microscopic level by scanning electron microscopy (SEM), and the micrographs obtained from samples (days 0, 60, and 180) are displayed in Figure 2.

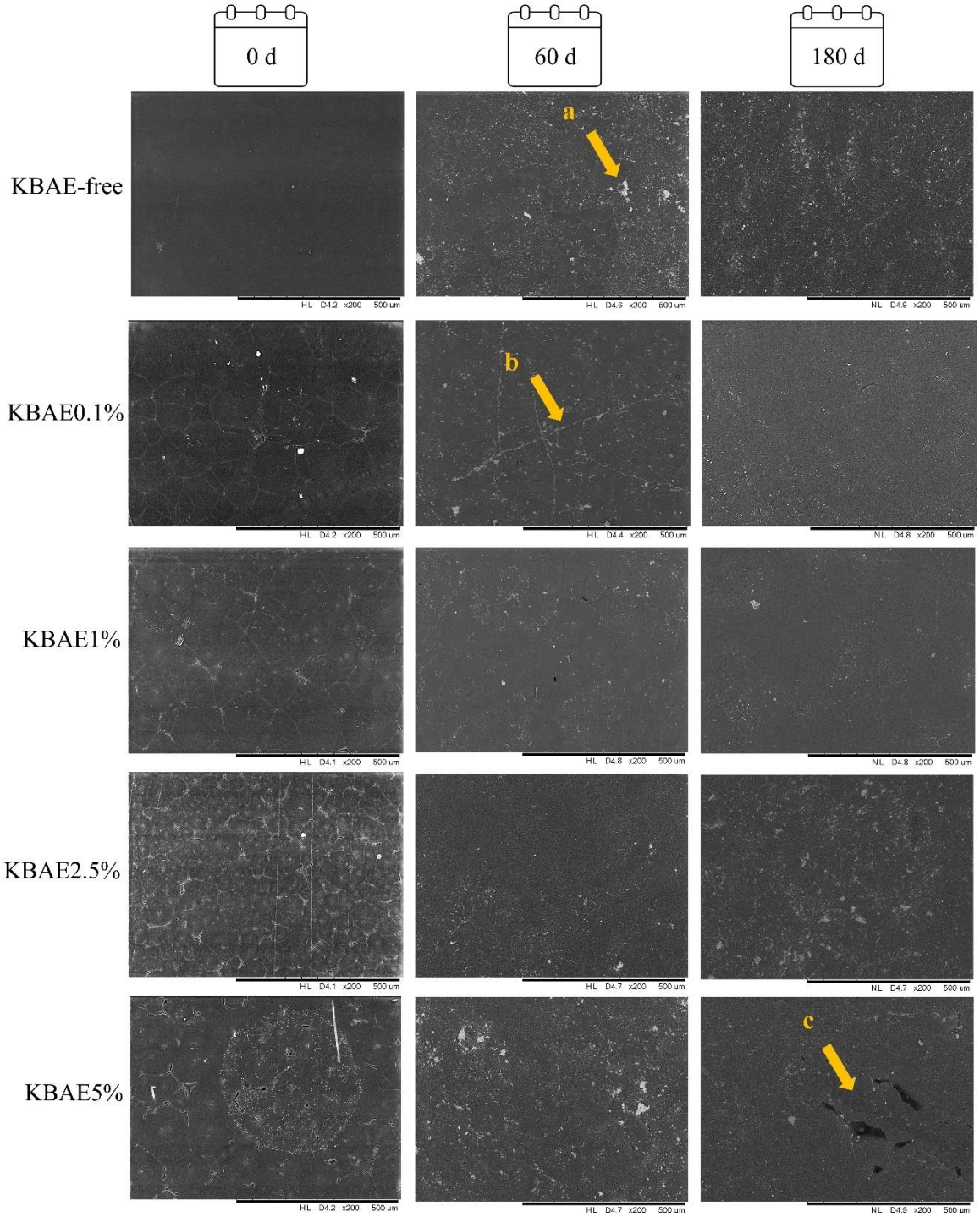

Figure 2. Micrographs of PLA sheets with different concentrations of KBAE (0 to 5%) before burial in soil (0 d) and retrieved after 60 and 180 days. Yellow arrows indicate soil particles adhered (a), scratches (b), and fissures (c).

The micrographs reveal that the KBAE incorporation changed the microscopic appearance of the PLA sheets, probably due to the presence of propylene glycol and the extract itself as discussed in a previous paper [10]. After burial in soil, the images suggest either the loss or

degradation of the additives in the KBA-added sheets over time. The presence of soil particles adhered to the sheets surface was also registered, as well as the appearance of signs of erosion, such as scratches and fissures, which are indications of degradation [5,6,9].

Besides the macro and microscopic changes, a fluctuation in the area and mass values of PLA sheets was observed during the burial trial (Figure 3). Loss of mass over time is a common occurrence during polymer degradation, and it is a consequence of the polymer chain cleavage by hydrolysis, oxidative reactions, enzymes, and microorganisms attack, and even loss of additives [9]. The events occur alongside shrinkage and fragmentation of the samples, as observed. Samples containing 0%, 0.1% and 2.5% w/w of KBAE lost their mass following an exponential behavior, which allowed the adjustment of non-linear models (Figure 3 and Table 1). No models were fitted for samples incorporated with 1% and 5% w/w of KBAE, which behaved inconsistently, gaining and losing mass over the burial period (data not shown). However, all samples had a maximum decrease of about 20% in mass and area compared to the initial values, regardless of the KBA incorporation.

Table 1 Non-linear models, and their respective adjusted R^2, obtained for mass (Ym, %) and area (Ya, %) reduction over time (x, day) during the 180-day period buried in soil, for each treatment.

Sample*	Model	R^2adj
KBAE0.1%	Ym = 7.486*exp(-x/0.751) + 92.451	0.849
	Ya = 11.449*exp(-x/21.833) + 88.527	0.527
KBAE1%	Unadjusted	-
	Unadjusted	-
KBAE2.5%	Ym = 18.547*exp(-x/101.653) + 81.337	0.771
	Ya = 7.672*exp(-x/28.068) + 92.649	0.396
KBAE5%	Unadjusted	-
	Unadjusted	-
KBAE-free	Unadjusted	-
	Ya = 8.401*exp(-x/17.187) + 91.628	0.831

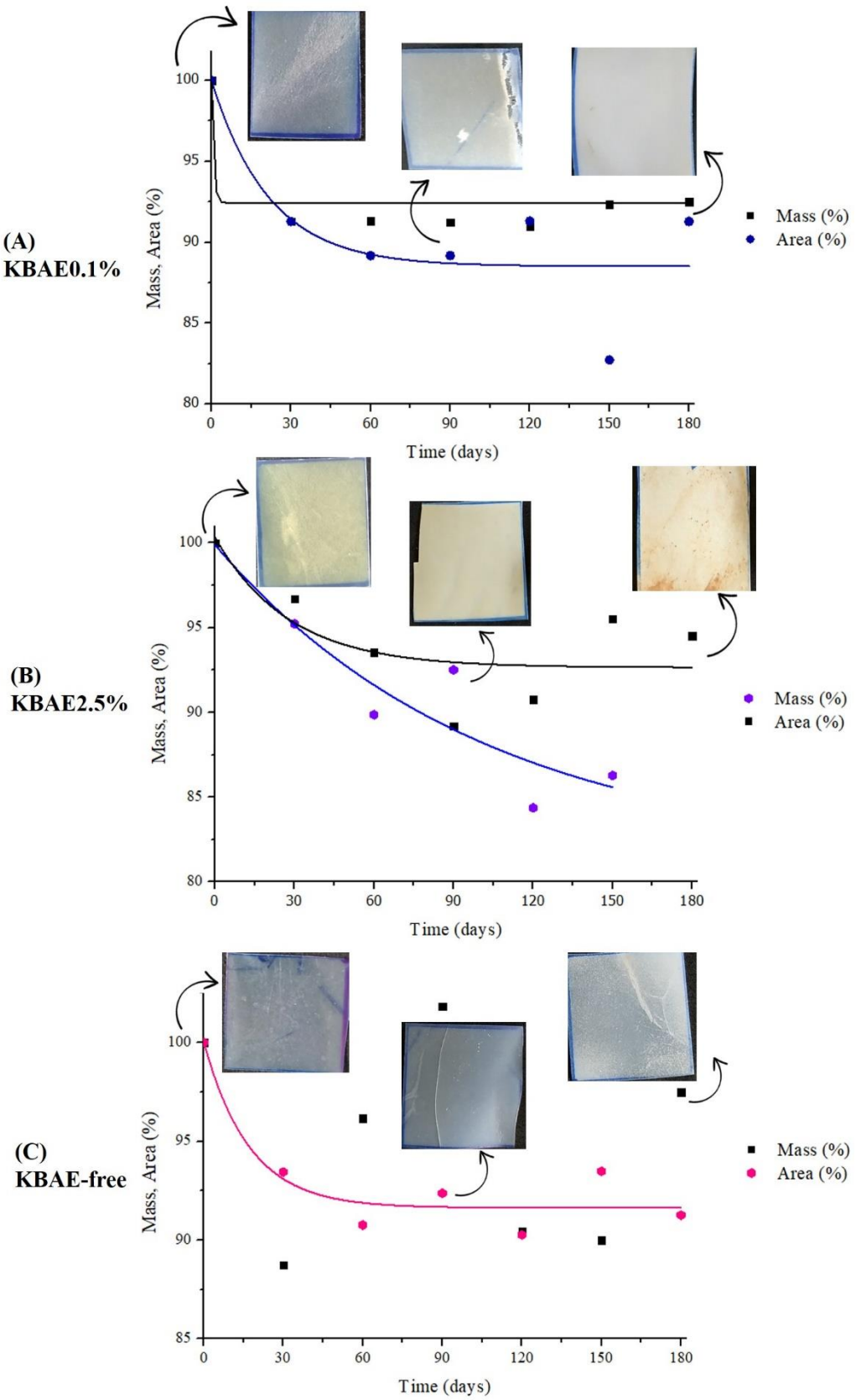

Figure 3. Mass and area reduction (%) over time plus photographs of PLA sheet samples on the initial day, 90th day and 180th day. (A) PLA film with 0.1% of KBAE (KBAE0.1%); (B) PLA film with 2.5% of KBAE (KBAE2.5%); (C) Control film (KBAE-free).

These results are an interesting outcome since they suggest two possibilities: one, the extract, albeit showing antimicrobial action and granting the sheets antimicrobial function [10], did not impact the soil microbiota responsible for PLA degradation; and two, in this case, the microbial attack did not play a role in PLA-sheets degradation. The second hypothesis is reinforced by earlier studies, in which the microorganisms related to PLA degradation are very specific and not widely distributed in natural soil environments (e.g., microorganisms from *Pseudonocardiaceae* family and related genera such as *Amycolatopsis, Lentzea, Kibdelosporangium, Streptoalloteichus, and Saccharothrix*) [4,5,16].

When buried in soil under ambient temperature, PLA samples degrade slowly, taking a long time for the material disintegration to begin [4]. Slezak et al. (2023) [6] reported that PLA blends evidenced a mass loss of about 0.5% after 6 months buried in soil, a value well below the one reported in the present study (ca. 20%). The authors reported that the average temperature during this period was 11 ºC, which can explain the difference in the results since the average temperature during the 180-day period in the present experiment was higher, around 25-30 ºC. According to Karamanlioglu and Robson (2013) [17], temperature is an important abiotic parameter that governs the degradation rate of PLA, and lower temperatures can decelerate the pace of polymer degradation.

On the other hand, Chuensangjun et al. (2013) [12] conducted a degradation study of PLA blends (PLA products from microbial lipase-catalyzed polymerization and commercial PLA beads, at 40:60, 60:40 and 70:30 ratios) buried for 14 days in soil under laboratory conditions (relative humidity around 40%, at room temperature). The obtained results verified that PLA blends presented a mass loss varying from 5% to 30% during the 14-days period, whereas the commercial PLA lost less than 5% of mass. Another important information to be pointed out concerns the nature of PLA sheets: they present a considerable thickness, i.e., ranging between approximately 319.63 μm and 334.94 μm [10], which can also affect the degradation rate of the plastic material. The thickness is described as inversely proportional to the rate of degradation, meaning that the thicker the film the lesser the degradation [1].

4.3.2. TGA

Samples of all treatments before and after 180-day burial trial presented a similar thermal degradation behavior, with two major degradation peaks being observed at TG and DTG curves, as displayed in Figures 4A and 4B, respectively. Figure 4A evidences that the first mass loss occurred around 100 ºC. Before the burial, the peak attributed to moisture [10], indicated 7.94%, 8.26%, 7.21%, 7.41%, and 6.73% w/w of mass loss for KBAE-free, KBAE0.1%,

KBAE1%, KBAE2.5% and KBAE5% samples, respectively. However, after 180 days of burial, these values shifted to 4.13%, 3.25%, 2.85%, 1.88%, and 1.17% w/w for the same samples, indicating a reduction in sheets' water content (Figure 4A). This behavior can be attributed to the participation of the water molecules in the hydrolytic degradation of the polymer [9], which is considered the first step of PLA degradation [8].

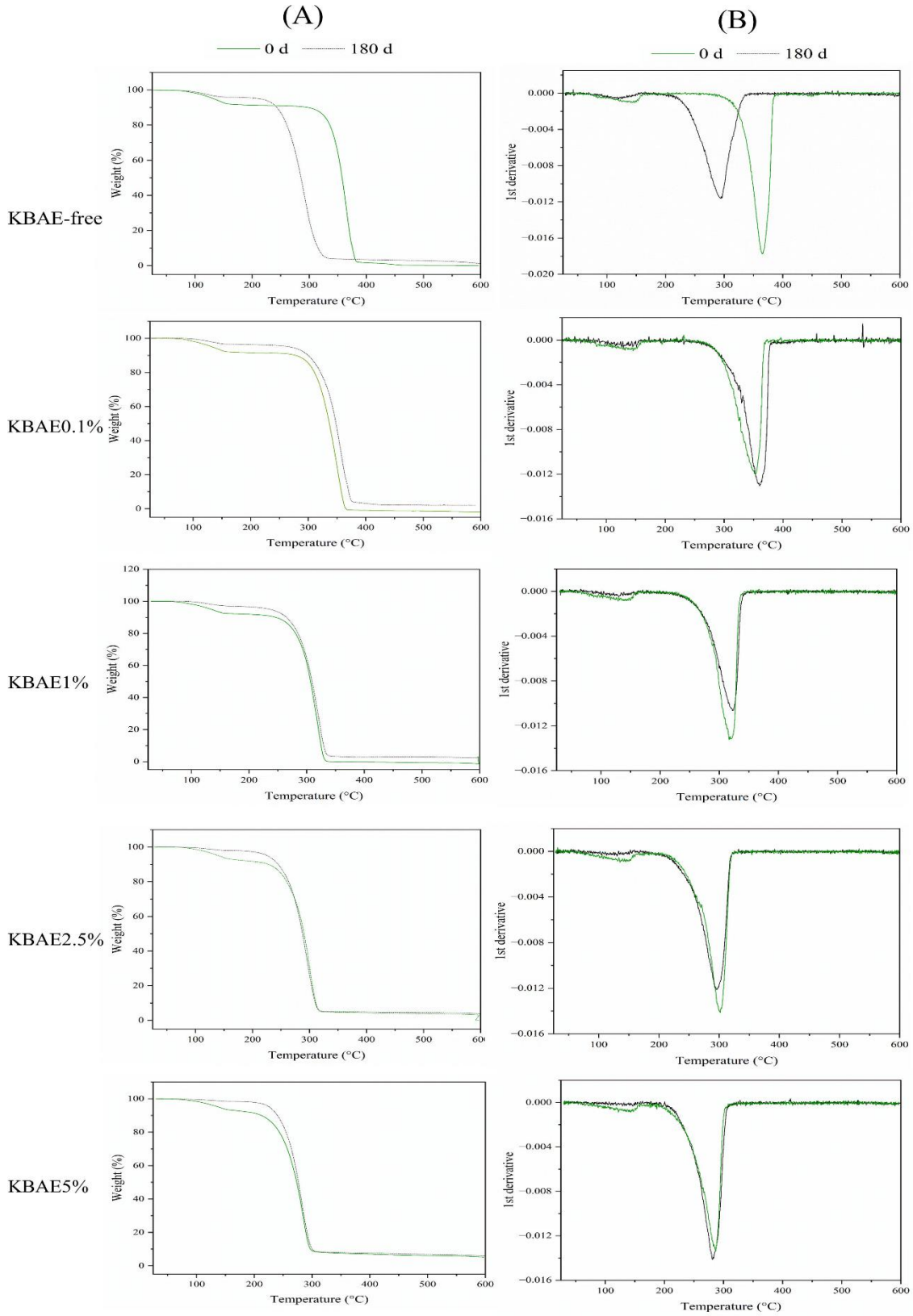

Figure 4. TG (A) and DTG (B) curves of PLA sheets added with 0% (KBAE-free), 0.1% (KBAE0.1%), 1% (KBAE1%), 2.5% (KBAE2.5%) and 5% (KBAE5%) w/w of KBAE before and after 180-day burial trial.

It is believed that the hydrolysis of polymeric materials occurs in three steps: the first one involves the degradation at the molecular level, within the reactions only being controlled by chemical reactivity; the second step is associated with molecular mobility and water:polymer interactions, also in a molecular level; and the final one corresponds to the macroscopic changes, where erosion and water diffusion are the governing parameters for degradation [18]. Furthermore, it is possible to infer concerning the water absorption by the polymer during the decomposition, as the moisture decreased accordingly with the KBAE incorporation after the 180 days of soil burial, possibly due to the presence of hydrophobic molecules (e.g., hop β-acids).

The second peak represents the main thermal degradation of the PLA-based sheets, in which most organic materials are decomposed and maximum weight loss occurs. Peak shift in DTG curves revealed changes in the maximum degradation temperature between the samples before and after the 180th day of the soil burial test (Figure 4B).

For the KBAE-free sheet, the onset and maximum degradation temperatures for the major peak were significantly affected, decreasing ca. 90 ºC and 70 ºC, respectively. The decrease of the thermal stability is possibly justified by the breakage of the bonds in the entire volume of the tested sample [6]. On the other hand, the sheets incorporated with lower amounts of KBAE (i.e., KBAE0.1% and KBAE1%) evidenced an increase of 7 ºC and 4 ºC in maximum degradation temperature, respectively. This observation can be related to the increase of the PLA crystallinity as a function of the reduction of amorphous regions due to the occurrence of hydrolytic degradation preferentially in areas less structurally organized [8]. The presence of higher concentrations of KBAE (2.5% and 5%) did not affect significantly the onset temperature but induced a slight reduction in the maximum degradation temperature, probably due to a higher mobility of the polymer chains, as the KBAE may exert a plasticizing effect in PLA sheets [10].

As presented in our previous work, the presence of KBAE reduces the thermal stability of PLA-based sheets [10]. Interestingly, in an overview of the results presented in Figure 4, it can be highlighted that the presence of KBAE helped to maintain the thermal stability of PLA sheets after 180 days of burial in soil. This maintenance of thermal stability can be explained by the fact that the hydrolytic degradation process involves the diffusion of water molecules, which begins in amorphous regions, and subsequently initiates ester bond cleavage, and continued along the crystalline region [19]. With the incorporation of KBAE, the presence of the hydrophobic β-acids can interfere with the water molecules' movement and their diffusion from the surface to the inner structure, restricting the hydrolysis degradation inside PLA sheets.

Nevertheless, as no differences were found for weight loss among the treatments, the thermal analysis results indicate that the incorporation of KBAE possibly interfered with how the degradation process occurred, especially considering the hydrolysis of the material. This hydrolytic decomposition can be developed either through surface or bulk erosion. In the first case, the degradation occurs primarily at the outer surface of the polymer, while the inner layers are degraded at last. The opposite happens with bulk erosion, where water molecules quickly diffuse into the amorphous regions of the polymer, resulting in a rapid loss of strength and structural properties [20]. In general, the higher the hydrophobicity of the polymer, the lower the rate of hydrolysis reactions, as it restricts the access of water molecules.

Our previous work revealed that the PLA-based active sheets herein studied present an outer surface more hydrophilic than the inside of the material due to the presence of the propylene glycol outside; meanwhile, the hop β-acids were located at the inner polymeric matrix. This hypothesis may justify the results found for thermal degradation. As the hydrophobic molecules present in the extract may decrease the water diffusion and mobility among the polymer chains, the hydrolysis of PLA sheets added with higher concentrations of KBAE (e.g., KBAE5%) mainly occurred at the surface of the material (i.e., surface erosion), improved by the hygroscopic propylene glycol. In turn, the changes in thermal composition found for the control sample are consistent with the effective breaking of polymer chains along with higher water absorption, which per self could reduce the temperature of decomposition of the polymer [21]; therefore, evidencing a higher bulk erosion than KBAE-added sheets.

4.3.3. FTIR-ATR

The FTIR-ATR spectra of KBAE-added and KBAE-free sheets during the burial trial can be found in Supplementary Material (Figure S1). In addition, KBAE-free and KBAE5% were specifically chosen to be evaluated during the degradation period spectra and helped elucidate the phenomenon that took place during the 180-day period (Figure 5).

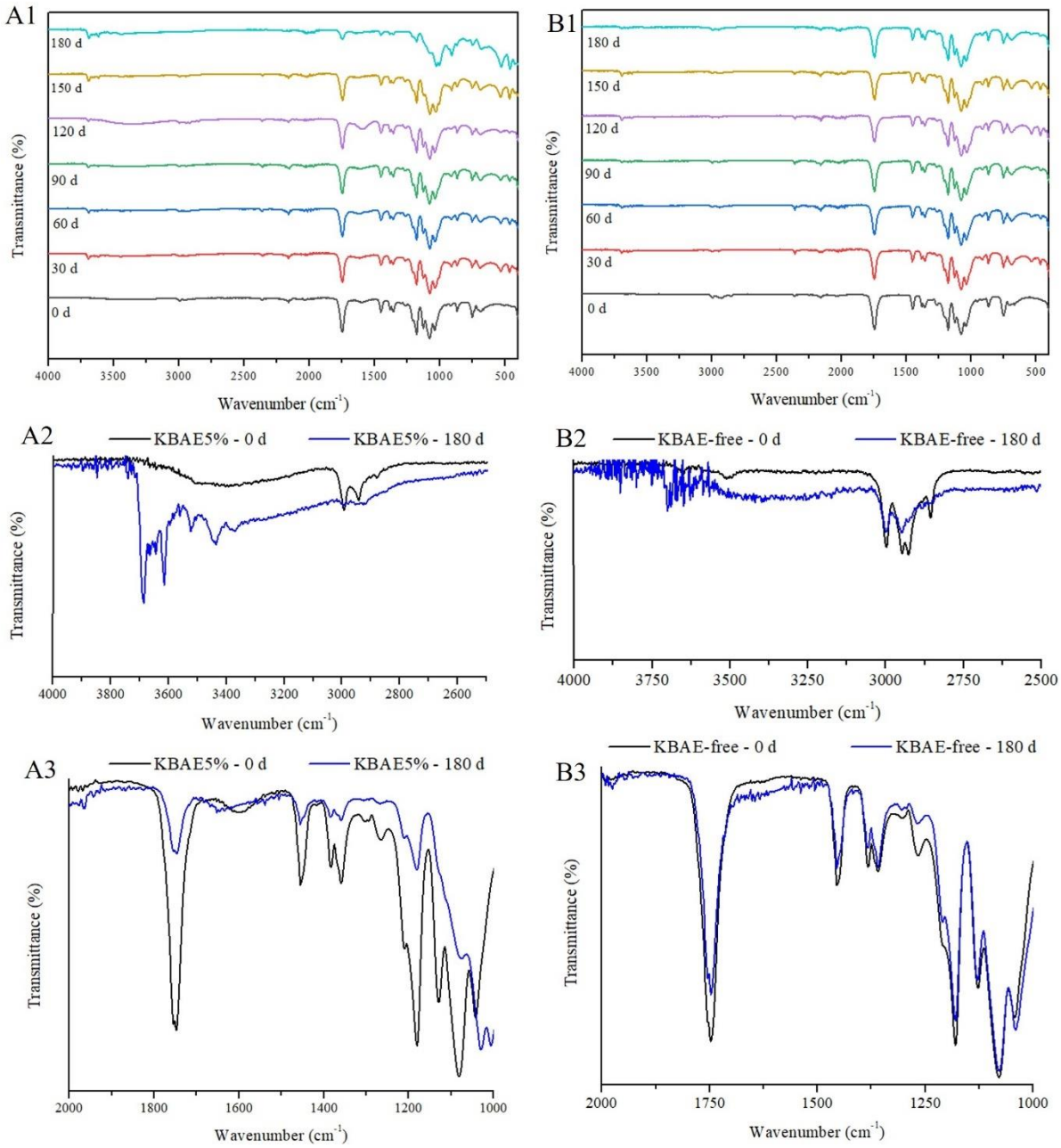

Figure 5. FTIR-ATR spectra of KBAE 5% (A1) and KBAE-free (B1) films after burial in soil for 0, 30, 60, 90, 120, 150, and 180 days. Zoom of FTIR spectra: 4000-2500 cm^{-1} region, corresponding to the O-H stretching and alkyl chain stretching in KBAE 5% (A2) and KBAE-free (B2) films, and from 2000 to 1000 cm^{-1}, focusing on the peak at 1750 cm^{-1} in KBAE 5% (A3) and KBAE-free (B3).

As observed in Figures 5A2 and 5B2, the increase in intensity at the 3600-3400 cm^{-1} band can be related to the lactic acid derived from PLA breakdown (i.e., through hydrolysis), allowing the hydroxyl groups to participate in hydrogen bonds, a behavior also encountered by

Rodrigues et al. (2015) [22]. These changes can be observed for both sheets, but in a less intensity for the control one. Furthermore, considering KBAE5% before the degradation trial (0 d), this region is occupied by a single band attributed to O–H groups stretching vibration in KBAE, as mentioned in our previous work [10]. After 180 days, some outstanding peaks (ca. 3700 cm^{-1}, 3600 cm^{-1}, 3550 cm^{-1}, 3430 cm^{-1}, and 3390 cm^{-1}) were also only when KBAE was incorporated into the polymeric matrices. This observation is another indicative of higher degradation of KBAE5% since more hydroxyl groups were exposed to interact through hydrogen bonding.

The decreased intensity in the 1185 and 1215 cm^{-1} doublet, assigned to symmetric C–O–C stretching modes of ester groups [23], herein observed for both treatments (KBAE-free being way less affected), was also found in previous studies for PLA films maintained in soil and is consistent with the formation of lactic acid [22]. In addition, as seen for both PLA sheets, changes in peaks at 3300-2800 cm^{-1} (C-H stretching) suggested that the initial long alkyl chains in the polymer have been broken [13] (Figures 5 A2, B2). Again, these changes are more intense for the KBAE5% sheet, with the appearance of an undefined band projected two peaks at 3000 cm^{-1} and 2940 cm^{-1}; meanwhile, for KBAE-free, an intensity decrease was found for 3000 cm^{-1}, 2950 cm^{-1}, 2935 cm^{-1} peaks and the disappearance of the peak at 2875 cm^{-1}.

Changes in peaks intensity at 1750 cm^{-1} (strong stretching vibration of C=O groups) verified in Figures 5 A3 and B3 were also identified by Chuensangjun et al. (2013) [12], Lv et al. (2017) [13], Rodrigues et al. (2015) [22], Rudnik and Briassoulis (2011) [4] when studying degradation of PLA, pure or blended with other polymers, in soil. This behavior can indicate that the polymer degradation occurred mainly in the ester group, resulting in the breaking down of the PLA long molecular chain in lower molecular weight PLA [22,24]. Other changes in FTIR spectra comprehend the decrease in intensity of peaks around 1450 cm^{-1}, 1390 cm^{-1} and 1350 cm^{-1} for KBAE5% sheets (Figure 5A3), representing bending of CH_2 and CH_3 (i.e., alkanes), in parallel with subtle (ca. 1450 cm^{-1} and 1325 cm^{-1}) or no changes (ca. 1320 cm^{-1}) for similar peaks in KBAE-free spectra.

Furthermore, after the 180-day burial trial, a decrease in the intensity was verified for two peaks around 1080 cm^{-1} and 1130 cm^{-1} exclusively for KBAE5%, corresponding to asymmetric C–O–C stretching of ester groups and CH_3 bending, respectively [23,25,26]. These changes in FTIR spectra are indicative of the cleavage of the PLA ester structure due to hydrolysis during the degradation of the polymer.

It is important to mention that the observed alterations in FTIR-ATR spectra are mainly reserved for the sheets' surface, considering the low penetration of IR through ATR associated with the considerable thickness of PLA sheets (>300 μm) [10]. In this sense, as changes in

FTIR-ATR spectra (especially at concerning the ester structure) were observed solely or in a higher intensity for KBAE5%, the findings obtained for TGA analysis are reinforced: with KBAE incorporation, the hydrolytic degradation mainly occurred on the surface of the materials, as the neat PLA sheet was affected by bulk erosion. This information is corroborated by the Figure 5B3, in which no further changes can be observed for peaks at approximately 1080 cm^{-1}, 1130 cm^{-1}, 1185 cm^{-1} and 1215 cm^{-1}.

Furthermore, the characteristic band at around 1650-1550 cm^{-1} referent to the stretching of the C=O bond present at hop β-acids molecules [27], and observed at KBAE5% FTIR-ATR spectra, was weakened after the 180-day period, corroborating with information that the KBAE was degraded or lost during the trial. After burial, this band was dislocated to approximately 1750-1600 cm^{-1}, which can be attributed to the formation of degradation products from hop β-acids (hulupones) [28]. These degradation structures can be formed through an oxidative process, mainly involving the double bonds of the prenyl side chains in the molecule. Therefore, the changes in FTIR spectra which can be explained by the formation of new carbonyl and carboxyl groups with the abstraction of the hydrogen [29,30]. Similar results pointed out the presence of broad and weak bands ca. 1650 cm^{-1} related to PLA oxidation after soil burial [30].

Another hypothesis would consider the peaks highlighted between 3700 cm^{-1} and 3390 cm^{-1}. As discussed, these outstanding peaks appeared only for KBAE5% after the burial period, not evidenced on KBAE-free spectra. The above mentioned peaks and the broad band in 1750-1600 cm^{-1}, representing the N-H bending, indicate the presence of amides, possibly concerning an interaction between the exposed hydrogen from β-acids molecules and the soil nitrogen [31].

4.3.4. XRD

XRD is a highly suitable method for monitoring polymer degradation through changes in crystallinity index. The XRD diffractograms of KBAE-added and KBAE-free sheets during the burial trial can be found in Supplementary Material (Figure S2). Figure 6A presents XRD patterns of the PLA control and active sheets with KBAE at initial (0-day) and final (180-day) time points. The main diffraction peaks associated with PLA at 2θ = 16.8°, 18.9°, and 22.3° were identified, corresponding to the (200)/(110), (203)/(113), and (015) planes, respectively, which were used to determine the crystalline fraction of the sheets [10,32–34]. For the control sheet, it was also identified a diffraction peak around 9.4º before the burial assay (0 d) [10]. In Figure 6B, it is possible to confirm that the PLA sheets present a semi crystalline character, and the control one (KBAE-free) exhibited the highest degree of crystallinity (ca. 58.77%) before the burial test. As supported by previous results [10], the incorporation of KBAE promoted a

decrease in the polymer's crystallinity, which remained at an average of 35.18 ± 0.69% among the active sheets.

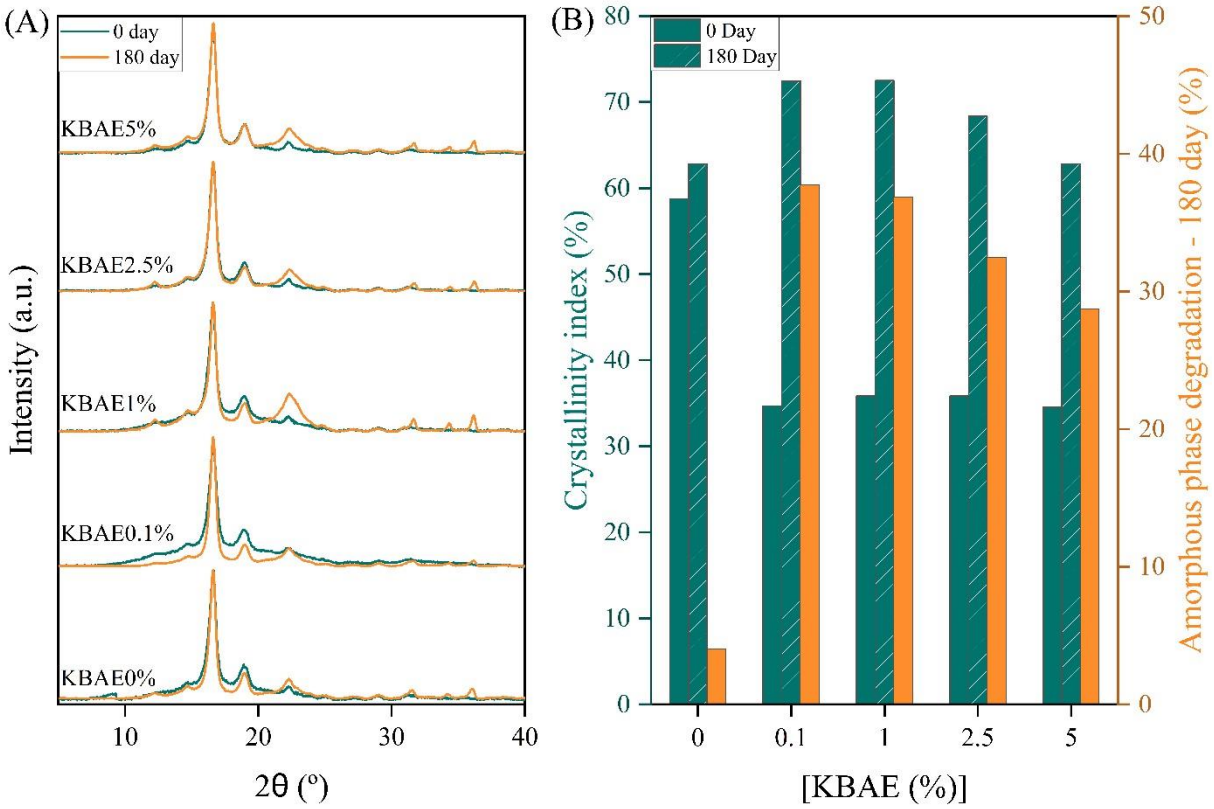

Figure 6. XRD curves of PLA sheets containing 0, 0.1, 1, 2.5, and 5% KBAE (A). The crystallinity index of the sheets at the initial time (0 d) and after degradation (180 d) (B).

After 180 days, all sheets evidenced an increase in the crystallinity index, followed by an amorphous phase degradation (Figure 6B). This effect was already expected as, during the PLA's hydrolysis, the chain cleavage reactions are firstly favored among amorphous regions, with an observed increase in the material crystallinity [8].

Nevertheless, PLA sheets with the lowest concentration of hop extract (KBAE0.1%) exhibited the highest degradation of the amorphous phase (ca. 36.8%). This observation can be attributed to both its higher percentage of initial amorphous phase and the probable lower protection of β-acids against bulk hydrolysis.

These results reinforce the findings presented in the previous Sections, whereas the presence of β-acids inside of the polymer chains of the active sheets can interfere with the diffusion of the water molecules and, therefore, suppress the hydrolytic process occurring at the inner

layers of the material. Consequently, the hydrolytic degradation that the amorphous phase during the burial time decreases proportionally accordingly with the concentration of KBAE added to the sheet. On the other hand, the inferior degradation of the amorphous phase demonstrated by KBAE-free can be assigned to the initially lower amorphous phase content. As mentioned, the PLA hydrolysis begins in amorphous regions, subsequently followed by ester bond cleavage and continues along the crystalline boundary [8]. It is important to emphasize that, despite the possible protective behavior presented by the hydrophobic β-acids against bulk hydrolysis, as discussed in Section 3.2, the active sheets mostly evidenced surface erosion, especially concerning the outer presence of propylene glycol.

4.4. Conclusion

The incorporation of hop β-acids extract did not interfere with the mass loss rate of PLA sheets during the 180-day burial in soil trial. Therefore, concerning the parameters coordinated (e.g., natural light incidence, water) and the soil used in the present study, no information regarding microbial degradation can be implied. It was herein considered that hydrolytically degradation was the main process during the burial assay. Nevertheless, the results indicated that the presence of the hydrophobic molecules from the extract restricted the hydrolytic degradation of the material, possibly by limiting the water diffusion through the polymer matrix. In this sense, meanwhile the PLA control sheet evidenced a bulk hydrolysis, within the degradation process beginning inner to the material; the active PLA sheets were more prone to surface erosion, also supported by the outer presence of propylene glycol (i.e., hydrophilic character).

FTIR-ATR spectra evidenced changes in the polymer structure, suggesting material degradation, especially for the sheets incorporated with the highest KBAE concentration. TGA findings indicated an increase at thermal stability of KBAE5%, which can be induced by increase on polymer crystallinity after the burial trial (i.e., as a result of the amorphous phase degradation), as supported by DRX diffractograms. DRX investigation was also useful to confirm that the bulk degradation was limited proportionally accordingly with the incorporation of KBAE, reinforcing the hypothesis of surface hydrolysis being the main degradation mechanism occurring for KBAE-added PLA sheets.

The study of degradation of active plastics is highly necessary, especially concerning the development of novel sustainable active packaging. These findings help to clarify the modifications that occurred during soil degradation of PLA control and active sheets, demonstrating

how the incorporation of a commercial hop-derived antimicrobial extract can interfere with the polymer degradation.

4.5. References

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4.6. Supplementary Material

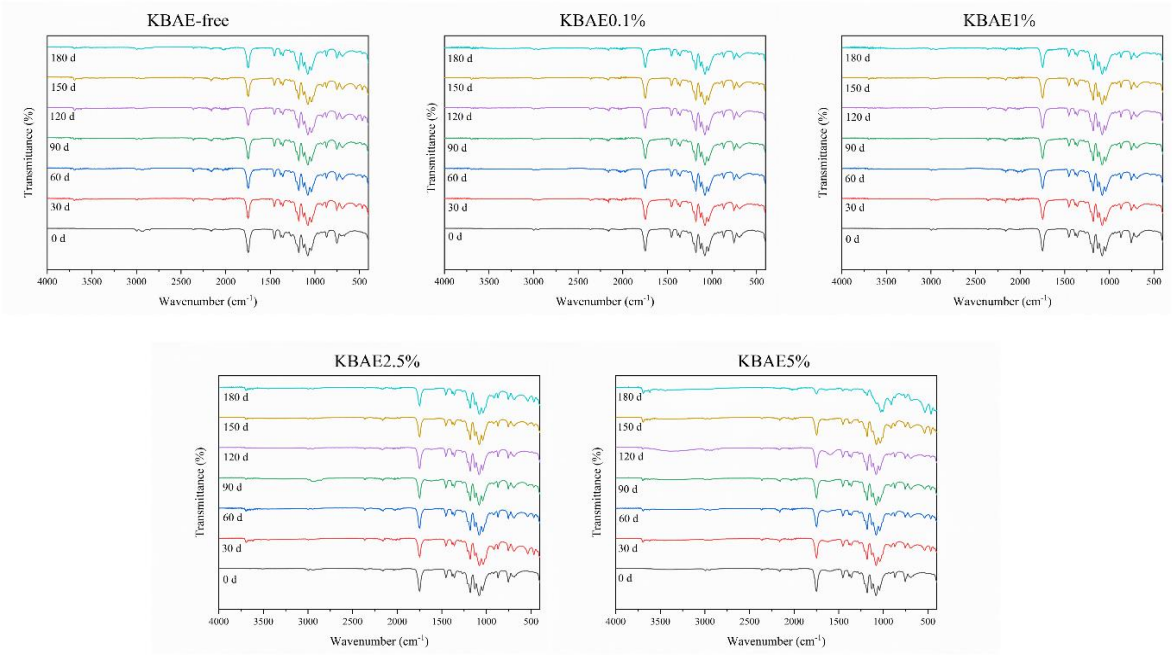

Figure S1. FTIR spectra of poly(lactic acid) sheets incorporated with 0% (KBAE-free), 0.1% (KBAE0.1%), 1% (KBAE1%), 2.5% (KBAE2.5%) and 5% (KBAE5%) of KBAE after burial in soil for 0, 30, 60, 90, 120, 150 and 180 days.

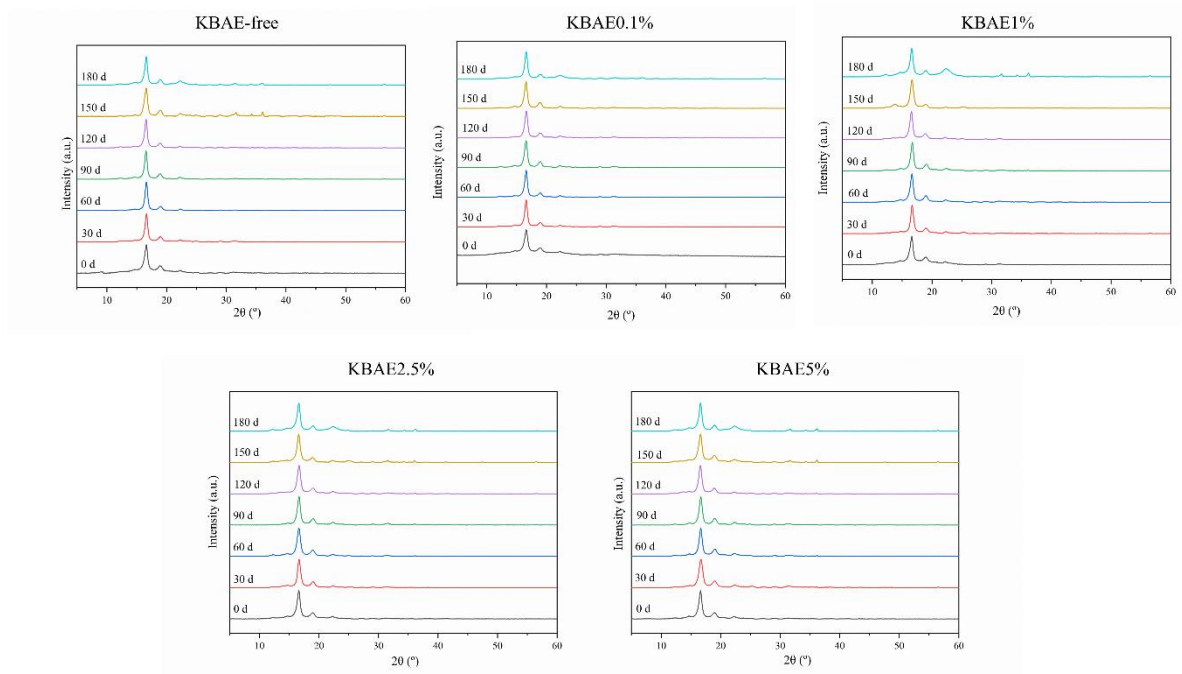

Figure S2. DRX Diffracttograms of poly(lactic acid) sheets incorporated with 0% (KBAE-free), 0.1% (KBAE0.1%), 1% (KBAE1%), 2.5% (KBAE2.5%) and 5% (KBAE5%) of KBAE after burial in soil for 0, 30, 60, 90, 120, 150 and 180 days.

5. ARTICLE 4: β-CYCLODEXTRIN *versus* HYDROXYPROPYL-β-CYCLODEXTRIN: IS INCLUSION COMPLEXATION A SUITABLE ALTERNATIVE TO IMPROVE THE PROPERTIES OF HOP-DERIVED β-ACIDS?

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5.1. Introduction

Several natural substances have been widely studied as possible ingredients to food and active packaging industry. Hop (*Humulus lupulus* L.) derived β-acids are one of them. These hydrophobic "Generally Recognized as Safe" (GRAS) compounds are important antimicrobial agents, with high activity against Gram-positive bacteria, also presenting some interesting antioxidant prospects. Recently, hop β-acids bioactivity have gained great attention and their properties have been studied beyond their traditional use in brewing industry, being applied directly to the food matrix (Kramer et al., 2021), or as a bioactive component in active food packaging (Arruda et al., 2023).

Nevertheless, hop β-acids present some limitations, such as unpleasant flavor and brownish color, along with significant instability in the presence of oxygen and susceptibility to oxidative reactions (i.e., products of oxidative deterioration generated are characterized by intense bitterness and reduced bactericidal and bacteriostatic effect) (Krofta & Mikyska, 2014). In addition, their high hydrophobicity can interfere with possible applications, especially concerning the release to/incorporation in food matrices. In this sense, commercial extracts are available comprehending β-acids in their alkali metal salt form (e.g., sodium, potassium) in hygroscopic propylene glycol (1,1-propanediol), aiming to provide a more soluble and stable presentation of hop β-acids (Wilson et al., 2003). However, these extracts are viscous liquids with intense brownish color and a strong flavor, which can interfere with food and food packaging applications. Furthermore, up to date, no data is available concerning their stability against external factors such as oxygen and temperature. To solve these problems, strategies such as encapsulation or complexation techniques can be applied using host molecules as, for example, cyclodextrins (CD).

CD are cyclic oligosaccharides formed by glucose subunits linked through α-1,4-glycosidic bonds, with a bucket-like shape. Some characteristics such as wide availability, biocom-

patibility and biodegradability have made CD valuable components in several commercial applications such as pharmaceutics, biomedicine, food (as additive), and food packaging. The ones directly obtained by enzymatic transformation of starch are described as natural CD, and their cone shapes can comprise 6, 7 or 8 glucopyranose units, named as α, β and γCD, respectively (Hădărugă et al., 2019).

Their configuration comprehends a hydrophobic inner cavity and a hydrophilic exterior, allowing the entrapment of nonpolar and geometrically compatible substances. This host:guest interaction results on the formation of inclusion complexes (IC), providing to the guest molecule some novel properties such as improved stability and solubility, also enabling their controlled release (Saffarionpour, 2019).

Among the CD used as host molecules for inclusion complexation, β-cyclodextrin (βCD) has been widely studied and applied (i.e., among the three native types, βCD is the most employed) (Arruda et al., 2021). The βCD's properties such as great ratio price and benefit, good entrapment efficiency, GRAS status, and cavity size capable to entrap several molecules are the main reasons for the growing industrial interest in this compound (Hădărugă et al., 2019). However, due to its low solubility in water (18.5 mg/mL at 25 °C) (Marques, 2010), various derivatives molecules were developed in order to circumvent this limitation. Among them, 2-hydroxypropyl-β-cyclodextrin (HPβCD) is one of the most investigated to be employed as a bioactive compounds carrier, mainly due to its high water solubility (>600 mg/mL at 25 °C), and bioavailability, also being recognized as a toxicologically benign derivative (Arruda et al., 2021; Hădărugă et al., 2019).

In this context, IC formation can be considered an alternative to minimize the drawbacks presented by hop β-acids (Gu & Liu, 2020), enabling a great range of host:guest possible combinations (including hop β-acids and CD derivatives). However, some questions about these systems remain unanswered. For example, are all CD appropriate for the improvement of β-acids applicability? Furthermore, is inclusion complexation suitable to transform a viscous commercial hop extract into a stable powdered active ingredient?

Therefore, the aim of this study was to investigate and compare βCD and its derivative HPβCD as host molecules as carriers to a commercial β-acid rich hop extract through IC formation. Besides, it was investigated the interaction between CD and β-acids, and their influence on the stability, bioactivity, and release properties of the obtained IC in a simulating food system.

5.2. Material and Methods

5.2.1. Material

β-acids rich hop extract (BetaBio45, Hopsteiner, Germany) (βHE) was kindly donated by Wallerstein Indl e Coml Ltda (São Paulo, Brazil). According to the manufacturer, βHE presents a pH of 10.7 ± 1.0 and density of 1.07 ± 0.03 g/mL at 20 ºC. In addition, βHE's composition comprehends 45.0% ± 2.5% w/w potassium salts of β-acids (mainly colupulone, lupulone and adlupulone), 22.5% ± 12.5% w/w food grade propylene glycol, 1.0–2.0 % w/w hop oils and corresponding residual moisture. For IC formation, β-cyclodextrin (97% purity degree, Sigma Aldrich, United States) and 2-hydroxypropyl-β-cyclodextrin (97% purity degree, Acros-OrganIC, Belgium) were used without further purification. The chemicals 99.8% ethanol (Neon Comercial, Brazil), 99.8% methanol (Merck, Germany), 2,2-diphenyl-1-picrylhydrazy (DPPH) (Sigma Aldrich, United States), and 2,2-azino-di-(3-ethyl-benzothialozine-sulphonic acid (ABTS) (Sigma Aldrich, United States) were also used during analysis.

Listeria monocytogenes ATCC 15313, *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 11229, *Pseudomonas aeruginosa* ATCC 15442, and *Bacillus cereus* ATCC 14579 used in the antimicrobial assays were obtained from the culture collection of the Laboratory of Food Packaging, Federal University of Viçosa.

5.2.2. Preparation of inclusion complexes (IC)

IC were prepared through the kneading method, according to a methodology adapted from Marques et al. (2019). 204.4 mg of βHE were macerated (through pestle and mortar aid) with 2 g of HPβCD or 1.85 g of β-CD (Gu & Liu, 2020) for 5 min. Then, 2.0 and 1.85 mL of ethanol was added to the systems containing HPβCD and βCD, respectively (1:1 m/v, for CD: solvent) (Marques et al., 2019). The mixtures were macerated for another 40 min after the addition of solvent. The IC (HPβCD:βHE and βCD:βHE) obtained were kept in a desiccator, under vacuum, at 7 ºC ± 2 ºC for 48 h. Finally, they were stored in amber bottles and packed in nylon polyethylene plastic bags, under vacuum, at -20 °C ± 2 ºC. For comparison purposes, the respective physical mixtures (PM) were also prepared, by briefly mixing CD and hop extract at the proportions used for IC preparation (PM HPβCD:βHE and PM βCD:βHE).

5.2.3. Entrapment efficiency (EE%) and loading efficiency (LE%)

The amounts of βHE in the IC of βCD and HPβCD was determined as described by Hill et al. (2013) with modifications. Initially, 50 mg of each IC was dispersed, separately, in 50 mL

of methanol. The suspensions were kept under magnetic stirring at 500 rpm, for 48 h at 25 °C. Then, they were centrifuged at 3200 x g for 15 min (model 4K-15; Sigma, Germany) and the supernatants were separated to determine the absorption at 354 nm in a UV-Vis spectrophotometer (model UV1800; Shimadzu, Japan). For quantification, a calibration curve was prepared. The percentage of βHE entrapped in IC was calculated using Equation 1, in which βHE_R and βHE_T comprehends the real and the theoretical amounts of βHE entrapped in the IC (g), respectively:

$$EE\% = \frac{\beta HE_R}{\beta HE_T} \times 100$$

The loading efficiency (LE%), representing the mass of compound as a percentage of the total mass of the delivery system, was also calculated as follows in Equation 2:

$$LE\% = \frac{\beta HE_R}{IC_T} \times 100$$

In which IC_T represents the total amount of the inclusion complexes (g).

5.2.4. Dynamic light scattering (DLS)

Using the DLS technique, size, the polydispersity index (PDI), and the zeta potential (ZP) of IC were measured. The samples were diluted in type 1 water (pH 6.0, Millipore Corporation), up to a concentration of 1 mg/mL, and analyzed in Zetasizer Nano ZS (Zen 3600, Malvern Instruments, United Kingdom) with a folded capillary cell (DTS 1070 cell) equipped with two electrodes, at 25 ºC.

5.2.5. Fourier-transform infrared spectroscopy (FTIR)

FTIR spectra were obtained for βCD, HPβCD, βHE and their respective PM and IC, using an FTIR spectrometer, model 660-IR (Varian Inc., United States). Attenuated Total Reflectance (ATR) spectra of the samples were obtained in the 4000–400 cm^{-1} region, using 16 scans and 4 cm^{-1} resolution.

5.2.6. X-ray Diffraction (XRD)

The X-ray patterns of IC manufactured from βCD or HPβCD, and their respective PM were measured using a D8 diffractometer (Bruker AXS, Germany), Cu-Kα radiation (λ = 0.1514 nm) in a 2θ range from 5 º to 60 º, with a scanning rate of $0.05º.s^{-1}$.

5.2.7. Thermogravimetric analysis (TGA)

TGA was performed with a thermogravimetric analyzer (Model DTG-60H, Shimadzu, Japan) under a nitrogen atmosphere (50 mL/min). Approximately 3 mg of each sample was weighed and heated from 25 ºC to 450 ºC, at a rate of 10 ºC/min. This analysis was also performed with βHE as well as IC and PM, respectively.

5.2.8. Scanning electron microscopy (SEM)

SEM images were acquired by a scanning electron Tabletop Microscope (model TM3000, Hitachi High-Technologies, Japan) using a secondary electron detector and operating under a low vacuum. Uncoated samples (βCD and HPβCD and their respective IC and PMs) were attached to the stubs' surface with a double-sided carbon tape aid, and an accelerating voltage of 15 kV was used.

5.2.9. Investigation of IC stability

βHE stability, as a pure extract and in IC with CD, was evaluated in dependency of the following factors: presence/absence of oxygen and temperature (Gu & Liu, 2020). In order to evaluate the oxidation stability of the complexes, the samples were placed individually in vials under two conditions (at 25 °C ± 2 ºC and in absence of light): packaged under vacuum in nylon polyethylene plastic bags, and opened under an atmospheric environment, aiming to simulate a conventional storage situation. Thermostability, in turn, was investigated under three different temperatures: 5 ± 2 ºC, 25 ± 2 ºC (room temperature) and 40 ± 2 ºC. Regarding thermostability, the vials were kept properly closed, avoiding contact with atmospheric air. The analysis was conducted for 3 months, with absorbance a periodically absorption measurement according to the following time interval: 0, 1, 2, 7, 15, 30, 45, 60, and 90 days. For all treatments, the samples were dissolved in distilled water, in the proportion of 14 µg/mL (calculated as a function of the concentration of β-acids) (Gu & Liu, 2020), and their absorbance was read in a UV-Vis spectrophotometer (model UV 1800, Shimadzu, Japan), at 354 nm. A standard curve was used to quantify the amount of β acids throughout the analysis and the results were expressed as a percentage of remaining β-acids (% w/w) (Gu & Liu, 2020).

5.2.10. Release test

The βHE release from IC was investigated *in vitro* according to the methodology described by Babaoglu et al. (2017). The release media used was a 23.75% v/v ethanol solution, as a simulant for meat products (fresh, chilled, salted, smoked or processed meats) (BRASIL, 2010). Approximately 0.1 g of the IC were placed, separately, with 1 mL of the simulant inside a high molecular weight dialysis membrane (14 kDa; Sigma Aldrich, Germany). The membranes were immersed in 135 mL of the simulant, and the systems were kept under agitation on a shaking table (Solab Científica, Brazil) at 200 rpm (25 ºC ± 2 ºC). Aliquots (1 mL) were periodically removed and had their absorbance measured at 354 nm in a spectrophotometer UV-Vis (model UV 1800, Shimadzu, Japan). After the measurement, the aliquots were returned to the respective systems to ensure the maintenance of analysis conditions. The *in vitro* release kinetics were monitored for 7 days. A calibration curve for β-acids was used to quantify the active component released from the complexes, and the results were expressed in cumulative release (% w/w).

5.2.11. Assessment of IC bioactivity

5.2.11.1. Antioxidant activity

The antioxidant activity of IC was determined by ABTS and DPPH radicals, as described by Re et al. (1999) and Brand-Williams et al. (1995), respectively, with modifications. Before the antioxidant assays, the βHE and IC were diluted in 99.8% ethanol up to a concentration of 45 ppm (Arruda et al., 2023). The absorbance of the solutions containing the radicals was adjusted to 0.700 (± 0.05), at 517 nm (using a 1 mmol/L DPPH solution) and 734 nm (using an ABTS solution, prepared from the mixture of 7 mmol/L ABTS with 2.45 mmol/L $K_2S_2O_8$, 1:1 v/v). For each assay, 3.5 mL of the respective radical solution was added to 0.5 mL of each dispersion (βCD:βHE, HPβCD:βHE and βHE). The absorbance was measured using a spectrophotometer (model UV 1800, Shimadzu, Japan), after 6 min at 734 nm, and 30 min at 517 nm for ABTS and DPPH analysis, respectively. The ABTS/DPPH radical-scavenging activity (%) was calculated as follows:

$$ABTS/DPPH(\%) = \frac{A_{control} - A_{sample}}{A_{control}}$$

In which $A_{control}$ is the absorbance of the adjusted radical solution without samples, and A_{sample} corresponds to the absorbance of radical added with the extraction solution after the corresponding time (Arruda et al., 2023).

5.2.11.2. Antimicrobial activity against Gram-positive and Gram-negative bacteria

Antibacterial activity of the obtained IC was investigated against Gram-positive (*L. monocytogenes, B. cereus* and *S. aureus*) and Gram-negative (*E. coli* and *P. aeruginosa*) bacteria, using the agar dilution method (CLSI, 2012), with modifications.

For inoculum preparation, bacterial strains were activated twice in brain heart infusion broth (BHI, Kasvi, Italy), incubated at 37 ± 2 °C, for 24 h, streaked over plate count agar (PCA, Oxoid, England), and incubated again under the same conditions. Selected colonies were taken from the plate and suspended in 0.85% (w/v) saline solution until obtaining a visual suspension turbidity similar to 0.5 McFarland standard (c.a. 10^8 CFU/mL) (Probac, Brazil).

The samples (HPβCD:βHE and βCD:βHE) were dispersed in 10 mL of liquefied BHI agar (approximately 45 °C), resulting in final concentrations ranging from 8.79 µg/mL to 11,250 µg/mL. Pure βHE was also tested using prepared dilutions in distilled water to achieve the same final concentrations of β-acids as evaluated for IC. The dispersions were subsequently poured into Petri dishes with a diameter of 60 mm. Pure BHI agar and BHI agar containing the maximum concentration of pure CD were considered as negative controls. After the agar solidification, 20 µL aliquots of the inoculum were dispensed onto the agar surface. The final bacterial concentration was approximately 10^4 CFU/mg per spot (Marques et al., 2022). The plates were incubated at 37 ºC ± 2 ºC for 24 h. The Minimal Inhibitory Concentration (MIC) was determined based on the lowest antimicrobial concentration capable of inhibiting the growth of microorganisms under the studied conditions.

5.2.12. Statistical analysis

The results were subjected to analysis of variance (ANOVA) followed by Tukey's test or linear regression ($p < 0.05$), when suitable. The software SigmaPlot 14.0 (Systat®) was used for the preparation of diagrams.

5.3. Results and Discussion

5.3.1. Characterization of the obtained IC

5.3.1.1. Entrapment and loading efficiencies

Figure 1 presents the physical appearance of the obtained inclusion complexes and enable a comparison with the pure βHE. As mentioned, one of the biggest drawbacks for incorporation of βHE in food/packaging industry is the intense brown feature that it presents. In addition, the high viscosity can also interfere with its manipulation and applicability, especially in dry foods. It is noticeable that the brownish color originally presented by βHE was attenuated when in the IC form (Figure 1). Both IC were presented as dry powders with a white aspect (derived from the CD). Therefore, the obtaining of a whitish fine powder may represent the broadening of application prospects for a commercial available hop extract. Further evaluation of the sensorial impact of IC's incorporation as active additives in food matrices and packaging materials are further encouraged.

Figure 1. Physical appearance of the pure βHE and the obtained inclusion complexes βCD:βHE and HPβCD:βHE.

The kneading was an efficient method for IC formation of both CD and β-acids, in which the entrapment efficiency calculated was 94.65% and 93.32% for βCD:βHE and HPβCD:βHE, respectively. These values are quite interesting considering that the ICs were obtained from a mixture of the active components in a given hygroscopic carrier (i.e., propylene glycol). Similar results were obtained by Lu et al. (2019), during the characterization of 2-O-methyl-β-cyclodextrin inclusion complex containing a derivative of hop β-acids (hexahydro-β-acids). The authors found an EE% of approximately 95%. It is important to emphasize, however, that in the mentioned study, the ICs were obtained from isolated and purified guest compounds. In parallel, the loading efficiencies for βCD:βHE and HPβCD:βHE were 5.34% and 4.40%, respectively. These last values represent the amount of drug loaded per unit weight of the entire

particle (IC), indicating the percentage of mass of the IC that is due to the entrapped guest compounds (hop β-acids).

Despite propylene glycol being the major component of βHE, since is used as a vehicle/solvent for the active compounds, Harada et al. (1995) mentioned in their study that βCD were not able to form IC with propylene glycol, except with its high molecular weight analogues. In fact, propylene glycol has been mentioned as a potential non-aqueous solvent for hydrophobic drugs, suggesting the presence of a synergy between non-aqueous solubility and inclusion complexation (Chang & Shojaei, 2004). Therefore, it is plausible to believe that propylene glycol was mainly directed to the hydrophilic outer surface of CD, and not entrapped into the cavity as expected to βHE's bioactive (i.e., hop β-acids).

5.3.1.2. Size, zeta potential and PDI characterization

The size, zeta potential, and PDI of IC particles were evaluated by DLS analysis. βCD:βHE had an average diameter of 187.3 nm (± 4.2 nm), a zeta potential of -59.2 mV (± 2.3 mV), and PDI of 0.136 (± 0.025). In turn, HPβCD:βHE particles presented a similar diameter of 184.1 nm (± 33.6 nm), along with similar zeta potential (-56.8 ± 4.2 mV) and PDI (0.171 ± 0.071) (t-Student test, $p > 0.05$). The outcomes evidenced that both complexes were of nanometric size with a homogeneous distribution and highly negatively charged. PDI values lower than 0.300 indicated that the system had a narrow size distribution, which is desirable for system stability. In addition, absolute zeta potential values higher than 30 mV (in modulus) are also desirable since the existence of repulsive forces hinders the aggregation of the particles, ensuring a good dispersion (Bi et al., 2021).

However, it is important to state that CD and their IC undergo through self-association to form aggregates or micelle-like structures. This process is responsible for the development of self-assembled structures that exhibits sizes of ca. 200–300 nm (González et al., 2002). Therefore, despite βCD and HPβCD presenting significantly different structures, the IC aggregation property was responsible for agglomerates with similar size and, consequently, comparable PDI values, as it is a measurement of the heterogeneity of a sample based on size (Mudalige et al., 2019). In general, the self-assembly of CD and IC occur due to intramolecular hydrogen bonds, and the substitution the βCD's hydroxyl groups (e.g., with methyl- or hydroxypropyl groups) results in significant decrease in the aggregate size (Coleman et al., 1992). However, with the IC formation, and the possible presence of propylene glycol and non-complexed bioactive components forming hydrogen bonds with the hydroxyl groups at the rim of

the CD cavity, the aggregation properties of each CD molecule must be affected, with a possible decrease in the self-assembly properties of βCD.

Due to its lower aqueous solubility, βCD has a high tendency for agglomerates formation. Nevertheless, it can be significantly affected after inclusion complexation. For example, the critical agglomeration concentration (cac, defined as the lowest CD concentration at which aggregates can be detected under some specific conditions) of natural βCD in pure aqueous solutions and ambient temperature is about 8 mg/mL (i.e. the lowest cac value among the natural CD) (Muankaew et al., 2020). After inclusion complex formation between βCD, polyethylene glycol and guanosine, the cac for the IC changed for about 4.018 µg/mL (J. Li et al., 2020). Alterations in aggregation tendency after inclusion complexation are governed by many factors (e.g., host and guest molecules, concentrations employed) and, therefore, are specific for each IC.

A study conducted by Rodrigues et al. (2019) investigated the inclusion complexation of benzimidazole-2-carbamates (albendazole and fenbendazole) with βCD and HPβCD. To illustrate the effects of self-assembly of ICs in the size of the particles, the authors found that systems obtained with βCD were formed by nanoclusters with a mean hydrodynamic radius of 188.6 ± 11.7 nm, meanwhile for the systems obtained with HPβCD, CD aggregates (180.9 ± 4.5 nm) and CD monomers (1.4 ± 0.3 nm) were observed. Therefore, due to this property, in aqueous solution, distinct IC manufactured with different CD can results in systems with similar particles size and/or PDI.

5.3.1.3. FTIR

To better understand the interactions between βHE and the studied CD, FTIR analysis was conducted, and the obtained spectra are displayed in Figure 2. βHE and both βCD and HPβCD spectra exhibited a broad band around 3600-3000 cm^{-1}, related to the O-H groups stretching vibrations, naturally present in the molecules as well as in the βHE solvent (propylene glycol). Small peaks at around 2920 cm^{-1}, related to C-H stretching, were also in common among these three samples, as well as the sharp peaks around 1045-1020 cm^{-1}, probably due to presence of C-O group (Arruda et al., 2023; Gu & Liu, 2020; Marques et al., 2019).

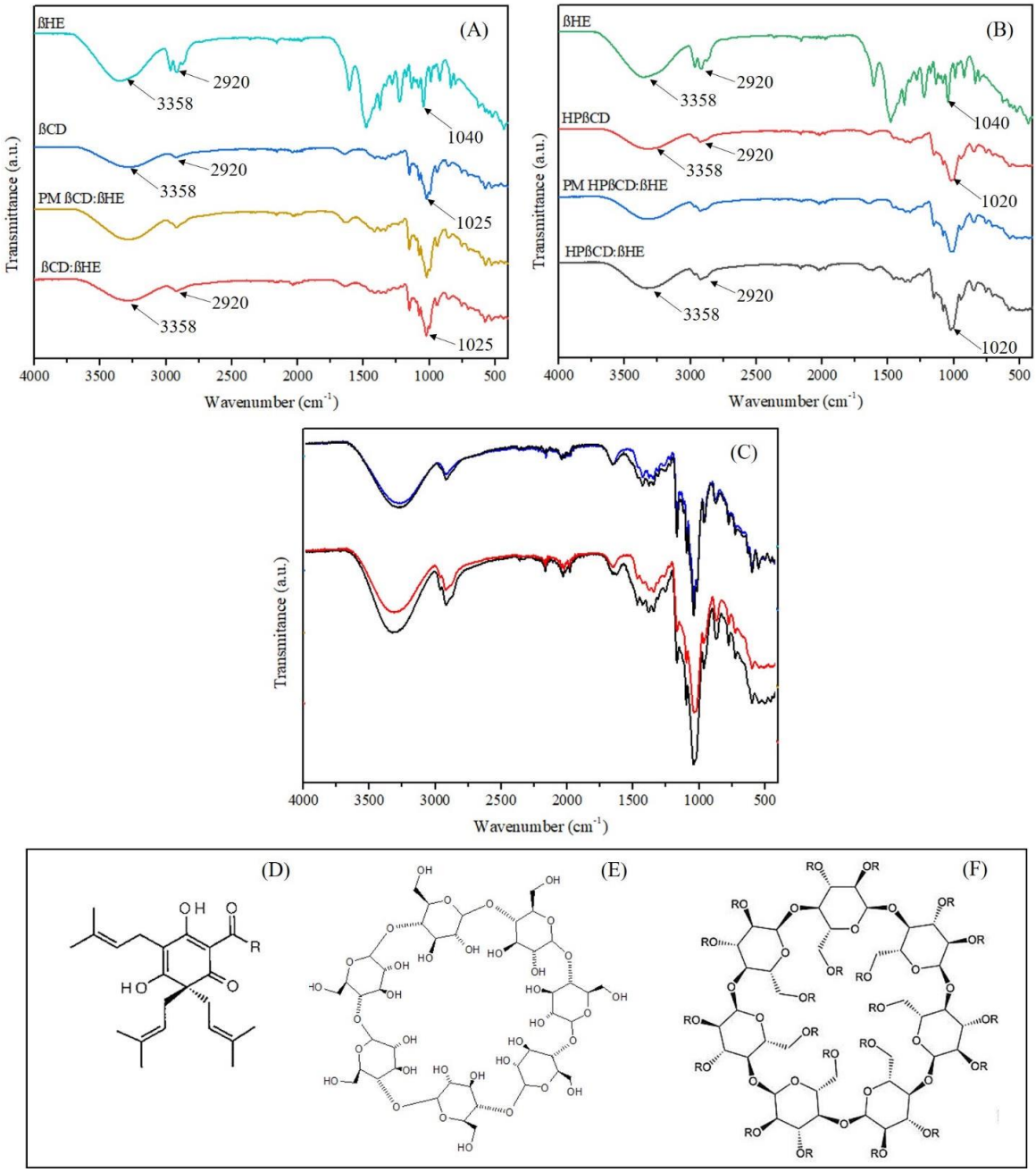

Figure 2. (A) ATR-FTIR spectra of β-acids rich hop extract (βHE), β-cyclodextrin (βCD), their physical mixture (PM), and their inclusion complex (βCD:βHE). (B) ATR-FTIR spectra of βHE, hydroxypropyl-β-cyclodextrin (HPβCD), their physical mixture (PM), and their inclusion complex (HPβCD:βHE). (C) Overlap of FTIR spectra of βCD (blue) and HPβCD (red) and their respective IC (both in black). Molecular structures of (D) β-acids, (E) βCD, and (F) HPβCD (ACD/ChemSketch 2017.2.1).

Complexation of βHE with the CD, resulting in βCD:βHE and HPβCD:βHE, led to narrow changes in FTIR spectra when compared with the pristine CD, mainly concerning an

increase of intensity, as verified more intensively for the HPβCD:βHE sample (Figure 2C), suggesting a stronger interaction between the molecules in this system. Changes in IC FTIR spectra, such as displacement and/or broadening of peaks, are often related to the host:guest interactions that occur during the complexation process (Gu & Liu, 2020). In addition, the absence of new bands in IC spectra indicates that no new chemical bonds were developed between βHE components and the CD, suggesting that the interactions occurred mainly via hydrogen bonds and the Van der Waals force. Similar behavior was found by Lu et al. (2019) during the characterization of 2-O-methyl-β-cyclodextrin IC containing hexahydro-β-acids.

The presence of hydroxypropyl groups in the HPβCD molecule (Figure 2F) can favor the occurrence of propylene glycol (i.e., a highly hygroscopic solvent) outer of HPβCD:βHE. Moreover, it is also possible to observe that the basic structure of β-acids contains a hydrophobic and a smaller hydrophilic region (Figures 2D, 2E, and 2F). During the complexation, the preference is for the entrapment of guest molecules (β-acids and other lipophilic components) into the host molecule cavity (βCD and HPβCD). However, due to their hydrophilic domain, an interaction of hop β-acids with the CD external face can also take place (i.e., associative complex). The feasible presence of these components on CD outer surfaces also justifies the observed results.

5.3.1.4. XRD

XRD is another valuable technique for investigating the complexation between CD and other molecules (Marques et al., 2019). The X-ray diffraction patterns obtained for the solid samples are displayed in Figure 3.

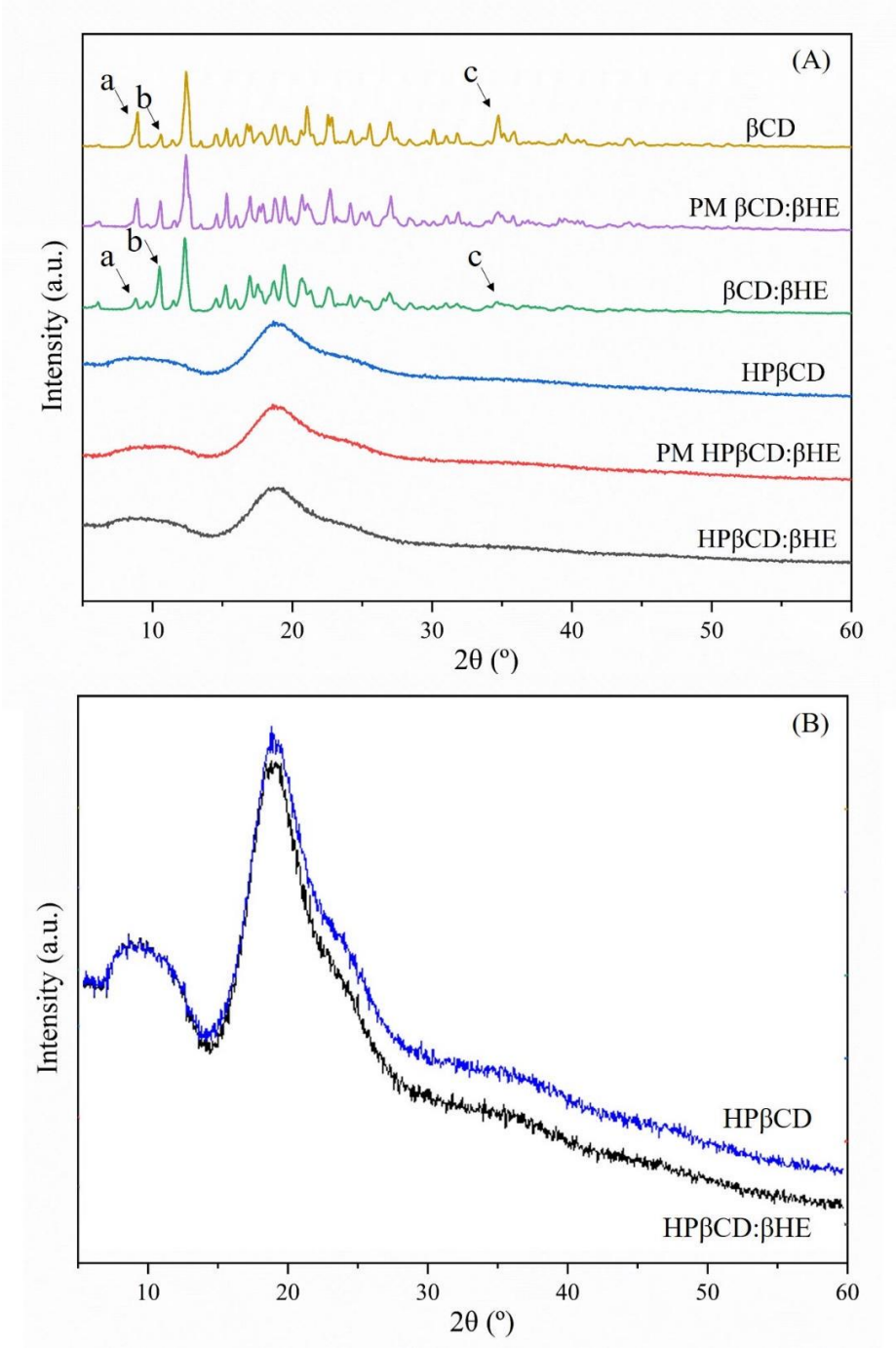

Figure 3. (A) X-ray diffraction patterns of β-cyclodextrin (βCD) and hydroxypropyl-β-cyclodextrin (HPβCD), their respective physical mixture with β-acids rich hop extract (βHE) (PM), and their inclusion complexes (βCD:βHE and HPβCD:βHE). Letters "a", "b", and "c" indicates peaks at 2θ = 8.90, 10.50, and 34.70º, respectively. (B) Zoom of HPβCD and HPβCD:βHE diffraction patterns.

The pristine CD showed distinct X-ray diffraction patterns: while βCD presented narrow peaks at 2θ (around 8.90, 12.40, 21.05, 22.55, 26.95, 34.70º), which is characteristic for

crystalline materials, the HPβCD pattern was of an amorphous one (Narayanan et al., 2017; Marques et al., 2019; Mendes et al., 2023). The complexation with βHE changed the pattern of the βCD sample, resulting in a diffractogram with a different profile. In Figure 3A, it is possible to compare the patterns of βCD and βCD:βHE, and verify a reduction in the intensity of peaks at 8.90º and 34.70º (marked with arrows) as well as broadening of peaks, such as the one at 10.50º, corroborating with the hypothesis that the interactions between the components occurred and the IC was successfully formed.

As stated before, the HPβCD diffractogram, in turn, exhibited broad bands, characteristic for amorphous materials. Besides even with the wide band's presence in IC diffractrogram, a slight narrowing around 25º (Figure 3B) was observed when compared with the pristine compound, corroborating with the formation of IC and reorganization of the molecules during their interaction (Mendes et al., 2023). The same narrowing in XRD pattern was not observed for the respective PM (not shown in the Figure).

As stated by Deng et al. (2016), the simple overlapping of native patterns would represent that the inclusion complexation was not achieved. This was confirmed by another study of IC formation for HPβCD and β-acids (Gu & Liu, 2020). Therefore, despite the changes in the amorphous XRD pattern not being that obvious, the maintenance of the amorphous structure observed for HPβCD:βHE, provides strong evidences of inclusion complexation between the molecules.

5.3.1.5. TGA

The thermogravimetric (TG) herewith their respective derivative (DTG) curves are presented in Figure 4. TG and DTG graphs for βCD:βHE (Figures 4A and 4B, respectively) demonstrated that the thermal degradation of this IC occurred in three main steps: the first one on the range of 25-90 ºC (ca. 8.7%), representing the disappearance of water moisture associated with βCD (Reddy et al., 2020) and βHE, along with the degradation of volatile compounds; the second one between 150-240 ºC (ca. 2.5%), which can be attributed to volatile compounds weakly bonded on CD outer surface and non-volatile compounds present in βHE (e.g., oils); and the final stage, comprehending 255-365 ºC (ca. 67.1%) that corresponds to the thermal degradation of βCD and the majority of βHE constituents. The DTG curve for this last stage (Figure 4B) evidenced that the principal degradation (ca. 328 ºC) occurred at a similar temperature than the evidenced to the pristine βCD, but also in a broader temperature range (possible overlapping of CD and bioactives degradation curves). This behavior reinforces the successful occurrence of inclusion complexation between βHE and βCD. It also infers that some βHE

compounds were bonded to the inner and outer surfaces of the IC, which may directly impacts the bioactive, stability and release behavior of the obtained molecule (to be discussed in the following Sections).

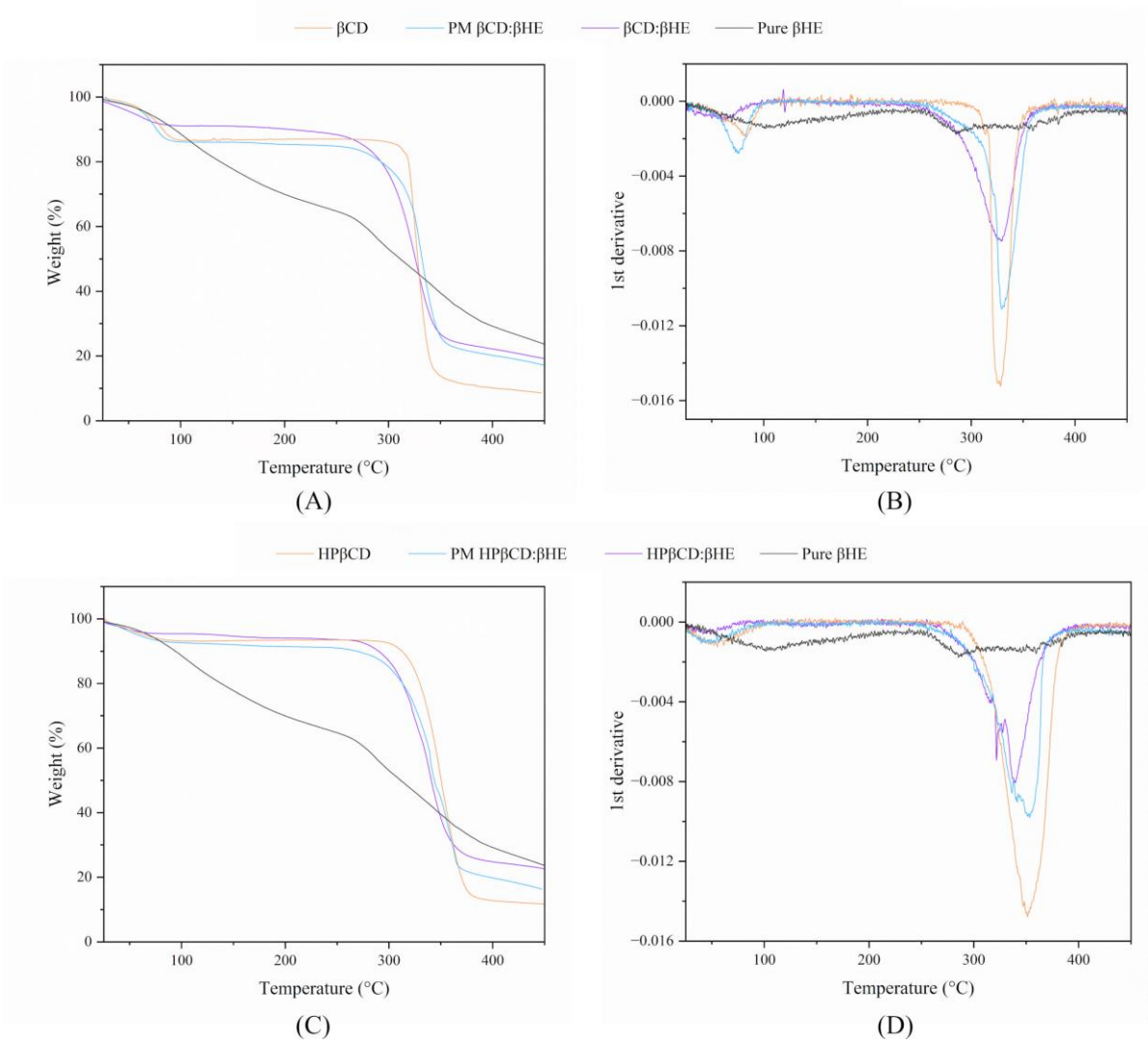

Figure 4. Thermogravimetric (TG) (A and C) and respective 1st derivative curves (DTG) (B and D) of: βCD, βHE and their PM and IC (βCD:βHE) (A and B); HPβCD, βHE and their PM and IC (HPβCD:βHE) (C and D).

On the other hand, the thermal degradation of HPβCD:βHE occurred with a different profile (Figures 4C and 4D). Within the DTG graph for this inclusion complex, it is possible to observe six distinct mass loss stages: the degradation started between 25-68 ºC, with water evaporation and degradation of volatile compounds present in βHE; later, between 113-180 ºC a subtle degradation occurred, with approximately 2% of mass loss; and finally, four distinct peaks were overlapped in the range of 315 ºC to 339 ºC (ca. 315 ºC, 321.5 ºC, 326.5 ºC and 339

ºC). These peaks indicated that the components were degraded separately, in a small temperature range, which can be attributed to βHE components that were interacting with the outer surface of HPβCD along with the major thermal degradation of the IC components. As demonstrated by FTIR analysis, the chemical structure of HPβCD favors the bonding of hydrophilic compounds (e.g., propylene glycol) with the outer surface of the molecule.

These results showed that complexation might have improved the thermal stability of the guest compounds, especially concerning the heat susceptibility of hop β-acids (Gu & Liu, 2020). The thermal degradation profile presented by the pure βHE evidenced a constant mass loss during the analysis, in which the mass loss peaks are overlapped, and three major peaks can be distinguished (Figures 4B and 4D): 105 ºC, 255ºC and 359 ºC, attributed to the mass losses of water and bioactive compounds, propylene glycol and carbonaceous matter, respectively.

It is important to emphasize the difference among the TG curves in each scenario. In both cases, the TG curves for IC evidenced a shift and a decrease on the first small peak that mainly corresponds to the evaporation of water (in comparison to the pristine CD), which can be attributed to the redistribution and reduction of water molecules and the water concentration, respectively. The phenomenon of substitution of water molecules primarily binding to the CD by an appropriate hydrophobic host molecule is the principal “driving force” for complex formation (Kringel et al., 2017). Which can also occurs for other remaining polar solvents used for inclusion complexation (e.g., ethanol). Furthermore, the occurrence of a decrease in the thermal stability of both obtained molecules indicates that changes occurred in the original structure of pristine CD, confirming that the complex formation was achieved (Reddy et al., 2020).

Therefore, the results herein obtained describe important prospects to hop bioactive compounds, as one of the main obstacles to their industrial application is their high thermal susceptibility. Demonstrating that it is possible to obtain a thermal stable bioactive product based on β-acids can significantly increase their introduction in industrial sectors other than brewing. Recent studies also evidenced that the inclusion complexation might expand the thermal stability of guest compounds. For example, Reddy et al. (2020) found that the IC formation between a catechin-rich green tea extract and βCD lead to an improvement of thermal stability of the bioactive host compounds. At the mentioned study, the pure extract exhibited a major weight loss at around 130 ºC, which was mainly assigned to the thermal decomposition of polyphenols; meanwhile, the IC complex evidenced a main thermal decomposition at approximately 265 ºC.

5.3.1.6. SEM

Aiming to evaluate possible alterations on molecules' morphology during inclusion complexation, micrographs of CD, PM and IC were taken through SEM (Figure 5). The differences between the images are noticeable. βCD and HPβCD are morphologically distinct, in which the first one is characterized mainly for block and the second for and spherical structures. The images obtained for PM indicated a morphology quite similar to the pristine CD, due to the higher ratio of CD in IC preparation. However, after inclusion complexation, IC presented irregular morphologies distinguishable from the CD and PM, along with size differences between the two structures (corroborated by DLS results). These results confirm the formation of a novel solid phase, further indicating that the complex formation was successfully achieved (Gu & Liu, 2020; Lu et al., 2019).

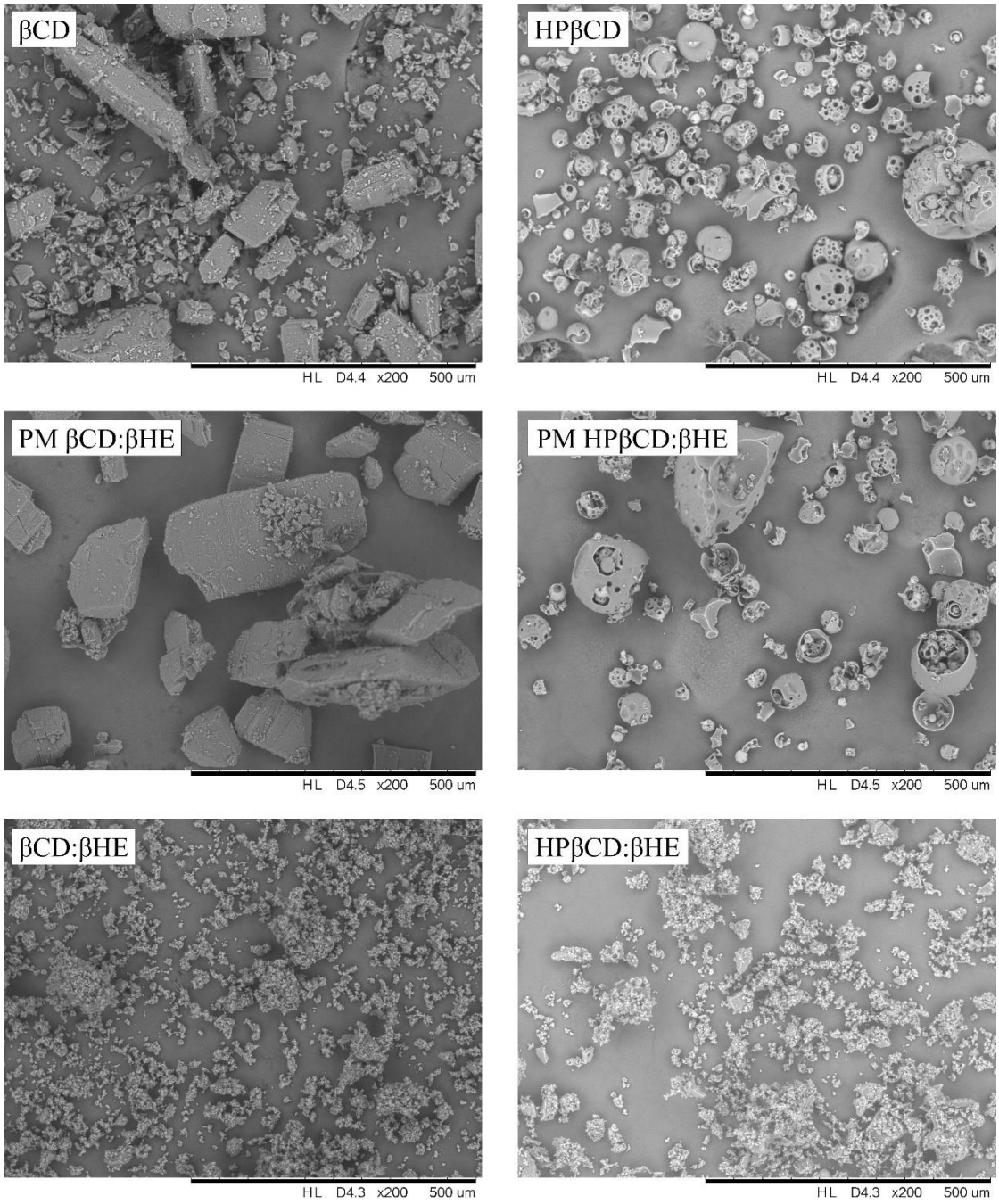

Figure 5. Scanning Electron micrographs of pristine βCD and HPβCD and its respective physical mixtures (PM) and inclusion complexes (βCD:βHE and HPβCD:βHE).

βCD:βHE and HPβCD:βHE exhibited small size particles with the tendency to cumulate. In both images, it is possible to observe the aggregation of the particles, a pattern also evidenced for other IC systems described in the literature (Arya & Raghav, 2021). Despite the Zeta potential values favoring the existence of dispersive forces to avoid aggregation of particles, agglomerates can be formed due possible H-bonding of the peripheral hydroxyl groups of

CD or propylene glycol present on their outer surface (Arya & Raghav, 2021). Prior DLS analysis, the IC were suspended in distilled water and mechanically agitated (vortex), which may resulted in segregation of the IC and retention of segregation (due to IC' considerably high Zeta potential), whereas during SEM methodology they were analyzed in powder form.

5.3.2. Release into meat simulant

The release profile of the β-acids was investigated before evaluation of the impact of CD structures on the antimicrobial and antioxidant activity. A controlled study was conducted to simulate compound migration into meat products, using conditions that closely resemble those in the food industry (Figure S1, Supplementary Material) (BRASIL, 2010). Despite previous works showing that pure βCD and HPβCD solutions do not present UV absorption between 200-500 nm (Deng et al., 2018; Gu & Liu, 2020), the UV-Vis spectra of their respective IC formed are similar to those of the pure active compounds (guest molecules) in solution, which could be responsible to interfere in the quantification of the released molecules from the respective host substances. Therefore, a dialysis membrane was employed to assist the methodology, avoiding an over quantification, as the non-released β-acids could interfere on the UV absorption, misleading the results. The membrane was responsible to retain the IC and pure cyclodextrins inside (due to the size of the molecules), allowing the migration of the released active compounds (i.e., β-acids) to the external environment through osmosis until reaching equilibrium (Mendes et al., 2023).

Both IC exhibited a typical release pattern characterized by an initial rapid release, i.e., burst effect, followed by a sustained release plateau. The sustained release plateau suggests strong host:guest interactions within the CD cavity (Ren et al., 2018). Notably, IC based on βCD demonstrated a more pronounced initial release of the bioactive compound (up to 10% of β-acids within the first 24 h), indicating a higher content of these substances on the external surface of βCD:βHE, in comparison to HPβCD:βHE. Therefore, the difference in the CD host structures clearly influenced the release behavior of βHE, as supported by the fitting of different non-linear models ($p < 0.05$) presented in Table S1 (Supplementary Material).

βCD is poorly soluble in ethanol and water (Zhu et al., 2019), meanwhile HPβCD evidences higher water solubility and is also more prone to dissolution in ethanol (Szejtli, 1992), both components of the simulated solution employed for the *in vitro* release investigation (23.75% ethanolic solution). Based on this, a solvation effect would be expected for HPβCD:βHE preceding to rapid release in the simulated media. In parallel, in theory, a slower release with lower burst effect would be presented by βCD:βHE, as insignificant matrix effect

(e.g., swelling and dissolution) would happen. However, the differences in binding ambiguities among both studied CD with βHE as discussed in previous Sections have proven to exerted significant influence on the release of the guest compounds. The chemical differences among βCD and its derivative HPβCD imparted not only in a higher presence of β-acids on the external surface of βCD, but also may have affected on how each CD interacted with βHE. As a direct consequence for these differences, important changes (e.g., release into food simulant) were triggered for the same guest compounds complexed with distinct CDs.

Hop β-acids are highly hydrophobic molecules and, therefore, their release in a hygroscopic media is remarkably low (Arruda et al., 2023). Their solubility is lower than 0.002 mg/mL when pH is greater than 7.0 in the beer matrix (Gu & Liu, 2020) and only 0.0085 mg/mL at 100 °C and pH of 5.6 in water (Krofta & Mikyška, 2014). Nevertheless, despite the lower solubility of βCD in comparison with HPβCD, the inclusion complexation with both CD represents an interesting increase in water solubility of the hop resins, improving their applicability range, especially in the food field.

Considering a release profile of active compounds from IC, both factors must be pointed out: the amount of active compounds attached to the CD outer surface *versus* the amount entrapped into their cavities, and also, the type and intensity of interactions between host and guest compounds. The intermolecular interactions that may occur during the IC formation are electrostatic interactions, weak van der Waals forces, hydrogen bonds with secondary and/or primary rims, and hydrophobic interactions with the cavity and the nonpolar groups or π electrons of the guest (Raffaini & Ganazzoli, 2020). The adsorption of host compounds on CD outer surfaces mostly occurs through relatively weak interactions, in a process called associative complex (Dailey et al., 1993). Thus, the higher amount of bioactives externally attached to the IC may also provide a faster profile release within the first hours of trial (as suggested for βCD:βHE).

The release rate of bioactive compounds from the film into the food must be specifically controlled to be in accordance with the growth rate of the target microorganisms and the oxidation rate of the target lipid compounds (Nogueira et al., 2020). Considering food packaging systems (but also inferring for food additives), a rapid initial release of bioactive compounds into the food is desired for products with a higher natural microbiological charge and/or did not pass through intense processing (high temperatures, freezing, moisture removal) and therefore, are more prone to spoilage. The released bioactive compounds may inhibit/retard the growth of microorganisms and control/prevent oxidation of lipids and other compounds as these food

systems possesses a fast-spoiling rate. Therefore, an IC with a more intense initial release may be adequate for foods with an expected smaller shelf life (e.g., βCD:βHE).

On the other hand, when the migration rate of a bioactive component is faster than the growth rate of target microorganisms or oxidation process, this active agent will be depleted before the expected storage period. Consequently, in terms of active packaging, the packaging system will lose its activity and promote microorganism's growth and/or lipid oxidation (Nogueira et al., 2020). A steady and controlled release is mostly benefic for foods with a higher shelf life, as the bioactives will contribute to maintenance of food quality for longer periods acting against spoiling and oxidation. It may be the case for HPβCD:βHE applications.

The present results provide evidence that is possible to obtain a sustainable release of the hop β-acids for food/food packaging applications. The cumulative release of these hop compounds after inclusion complexation continued increasing for over 48 h in both CD samples, meanwhile other studies verified that the total release of other bioactive substances might occur within the first 24 h.

It was the case of tea polyphenols and sulfobutyl ether βCD inclusion complexes, that presented a high cumulative release of tea polyphenols, but over 90% of the release occurred within the first 10 h of *in vitro* evaluation on water, and buffer solutions (pH 1.2, 4.0 and 6.8), with a rapid and significate release between 0 and 3 h (N. Li et al., 2023). Another study suggested that curcumin was released from its inclusion complex with βCD over a period of 5h in a sustained release manner (Arya & Raghav, 2021). As mentioned, a steady long-term release can implicate in a controlled antibacterial/antioxidant activity and, consequently, represent a strong potential for the extension of shelf life of the food product, as demonstrated by Dai et al. (2022) in a study with polyethylene oxide/casein nanofibers loaded with thymol:βCD inclusion complexes in beef preservation.

5.3.3. Stability against heat and O_2

The stability of β-acids against heat and oxygen is crucial for improving the shelf life, processing versatility, and bioactivity of their products. In order to comprehensively investigate these effects across different host systems, the thermal and oxidative stability of pure βHE, HPβCD:βHE, and βCD:βHE were systematically evaluated (Figure 6). Based on the findings, it is evident that pure βHE exhibited the highest mass loss, reaching up to 72% when stored at 40 °C, and 82% when exposed to oxygen for 90 days at room temperature (ca. 25 ºC) (Figure 6F). These results indicate that β-acids are more susceptible to oxidation than temperature ef-

fects up to 40 °C, consistent with prior research by Krofta et al. (2013). Furthermore, this susceptibility is further underscored by the fact that 80% of βHE β-acids are lost under aerobic conditions within the first 45 days of storage.

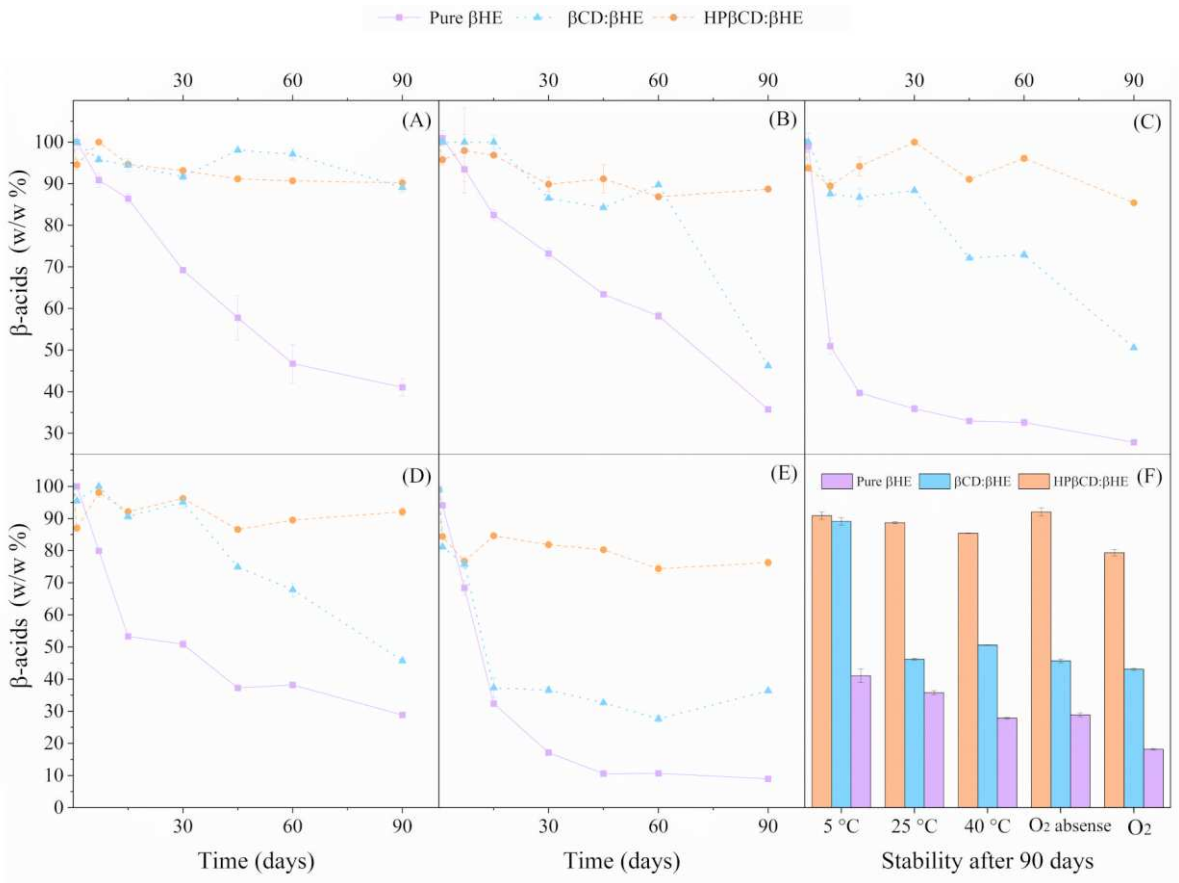

Figure 6. Thermostability of pure βHE, HPβCD:βHE and βCD:βHE at temperatures of (A) 5 °C, (B) 25 °C or (C) 40 °C. Oxidative stability of Pure:βHE, HPβCD:βHE and βCD:βHE at room temperature (25 °C) under (D) absence of atmospheric oxygen or (E) with access to oxygen. (F) Bar graph of temperature and oxygen stability after 90 days. All samples were kept in the dark.

The observed changes in β-acids content appears to be closely associated with their oxidation, as these substances are highly prone against oxygen. The increase in storage temperature also affects the rate of oxidative reaction, but does not essentially change the oxidation products (Krofta & Mikyška, 2014). These oxidation products are mainly characterized by strong bitterness and attenuated bioactive properties, with hulupons being the main products of β-acids oxidation under air conditions. Luputrions, lupoxes, lupdoxes, lupdols, lupdeps are also

mentioned as oxidized β-acids degradation products. These derivatives are formed by cyclization and epoxidation of isopentenyl side chains while maintaining full six-membered aromatic ring (Krofta & Mikyška, 2014).

In contrast, results shown that significant protection was imparted through inclusion complexation in all conditions evaluated, especially considering the HPβCD:βHE (Figure 6). This represents an important achievement in terms of stability of hop β-acids. As mentioned, β-acids present a very poor oxidative stability, and even the employment of very low temperatures (-20 ºC) cannot interrupt the oxidative reactions (De Almeida et al., 2012).

It is, however, noticeable the differences among the protective effect displayed by each CD, with HPβCD:βHE exhibiting higher thermal and oxidative stability in comparison to the βCD-based IC. For example, after 90 days, HPβCD:βHE achieved protection levels of up to 85% at 40 °C and 92% under vacuum (at 25 °C), while βCD:βHE reached only 50% and 45%, respectively (Figure 6F). A possible hypothesis for this phenomenon is supported by the interactions involving propylene glycol localization. Despite being used as a solvent for IC formation, this substance can act as a competing agent during IC formation, as corroborated by Miyake et al. (1999). The substitution of hydrogen for a hydroxypropyl group in native βCD not only improved the water solubility of the native CD, but also strongly influenced the interactions between the host and guest molecule during the inclusion complexation. In fact, structural changes in CD's cavity have been proven to influence their interactions with guest substances and the surrounding solvents also affecting the intra-molecular interactions (Yong et al., 2008). In this case, as the hydroxypropyl radicals of HPβCD have a preference for interacting with hydroscopic molecules rather than hydrophobic ones (Azarbayjani et al., 2010), the binding of propylene glycol on the outer surface of the IC may be favored when employing HPβCD as host molecule.

Whereas in βCD:βHE this excipient may act more intensively as a competitor for the host cavity, with the inner interactions between βCD and the entrapped β-acids being weakened, or even a direct competition between the hydrophobic bioactives and propylene glycol for the host's cavity may occur more often. Thus, despite IC exhibiting a high and quite similar entrapment efficiency, β-acids may be attached to βCD's external surface to a greater extent, as supported by release results described in the previous section (i.e., external association).

It is important to emphasize that the cavity of CD is extended by hydroxypropyl substitution. According to previous studies, this morphological change can impart either in (i) the protection of the guest substances or (ii) the sterical hindrance of the inclusion or (iii) the possibility of the formation of new H-bonds, when the guest possesses any H-donor or H-acceptor

abilities; with these new H-bondings also influencing the stability of the inclusion complex (Gnes Buvári-Barcza & Barcza, 1999). Therefore, in addition to the differences in the release behavior previously discussed (Section 3.2), the biding ambiguities between βCD and HPβCD with βHE effectively impacted the protection and stability of the guest ligands.

On the other hand, no significant differences were observed among the thermal stability of the studied IC at low temperatures under vacuum (5 °C). This fact suggests that, at this temperature, the intermolecular interactions between βCD and β-acids were not affected, and both the outer and inner surface interactions provided protection against the bioactives loss. The temperature in this case appeared to be an important factor to βCD:βHE stability, since higher temperatures affected β-acids content during the trial, even if the loss was lower than the verified to the pure βHE. Thus, refrigerated storage would be a good option to the maintain of βCD:βHE shelf life. Similar refrigeration effects were found for storage stability of βCD:curcumin IC at -15 °C, 4 °C and 25 °C for 90 days (Mangolim et al., 2014). The color retention values of the IC were 9% better at -15 °C and 4% better at 4 °C, when compared to the pure curcumin. Meanwhile, no differences were found between IC and pure compound stored at 25 °C.

5.3.4. Bioactivity of IC

5.3.4.1. Antioxidant activity

In addition to the known antimicrobial effects, α- and β-acids have also evidenced some antioxidant activity presenting moderate anti-radical activity and interesting *in vitro* antioxidant properties (Arruda et al., 2023; Gu & Liu, 2020).

The antioxidant activity of βHE and their IC with βCD and HPβCD was assessed through ABTS and DPPH assays (Figure S2, Supplementary Material). The results indicated that no significate difference was observed between βCD:βHE and HPβCD:βHE, indicating that the type of CD did not interfere with the antioxidant potential of the bioactive samples. However, the radical scavenging activity of pure βHE was higher than for the IC against both radicals ($p < 0.05$), suggesting that the inclusion complexation reduced the overall antioxidant activity of βHE bioactives.

The amount of β-acids present in the IC is considerably low (ca. 1:20 ratio). Thus, the amount released to the media was not enough to exhibit higher radical scavenging activity. Furthermore, with the entrapment of βHE hydrophobic bioactive compounds into the CD cavity and other chemically bonded on the CD's outer surface, the bioactive release was not rapid enough to provide a fast response against the tested radicals during the reaction time (30 min).

According to Liu et al. 2019, the presence of hydroxyl groups is the main reason for β-acids antioxidant properties (Liu et al., 2019). During inclusion complexation, these groups may have interacted with CD molecules (e.g., hydrogen bonds). This could explain the lower scavenger activity. Similar results were found by Żyżelewicz et al. (2018) during the investigation of the antioxidant capacity of the complex of (+)-catechin with βCD. The authors found that the ORAC-FL assay was lower in the complexes of (+)-catechin with βCD compared with the free bioactive substances, justified by the fact that the 3-OH group responsible for the antioxidant activity of flavonoids was completely included in the host cavity, decreasing the hydrogen atom donating capacity of the inclusion complex.

5.3.4.2. Antibacterial activity

The results concerning the antimicrobial activity of βHE and IC (βCD:βHE and HPβCD:βHE) against pathogenic bacteria are displayed in Table 1. The results for βHE were expressed in terms of β-acids (the major bioactive on hop extract). The first observation clearly demonstrated higher activity against Gram-positive non-sporulated bacteria (*S. aureus* and *L. monocytogenes*). This agrees with similar results found for hop β-acids, indicating higher anti-microbial efficacy against Gram-positive bacteria (Arruda et al., 2023).

Table 1. Minimal inhibitory concentration (MIC) of pure β-acids present in rich hop extract (βHE) and obtained inclusion complexes with βCD (βCD:βHE) and HPβCD (HPβCD:βHE) against Gram-positive and Gram-negative bacteria incubated at 37 ºC/24 h.

Bacteria	MIC (µg/ml)		
	β-acids (pure βHE)	βCD:βHE	HPβCD:βHE
L. monocytogenes	<8.789[a]	31.56	70.31
S. aureus	<8.789	31.56	70.31
E. coli	11250	>11250[b]	>11250
P. aeruginosa	5625	>11250	>11250
B. cereus	11250	>11250	>11250

[a] Minimum concentration tested. [b] Maximum concentration tested.

The differences in susceptibility of Gram-negative and Gram-positive bacteria can be attributed to the different cellular structure. The outer membrane present on Gram-negative bacteria can imply the inhibition of β-acids diffusion and/or inactivation by serum phosphatides (Sacks & Humphreys, 1951; Teuber & Schmalreck, 1973). Furthermore, this outer membrane has transmembrane channels (i.e., porins) and lipopolysaccharides with polar ends that allow the passage of hydrophilic compounds while impeding the diffusion of hydrophobic molecules into the cytoplasm (Ertan et al., 2023). In this sense, despite the possible improvement of βHE hydrophobic compounds' solubility trough inclusion complexation, IC presented no enhanced activity against Gram-negative bacteria. A feasible explanation concerns the size of the porins: the porin trimers most likely contain only one large but well-defined pore with a diameter between 1.2 and 2 nm, smaller than the verified IC size (Section 3.1). On the other hand, the greater resistance of *B. cereus* against βHE could be justified by the fact that, despite belonging to the Gram-positive group, this bacterium possesses the capacity of sporulating (Arruda et al., 2023).

The MIC for βHE against *S. aureus* and *L. monocytogenes* was below the lowest concentration tested in this study (8.789 µg/mL), while βCD:βHE and HPβCD:βHE evidenced MIC of 31.56 µg/mL and 70.31 µg/mL, respectively. Even higher than those found for the pure β-acids, these values are considerably low, indicating good perspectives for application of the studied IC against foodborne Gram-positive pathogens. Comparatively, Marques et al. (2022) evaluated the activity of garlic essential oil and its IC with βCD against *S. aureus* and reported MIC of 78.12 µg/mL and 4800 µg/mL, respectively. It is important to remember that in our study, the amount of β-acids present in IC corresponded to only approximately 5% w/w of the total IC weight, thus reducing the discrepancy between the MIC values found for them. Furthermore, the lower MIC value found for βCD:βHE is probably associated with the β-acids release profile discussed previously (Section 5.3.2), as this IC exhibited a more pronounced release of the bioactive compound into the media within the first 24 h.

5.4. Conclusion

The observed results evidenced the successful inclusion complexation of βHE with both βCD and HPβCD. The IC can be characterized as nanosized molecules with a narrow size distribution in water dispersion. HPβCD nanoparticles manufactured, due to the more hydrophilic outer surface, probably favored the immobilization of propylene glycol (vehicle for β-acids in βHE) to the external face of the HPβCD:βHE. For βCD:βHE nanoparticles, the concentration

of outer propylene glycol was lower, leading to a higher competition between propylene glycol and the βHE bioactive compounds for the CD's cavity. This resulted in a weakened interaction between βCD and β-acids and a higher content of this bioactive on the outer surface of the IC. This behavior corroborated the results found for the stability test, along with the release behavior and antibacterial activity of βCD:βHE and HPβCD:βHE. In this sense, βCD:βHE evidenced a faster and higher release into the meat simulant but lower stability than HPβCD:βHE. The inclusion complexation demonstrated a promising alternative to maintain the stability of βHE against oxygen and temperature (until 40 °C). As oxygen is an important factor concerning the stability of the β-acids, HPβCD:βHE can be considered an useful ally to these bioactive compounds, helping to expand their application fields.

Both IC exhibited a certain antioxidant activity and an interesting overall antimicrobial activity against Gram-positive non-sporulated bacteria. As an overall conclusion, βCD:βHE may be a good option to perishable foods with a small shelf life (fresh sausages, pasteurized milk, cheese, fresh vegetables), since it presented faster release in the food simulant, but a lower thermal/oxidative stability. Meanwhile, HPβCD:βHE presented itself as an interesting tool food products which shelf life is intended for longer periods (dried and/or maturated foods), as this IC exhibited great storage stability and a slower release.

In a general overview, significant differences were found between the CD studied. HPβCD presented great performance for inclusion complexation of the βHE, with its structure favoring high and strong interactions between hop β-acids and the CD's cavity. As a result, slower and steady release was achieved into the meat food simulant and a protective effect was provided to β-acids' biggest villains: temperature and oxygen.

By transforming a liquid substance (βHE) into a powdered one, the IC formation demonstrated to be a suitable technology to deliver bioactive compounds even in a dry form, with improved properties (e.g., stability). This study showed that these molecules possess promising prospects in terms of food applications, with tests in real food systems being necessary to attest their functionality. It is important to emphasize that this powdered substances not only offers the possibility of being used as a natural food additive with bioactive properties, as substitutes to the synthetic ones, but also are potential candidates to be employed in active food packaging/coating. Further investigation concerning thermodynamic evaluation and molecular docking are further encouraged to a better understanding of the structures herein studied.

5.5. References

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5.6. Supplementary Material

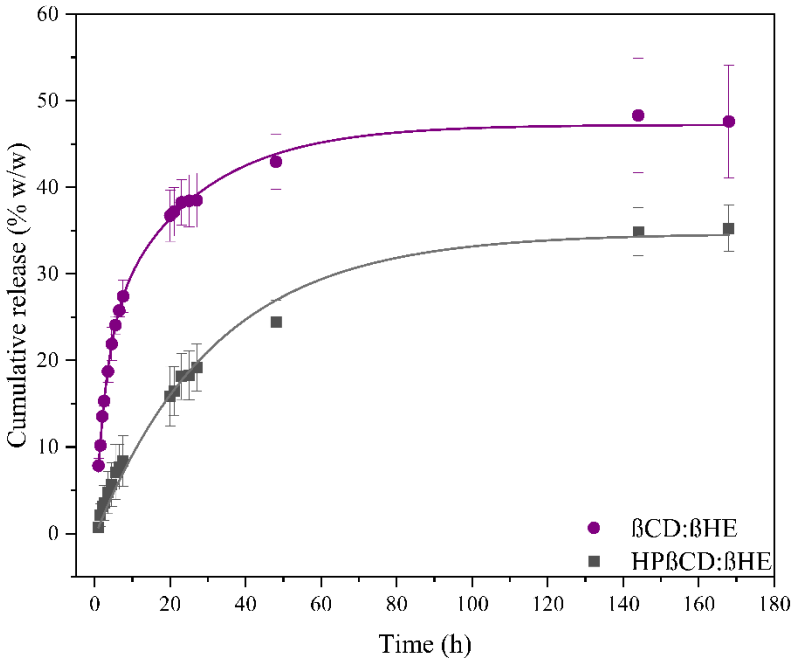

Figure S1. The cumulative release of βHE from inclusion complexes of HPβCD and βCD. Error bars represent the standard deviation.

Table S1. Adjusted models for *in vitro* release of the βHE from inclusion complexes of HPβCD and βCD. Adjusted coefficient of determination (R^2_{adj}).

Inclusion complexes	**Models**	**R^2_{adj}**
βCD:βHE	$Y_{CR} = 47.19 - 24.84e^{(-Time/24.01)} - 22.23e^{(-Time/2.92)}$	0.998
HPβCD:βHE	$Y_{CR} = 34.66 – 33.97^{\wedge}Time$	0.992

Y_{cr}: cumulative release.

6. ARTICLE 5: β-CYCLODEXTRIN AND 2-HYDROXYPROPYL-β-CYCLODEXTRIN AS CARRIERS OF HOP β-ACIDS IN THE DEVELOPMENT OF POLYLACTIC ACID-BASED ACTIVE PACKAGING FOR A READY-TO-EAT MEAT PRODUCT

This article will be submitted to the Journal *Carbohydrate Polymers*.

6.1. Introduction

Cyclodextrins (CD) are cyclic oligosaccharides consisting of α-(1,4)-linked glucopyranose units. They are physically and chemically stable compounds whose structure is ring-shaped, allowing them to entrap several guest substances, forming inclusion complexes (IC) (Liu et al., 2022). Different types of CD are currently available, derived from the three natural ones, such as α-CD, β-CD, and γ-CD, that present 6, 7, and 8 glucose units, respectively. Among these, β-CD is the most commonly employed, due to its relatively lower price and Generally Recognized as Safe (GRAS) concept (Arruda et al., 2022).

Their particular structure presents a hydrophilic exterior with a hydrophobic inner surface, and the inclusion complexes, with non-polar molecules, can provide a higher water solubility, bioavailability, and stability to the guest substance (Arruda et al., 2024; Carneiro et al., 2019). Therefore, CD are applicable in several industrial areas, including pharmaceutical, medical, and food/packaging production. In special, CD have been exceptionally explored in the food packaging industry, triggered by the development of innovative active packaging systems (Liu et al., 2022).

Active packaging is a concept that involves the interaction between the packaging material and the packaged food. In release systems, such as antimicrobial ones, bioactive substances are incorporated/attached to the barrier material and intentionally released to interact with the food matrix, in order to extend its shelf life (Yildirim et al., 2018). To meet the current market tendencies and consumer preferences, natural bioactives have been extensively employed in active packaging development, along with the usage of biodegradable polymers as substitutes for conventional fossil-based plastics (Arruda et al., 2022). Hop (*Humulus lupulus* L.) and its antimicrobial/antioxidant constituents are pertinent examples in this context (Arruda et al., 2023a; Tian et al., 2022; Tian, Xu, et al., 2021).

Hop is a well-recognized plant used in brewing. It presents several bioactive compounds including phenols, essential oils, and bitter acids (i.e., α- and β-acids) (Carbone & Gervasi, 2022). Due to their high antimicrobial and antioxidant potential, hop and hop-derived constituents may offer opportunities in food and non-food applications, with increased interest as a replacement for synthetic additives and/or ingredients in food and packaging industries (Tatasciore et al., 2024; Tian et al., 2022). Moreover, some studies revealed that hop compounds may interfere with bacterial quorum sensing and adhesion, decreasing biofilm formation (Bogdanova et al., 2018; Di Lodovico et al., 2020).

Hop β-acids (also known as lupulones) and their derivatives are among the key antimicrobial substances present in the hop cones. However, despite the good prospects concerning their bioactivity, these components present some pitfalls that limit their applicability such as the high sensitivity against extrinsic factors (e.g., temperature, oxygen, light), low water solubility, and pronounced sensorial aspects (e.g., bitter taste and characteristic aroma) (Carbone & Gervasi, 2022). In this regard, complexation with cyclodextrins arises as a viable strategy to circumvent these problematics and broaden the applicability of hop β-acids (Arruda et al., 2024).

Few studies have approached the incorporation of hop β-acids in active food packaging (Arruda et al., 2023a; Chen et al., 2020; Tian et al., 2022; Tian, Wang, et al., 2021; Tian, Xu, et al., 2021), and even fewer studied the incorporation of IC based on β-acids for film production (Lu & Liu, 2020). Nevertheless, in most cases, the packaging material was developed, by associating a natural bioactive with a biodegradable polymer. Polylactic acid (PLA) is an example of a biopolymer with good prospects in active packaging development. Some outstanding features of PLA make it targeted for application at the industrial level, such as high transparency, easy processability, chemical resistance, high mechanical strength, low toxicity, and homogeneous and smooth appearance (Rojas et al., 2021).

Our previous work has already proven the benefits induced by the complexation of a commercial hop extract rich in β-acids with β-cyclodextrin (βCD) and its more water-soluble derivative, 2-hydroxypropyl-β-cyclodextrin (HPBβCD) (Arruda et al., 2024). Furthermore, some differences were observed in the properties of the two obtained IC. The present work aimed to develop and characterize PLA-based films incorporated with distinct IC prepared with a β-acids rich hop extract and two CD (βCD and HPBβCD). Both films were evaluated concerning their performance (mechanical, thermal, structural) and bioactive (antimicrobial, antioxidant) properties. The quorum quenching and antibiofilm activities were also evaluated. The

antimicrobial activity of PLA-based active films was tested *in vitro*, and *in situ* to investigate the impacts of the develop films on bologna shelf-life and *Listeria monocytogenes* growth.

6.2. Material and Methods

6.2.1. Material

Amorphous PLA LX930 with a 1.24 g/cm^3 density, 92% purity (L-isomer) (Total corbion, Netherlands), and ethyl acetate 99,5% (Chemsolute, Germany) were used to prepare the PLA films. Poly(ethylene glycol) (PEG) (Aldrich Chemistry, Belgium) was used as a plasticizer. The β-acid rich hop extract (βRHE) was kindly donated by Hopsteiner (Mainburg, Germany). According to the manufacturer, it contains 40 ± 1.5% potassium salts of β-acids, 0.5 ± 0.2% alpha acids, 1.5 ± 0.3% hop oils, 27 ± 8% propylene glycol, and corresponding residual moisture; the pH value was 11.0 ±0.5 and the density 1.07 ± 0.01 g/mL at 20 ºC. β-cyclodextrin (97% purity degree, Sigma Aldrich, United States) and 2-hydroxypropyl-β-cyclodextrin (97% purity degree, Acros-OrganIC, Belgium) were used without further purification during IC formation.

The chemicals 99.9% ethanol (Chemsolute, Germany), 99.95% methanol (Chemsolute, Germany), 2,2-diphenyl-1-picrylhydrazy (DPPH) (Sigma Aldrich, United States), crystal violet (Sigma-Aldrich, United States) and dimethyl sulfoxide (DMSO) (Merck, Germany) were also used during analysis. Tryptic soy agar (TSA) (Oxoid, UK), tryptic soy broth (TSB) (Oxoid, UK), plate count agar (PCA) (Merck, Germany), Listeria Agar (Base) (LA) acc. OTTAVIANI and AGOSTI (Merck, Germany), OCLA supplement (Oxoid, UK), Luria Bertani (LB) broth and agar (Sigma, USA), ringer solution (Oxoid, UK), nutrient broth (Merck, Germany) and Man-Rogosa-Sharpe agar (MRS) (Merck, Germany) where employed in microbiological evaluations.

Listeria monocytogenes (DSM 15675), *Staphylococcus aureus* (DSM 346), *Escherichia coli* (DSM 1576), *Bacillus subtilis* (DSM 4181), *Bacillus cereus* (DSM 345) and *Chromobacterium violaceum* (DSM 30191) were the bacterial strains tested during the antimicrobial experiments.

6.2.2. IC preparation

The IC were obtained through kneading methods. The IC employed were prepared and characterized in our previous study (Arruda et al., 2024). Approximately 233 mg and 227 mg of βRHE were respectively macerated (through pestle and mortar aid) with 2 g of HPβCD and 1.85 g of β-CD (Gu & Liu, 2020) for 5 min. Then, ethanol was added to the systems containing

HPβCD and βCD, respectively in a ratio of 1:1 m/v, for CD:solvent). The mixtures were again macerated for 40 min, until obtaining a homogeneous powder. The respective ICs from HPβCD (HPIC) and β-CD (βIC) were subsequently kept in a desiccator, under vacuum, at 7 ºC ± 2 ºC for 48 h. Finally, they were stored in amber bottles and packed in polyamide/polyethylene plastic bags, under vacuum, at 20 ºC ± 2 ºC.

6.2.3. PLA-based films elaboration

PLA-based films were prepared through casting. PLA pellets were dispersed in ethyl acetate (10% w/v) under magnetic agitation (300 rpm, 5 h, 25 ± 2 ºC). After complete dispersion of the polymer, PEG and the respective IC, βIC and HPIC, were added at 20% w/w rate regarding the polymer mass. The polymer dispersions were then homogenized in Ultra-turrax (1 min, 10500 rpm) (D-8, Miccra, Germany), degassed, and cast with the aid of a coating unit (CUF 5, Sumet Messtechnik, Germany) (20 mm/s, 30 N; drying at 40 ºC/3 min with air circulation) onto polytetrafluoroethylene plates (29 cm x 21 cm). The films were left under atmospheric conditions for 24 h to ensure the complete evaporation of the solvent, peeled from the trays, and cut before the testing.

The resulting films were PLA-βIC (prepared with βCD-based ICs) and PLA-HPIC (prepared with HPβCD-based ICs); IC-free formulation served as control (PLA-control).

6.2.4. Films characterization

6.2.4.1. Scanning Electron Microscopy (SEM)

The surface and cross-sectional area of the films were examined with a Scanning Electron Microscope (JSM-7200F Schottky Field Emission, Jeol, Japan), using a secondary electron detector and operating under a low vacuum and an accelerating voltage of 5 kV. Samples were sputtered with a golden layer and attached to the stubs' surface with a double-sided carbon tape aid.

6.2.4.2. Thickness and mechanical properties

The thickness (μm) of PLA-based films was evaluated in five different points, randomly selected, with a precision thickness gauge (FT3, Hanatek Instruments, UK), according to the DIN ISO 4593. The mechanical properties evaluation was conducted on a Universal Testing Machine (BT2-FR005TH.A50, ZwickRoell, Germany), following the DIN EN ISO 527-1/-3. Five specimens of each treatment were tested on each repetition. The tensile properties analyzed were yield strength (YS, in MPa), elongation at yield strength (EYS, in %), breaking strength

(BS, in MPa), elongation at break (EB, in %), and modulus of elasticity (Young's modulus, YM, in MPa).

6.2.4.3. Fourier Transform Infrared Spectroscopy (FTIR-ATR)

FTIR analysis was performed on a compact spectrometer model Alpha II (Bruker Corporation, USA) equipped with a platinum ATR accessory. Spectra were obtained at a resolution of 4 cm^{-1} with wavenumber ranging from 4000 to 400 cm^{-1} and 24 scans.

6.2.4.5. Thermogravimetric analysis (TGA) and Differential Scanning Calorimetry (DSC)

TGA of the PLA-based films was performed on a thermogravimetric analyzer (Model DTG-60H, Shimadzu, Japan), under a nitrogen atmosphere (50 mL/min). Samples were weighted heated to 600 ºC, at a rate of 10 ºC/min.

DSC measurements were performed with a calorimeter, model DSC 3+ (Mettler Toledo, United States). Samples were placed in aluminum crucibles and heated to 200 ºC, also under a nitrogen atmosphere (50 ml/min), at a rate of 10 ºC/min. The degree of crystallinity was determined using Equation 1 and the thermal fusion enthalpy of 100% crystalline PLA of 93.7 J $g^{-}1$ (Garlotta, 2001).

$$\%Crystallinity = \frac{(\Delta H_m - \Delta H_c)}{\Delta H_m^{\circ}} \times 100 \qquad \text{(Equation 1)}$$

In which ΔH_m° is the thermal fusion enthalpy of 100% crystalline PLA, ΔH_c the enthalpy of cold crystallization, and ΔH_m is the thermal fusion enthalpy of the developed film samples.

6.2.4.6. Visual characterization: color and optical transmittance

The colors of the PLA films were measured using the Digi Eye system (VeriVide Limited, Leicester, UK) and a Nikon D90 camera (Nikon Metrology GmbH, Düsseldorf, Germany). The International Commission on Illumination (CIE) L*a*b* method was used to measure the parameters lightness (L*), chroma (C*), hue angle (h), and yellowness. The white color from the calibration board was used as the white reference for comparison among samples. The total color difference (ΔE_{ab}^{*}) compared to the white reference board was calculated according to the Equation 2.

$$\Delta E_{ab}^{*} = \sqrt{(L_2^* - L_1^*)^2 + (a_2^* - a_1^*)^2 + (b_2^* - b_1^*)^2} \quad \text{(Equation 2)}$$

The light transmission (T%) of the films was also measured through a UV-Vis spectrophotometer (Bio-Tek Uvikon XS, Goebel Instrumentelle Analytik, Germany), with wavelengths ranging from 200 to 800 nm, with a step size of 5 nm.

6.2.4.7. Controlled release

Migration properties of the active films were evaluated according to the methodology described by Arruda et al (2023), with modifications. Two kinds of standard food simulants were used: 23.75% (v/v) and 95 % (v/v) ethanol solution, as a simulant for meat products and fatty foods, respectively (Brasil, 2010; European Union, 2011). The film samples (3 cm × 4 cm) were immersed, separately, in 50 mL of the simulating solution, under agitation (200 rpm, 20 ± 2 ºC) (C. Gerhardt Analytical Systems, Germany). An aliquot of the sample solutions was taken at intervals at first 7 h (30 min, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h) and daily on the subsequently 13 days to measure its absorbance at 354 nm, in a UV–Vis spectrophotometer. After the absorbance reading, the aliquots were returned to the simulating solution. The accumulative release percentage was calculated by the ratio of the release determined by the calibration curve (based on βHE sequentially diluted on the respective simulation solutions) and the theoretical total release.

6.2.4.8. *In vitro* bioactive properties

6.2.4.8.1. Antioxidant activity

The antioxidant activity of the PLA films was investigated through DPPH radical cation-based assays, with modifications (Arruda et al., 2023). Squared samples of each treatment (1 cm^2) were immersed in 10 mL of ethanol and stored under agitation (200 rpm, 25 ±2 ºC), in the dark, for 24 h. Subsequently, 0.5 mL extraction solution was added to 3.5 mL free radical solution (1 mmol/L) and the absorbance was measured at 517 nm after 30 min using a UV-Vis spectrophotometer. The DPPH radical-scavenging activity (%) was calculated as described in Equation 3.

$$DPPH(\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \quad \text{(Equation 3)}$$

In which $A_{control}$ is the absorbance of the adjusted radical solution, and A_{sample} corresponds to the absorbance of radical added with the extraction solution.

6.2.4.8.2. Antimicrobial activity

The antimicrobial properties of the active films were tested against Gram-positive (*L. monocytogenes*, *S. aureus*, *B. cereus,* and *B. subtilis*) and Gram-negative (*E. coli*) bacteria. *B. cereus* and *B. subtilis* spores were also used during antimicrobial investigation.

6.2.4.8.3. Agar diffusion test

Bacterial (c.a. 1.5 x 10^8 CFU/mL) and spore (c.a. 2 x 10^8 CFU/mL) suspensions were individually spread onto solidified TSA using a swab. Disk samples (Ø =10 mm) were placed at the center of the agar surface. The plates were incubated at 37 ºC/24 h. For *L. monocytogenes*, the antimicrobial activity was further investigated under refrigeration, at 7 ºC/10 d. Inhibition zones were measured and presented in mm.

6.2.4.8.4. Antimicrobial activity according to ISO 22196:2011.

To check the antibacterial properties of the films quantitatively, an evaluation was performed using the ISO 22196:2011 standard with minor modifications. *E. coli*, *L. monocytogenes,* and *S. aureus* bacterial inoculums were prepared in nutrient broth to achieve approximately 6 x 10^5 CFU/mL. The test samples (50 mm x 50 mm) were placed into Petri dishes and 0.4 mL of bacterial inoculum was dropped onto their surfaces. Then, a 40 mm × 40 mm polypropylene film was placed on the bacterial inoculum. After 0 h for the reference sample and 24 h for the reference and tested samples (incubated under 35 ºC and 95% RH), the bacteria were retrieved from the films' surfaces and placed in a SCDLP broth (used as a neutralizer). The total bacterial growth was determined by placing them in PCA. The following incubation was carried out at 37 ºC/48 h. Bacteriostatic/bactericidal rate (%) was determined by the bacterial colonies of the testing groups and the control group (Wu et al., 2021).

6.2.4.8.5. Quorum quenching activity

The biomonitoring bacterium *C. violaceum* was used to evaluate the quorum quenching properties of PLA films. The qualitative analysis was carried out as described by Luís et al. (2020), with modifications. *C. violaceum* was incubated under aerobic conditions in LB broth at 30 ºC/18 h, with shaking at 250 rpm, to achieve an inoculum within a range of 1.5 x 10^8 CFU/mL (Alvarez et al., 2014). The inoculum was spread onto LB agar plates using a swab.

The circular-shaped films (Ø = 6 mm) were placed on inoculated plates and then incubated at 30 ºC/24 h. The quorum quenching activity was determined by measurement of the prevention of violacein production within the violacein inhibition halo, without staining, but with viable cells, in mm.

A quantitative assay was also performed according to Silva et al. (2016). The inoculum (1.5 x 10^8 UFC/mL) was prepared as described previously. The film samples (4 cm x 2 cm) were added into tubes containing 10 mL of LB broth, and the system was subsequently added with a 100 µL inoculum. The inoculated tubes were incubated at 30 ºC/24 h. Then, 1 mL of the inoculated LB broth was removed and centrifuged (Universal 320R; Hettich, Germany) at 9000 x g for 10 min, to precipitate the violacein. The supernatant was discarded and the violacein was solubilized by adding 1 mL of DMSO. The DMSO/violacein mixture was vortexed for 30 s and centrifuged (9000 x g, for 10 min). Finally, the absorbance of the supernatant was measured at 585 nm in microplates. The film without the addition of active components was considered a control. The results were expressed as a percentage of violacein inhibition (Equation 4) (Mohan et al., 2019):

$$Violaceininhibition(\%) = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \quad \text{(Equation 4)}$$

In which $Abs_{(control)}$ is the absorbance from the systems containing the PLA control films and $Abs_{(sample)}$ the absorbance read from the wells with the active films.

6.2.4.8.6. Antibiofilm activity

Antibiofilm activity was evaluated against Gram-positive (*S. aureus* and *L. monocytogenes*) and Gram-negative (*E. coli*) bacteria (Qu et al., 2022; Tarawneh et al., 2022). The inoculum was prepared as previously described, using the McFarland standard, to achieve ca. 1.5 x 10^8 UFC/mL.

A 40 µL inoculum was added into each well of 6 wells plate and complemented with TSB, supplemented with 1% w/v glucose until a 4 mL volume. Squared film samples (1 cm²) were then put into the inoculated media (ca. 10^6 CFU/mL), and the plates were incubated at 37 ºC/48 h. After the incubation period, the films were removed and washed three times with sterile water to remove planktonic cells. The films were added again into sterile wells, immersed in 2 mL crystal violet solution, and kept for 15 min, at room temperature. Subsequently, the films were washed three times with sterile water and added to new plates containing 2 mL ethanol 95% (v/v), remaining for 15 min. 200 µL were collected from each well and added into 96 wells

microplate for reading in a spectrophotometer, at 600 nm (Sharma et al., 2020). Films without the IC incorporation were used as controls. Commercial polypropylene (PP) was also tested to verify the visual growth of the bacteria (changes in the turbidity). Results were expressed as a percentage of biofilm inhibition, as follows:

$$Biofilminhibition(\%) = \frac{Abs(control) - Abs(sample)}{Abs(control)} x100 \quad \text{(Equation 5)}$$

In which $Abs_{(control)}$ is the absorbance from the systems containing the PLA control films and $Abs_{(sample)}$ the absorbance read from the wells with the active films.

6.2.4.9. *In situ* evaluation in bologna: effect on natural microbiota and *L. monocytogenes*

An *in situ* experiment was also conducted by applying the PLA-based films as packaging materials for sliced Dulano Lyoner Bologna (The Family Butchers GmbH, Germany) acquired from a local supermarket in Freising, Germany. According to the manufacturer, the bologna composition comprehends 23% w/w fat, 13% w/w protein, 1% w/w carbohydrate, and 1.9% salt. The product was packaged under a modified atmosphere at the moment of purchase. The bologna samples were cut into rectangular slices (ca. 5.5 cm x 7.5 cm) with approximately 10 g and inoculated with a *L. monocytogenes* bacterial suspension (previously prepared in ringer solution) to achieve ca. 2.5 log CFU/g, without previous sterilization to simulate the contamination with the pathogenic bacteria and the effects on/with the natural microbiota of the meat product.

The inoculated bologna slices were positioned between the active PLA films (PLA-HPIC and PLA-βIC) and the slices involved by films without CI (PLA-control) were used as control. For this analysis, 6.5 cm x 10 cm films were employed. Then, the systems were packaged in polyamide/polyethylene bags and vacuum sealed. The samples were maintained under 7 ± 2 ºC for 28 days. Microbial counts were made at 3, 7, 14, 21, and 28 d for evaluation of lactic acid bacteria, total mesophiles, and *L. monocytogenes*, using MRS (30 ± 2 ºC/72 h, under anaerobiosis), PCA (37 ± 2ºC/48 h) and LA with OCLA supplement (37 ºC/48 h), respectively. The microbial counting was also performed at 0 d.

6.2.5. Statistical analyses

Each experiment was repeated three times. Results were expressed as mean ± standard deviation (SD). When applicable, the results were subjected to analysis of variance (ANOVA)

followed by Tukey's test ($p < 0.05$) or non-linear regression analysis (controlled release and *in situ* experiments). The free software R was used (R Core, 2021).

6.3. Results and Discussion

6.3.1. Macro and microscopical features of PLA-based films

The physical and microscopic features of PLA-based films are presented in Figure 1. The images evidenced that the incorporation of IC induced changes in the appearance and homogeneity of the PLA matrix since PLA-βIC and PLA-HPIC presented more irregular surfaces in comparison with the control film. A slightly yellowish character can also be observed for both IC-added films, probably attributed to the presence of non-complexed βRHE (94.01 ± 5.69% and 90.26 ± 2.97% of entrapment efficiency for inclusion complexes prepared with βCD and HPβCD, respectively). Films incorporated with IC presented clusters of non-dispersed particles with a less smooth texture compared to PLA-control film. As discussed previously, βIC and HPIC exhibit small-size particles with the tendency to form aggregates in solution due to possible H-bonding of the peripheral hydroxyl groups of CD or propylene glycol (from βRHE) present on their outer surface (Arruda et al., 2024). Therefore, these aggregates were also formed in the polymeric dispersion and maintained after the evaporation of the solvent. Similar results were found by Marques et al. (2022) when added βCD-based IC into cellulose acetate films.

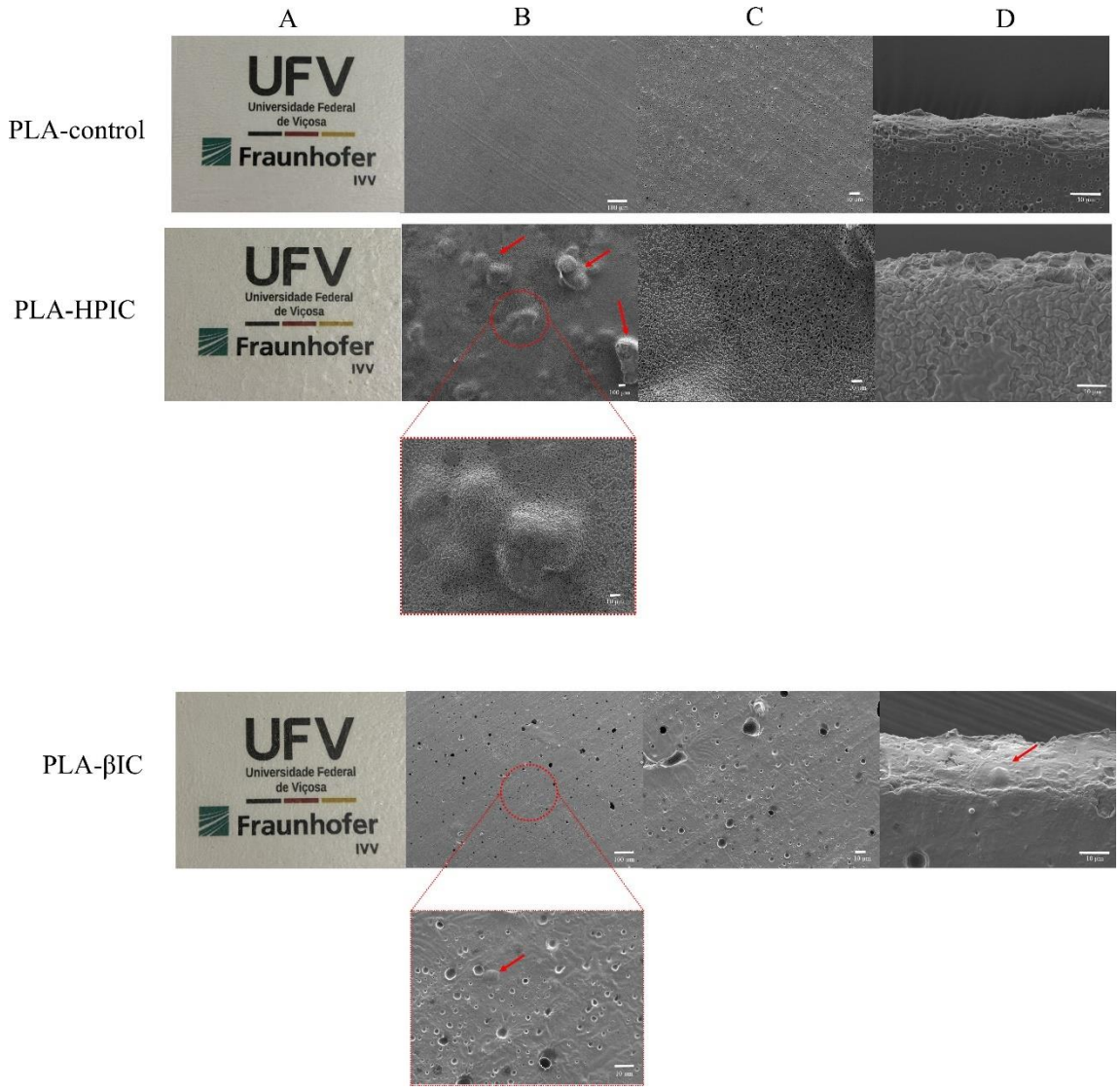

Figure 1. Physical appearance (A) and SEM images of surface at 100x (B), 500x (C), and cross-section (D) of PLA-based films: PLA-control, PLA-βIC and PLA-HPIC.

A relatively porous surface can be observed for PLA-control in SEM microphotographs (Figure 1C), possibly attributed to low compatibility between the polymeric matrix and the plasticizer. However, the microporous diameter increased with the addition of IC, which can be indicative of a further incompatibility between PLA and the outer face of the CD studied.

The films aspect also changed according to the type of IC added. Considering PLA-βIC, an increased micropore formation and the presence of some small lumps can be observed on the films' surface, even in the smaller magnification used (100x) (Figure 1B). Meanwhile, films produced with HPIC incorporation (PLA-HPIC) exhibited a different superficial profilometry, with a high number of lumps and aggregates. A thin layer of sputtered gold can also be observed on the surface of the HPIC agglomerates (Figure 1C). These differences probably can be attributed to the structural singularities of each CD employed.

Previous results had shown that due to the more hydrophilic outer surface, HPβCD probably favored the immobilization of propylene glycol (vehicle for β-acids in βRHE) to the external face of the IC (Arruda et al., 2024). A different outcome was observed for βCD-based IC, evidencing a higher amount of β-acids externally located. These discrepancies possibly influenced the location of IC agglomerates in the PLA polymeric matrix: lower miscibility between the PLA and the more hydrophilic IC (HPIC) have induced their increased external presence. Small IC agglomerates can also be observed internally in the PLA-βIC matrix, presented in the cross-sectional images (Figure 1D). These results corroborate with the hypothesis discussed in another study incorporating the pure hop extract into PLA-based sheets, where the results indicated an external immobilization of propylene glycol and the presence of β-acids in the inner structure of PLA matrix (Arruda et al., 2023).

6.3.2. Thickness and mechanical properties

The findings obtained for the PLA-based films incorporated or not with the ICs are summarized in Table 1. All mechanical properties investigated, as well as thickness and area, were significantly affected by the incorporation of ICs into the PLA matrix ($p < 0.05$). However, the type of IC included in the films did not affect these properties, as both treatments yielded similar results ($p > 0.05$).

Table 1. Mechanical properties of polylactic acid based-films (PLA) elaborated with polyethylene glycol and inclusion complexes of β-acids rich hop extract with β-cyclodextrin (PLA-βIC) or 2-hydroxypropyl-β-cyclodextrin (PLA-HPIC).

Treatment	**Thickness (µm)**	**Yield Strength (MPa)**	**Elongation at Break (%)***	**Young Modulus (MPa)**	**Breaking Strength (MPa)**
PLA-control	49.4 ± 4.2 a	15.16 ± 1.03 a	6.42 ± 6.84 a	1263.6 ± 91.6 a	15.15 ± 1.03 a
PLA-HPIC	93.3 ± 6.8 b	5.10 ± 1.06 b	0.97 ± 0.04 b	538.2 ± 75.7 b	5.09 ± 1.06 b
PLA-βIC	90.0 ± 13.0 b	5.46 ± 0.26 b	0.96 ± 0.03 b	593.7 ± 76.5 b	5.46 ± 0.26 b

Means ± standard deviation; means followed by the same letter (column) did not differ by Tukey's test ($p < 0.05$); *ANOVA and Tukey's test calculated with transformed data (1/x).

The samples' thickness and area practically doubled with the incorporation of HPIC or βIC compared to the control (Table 1), likely due to the presence of clusters of non-dispersed particles, as shown in Figure 1 (Section 3.1). This behavior is consistent with findings reported by other authors when investigating the addition of cyclodextrins IC into polymeric matrices, given rise to thicker and roughened films with a non-homogeneous surface (Dias et al., 2018; Friné et al., 2019; Marques, Silva, et al., 2022; Wang et al., 2017). This could be attributed to low compatibility between the polymer and the cyclodextrin or between the solvent and cyclodextrin since βCD and HPβCD are both insoluble in ethyl acetate, the solvent used for film elaboration (Arruda et al., 2021).

The incorporation of IC also had a negative impact on the mechanical features of the films (Table 1). Elongation at break, Young modulus, yield and breaking strength values decreased with the addition of either HPIC or βIC, indicating that the material became less rigid and more brittle than the control, easily breaking when submitted to a tensile force.

One of the main drawbacks of the application of bio-based polymers as food packaging is their inferior mechanical performance compared to petroleum-based packaging. Several studies focused on improving their performance in order to reach similar or better features than common plastics. In this sense, several strategies are considered, such as the incorporation of nanoparticles and plasticizers. Cyclodextrins, however, despite their interesting advantage regarding the controlled release aspect of bioactive compounds, providing them protection against external factors, seem to have a detrimental effect on the PLA mechanical properties. For example, Joo et al. (2011) fabricated extruded sheets from pellets of PLA mixed with βCD and also verified poor mechanical performance due to incompatibility between the cyclodextrin and the polymer. Similarly, Friné et al. (2019) produced PLA and βCD boxes and reported lower Young's modulus and yield strength the higher the amount of βCD in the samples.

6.3.3. FTIR

FTIR spectra of the three treatments are displayed in Figure 2A. The analysis is an important tool to better understand the molecular interactions among the films' components: PLA, IC (HPIC or βIC), and polyethylene glycol. The pristine PLA, βRHE, and IC FTIR spectra can be seen in previous works (Arruda et al., 2023, 2024).

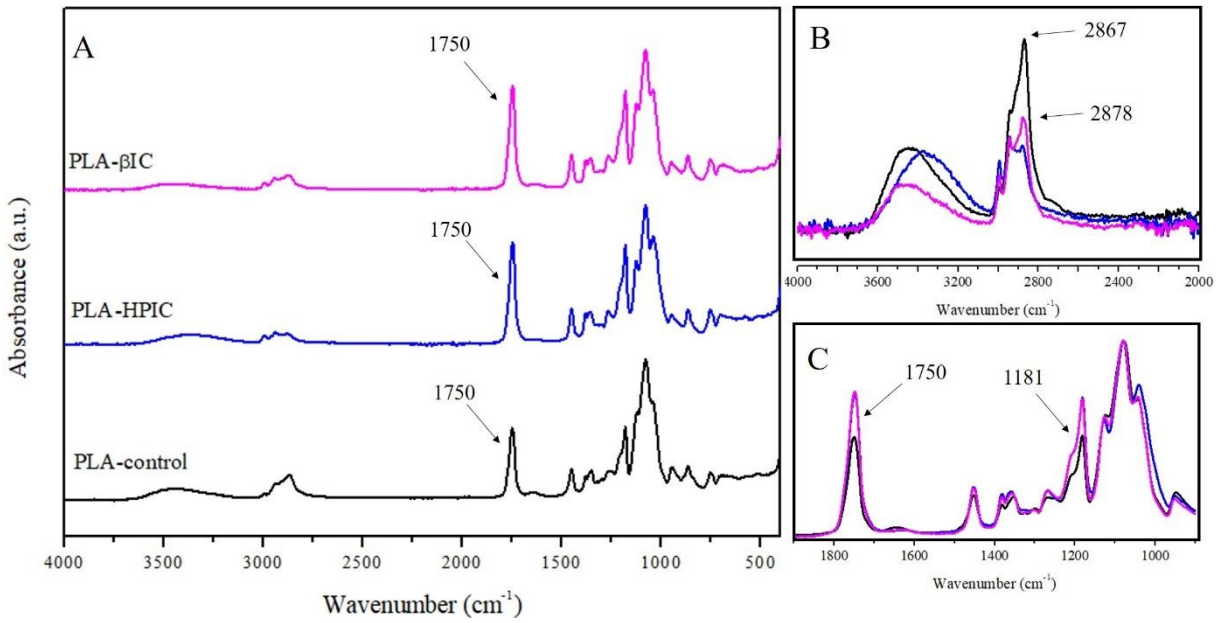

Figure 2. FTIR Spectra of polylactic acid based-films (PLA) elaborated with glycerol and inclusion complexes of β-acids rich hop extract with β-cyclodextrin (βIC) or 2-hydroxypropyl-β-cyclodextrin (HPIC) (A). Zoom in FTIR spectra: 3600-2500 cm^{-1} region (B), corresponding to the O-H stretching (broadband) and C-H bond stretches (peaks). Zoom in FTIR spectra: 1750 cm^{-1} peak (C), corresponding to C=O vibration, and 1181 cm^{-1} peak (C-O stretching).

All films presented a broad band between 3600-3000 cm^{-1} (Figure 2B), typical of O-H stretching, likely related to polyethylene glycol since this band is not present in pristine PLA (Arruda et al., 2023; R. Li et al., 2020). Other characteristic peaks related to both PLA and PEG can be seen in 2943 and 2867 cm^{-1} (symmetrical and asymmetrical stretching vibration of CH_3), and 1750 cm^{-1} (C=O stretching vibration), displayed in the FTIR spectra magnification (Figure 2B and 2C).

The incorporation of both IC into the PLA matrix did not lead to the appearance of new peaks, however it induced changes in the intensity of the already present peaks, as well as slightly wavenumber shifts, suggesting a lower hydrogen bonding between the polymeric chains and the occurrence of interactions between PLA and the IC. For example, the peak at 2867 cm^{-1}, had its intensity reduced and its frequency shifted to 2878 cm^{-1}, a higher wavenumber (Figure 2B). On the other hand, the narrow peak at 1750 cm^{-1} and the one at 1181 cm^{-1} (ether C-O stretching) had their intensity increased due to IC incorporation (Figures 2A and 2C), corroborating with the hypothesis of βRHE externally located, as this duplet is characteristic from the hop extract spectra. Furthermore, The O-H characteristic peak slightly shifted for a lower wavenumber in the PLA-HPIC spectra, whilst the presented by PLA-βIC had its intensity reduced. This feature is an indicative of presence of higher intermolecular

hydrogen bonds in films incorporated with HPIC (Silva et al., 2022), and could possibly be explained by an increased interaction between the plasticizer (i.e., that can also be partially be allocated in the films' surface) and the inclusion complex, or likely due to an enhanced interaction between the HPIC and the polymer, in comparison to PLA-βIC. Shifts and intensity changes were also verified by Li and Zhen (2017), with a derivatized PLA and γCD composite, by Wang et al. (2017) when investigating PLA-based films with allyl isothiocyanate-βCD IC, and by Liu et al. (2017) when studying composite fibers of PLA, βCD, and cinnamaldehyde.

6.3.4. Thermal analyses

Thermal analyses provide not only information on thermal resistance, but also insights into how additives interact, e.g., active compounds with PLA matrix, contributing to understanding their impacts on mechanical and antibacterial performance. Results of thermal analysis, including the thermogravimetric (TG), their respective derivatives, and the DSC curves are presented in Figure 3. TG analysis revealed three main stages of thermal mass loss (Figure 3A). In the first stage (25-110 °C), there was a slight mass loss due to water evaporation and volatilization of compounds present in βRHE and polyethylene glicol (Arruda et al., 2024). Furthermore, PLA-HPIC films showed a higher mass loss (2.9%) in this temperature range, when compared to PLA-βIC (1.8%) and PLA-control (0.9%). This is likely due to HPβCD's higher hygroscopicity (with a consequent increased water absorption) and possible higher external location of propylene glycol (from βRHE) on IC surface, in addition to a greater loss of non-complexed βRHE (i.e., slightly lower entrapment efficiency, as mentioned in Section 6.3.1).

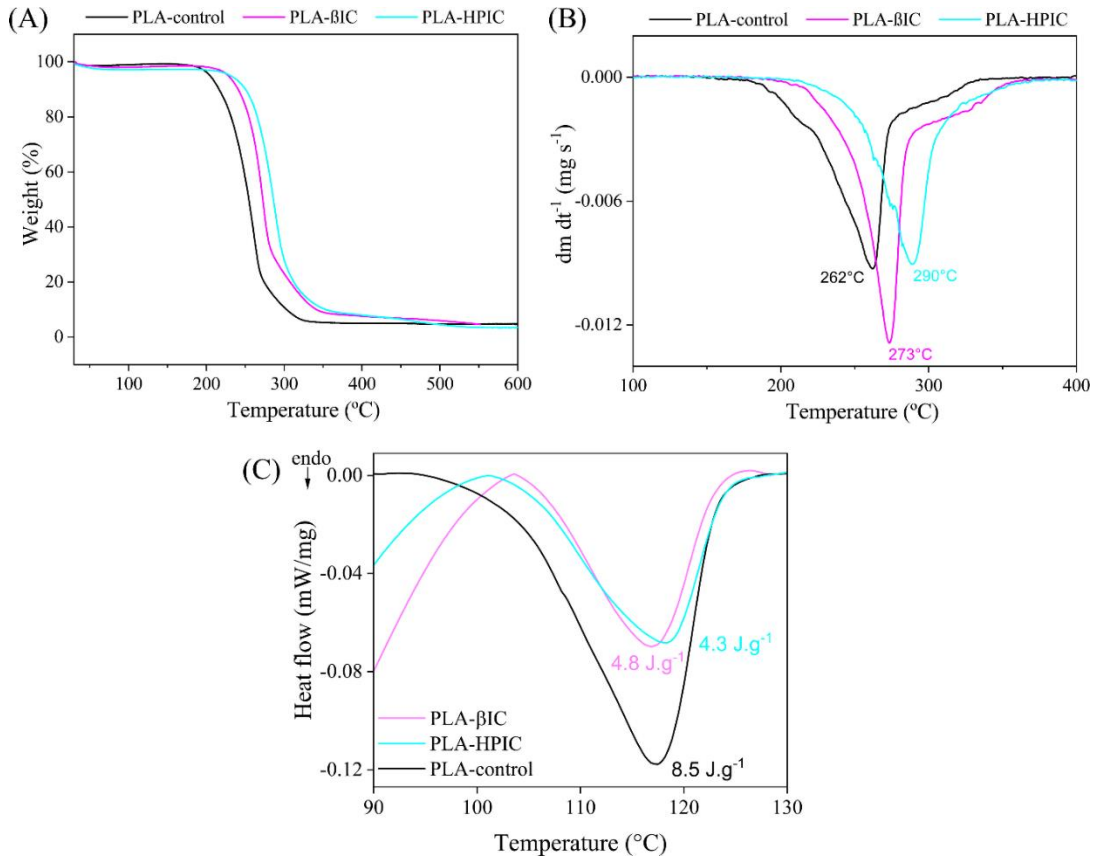

Figure 3. TG curves of PLA-control film, PLA-βIC, PLA-HPIC (A), their respective derivatives (B), and DSC curves (C).

The second stage (110-340 °C) is mainly related to PLA and some IC degradation (Figure 3B) (Arruda et al., 2023). The incorporation of IC improved the thermal stability of PLA, in which the temperature of maximum degradation was from 262 °C to 273 °C and 290 °C with βIC and HβIC incorporation, respectively. Moreover, the initial degradation temperature of PLA-control films (174 °C) was notably lower than those containing IC (196 °C for βCD and 216 °C for HPβCD), indicating the stability promoted be the enhanced resistance to the mass loss of IC, whose mass loss occur in higher temperatures (over 300 ºC). Additionally, the first derivative of TG confirmed the higher degradation resistance of PLA-HPIC compared to PLA-βIC (up to 25 °C). This enhanced thermal resistance might be attributed to HPβCD's free hydroxyl groups, which likely strengthen intermolecular interactions like hydrogen bonds with PLA chains, as discussed in FTIR analysis. Finally, the third stage of thermal degradation occurred between 340 °C and 600 °C, owing to the thermal degradation of cyclodextrins and complexed βRHE components.

DSC curves confirm a significant reduction in PLA melting enthalpy upon the incorporation of IC into PLA films (Figure 3C). Consequently, the degree of crystallinity in PLA films decreased from 9.1% to 5.6% and 4.6% after incorporating βIC and HPIC,

respectively. This effect arises from the interaction of additives with polymer matrices, which can function as steric hindrances, reducing the arrangement of polymer chains into a regular and ordered pattern. Therefore, this diminishes the molecular organization capacity between PLA polymer chains, thereby lowering the degree of crystallinity. The observed shift in melting temperature (T_m) to higher values by incorporating CD into polymeric matrices has also been reported by other studies in the literature (Byun et al., 2015; Niazmand & Razavizadeh, 2021).

Nevertheless, the lower degree of crystallinity of PLA-HPIC compared to PLA-βIC is attributed to the larger presence of branched structures (hydroxypropyl groups) in HPIC, which hinder the packaging of PLA polymer chains due to steric hindrance. Furthermore, the lower crystallinity evidenced by PLA-HPIC can be induced by a possible greater interaction between the plasticizer and propylene glycol externally located on HPIC, increasing the distance between the PLA polymeric chains (as discussed in the previous Section). Therefore, the reduction in crystallinity directly impacts several films' properties, e.g., by reducing film stiffness and enhancing gas and vapor permeability. These results are further corroborated by the findings for the mechanical properties of the films herein developed (Section 6.3.2). Thus, the incorporation of active IC into PLA films not only aids in the development of antimicrobial films but also modulates important properties for food packaging, e.g., flexibility and gas permeation.

6.3.5. Color and optical transmittance

The developed films were also evaluated for color characteristics (Table 2). The type of IC directly influenced the color attributes of PLA-based films ($p < 0.05$). The IC-added films did not differ from the control ($p \geq 0.05$), however, when comparing the films incorporated with HPIC and βIC, PLA-HPIC evidences higher luminosity (L*) than the one presented by PLA-βIC ($p < 0.05$). On the other hand, PLA-βIC exhibited a higher C* and yellowness, followed by PLA-HPIC and the control film ($p < 0.05$). The type of IC did not influence the hue angle (h), but the incorporation of either IC led to a significant increasing in this property ($p < 0.05$).

Table 2. Color parameters of PLA-based films: PLA-control, PLA-HPIC and PLA-βIC

Treatment	**L***	**C***	**h**	**Yellowness**	**ΔE*[a]**
PLA-control	95.31 ± 0.26 ab	2.41 ± 0.20 c	86.9 ± 1.89 b	12.49 ± 0.28 c	-
PLA-HPIC	95.76 ± 0.57 a	4.34 ± 0.54 b	92.36 ± 2.72 a	15.11 ± 0.68 b	2.07 ± 0.68 b
PLA-βIC	94.97 ± 0.49 b	5.2 ± 0.54 a	90.78 ± 2.12 a	16.31 ± 0.73 a	2.87 ± 0.61 a

Means ± standard deviation; Means with a different letter within columns differ significantly by Tukey's test ($p < 0.05$). [a] Means tested with the Student's T-Test ($p < 0.05$).

The incorporation of both IC significantly induced a slightly more yellowish tone in the PLA films, supported by the hue angle of approximately 90º. Nevertheless, the presence of βIC led to the production of films with an increased yellowness and higher color intensity (i.e., as indicated by the C* parameter) compared to HPIC. These findings corroborate previous discussions concerning the IC structure and position on PLA-based films. β-acids are yellow-brownish compounds known to induce the development of a yellow tone when incorporated into polymeric matrices (Arruda et al., 2023; Kowalczyk et al., 2020). Therefore, the increased presence of these hop constituents in the outer surface of βCD during the inclusion complexation can be responsible for these color features in PLA- βIC. The more significative changes in the global color of films were confirmed by the higher ΔE value evidenced by films incorporated with βIC ($p < 0.05$).

In parallel, the optical transmittance of PLA-based films can be observed in Figure 4. The presence of both IC promoted a significant decrease in light transmittance between ca. 220 and 400 nm, corresponding to the UV region. A more pronounced UV-blocking effect was observed for PLA-βIC films, probably attributed to a greater fraction of β-acids externally located on the IC. For both IC-added films, the transmittance in the visible light range increased within the wavelength, with PLA-HPIC exhibiting similar transmittance values to the control film up from 450 nm.

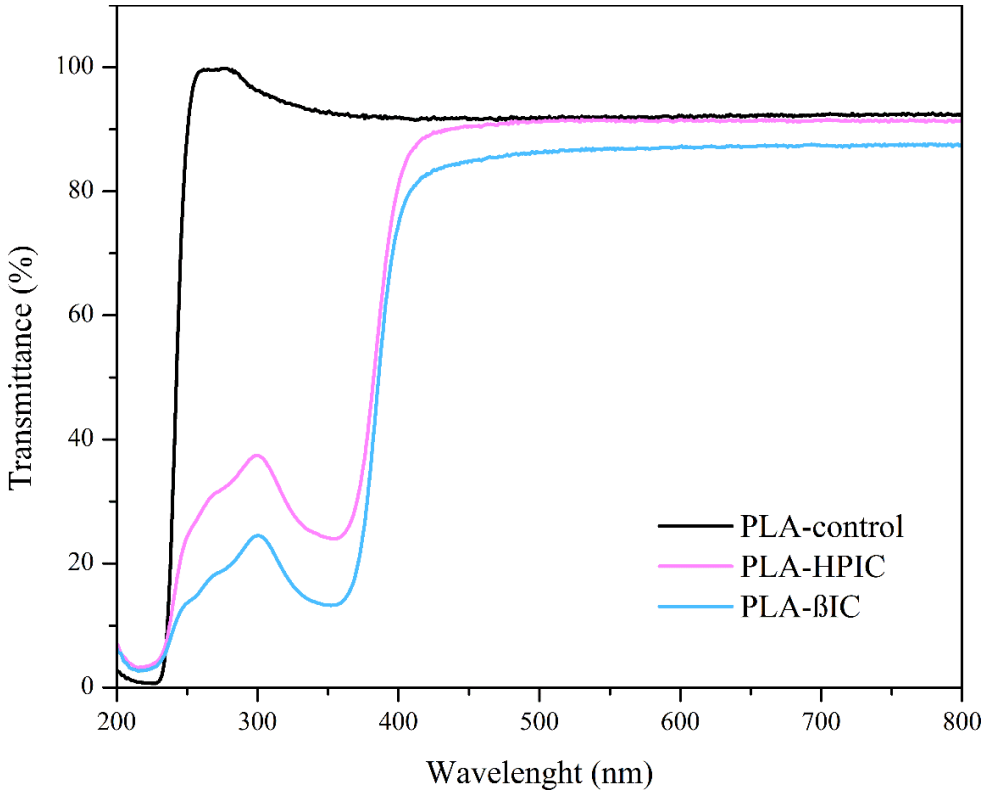

Figure 4. Optical transmittance of PLA-control, PLA-HPIC, and PLA-βIC.

Hop β-acids are known for UV absorption at approximately 224 nm and 356 nm (Gu & Liu, 2020), similar to the ones whose lowest transmittance was observed in the present study (Figure 4). From an overall perspective, the optical transmittance findings for PLA-based films demonstrated that the inclusion complexation of hop β-acids with both cyclodextrins (βCD and HPβCD) did not hinder this property, enabling the development of films with UV-blocking activity. This result is of great interest for packaging food products proner to oxidation, such as the ones with a high-fat content, as the oxidation process can be triggered by UV light incidence (Ezati et al., 2023).

6.3.6. Controlled release

The bioactivity of an active film is highly influenced by the release of the bioactive substance used during the development of the material. The active substance must be promptly available to exert its bioactivity according to the shelf life of the desired packaged food (Yildirim et al., 2018). Therefore, the release rate of these bioactives shall follow a behavior that attends to the food's requirements in the timeframe. The curves representing the cumulative release of hop β-acids into two distinct food simulants are shown in Figure 5, and their respective adjusted (non-linear) models can be observed in Table S1 (Supplementary Material).

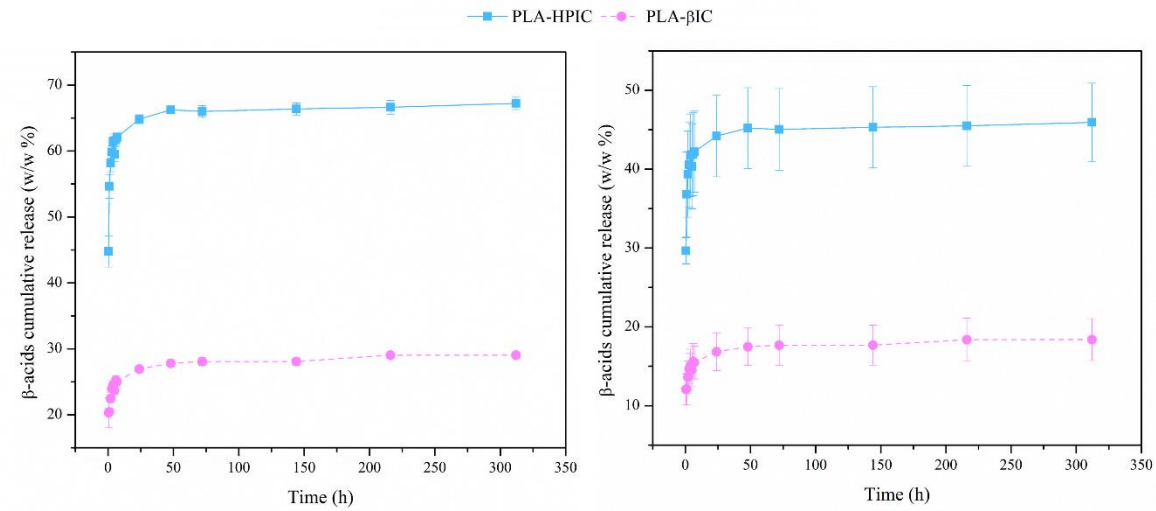

Figure 5. Cumulative release of βRHE from PLA-HPIC and PLA-βIC into (A) fatty food simulant and (B) meat products simulant. Error bars represent the standard deviation.

Concerning the incorporation of IC into plastic films, several factors must be considered besides the interaction between the active substance and the cyclodextrin (host:guest), including

the aspects involved in the interaction between the IC and the polymeric matrix. In both scenarios, it can be observed a typical release pattern characterized by an initial rapid release (burst effect, within the first ca. 5h), followed by a more controlled release (ca. up to first 50h), and finally by a sustained release plateau. A similar study conducted with zein edible films incorporated with catechin/β-cyclodextrin IC also evidenced a controlled release of the bioactives in phosphate buffer solution (PBS, pH = 7.4) containing 20% ethanol in a 132 h experiment after an initial burst (12 h) (Jiang et al., 2021). As expected, due to the highly hydrophobic profile of β-acids, an overall release was observed into the fatty food simulant after 350 h (14 days), with approximately 68% w/w and 28% w/w release from PLA-HPIC and PLA-βIC, respectively. Considering the meat products simulant, the release was lower, with 46% w/w from HPIC-added films and less than 20% w/w released from films incorporated with βIC at the end of the experiment (Figure 5).

A previous study evaluated the release of β-acids from βCD and HPβCD IC into a meat simulant solution (23.75% ethanolic solution) and the results pointed out a more pronounced initial release of the bioactive compounds from βCD IC (up to 10% of β-acids within the first 24 h) (Arruda et al., 2024). In the present study, a different outcome was observed, with PLA-HPIC exhibiting a higher cumulative release of hop compounds in the two food simulants tested, in comparison to PLA-βIC. This can be attributed to a higher amount of IC agglomerates on the surface of PLA films, favoring the release of the active compounds from the IC cavity.

Up to date, only a few studies covered the release of hop β-acids (or its derivatives) from polymeric films (Arruda et al., 2023a; Tian et al., 2022; Tian, Wang, et al., 2021) and even fewer investigated the release from nanocomposites after inclusion complexation with cyclodextrins. Although it is important to emphasize that a decreased cumulative release was found for PLA-βIC into the meat simulant compared to the respective pure IC (Arruda et al., 2024), the contrary was observed for PLA-HPIC. A higher interaction between the β-acids and the PLA can be hypothesized to explain this fact, corroborated by the possible higher presence of these bioactive on the outer surface of βIC and a more inner location in the polymeric matrix (Section 3.1).

6.3.7. Antioxidant activity

In addition to light-blocking properties, the capacity to block free radicals and/or interrupt oxidative reactions is of great interest concerning the active packaging of fatty food products. The incorporation of IC into the PLA films led to improved antioxidant activity in comparison to the control film ($p < 0.05$) despite the type of cyclodextrin used. PLA-HPIC and

PLA-βIC exhibited similar DPPH radical scavenging activity of 11.96 ± 0.14% and 11.48 ± 0.15%, respectively ($p \geq 0.05$); meanwhile, the scavenging activity exhibited by PLA-control was significantly lower (10.44 ± 0.28%, $p < 0.05$).

Hop β-acids have recognized antioxidant property induced by the presence of active hydroxyl groups in their structure (C. N. Wu et al., 2020). However, the inclusion complexes with βCD and HPβCD can hinder this bioactivity. Previous findings have shown that, possibly due to a considerably low ratio of hop bioactives in each IC and the interactions with cyclodextrins, these nanosized substances evidenced a decreased antioxidant capacity in comparison to the free hop compounds (Arruda et al., 2024). The present study showed that despite the lowered radical scavenging capacity described to the IC, the developed active films still exhibited significant antioxidant activity in comparison to the PLA control film ($p \geq 0.05$). It is worth mentioning that along with the entrapment by the cyclodextrins, interactions of the bioactives with the polymeric matrix can also reduce the activity potential. To exert their radical scavenging activity against the DPPH molecule, the hydroxyl groups might be out of the cyclodextrin cavity, otherwise, the hydrogen donation to reduce and neutralize the free radical is not possible (Aytac et al., 2016).

6.3.8. Antimicrobial properties

6.3.8.1. Agar diffusion test

The *in vitro* antimicrobial activity of the films incorporated with the two IC studied was assessed through the agar diffusion test. The results of inhibition zones evidenced against Gram-positive and Gram-negative bacteria are presented in Table 3 and Figure S1 (Supplementary Material).

Table 3. Mean diameter (mm) of inhibition zones for the PLA-based films towards Gram-positive (*S. aureus*, *L. monocytogenes*, *B. subtilis,* and *B. cereus)* and Gram-negative (*E. coli*) bacteria under optimum temperature (37 ºC/24 h), with *L. monocytogenes* also tested under refrigeration conditions (7 ºC/10 d).

Bacteria	**Film**		
	PLA-control	**PLA-HPIC**	**PLA-βIC**
S. aureus	0.00 ± 0.00 aC	21.53 ± 4.43 dB	24.33 ± 3.79 aA
L. monocytogenes	0.00 ± 0.00 aC	23.97 ± 2.68 cA	21.37 ± 3.96 bB
L. monocytogenes (7 ºC/10 d)	0.00 ± 0.00 aC	19.71 ± 6.85 fB	20.46 ± 5.72 cA
E. coli	0.00 ± 0.00 aA	0.00 ± 0.00 hA	0.00 ± 0.00 gA
B. subtilis	0.00 ± 0.00 aC	26.50 ± 4.30 bA	15.92 ± 3.72 eB
B. subtilis (spores)	0.00 ± 0.00 aC	19.25 ± 3.40 gB	22.53 ± 2.96 bA
B. cereus	0.00 ± 0.00 aC	26.50 ± 1.87 aA	17.3 3± 1.75 dB
B. cereus (spores)	0.00 ± 0.00 aC	19.80 ± 4.10 eA	13.83 ± 1.78 fB

Means ± standard deviation (n = 3); Means with a different letter (a, b, c, d, e, f, g) within columns differ significantly by Tukey's test ($p \leq 0.05$). Means with a different letter (A, B, C) within rows differ significantly by Tukey.

No activity was evidenced by PLA-control against all the bacteria tested. As expected, no inhibition zone was observed against the Gram-negative bacteria (*E. coli*) for either of the IC-added films. Several studies in the literature have pointed to similar results due to structural singularities: the outer cell membrane of Gram-negative bacteria acts as a barrier that can impair the permeability of hydrophobic substances (e.g., hop β-acids) and/or enable their inactivation by serum phosphatides (Sacks & Humphreys, 1951; Teuber & Schmalreck, 1973). On the contrary, hop β-acids are well-known for their activity against Gram-positive non-sporulated bacteria (Larson et al., 1996).

In the present study, no pattern was observed for the Gram-positive bacteria, as PLA-HPIC and PLA-βIC alternated in terms of higher bioactivity among the tested bacteria ($p < 0.05$) (Table 3). Previous results evidenced no differences in the minimal inhibitory concentration of βRHE complexed with βCD and HPβCD against Gram-positive bacteria (*L. monocytogenes* and *S aureus*) (Arruda et al., 2024). Thus, the discrepancies between PLA-βIC and PLA-HPIC can be attributed to the possible distinct interactions with the film constituents and the different distribution of IC in the PLA matrix. Furthermore, the differences in susceptibility between the Gram-positive bacteria tested can be attributed to the structural singularities of each bacteria tested, especially concerning the composition of bacterial cell surfaces (Cabanes et al., 2002).

The inclusion complexation with βCD and HPβCD also did not prevent the release of compounds of bacteria under refrigeration temperatures, as *L. monocytogenes* exhibited a certain susceptibility towards both active PLA films (Table 3). Despite the inhibition zones being smaller than the presented by *L. monocytogenes* tested at 37 ºC, similar results were found for the listerial activity of PLA sheets added with the pure β-acid rich hop extract when tested under 7 ºC (Arruda et al., 2023).

Interestingly, both active films tested exerted antimicrobial effects against the sporulated bacteria and their respective spores. Despite some studies in the literature confirming lower bioactivity of β-acids against spore-forming Gram-positive bacteria, such as *B. cereus* (Arruda et al., 2023; Pollach et al., 2002), other studies have shown different outcomes, with proven membrane leakage in *B. subtilis* 168 induced by these hop constituents (Teuber & Schmalreck, 1973). Moreover, differences among the inhibition zones exhibited by both bacilli spores are possibly due to different spore structures (e.g., the presence of exosporium surrounding *B. cereus* spores) (Koshikawa et al., 1989; Todd et al., 2003) and some important differences between *B. cereus* and *B. subtilis* sporulation genes and their expression. It is also im-

portant to point out that bacterial spore properties are affected by the conditions during sporulation. In this sense, the usage of fortified agar or rich liquid media can result in heterogeneous sporulation conditions for the individual cells, thus impacting the comparability between results obtained in the literature (De Vries et al., 2004). The results herein obtained are promising, as a sporicidal activity is of great interest to the food industry, not only towards the spoilage of packaged food but against foodborne pathogens.

6.3.8.2. ISO 22196

The three bacteria strains (i.e., *S. aureus*, *L. monocytogenes,* and *E. coli*) were first tested following the methodology described in ISO 22196. However, the growing conditions during the 24-hour incubation did not allow a proper quantification of *L. monocytogenes* and *S. aureus.* Therefore, the test was conducted using not-diluted nutrient broth as incubation media. This methodology has been widely employed to verify the antimicrobial efficacy on plastics and other non-porous surfaces. Nevertheless, the protocol has been extensively modified according to the study, with the reporting of the results varying greatly and a different expression of results than that requested by the standard (Bento de Carvalho et al., 2024)

No significative bacteriostatic effect was found for PLA-HPIC and PLA-βIC ($p \geq 0.05$) against *E. coli*, with bacterial count similar to the one found for the control film after the 24 h incubation (4.04 ± 0.32 log CFU/cm²). However, both IC-added films evidenced a maximum bactericidal rate (100%) against both Gram-positive bacteria (*L. monocytogenes* and *S. aureus*), as no bacterial counting was found after 24 h; meanwhile, the control samples (incubated with PLA-control) evidenced an increase of 2.05 ± 0.21 log CFU/cm² and 2.17 ± 0.20 log CFU/cm² after incubation, respectively. The percent reduction of bacteria of nearly 100% was also found by Winotapun et al. (2022) when testing paper packages coated nanocomposites with chitosan containing lignin at varied content ranging from 6% w/w to 33% w/w against *S. aureus* and *E. coli*. Lower values were found when testing cerium lactate (1.8% w/w) as an antibacterial nucleating agent for PLA-based films, in which the antibacterial rates against *S. aureus* and *E. coli* were 93% and 85%, respectively (Y. Wu et al., 2022).

6.3.8.3. Quorum quenching and antibiofilm activities

Quorum sensing is a bacterial cell-to-cell communication mechanism dependent on cell population density, responsible for mediating several biological activities such as swarming motility, aggregation, horizontal gene transfer, competence, production of antibiotics, luminescence, biofilm growth, synthesis of virulence factors, and sporulation (Kameswaran et al.,

2024). In this sense, finding strategies to disrupt this bacterial communication system (quorum quenching) is important to control these features involved and mediated by quorum sensing. Usually, the quorum quenching is achieved by the induction of conformational changes in the mediators and receptors (Raya et al., 2022).

The evaluation of violacein production by *C. violaceum* can be used to verify the quorum-quenching properties of active films (Luís et al., 2020). The production of violacein is mediated by N-acyl-homoserine lactones (AHLs), part of the quorum-sensing mechanism in many Gram-negative bacteria (Morohoshi et al., 2008). The PLA-based films were tested concerning their quorum quenching capacity qualitatively and quantitatively. The qualitative results (Figure S2, Supplementary Material) evidenced no inhibition zone for the production of violacein, therefore indicating no interruption in the quorum sensing mechanism by neither of the IC-added films. On the other hand, the quantitative investigation had shown a potential quorum quenching activity, with higher violacein inhibition for PLA-βIC (77.49 ± 13.65%) in comparison to the exhibited by PLA-HPIC (43.44 ± 7.97%) ($p < 0.05$, T Student Test).

A previous study had shown that hop compounds (humulone, lupulone, and xanthohumol) have the capability of inhibiting biofilm production in Gram-positive bacteria, possibly by interfering with the quorum sensing mechanism (Bogdanova et al., 2018). Therefore, despite showing higher activity against Gram-positive bacteria, evaluating the capability of nanosized compounds containing β-acids in quorum quenching of Gram-negative can help to elucidate the possible activity of these hop compounds in their biofilm formation. Other studies revealed similar outcomes, for example, rockrose essential oils. Pullulan films containing rockrose essential oil exhibited significative enhanced activity against Gram-positive bacteria in comparison with Gram-negative (no inhibition zones observed against *E. coli*, *Salmonella* Typhimurium, and *Pseudomonas aeruginosa*) (Luís et al., 2020). However, the authors found significative inhibition of the violacein production when the films were tested against *C. violaceum* ATCC 12472.

As for the antibiofilm properties of the IC-added PLA films, the results of the quantitative test can be observed in Table 4 (the visual results are shown in Figure S3, Supplementary Material).

Table 4. Antibiofilm activity of PLA-based films against Gram-positive (*S. aureus* and *L. monocytogenes*) and Gram-negative (*E. coli*) bacteria

Bacteria	**Biofilm inhibition (%)**		
	PLA-control	**PLA-HPIC**	**PLA-βIC**
S. aureus	0.00 ± 0.00 aC	81.79 ± 3.39 aA	56.64 ± 11.20 bB
L. monocytogenes	0.00 ± 0.00 aC	71.91 ± 6.90 cA	63.50 ± 13.22 aB
E. coli	0.00 ± 0.00 aC	76.16 ± 8.93 bA	55.93 ± 12.17 bB

Means ± standard deviation (n = 3); Means with a different letter (a, b, c) within columns differ significantly by Tukey's test ($p \leq 0.05$). Means with a different letter (A, B, C) within rows differ significantly by Tukey 's test ($p \leq 0.05$).

The findings indicated that HPIC and βIC possess antibiofilm properties not only against Gram-positive bacteria (*S. aureus* and *L. monocytogenes*) but also against the Gram-negative bacteria tested (*E. coli*). As biofilm production is mediated by a quorum sensing mechanism, the data collected support the hypothesis that the IC herein studied possibly have properties of quorum quenching against Gram-negative bacteria, that should be further investigated. The antibiofilm assay evidenced that PLA-HPIC had an improved influence on the biofilm formation capacity of the three tested bacteria (Table 4), which can be attributed not only to the release mechanism of the active agents from the polymer matrix but also to the particular structure of the IC (Arruda et al., 2024). The confocal microscopical images from biofilm formation are shown in Figure 6 and confirm the antibiofilm properties of IC-added films.

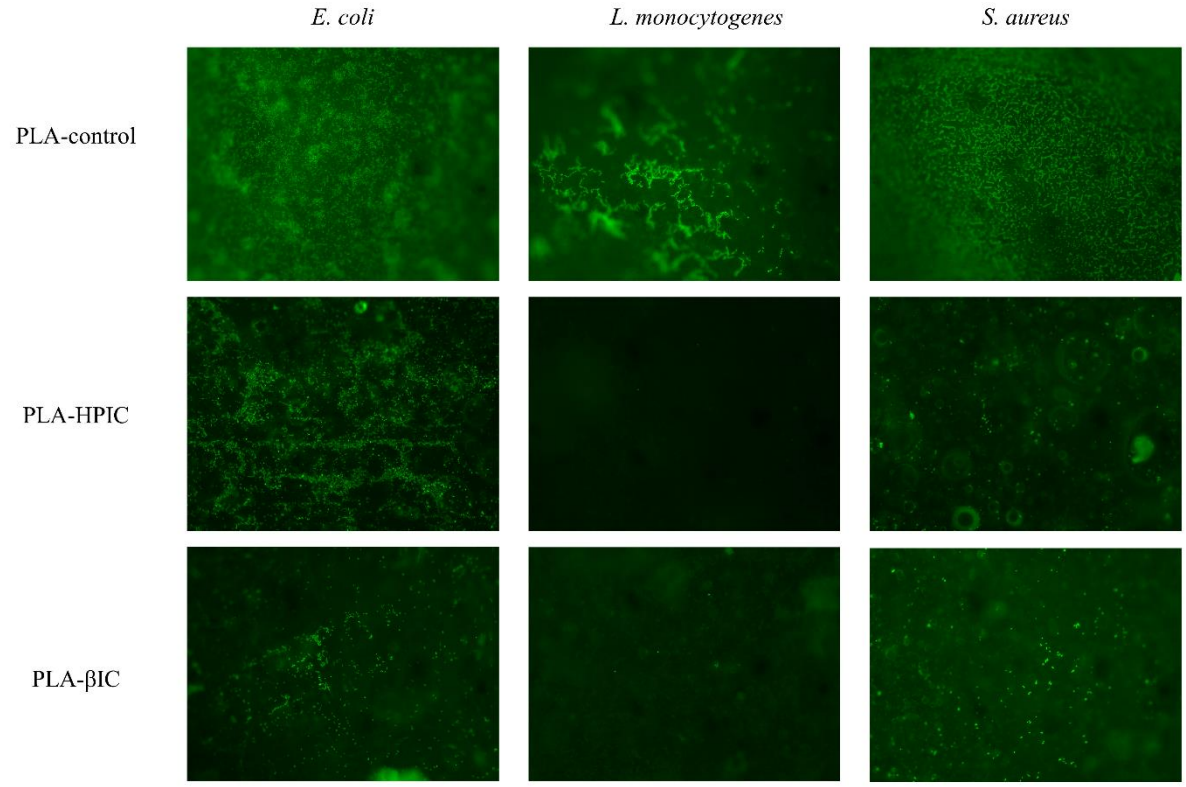

Figure 6. Biofilm inhibition of active PLA-based films (PLA-HPIC and PLA-βIC) in comparison to the effects of PLA-control assessed through confocal microscopy imaging against Gram-negative (*E. coli*) and Gram-negative bacteria (*S. aureus* and *L. monocytogenes*).

Despite the rougher surface evidenced by macro and microscopical evaluation (Section 3.1), no increase in biofilm formation was observed for IC-added samples when compared to the control PLA film. These results are different from those observed by other authors in the literature (Severo et al., 2021). Furthermore, the differences between the findings of the agar diffusion test and the antibiofilm assays can be attributed to the distinct media employed on each test (i.e., solid and liquid media, respectively), which can dictate the diffusion of the active compounds from the films. The biofilm production rate of each microorganism must also be taken into consideration.

6.3.9. *In situ* test in bologna

Meat products are highly prone to microbial deterioration due to their nutritional content (Panea & Ripoll, 2020). In special, ready-to-eat (RTE) meat products are susceptible to contamination during and/or post-processing, representing a challenge for the food industry not only in terms of microbial food spoilage but also considering the contamination by pathogens. Therefore, the antimicrobial activity of IC-added films was further evaluated in an *in situ* test

sing bologna as the food matrix. The results of the 28-day experiment can be observed in Figure 7.

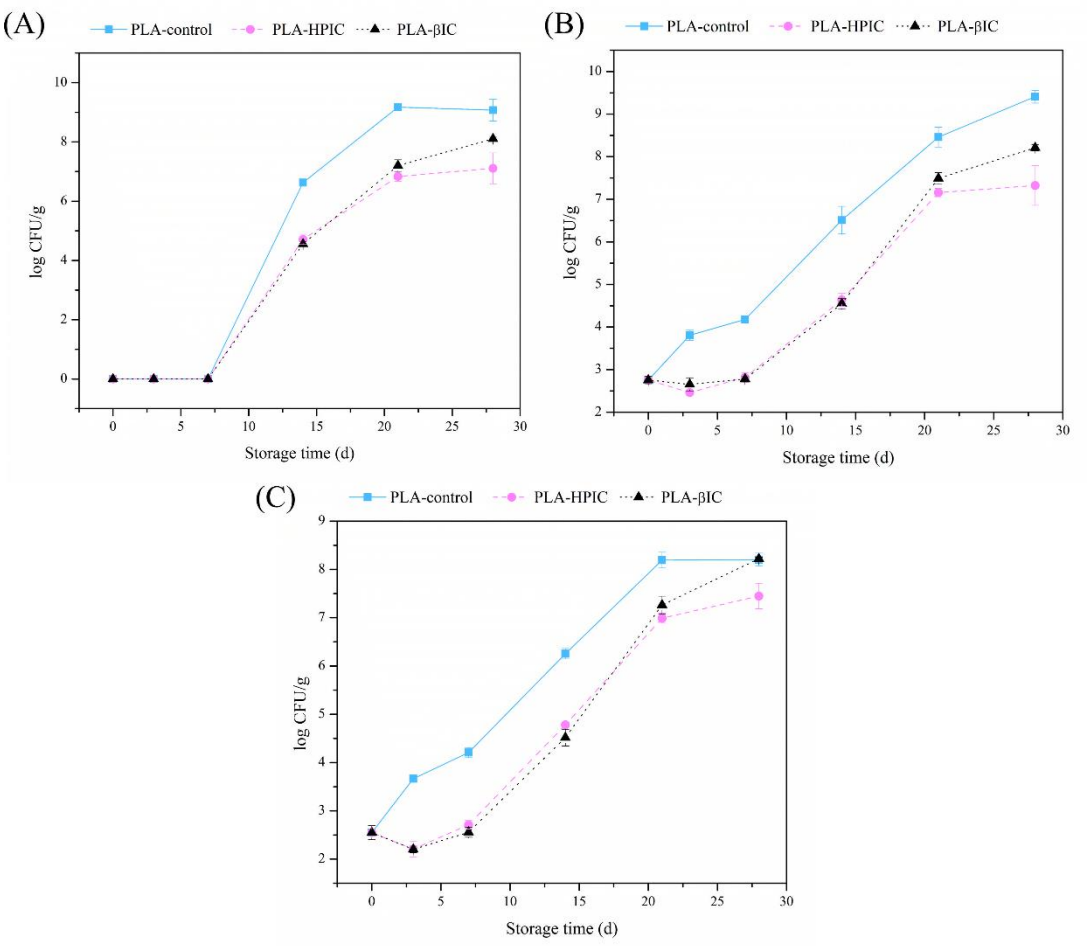

Figure 7. Effects of PLA-based films without active additive incorporation (PLA-control) and incorporated with HPIC (PLA-HPIC) and βIC (PLA- βIC) on growth of lactic acid bacteria (A) and total mesophiles (B) present on natural microbiota of bologna; and inoculated *L. monocytogenes* (C) during 28 days at 7 ± 2 ºC, under anaerobic conditions (vacuum packaging).

Total mesophiles and *L. monocytogenes* growth followed similar behavior in contact with the films tested, and the respective adjusted models for the microbial growth curves are presented in Table S2 (Supplementary material). However, no models were able to be adjusted for the lactic acid bacteria growth over time, possibly due to the growth delay observed until the 7th day of the experiment. As this delay was also found for the control PLA film, it was likely due to the natural microbiota of the bologna and/or higher counts of *Pseudomonas fluorescens* (not evaluated), which can hinder the lactic acid bacteria growth.

Both IC-added films exhibited an interesting effect on the microorganisms evaluated. An extension of the lag phase can be observed for *L. monocytogenes* and total mesophiles until the 7th day of the study for samples packaged with either PLA-HPIC or PLA-βIC. The two IC-added films had shown similar behavior against all the microorganism groups until the third week (21 days) of the experiment, with differences of ca. 1.5-2 log cycles from samples packaged with PLA-control. These findings changed during the last week of the trial (28 days), when PLA-βIC evidenced higher counts for the three tested microbial groups, comparable to the PLA-control for *L. monocytogenes*. Nevertheless, after 28 days of the test in the food system, PLA-HPIC and PLA- βIC induced a decrease of ca. 2 log CFU/g and 1 log CFU/g in lactic acid bacteria and the total mesophiles of the packaged bologna, in comparison with the control samples, respectively (packaged with PLA-control) (Figures 8A and 8B).

Concerning the application of active packaging, several studies in the literature have evidenced discrepancies between the *in vitro* and *in situ* results: a lower antimicrobial effect of active packaging is found when applied in a food matrix (Marques, Arruda, et al., 2022). This behavior is mainly due to the matrix effect since the food components can act by protecting the microorganisms and/or entrapping the antimicrobials (Farbood et al., 1976). Bologna contains ca. 23% fat, which can induce a higher diffusion of the lipophilic β-acids from the packaging material, as observed in the previous Sections. However, the absorption of the antimicrobial agent by the lipid in the food matrix can impair their bioactivity, as it can decrease their concentration in the aqueous phase, which was already observed for hop resins as food ingredients in previous studies (Kramer et al., 2021; Larson et al., 1996). Therefore, the results herein collected are promising as they evidence a potential application of IC-added PLA-films in real food systems.

6.4. Conclusion

HPIC and βIC were successfully incorporated into the PLA-based films. Both films evidenced the presence of clusters, indicating poor compatibility between the polymer and additives, which significantly increased their thickness and reduced the films' stiffness. The differences in the structure of each IC led to different outcomes in the PLA films: due to a more hydrophilic external surface, HPIC possibly had an increased migration to the films' surface, in comparison to the films manufactured with βIC. The presence of both IC increased the thermal stability and induced a decrease in the crystallinity of PLA films, in special of films con-

taining HPIC, as evidenced by thermal analysis results. A UV-blocking activity was also verified after the incorporation of both IC tested, with PLA- βIC promoting a higher absorption of light.

Despite both films exhibiting antioxidant and antimicrobial features, the differences between both studied IC significantly interfered with their performance as bioactive additives for active packaging. For example, different release behaviors were found and PLA-HPIC exhibited a higher release of hop β-acids in the two simulated solutions tested (i.e., meat and fatty food products), over time. On the other hand, PLA-βIC demonstrated improved quorum quenching properties for Gram-negative bacterial mechanism, with greater violacein production inhibition in *C. violaceum*. An antibiofilm property was also confirmed for both IC-added films (in special for PLA-HPIC, $p < 0.05$), with higher inhibition against Gram-positive bacteria, as supported by the *in vitro* antimicrobial results (agar diffusion and ISO 22196 tests). PLA-HPIC and PLA-βIC evidenced interesting antibacterial and antibiofilm activities, with potential for application in real food systems. The *in situ* test indicated that both IC possibly induced an increase in the lag phase of microorganisms of the natural microbiota of bologna (total mesophiles), and the same was observed for *L. monocytogenes* growth after inoculation on bologna slices. A retarded growth was evidenced in the first 21 days of experiment for all tested bacteria, including the pathogen, in comparison to the control samples. At the end of the trial (28^{th} day), a decrease of ca. 2 and 1 log cycles was observed in the growth of microorganisms from the bologna's natural microbiota for PLA-HPIC and PLA-βIC, respectively. The results confirm the usage of βCD and HPβCD as complexation agents for a commercial β-acids rich hop extract and their potential application in active packaging development. Further studies should be conducted to evaluate the up-scaling and the efficacy of the materials developed under different conditions and food matrices.

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6.6. Supplementary Material

Table S1. Adjusted models for *in vitro* release of the βRHE from PLA-based films (PLA-βIC and PLA-HPIC) into fatty foods and meat products simulant solutions and adjusted coefficient of determination (R^2_{adj}).

Film	Models	R^2_{adj}
Fatty foods simulant		
PLA-βIC	$Y_{CR} = 30.221\ x^{0.419}/0.458+x^{0.419}$	0.981
PLA-HPIC	$Y_{CR} = 67.463x^{0.624}/0.273+x^{0.624}$	0.959
Meat products simulant		
PLA-βIC	$Y_{CR} = 19.782\ x^{0.349}/0.549+x^{0.349}$	0.967
PLA-HPIC	$Y_{CR} = 45.503\ x^{0.802}/0.303+x^{0.802}$	0.991

Y_{cr}: cumulative release, x: time.

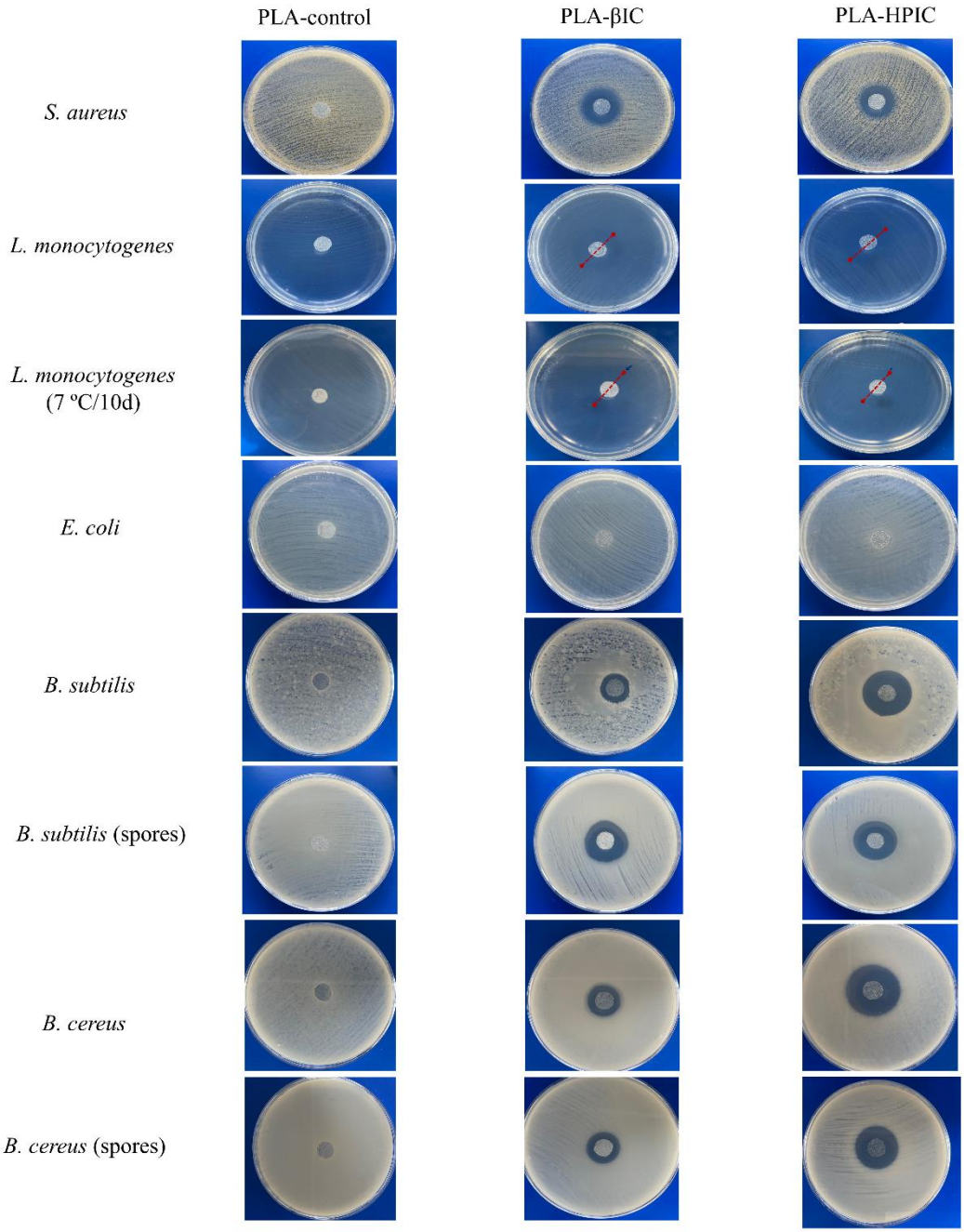

Figure S1. Agar diffusion test of PLA-based films incorporated with βIC (PLA- βIC) and HPIC (PLA-HPIC) against Gram-positive and Gram-negative bacteria under 37 ºC/24h. *L. monocytogenes* was also tested under refrigeration temperature (7 ºC/10 d).

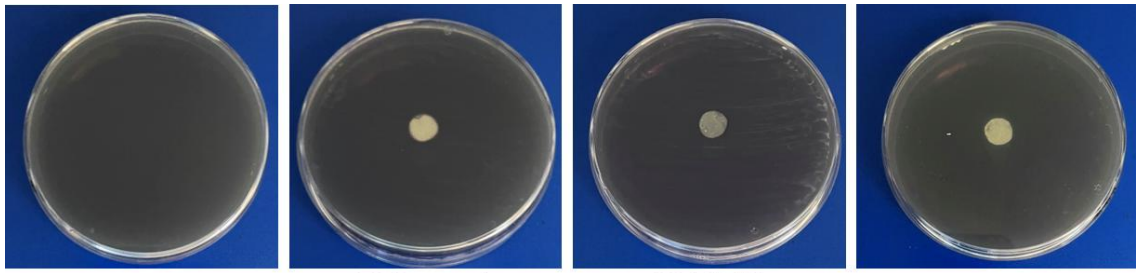

Figure S2. Plate diffusion assay for the quorum sensing inhibition.

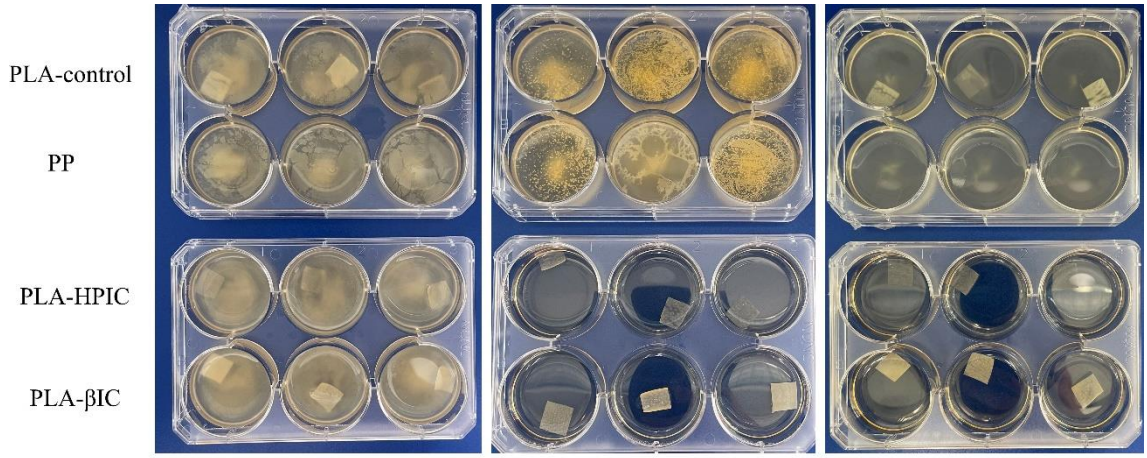

Figure S3. Visual appearance after incubation for the antibiofilm quantitative assay.

Table S2. Adjusted models (SGompertz) for the microbial growth of *Listeria monocytogenes* and total aerobic counting during the *in situ* experiment in sliced vacuum-packaged bologna (28 days)

Film	Models	S	R^2_{adj}
L. monocytogenes			
PLA-control	$Y=9.66*e^{(-e(-0.293(x-0.0835)))}$	0.367	0.968
PLA-βIC	$Y=23.21*e^{(-e(-0.92(x-0.0327)))}$	0.556	0.915
PLA-HPIC	$Y=12.72*e^{(-e(-0.638(x-0.0479)))}$	0.533	0.985
Total aerobic counting			
PLA-control	$Y=13.02*e^{(-e(-0.442(x-0.0576)))}$	0.337	0.929
PLA-βIC	$Y=27.94*e^{(-e(-0.933\ (x-0.0273)))}$	0.589	0.978
PLA-HPIC	$Y=13.78*e^{(-e(-0.62(x-0.0416)))}$	0.622	0.928

Y: microbial growth (logCFU/g); S: Standard Error of Regression.

7. GENERAL CONCLUSION

Active PLA sheets and films were successfully prepared with the incorporation of hop β-acids, complexed or not with cyclodextrins (βCD and HPβCD). Results evidenced the potentiality of β-acids beyond their traditional use (i.e., brewing), opening the prospects of industrial applications. Pure β-acids rich hop extract provided bioactivity to PLA casted sheets, with great antioxidant and antimicrobial activity against Gram-positive bacteria, without interfering with the biodegradable character of the polymer. The degradability of films in soil was although affected since the degradation of the active sheets mainly occurred through surface erosion.

The complexation with cyclodextrins also proved to be an interesting strategy to convert a strong-colored viscous extract into a powdered form, increasing the applicability range also enabling the possible usage in the upscaling process of active film production (e.g., extrusion). The IC produced with βCD and HPβCD exhibited different features, which demonstrated the possibility of tailoring the physicochemical and release properties of hop β-acids according to the CD employed. βCD and HPβCD-based IC presented interesting *in vitro* antimicrobial activity, supported by increased stability against external factors, such as oxygen and temperature.

The development of PLA films within the addition of both IC seemed promising, as, despite the poor compatibility between the polymer and the IC (observed in the mechanical and morphological findings), the active materials evidenced a controlled release of bioactives in food simulant medias (fatty foods and meat products). Furthermore, the *in vitro* antimicrobial results were corroborated by the *in situ* findings in bologna as, despite being a high-fat food matrix, which can impair the bioactivity of hydrophobic compounds (i.e., hop β-acids), the presence of IC extended the lag phase of the natural microbiota and *Listeria monocytogenes* intentionally inoculated. A potential quorum quenching activity and antibiofilm were additionally confirmed.

The studies herein conducted are of great asset for the sustainable approaches towards the reduction/substitution of fossil-based polymers, using PLA as polymeric matrix to develop active packaging materials. Moreover, the overview of hop compounds' bioactivity in a different area than beer industry expands their applicability, especially considering the more stable powdered form obtained by the inclusion complexation with CD. Further investigations should be conducted in order to evaluate the upscaling process, and obtention of IC-added PLA films with the IC herein studied.