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Development of a biodegradable plastic film extruded with the addition of a Brazilian propolis by-product

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ABSTRACT

The development of new materials environmentally friendly has become an important market niche for the food industry. The agro-industrial wastes and by-products can be an alternative for the production of biodegradable food packaging. The work aimed to produce biodegradable plastic film extruded with antioxidant and antibacterial properties by the joining of cassava starch and Brazilian propolis by-product (BPB). The volatile profile of BPB, Brazilian propolis by-product film (BPBF), and control film (CF) were analyzed by gas chromatography. The mechanical, antioxidant, and antibacterial properties of the films were also assessed. Eighty-seven volatile compounds were detected with aldehydes and terpenoids predominating in the samples. The major terpenoid detected in the samples was the 2-pinen-10-ol followed by α-copaene. Benzaldehyde and benzenepropanoic acid ethyl ester were the major aldehyde and ester classes present in the films. The BPBF exhibited promising mechanical properties showing the highest Elasticity modulus (11.96 Mpa) and excellent antioxidant (8.45 mmol Trolox equivalent/g) and antibacterial (12.5 mg/mL against *Staphylococcus aureus* and *Salmonella* Typhimurium) activities. The valorization of agro-industrial by-products following the current trends to environmental care can be a sustainable alternative for the development of a plastic into which the propolis by-product is incorporated in biodegradable packaging.

1. Introduction

In the current years, the research and utilization of agro-industrial waste have increased fueled by greater environmental awareness, legislative control, as well as economic factors (Nayak & Bhushan, 2019; Pham, Kaushik, Parshetti, Mahmood, & Balasubrmanian, 2015). In this sense, the use of valorized resources from agro-industry residues as sustainable new raw materials for application in extruded biodegradable films is a potential use for propolis by-product. According to several authors, cassava starch is the most used material for the preparation of biodegradable film (Assis, Lopes, Costa, & Flôres, 2017; Pagno, de Farias, Costa, Rios, & Flôres, 2016). At the same time, due to the chemical composition of the raw propolis, the addition of it in biodegradable films can improve the mechanical (Siripatrawan & Vitchayakitti, 2016) and functional (Rizzolo et al., 2016) properties, making them a sustainable alternative to conventional plastics.

Propolis is a resinous matter handled by *Apis mellifera* bees from various plants parts, including buds and exudates (Bankova, Popova, & Trusheva, 2018). The resin undergoes a reaction when mixed with the bee's salivary enzymes, beeswax and pollen (Rufatto et al., 2018) and is used as a cementing material in the construction of their hives and as a defensive agent against invaders (Król et al., 2013). Hundreds of compounds have already been reported to occur in propolis including flavonoids, phenolic acids, alcohols, esters and terpenoids (Madrigal-Santillán et al., 2014; Soltani et al., 2017; Yang et al., 2015). However, each propolis composition varies largely because it depends on the plants from which the resinous substance was collected, the chemical composition, the geographical location, phase of reproduction

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cycle and collection season (Yang et al., 2015). Besides, the chemical composition of the propolis is conditioned on the hereditary of the queen bee (Bittencourt et al., 2015; Cheng, Qin, Guo, Hu, & Wu, 2013). In fact, studies of genetic improvement have been used to influence the bees' preference at the time of resin collection for the production of a specific type of propolis (Nakamura & Seeley, 2006).

Propolis has gained widespread attention due to its purported antibacterial (Popova et al., 2011), antifungal (Ristivojević et al., 2018), anti-inflammatory (Franchin et al., 2016), antioxidant (Bittencourt et al., 2015) and immunomodulatory therapeutic (Chan, Cheung, & Sze, 2013) properties. This has resulted in the development of several popular pharmaceutical drugs, cosmetics formulations and food additives based on propolis microorganisms (Bankova et al., 2018). Indeed, new applications for propolis are currently being developed such as its use in the manufacture of an environmentally benign coating containing propolis extract for protection against marine fouling in ship hulls (Peres et al., 2018). Additionally, bio-based packaging materials containing propolis designed to transfer their antioxidant and antimicrobial properties to the food they are in contact with, also being developed (Rizzolo et al., 2016; Siripatrawan & Vitchayakitti, 2016).

However, the most used form of propolis is as a crude ethanolic extract of the parent raw material (De Francisco et al., 2018). During the industrial production process of this extract, huge quantities of a by-product are generated. In fact, 90% of the total propolis used during manufacturing processes of propolis ethanol extract remains as a by-product, which is usually discarded or used as a low-value additive to enrich animal nutrition (Soltani et al., 2017). In line with the ever-rising need for the application of sustainable approaches to product manufacture (Lopez-Hidalgo, Alvarado-Cuevas, & De Leon-Rodriguez, 2018), awareness is arising that this by-product may still possess useful biological properties such as antimicrobial and antioxidant activities. The antioxidant capacity of propolis is generally closely associated with some volatile and non-volatile phenolic compounds that can also exert a synergistic effect with each other, as well as with some lipophilic compounds. Phenolic compounds are considered excellent antioxidants due to their molecules' structure, facilitating the electron donation of the hydroxyl portion to oxidize radical species. Besides, it is recognized that these compounds can reduce oxidative stress during carcinogenesis by inhibiting reactive oxygen/nitrogen species. These species are found in the complex biological system and exert constant human body activity. Besides, according to De Francisco et al. (2018), a propolis by-product-based extract demonstrated antioxidant properties and antifungal activity against *Candida albicans* of a similar magnitude to the original raw propolis extract.

An increase in the demand for propolis is expected in the coming years. Consequently, this will also lead to an increase in the amount of waste generated, a thus greater appreciation of the propolis by-product will be necessary. Previous studies, already described the use of raw propolis in laboratory processes for the production of chitosan-propolis extract edible coating or biopolymer coatings in combination with propolis extract (Irigoiti et al., 2021; Peres et al., 2018; Skowron et al., 2019). Nonetheless, the propolis by-product is little explored, mainly in the production of biodegradable films based on cassava obtained by the industrial process extrusion.

In order to drive new applications in the food packaging industries, biodegradable films with added propolis by-products may create new opportunities for the marketing of plastic films. Thus, this research study aims to synthesize biodegradable films with antioxidant, antibacterial, and mechanical properties employing biodegradable polymer blend, cassava starch, and propolis by-product. Furthermore, plastic films were characterized in terms of volatile compounds by gas chromatography to determine their potential applications in food packaging.

2. Material and methods

2.1. Chemicals

The Poly(butylene adipate-co-terephthalate) (PBAT) Ecoflex® was purchased from Basf (Ludwigshafen, Germany); the starch from cassava was obtained from Yoki Inc. (São Paulo, Brazil), glycerol from Dinamica Química (Diadema, Brazil). At the same time, the Folin–Ciocalteu, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′- azino-bis (3-ethylbenzothiazoline-6-sulfphonic acid) (ABTS), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), brain heart infusion broth (BHI), and BHI agar were acquired from Sigma-Aldrich (Saint Louis, MO, USA).

2.2. Propolis by-product

Approximately 1 kg of propolis by-product (BPB) was generously donated by Breyer & Cia Ltda (União da Vitória, Paraná, Brazil) and stored at −15 °C prior to use. The Brazilian brown propolis used in this study was originated from *Apis mellifera* hives and was collected in Spring 2017 in União da Vitória, Paraná, Brazil (latitude 26°11′48.8″S, longitude 51°06′48.4″W).

2.3. Production of biodegradable active packaging

Pellets were prepared to consist of 4% of Brazilian propolis by-product, 19% of glycerol, 30% of PBAT, and 47% of cassava starch. The content of 4% propolis residue was the upper limit achieved to carry out the extrusion process. Above 4% the films could tear easily due to fibrous material, characteristic of the propolis by-product. This formulation was incorporated at the time of extrusion, and the pellets were produced on a single-screw extruder (model EL-25, BGM, Brazil). All the film extrusion process conditions, such as temperature profile from the feeding zone to the matrix zone, screw speed, intern air temperature, thickness, and mould type were the same as those recommended by Bilck et al. (2015). A control film composed of 19% of glycerol, 30% of PABT, 51% of cassava starch without Brazilian propolis by-product was also produced in the same manner as described above.

2.4. HS-SPME procedure and GC/MS analysis

The HS-SPME sampling conditions were used as they were previously described in Pellati, Prencipe, and Benvenuti (2013) for the characterization of volatile compounds in propolis from Italy by HS-SPME-GC/MS method. Desorption and analysis of volatile components were performed in a Varian GC systems coupled to an ion trap mass spectrometer (Varian Chromatography Systems, Walnut Creek, USA). Additionally, the parameters used in this analysis were reported previously by the team in Carpes et al. (2021) for the determination of volatile compounds of films with grape pomace.

Tentative identifications of the main volatile compounds were achieved by comparison of the mass spectra obtained from the analyses of the BPB, BPBF and CF with the reference mass spectra of National Institute of Standards and Technology, Environmental Protection Agency and National Institutes of Health libraries (Version 2.0 g, 2011). Besides, *n*-alkane standards (C_7–C_{30}) were previously injected under the same condition on this instrument by Gkarane et al. (2019). In this study, only compounds with a similarity of more than 87% were recorded. The LQ (limit of quantification) was set as a signal to noise ratio of approximately less than 10. The percentage composition of the volatile components was calculated from the GC peak areas using the normalization method. The analyses were carried out in triplicate on three separate batches of the sample.

2.5. Extracts

The procedure described by Reis et al. (2017) with modifications was used to prepare an ethanolic extract of propolis by-product. The extracts were prepared separately with 5 g of each sample BPB, BPBF and CF with 20 mL of ethanol 800 g/L in bathwater with agitation at 70 °C for 1 h. The samples were centrifuged for 15 min at 112×*g* (Hermle Z 200 A, Wehingen, Germany). The supernatants were stored at −12 °C until further analysis. Each sample was extracted in triplicate.

2.6. Total phenolic content (TPC)

TPC of samples was assessed employing a colorimetric test reported by Singleton, Orthofer, and Lamuela-Raventós (1999). An aliquot of 500 μL of each extract was mixed with 2.5 mL of Folin-Ciocalteu, and after 6 min, 2.5 mL of Na_2CO_3 40 g/L (v/v) was added. Absorbance was measured in a spectrophotometer (BelPhotonics 2000; Piracicaba, Brazil) at 765 nm after 2 h in darkness. The gallic acid was used for the standard curve the TPC values were expressed as mg GAE/g of sample (GAE: Gallic acid equivalent). All test were carried out in triplicate.

2.7. Antioxidant capacity assays

Quantitative evaluation of the antioxidant activity of BPB, BPBF, and CF was reported by three methods, namely DPPH, ABTS and Ferric reducing antioxidant power (FRAP) assays. These spectrophotometric methods are complementary and have different mechanisms of action, which can depend on the reaction of an organic radical, a cation radical, or a complex with an antioxidant molecule capable of donating a hydrogen atom (Pisoschi & Negulescu, 2011). Besides, by colorimetric assay, the amount of iron reduced can be associated with the amount of antioxidants in several matrices. All this to be enabled to report complete information on the antioxidant capacity of these samples.

2.7.1. DPPH assay

Antioxidant activity by DPPH assay was realized according to the method of Brand-Williams Cuvelier, and Berset (1995). Initially, 0.5 mL of each extract BPB, BPBF and CF (0.25 g/mL, ethanol) was added separately to 0.3 mL of DPPH (0.5 mM, ethanol) and 3.0 mL of ethanol. The mixture was left to stand for 45 min at room temperature in the darkness, and the absorbance values were measured against ethanol at 517 nm (Bel Photonics 2000; Piracicaba, Brazil). The antioxidant capacity was determined by a standard curve of Trolox (10–100 mmol of Trolox/mL). The results were expressed as mmol equivalent of Trolox (TE) per gram of the sample. All analyses were carried out in triplicate.

2.7.2. ABTS assay

The absorbance diminution of ABTS cation radical was measured as described by Re et al. (1999). The stock solution was prepared with 7 mM $ABTS^{\cdot+}$ and 140 mM $K_2S_2O_8$. This solution was held in the darkness at room temperature for 16 h before use. After this time, an aliquot of 1 mL $ABTS^{\cdot+}$ solution was diluted with 60 mL ethanol to achieve an absorbance of 0.70 at 734 nm in a spectrophotometer. Trolox was used as a standard, and the result was expressed as mmol of Trolox equivalent (TE) per gram of the sample. All analyses were performed in triplicate.

2.7.3. FRAP assay

The antioxidant activity through the ferric reducing antioxidant power was performed as described by Pulido, Bravo, and Saura-Calixto (2000). The stock solution was prepared by mixing 2.5 mL of TPTZ solution (10 mM TPTZ in 40 mM HCl), 2.5 mL of ferric chloride (20 mM), and 25 mL of acetate buffer (pH 3.6). 3 mL of the FRAP solution was maintained in bath water at 37 °C for 30 min with 100 mL of each extract. The absorbances were measured at 593 nm and the standard ferrous sulphate was used as a reference solution. The results were expressed as mmol of ferrous sulphate equivalent (FSE) per gram of the sample. All analyses were performed in triplicate.

2.8. Antibacterial activity

Important foodborne pathogens, such as *Staphylococcus aureus* (ATCC 25923), *Salmonella* Typhimurium (ATCC 14028), and *Escherichia coli* (ATCC 25922) were used to determine the antibacterial activity of the films containing the propolis by-product. The bacteria were reactivated overnight at 37 °C in nutrient agar slants. The minimum inhibitory concentration (MIC) of the extracts was performed by using the successive micro-dilution assay in 96-well plates according to the protocol of Clinical and Laboratory Standards (CLSI, 2015). Brain Heart Infusion (BHI) broth and chloramphenicol were used as culture media and a positive control, respectively. The suspension of the test microorganisms was adjusted according to McFarland 0.5 and 50 μL of the bacterial suspensions were inoculated into 50 mL of BHI broth. 190 μL of BHI broth previously inoculated and 10 μL of the extracts were added into well plates. The tested concentration of the BPB, BPBF and CF ranged from 12.5 mg/mL to 0.38 mg/mL and 1.2 mg/mL for the positive control. Samples were incubated in a microplate shaker for 24 h at 37 °C, and at the end, 30 μL of resazurin an oxidation-reduction indicator at 0.1 mg/mL was added to each well. MIC values were determined as no change in colour. That is, the extracts were considered active when growth inhibition at concentrations below or equal to 12.5 mg/mL was observed. MIC positive results were subjected to analysis of the minimum bactericidal concentration (MBC), which was carried out in Petri dishes containing BHI agar. MIC values were defined as the lowest concentration of each extract, which completely inhibited microbial growth. All analyses were performed in triplicate.

2.9. Mechanical properties

The mechanical properties of the films were determined in a texture analyser (TA.TX2 *plus,* Stable Micro Systems, Surrey, England), according to standard method ASTM D882-02 (ASTM, 2018). Fifteen repetitions of each film in the direction longitudinal of the film were tested. The films were cut into strips in a dimension of 50 mm × 20 mm and conditioned in a relative humidity of 53% (saturated magnesium nitrate solution) at 25 °C for 48 h. The strips were adjusted to the equipment's pneumatic grips. The initial gauge length was set to 30 mm and the films were tensioned at a speed of 0.8 mm/s. The maximum tensile strength (MPa), elongation at break (%) and elasticity modulus (MPa) were assessed.

2.10. Statistical analysis

All tests were performed in triplicate and the data were expressed as mean ± SD (standard deviation). Data was evaluated by variance analysis (ANOVA) and the averages were compared by Tukey test, considering the significance level of 95% ($p < 0.05$) using the STATISTICA program 8.0 version (StatSoft Company, Tulsa, OK, USA).

3. Results and discussion

3.1. Volatile compounds

The use of headspace solid-phase microextraction-gas chromatography-mass spectrometry (HS-SPME-GC/MS) has been widely applied to determine the volatile compounds of medicinal plants (Taherpour et al., 2017), food (Caporaso, Whitworth, Cui, & Fisk, 2018; Rizzolo et al., 2016) and pharmaceutical products (Rufatto et al., 2018). Besides, it is used to characterize complex biological samples such as hair, blood, plasma, and urine (Hashemi, Zohrabi, & Shamsipur, 2018).

In this study, 87 volatile compounds were tentatively identified using HS-SPME-GC/MS, as shown in Table 1. Out of the many compounds detected, 65 were identified in the propolis by-product, 52 in the

Table 1
Volatile components of Brazilian propolis by-product (BPB), Brazilian propolis by-product film (BPBF) and control film (CF) quantified in relative percentages (mean ± standard deviation ($n = 3$)).

Compounds	Linear retention index (LRI)	BPB	BPBF	CF	Previously reported*
Aldehydes					
2-Butenal	621	0.42 ± 0.03^{j}	0.14 ± 0.01^{k}	–	Kamatou et al. (2019)
Pentanal	666	–	–	0.24 ± 0.08^{i}	Kamatou et al. (2019), Jerković et al. (2016)
3-Methyl-2-butenal	748	0.24 ± 0.03^{m}	0.50 ± 0.07^{h}	–	Kamatou et al. (2019), Cheng et al. (2013), Pellati et al. (2013)
Hexanal	770	–	0.72 ± 0.27^{gh}	1.05 ± 0.10^{e}	Jerković et al. (2016), Cheng et al. (2013), Madrigal-Santillán et al. (2014)
2,4-Hexadienal	877	0.26 ± 0.02^{m}	–	–	NPR
Heptanal	900	–	0.26 ± 0.17^{jl}	0.42 ± 0.01^{h}	Kamatou et al. (2019), Jerković et al. (2016)
Benzaldehyde	957	2.04 ± 0.03^{f}	1.59 ± 0.09^{e}	–	Cheng et al. (2013), Pellati et al. (2013), Madrigal-Santillán et al. (2014), Yang et al. (2015), Melliou, Stratis, and Chinou (2007)
Methyl glyoxal	970	–	–	0.16 ± 0.02^{j}	NPR
Octanal	981	–	0.55 ± 0.07^{h}	1.57 ± 0.08^{d}	Kamatou et al. (2019), Jerković et al. (2016), Cheng et al. (2013), Pellati et al. (2013), Melliou et al. (2007), Nunes et al. (2009)
Nonanal	1101	–	1.25 ± 0.04^{e}	–	Jerković et al. (2016), Pellati et al. (2013), Melliou et al. (2007), Nunes et al. (2009), Nunes and Guerreiro (2012)
Decanal	1204	0.67 ± 0.01^{h}	0.51 ± 0.11^{h}	–	Jerković et al. (2016), Pellati et al. (2013), Melliou et al. (2007), Nunes et al. (2009)
Vanillin	1394	0.76 ± 0.10^{h}	–	–	Jerković et al. (2016), Pellati et al. (2013), Madrigal-Santillán et al. (2014), Tomaszewski et al. (2019), Król et al. (2013), Ristivojević et al. (2018)
Ketones					
Cyclopentanone	757	-	1.36 ± 0.10^{e}	1.88 ± 0.04^{c}	NPR
6-Methylhept-5-en-2-one	959	0.21 ± 0.03^{m}	1.14 ± 0.02^{f}	–	Jerković et al. (2016)
Acetophenone	1050	1.24 ± 0.18^{g}	1.08 ± 0.07^{f}	–	Cheng et al. (2013), Pellati et al. (2013), Madrigal-Santillán et al. (2014), Yang et al. (2015)
5,9-Undecadien-2-one, 6,10-dimethyl-	1427	–	0.50 ± 0.12^{h}	1.67 ± 0.06^{cd}	NPR
Ethanone, 1-[2,3-dihydro-2,3-dihydroxy-2-(1-methylethenyl)-5-benzofuranyl]-	1950	0.60 ± 0.12^{h}	0.23 ± 0.06^{jl}	–	NPR
Alcohols/phenols-					
1,4-Dichlorobenzene	991	–	–	1.41 ± 0.10^{d}	NPR
Benzyl alcohol	1005	0.45 ± 0.05^{j}	–	–	Jerković et al. (2016), Cheng et al. (2013), Pellati et al. (2013), Madrigal-Santillán et al. (2014)
Phenylethyl Alcohol	1083	0.38 ± 0.01^{l}	0.23 ± 0.01^{jl}	–	Kamatou et al. (2019), Jerković et al. (2016), Pellati et al. (2013), Yang et al. (2015), Tomaszewski et al. (2019), Król et al. (2013), Ristivojević et al. (2018)
3-Phenylpropanol	1233	0.30 ± 0.08^{l}	–	–	Bankova et al. (2000)
Acids					
Benzoic acid	1167	0.54 ± 0.07^{i}	–	–	Jerković et al. (2016), Pellati et al. (2013), Yang et al. (2015), Ristivojević et al. (2018), Soltani et al. (2017), Alencar et al. (2007)
Decanoic acid	1545	0.11 ± 0.00^{o}	–	–	Pellati et al. (2013), Król et al. (2013)
Dodecanoic acid	1555	1.30 ± 0.17^{g}	–	–	Pellati et al. (2013), Król et al. (2013)
Ester					
Acetic acid, ethyl ester	587	–	0.11 ± 0.01^{k}	0.26 ± 0.01^{i}	NPR
2-Butenoic acid, ethyl ester, (Z)-	812	0.12 ± 0.00^{o}	0.31 ± 0.04^{ij}	–	NPR
Butanoic acid, 3-hydroxy-, ethyl ester	946	0.21 ± 0.01^{m}	0.37 ± 0.04^{i}	–	NPR
Hexanoic acid, ethyl ester	978	0.16 ± 0.08^{o}	1.25 ± 0.28^{e}		NPR
Benzoic acid, methyl ester	1072	0.08 ± 0.00^{p}	–	–	Nunes et al. (2009)
Heptanoic acid, ethyl ester	1082	–	0.31 ± 0.09^{ij}	–	NPR
Benzoic acid, ethyl ester	1142	12.52 ± 2.02^{c}	13.65 ± 0.32^{b}	–	Yang et al. (2015), Cheng et al. (2013),
Butanedioic acid, diethyl ester	1144	2.16 ± 0.44^{f}	5.13 ± 0.11^{c}	–	Alencar et al. (2007)
Benzeneacetic acid, ethyl ester	1210			–	NPR

(*continued on next page*)

Table 1 (*continued*)

Compounds	Linear retention index (LRI)	BPB	BPBF	CF	Previously reported*
		0.13 ± 0.03^{o}	0.36 ± 0.06^{i}		
Benzenepropanoic acid methyl ester	1263	1.32 ± 0.08^{g}	0.65 ± 0.09^{g}	–	Bittencourt et al. (2015), Nunes and Guerreiro (2012)
Nonanoic acid methyl ester	1285	0.27 ± 0.06^{lm}	0.33 ± 0.00^{ij}	–	Jerković et al. (2016)
Benzenepropanoic acid, ethyl ester	1320	25.13 ± 1.59^{a}	32.93 ± 4.42^{a}	–	Tomaszewski et al. (2019), Bittencourt et al. (2015)
Decanoic acid ethyl ester	1374	3.21 ± 0.28^{e}	2.21 ± 0.33^{d}	–	NPR
Benzoic acid, 4-methoxy-, ethyl ester	1426	0.66 ± 0.08^{h}	–	–	NPR
2-Propenoic acid, 3-phenyl-, ethyl ester	1430	4.01 ± 0.20^{d}	1.41 ± 0.53^{e}	–	Tomaszewski et al. (2019)
Dodecanoic acid, ethyl ester	1573	4.58 ± 0.40^{d}	1.37 ± 0.44^{e}	–	NPR
1,2-Benzenedicarboxylic acid, diethyl ester	1578	–	–	0.52 ± 0.00^{g}	NPR
Hexanoic acid, 2-phenylethyl ester	1611	–	–	0.10 ± 0.00^{l}	NPR
Benzyl Benzoate	1745	0.12 ± 0.02^{n}	0.13 ± 0.01^{k}	–	Jerković et al. (2016), Cheng et al. (2013), Pellati et al. (2013), Melliou et al. (2007)
Hexanedioic acid, 1,4-butanediol ester	1890	–	13.25 ± 1.99^{b}	77.86 ± 1.35^{a}	NPR
Linoleic acid ethyl ester	2158	0.45 ± 0.06^{j}	–	–	NPR
Ether					
1-Methoxy- 2-propanol,	673	–	–	0.21 ± 0.02^{ij}	NPR
Methane oxybis dichloro	987	–	0.43 ± 0.13^{i}	0.10 ± 0.00^{l}	NPR
Acetic anhydride (Acetyl ether)	706	–	–	0.18 ± 0.01^{j}	NPR
Diglycerol	1504	-	–	11.41 ± 0.09^{b}	NPR
Hydrocarbon					
Cyclobutene, 2-propenylidene-	735	–	0.27 ± 0.06^{j}	–	NPR
Toluene	747	–	–	0.89 ± 0.01^{f}	NPR
Phenylethane	854	–	–	0.10 ± 0.00^{l}	Yang et al. (2015)
7-Methyl-3-octyne	869	0.07 ± 0.00^{p}	–	–	NPR
Anthracene	1754	16.31 ± 1.47^{b}	–	–	Moret et al. (2010), Porrini et al. (2003)
Terpenoids					
(2,4(10)-Thujadiene)	947	0.17 ± 0.03^{n}	0.66 ± 0.19^{g}	–	NPR
Eucalyptol (1,8-Cineole)	1021	0.27 ± 0.05^{lm}	–	–	Tomaszewski et al. (2019), Jerković et al. (2016), Yang et al. (2015), Cheng et al. (2013), Nunes and Guerreiro (2012), Melliou et al. (2007)
o-Cymene (ortho-Cymene)	1038	0.11 ± 0.00^{o}	1.06 ± 0.03^{f}	–	Cheng et al. (2013)
cis/trans-Pinocarveol	1143	0.54 ± 0.04^{i}	0.81 ± 0.04^{g}	–	Melliou et al. (2007)
Terpinen-4-ol	1161	0.53 ± 0.08^{i}	1.16 ± 0.10^{f}	–	Jerković et al. (2016), Pellati et al. (2013)
p-Cymen-8-ol	1165	0.14 ± 0.08^{o}	–	–	Pellati et al. (2013), Melliou et al. (2007)
2-Pinen-10-ol (Myrtenol)	1182	3.03 ± 0.17^{e}	1.47 ± 0.09^{e}	–	Pellati et al. (2013)
Verbenyl, ethyl ether	1184	0.11 ± 0.01^{o}	0.49 ± 0.07^{h}	–	NPR
cis/trans-Carveol	1207	0.57 ± 0.07^{i}	0.19 ± 0.01^{j}	–	Melliou et al. (2007)
Verbenone	1212	1.07 ± 0.03^{g}	0.77 ± 0.05^{g}	–	Jerković et al. (2016), Pellati et al. (2013)
α-Copaene	1221	0.20 ± 0.03^{mn}	1.47 ± 0.07^{e}	–	Jerković et al. (2016), Yang et al. (2015), Madrigal-Santillán et al. (2014), Pellati et al. (2013), Cheng et al. (2013), Nunes and Guerreiro (2012)

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Table 1 (*continued*)

Compounds	Linear retention index (LRI)	BPB	BPBF	CF	Previously reported*
Bornyl acetate	1269	0.38 ± 0.04^{l}	0.23 ± 0.01^{j}	–	Kamatou et al. (2019), Jerković et al. (2016), Melliou et al. (2007)
β-Cubebene (β-Cuvebene)	1382	0.25 ± 0.01^{m}	–	–	Melliou et al. (2007)
β-Bourbonene	1397	0.47 ± 0.03^{j}	0.71 ± 0.10^{g}	–	Jerković et al. (2016), Pellati et al. (2013), Melliou et al. (2007)
Caryophyllene	1422	0.63 ± 0.12^{h}	0.81 ± 0.00^{g}	–	Jerković et al. (2016), Bittencourt et al. (2015), Pellati et al. (2013), Melliou et al. (2007)
Aromadendrene	1447	–	0.37 ± 0.02^{i}	–	Nunes and Guerreiro (2012)
Humulene (α-Caryophyllene)	1455	0.79 ± 0.13^{h}	–	–	Melliou et al. (2007)
α-Curcumene	1470	0.14 ± 0.00^{o}	–	–	Jerković et al. (2016), Pellati et al. (2013), Cheng et al. (2013), Melliou et al. (2007)
γ-Gurjunene	1473	0.61 ± 0.07^{h}	–	–	NPR
Alloaromadendrene	1474	0.78 ± 0.13^{h}	1.10 ± 0.02^{f}	–	Jerković et al. (2016), Melliou et al. (2007)
γ-Muurolene	1482	1.05 ± 0.12^{g}	0.67 ± 0.04^{g}	–	Bittencourt et al. (2015), Pellati et al. (2013), Nunes and Guerreiro (2012)
α-Muurolene	1492	0.69 ± 0.09^{h}	0.71 ± 0.05^{g}	–	Jerković et al. (2016), Pellati et al. (2013), Melliou et al. (2007)
β-Cadinene	1499	2.02 ± 0.13^{f}	–	–	NPR
γ-Cadinene	1506	0.51 ± 0.05^{i}	0.54 ± 0.04h	–	Jerković et al. (2016), Pellati et al. (2013), Melliou et al. (2007)
Calamenene	1509	0.11 ± 0.01^{o}	0.33 ± 0.06^{ij}	–	Jerković et al. (2016), Pellati et al. (2013), Cheng et al. (2013)
δ-Cadinene	1525	0.57 ± 0.05^{i}	1.21 ± 0.10^{e}	–	Bittencourt et al. (2015), Cheng et al. (2013), Nunes and Guerreiro (2012)
α-Calacorene	1534	0.14 ± 0.03^{o}	–	–	Jerković et al. (2016), Pellati et al. (2013)
Spathulenol	1566	1.49 ± 0.10^{g}	0.71 ± 0.12^{g}	–	(18,15,9) Bittencourt et al. (2015), Nunes and Guerreiro (2012), Bankova et al. (2000)
Globulol	1594	0.23 ± 0.03^{m}	–	–	Melliou et al. (2009)
t-Cadinol	1632	0.02 ± 0.01^{q}	–	–	Jerković et al. (2016), Pellati et al. (2013),
Cubenol	1641	0.49 ± 0.05^{i}	–	–	Pellati et al. (2013)
Caryophyllene oxide	1575	0.51 ± 0.05^{i}	–	–	Jerković et al. (2016), Bittencourt et al. (2015), Pellati et al. (2013)
α-Bisabolol	1674	0.35 ± 0.05^{l}	–	–	Jerković et al. (2016), Yang et al. (2015), Pellati et al. (2013)

* Other authors that have reported these compounds in gross propolis. ** NPR = Not Previously Reported. Different letters in the same column mean significant difference at $p < 0.05$.

propolis by-product film, and 18 compounds in the control film (Table 1, Fig. 1ab). To date, no other author has reported on the volatile constituents of a propolis by-product. Therefore, in comparison to other author' work is limited to those concerning the native raw product and not the by-product.

Most of the volatile compounds found in BPB and BPBF were terpenoids, esters or aldehydes. Terpenoids were the first most numerous class of compounds found in this study with 33 compounds identified

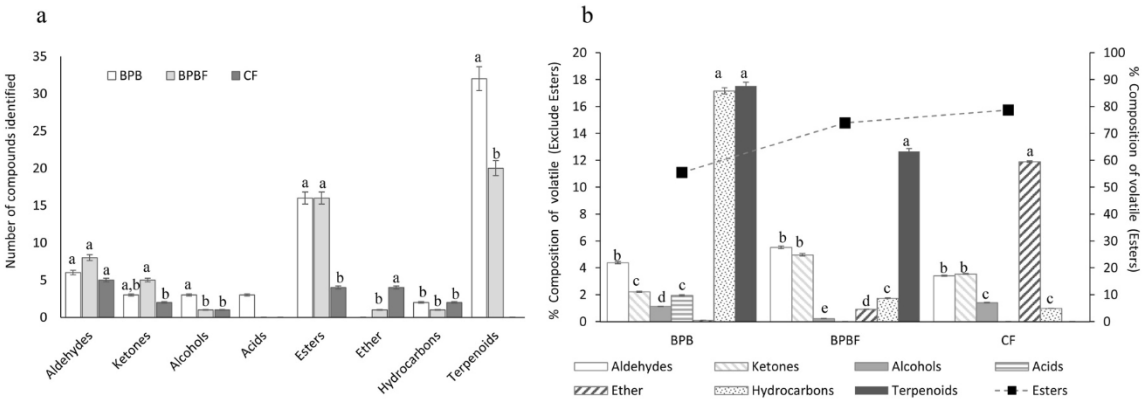

Fig. 1. Compounds classes identified in Brazilian propolis by-product (BPB), Brazilian propolis by-product film (BPBF) and control film (CF). (a) Number of compounds identified for each class and (b) % Composition of volatile component classes. Different letter in the same compound class denote statistical differences at $p<0.05$

(Table 1, Fig. 1ab). However, many of these compounds were present in very low amounts in the BPBF. Furthermore, no terpenoids was found in the control film (Table 1).

The main representatives of this class in the BPB were 2-pinen-10-ol, followed by β-cadinene, spathulenol and verbenone. In contrast, the major terpenoids in the BPBF were α-copaene, 2-pinen-10-ol, δ-cadinene, and terpinen-4-ol (Table 1). According to Bankova et al. (2018) the spathulenol is found only in propolis from tropical regions.

The terpenoid qualitative volatile composition of propolis by-product reported in the present study was similar to the volatile propolis profile obtained by other authors (Bankova, Castro, & Marcucci, 2000; Bittencourt et al., 2015; Cheng et al., 2013; Jerković, Marijanović, Kuś, & Tuberoso, 2016; Madrigal-Santillán et al., 2014; Pellati et al., 2013). However, three terpenoids as γ-gurjunene, β-cadinene and Guajazulene, the first two with a substantial concentration in the BPB, have never been previously reported as Brazilian propolis constituents.

Twenty-one esters were identified in this study, making them an important compound class detected in the BPB (16) and BPBF (16). The main compounds detected in this class include benzenepropanoic acid ethyl ester, and benzoic acid ethyl ester. In the control film, only four esters, namely acetic acid ethyl ester, 1,2-benzenedicarboxylic acid diethyl ester, hexanoic acid 2-phenylethyl ester, and hexanedioic acid 1,4-butanediol, ester were identified. However, these compounds were not present in the BPB (Table 1, Fig. 1ab). In particular, the benzenepropanoic acid ethyl ester was the principal constituents of BPB and BPBF, accounting for 25.1 ± 1.6% and 32.9 ± 4.4% of all volatiles identified, respectively (Table 1). This ester was also reported to be abundant in ethanol, dichloromethane and hexane extracts of green and brown Brazilian propolis (Bittencourt et al., 2015).

Madrigal-Santillán et al. (2014) conducted a review of diverse natural products, including propolis with hepatoprotective effects attributed to the presence of phytochemicals. In this study, the authors showed the biological activity of aromatic esters of propolis, such as benzoic acid ethyl ester (ethyl benzoate). This compound was found in high proportion in the BPB and BPBF, accounting for 12.5 ± 2.0% and 13.7 ± 0.3% of all volatiles identified, respectively.

Twelve aldehydes were identified in the BPB, BPBF and CF, typically at a low level (on average < 0.7% of all volatiles identified) (Table 1, Fig. 1ab). Benzaldehyde was the main aldehyde present in the BPB (2.0 ± 0.0% of all volatiles identified) and in the BPBF (1.6 ± 0.1% of all volatiles identified). These results are in good agreement with the results reported for native propolis by Cheng et al. (2013) and Jerković et al. (2016). Small proportions of decanal and nonanal were detected in the BPB and BPBF (Table 1). Moreover, only two (hexanal and octanal) of the 12 compounds found in the control film were present in the BPB and BPBF (Table 1).

Ketones, alcohols, acids, hydrocarbons and ether groups were detected at low percentages in all the samples. According to Cheng et al. (2013), thirteen components contributed most to the aroma profile of Chinese propolis including acetic acid, propanonic acid, 2-butenoic acid, benzyl alcohol, phenylethyl alcohol, benzaldehyde, nonanal, vanillin and guaiol. Among these compounds, benzyl alcohol, phenylethyl alcohol, benzaldehyde and nonanal were present in the BPB and BPBF, while vanillin was present only in the BPB.

Moret, Purcaro, and Conte (2010) reported the presence of anthracene in six raw propolis and three propolis extract on the Italian market. Anthracene, is a polycyclic aromatic hydrocarbon (PAH) and may have arisen via contamination of the samples as reported by Porrini et al. (2003). The *Apis mellifera* bees travel miles to find their food, making it a possibility that the sample containing anthracene came from an industrial site. In that case, we would have to look more carefully at the causes of this contamination with beekeepers so that they take good practice measures to avoid this problem. And although we did not find the anthracene in the films, it will also be necessary to investigate the migration of this compound into food. However, reports of PAH content in propolis are rare and further research is required to identify the main sources of contamination not only in the present case but in propolis and its products in general.

3.2. Antioxidant and antibacterial activities

One of the main problems affecting the quality and safety of food is the lipid oxidation and microbial contamination. In this way, natural products with antioxidant and antimicrobial properties can be incorporated into the packaging to reduce these problems. Besides, it can become an alternative to the overuse of synthetic antioxidants, which are harmful to human health. Skowron et al. (2019), showed the effects of propylene film coated with a solution of chitosan and ethanolic extract of propolis that helped to reduce the lipid oxidation in refrigerated wrapped fishes and cheese. In this sense, the propolis by-product seems to be a good source of bioactive compounds and has high potential to be incorporated into films.

In this study, the antioxidant activity of the BPB, BPBF and CF extracts was evaluated through the scavenging activity by DPPH and ABTS methods as well as through FRAP assay, as shown in Table 2. The BPB extract obtained the highest antioxidant activity by DPPH, ABTS and FRAP methods with values of 28.55 mmol TE/g, 8133.33 mmol TE/g and 60259.05 mmol FSE/g, respectively. That was followed by BPBF and CF with values of 8.45 and 0.09 mmol TE/g. At the same time, the antioxidant activity by ABTS methods for these samples were 317.58 and 6.30 mmol TE/g, respectively. The FRAP values for BPBF and CF were 1217.75 and 10.50 mmol FSE/g, respectively. The levels of TPC present in BPB, BPBF and CF were, respectively, 99.34, 4.99 and 0.69 mg GAE/g of sample, with statistical difference ($p < 0.05$) between them (Table 2). The TPC and FRAP result for BPB agrees with those reported by De Francisco et al. (2018) upon assessing the total phenolic compounds in raw propolis and propolis by-product from Maringá, Brazil, who found 100.7 mg GAE/g and 1273.25 μmol FSE/g respectively.

Several authors have reported that the terpenoids may be of practical significance since many of these compounds could inhibit the lipid oxidation of the food packaged in plastic film with propolis (Rezaeigolestani et al., 2017; Silici & Kutluca, 2005). In a previous study, Rizzolo et al. (2016) used paper sheets obtained by incorporating propolis to package cooked ham slices and to determine the possible changes in the volatile profile during storage. The terpenoids and other phenolic compounds gradually migrated from packaging into the fat portions of cooked ham slices. In fact, the antioxidant properties of propolis may have been responsible for the decrease in lipid oxidation and the gradual migration of compounds from packaging into ham slices did not influence the sensory properties.

Vanillin was the second major aldehyde found in the BPB and previous studies confirm that vanillin is a common compound in raw propolis (Kamatou, Sandasi, Tankeu, Vuuren, & Viljoen, 2019; Tomaszewski et al., 2019). Another study showed propolis phenolic acids and vanillin's ability to enter the skin and contribute to skin protection from free radicals formed beneath UV and early skin ageing (Król et al., 2013).

Several authors have studied the physical, antioxidant and

Table 2
Total phenolic content (TPC) and antioxidant activity of Brazilian propolis by-product (BPB), Brazilian propolis by-product film (BPBF) and control film (CF).

Analysis/samples	BPB	BPBF	CF
TPC (mg GAE/g)	99.34 ± 1.21[a]	4.99 ± 0.47[b]	0.74 ± 0.05[c]
DPPH (mmol TE/g)	28.55 ± 0.03 [a]	8.45 ± 0.54 [b]	0.09 ± 0.03 [c]
FRAP (mmol FSE/g)	60259.05 ± 7.42[a]	1217.75 ± 0.13[b]	10.05 ± 1.64[c]
ABTS (mmol TE/g)	8133.33 ± 4.17 [a]	317.58 ± 1.13[b]	6.30 ± 0.55[c]

GAE: Gallic acid equivalent. TE: Trolox equivalent. FSE: Ferrous sulphate equivalent.
Mean followed by different letters in the same line indicate significant differences ($p < 0.05$).

antimicrobial properties of films based on hydroxymethylcellulose including propolis made by solvent casting method (Rezaeigolestani et al., 2017; Rizzolo et al., 2016; Siripatrawan & Vitchayakitti, 2016). Nevertheless, few efforts have been performed to study the addition of propolis by-product and other agro-industrial by-products to food packaging materials by extrusion process.

Regarding antibacterial activities, the bacteria and molds are the main spoilage agents in foods and are the causative agents of several food-related diseases. The propolis has an important antimicrobial effect on several bacteria such as *Staphylococcus aureus* (Bittencourt et al., 2015), *Photobacterium damselae* (Soltani et al., 2017), *Streptococcus mutans* (Ristivojević et al., 2018) and *Pseudomonas aeruginosa* (Siripatrawan & Vitchayakitti, 2016). This inhibitory potential of propolis against a set of pathogenic microorganisms is due to their chemical composition such as aromatic compounds, phenolic acids, flavonoids and the interaction between them. Thus, propolis can be used against foodborne pathogens to reduce synthetic preservatives and increase the assertive result of food on the people's well-being. In fact, the chemical composition of different propolis and its antibacterial and antioxidant potential have been extensively studied (Siripatrawan & Vitchayakitti, 2016; Tomaszewski et al., 2019). However, in by-product and plastic films into which it is incorporated, these results are scarce, which makes comparison difficult.

Therefore, inhibitory potential of the BPB, BPBF, and CF against a Gram-positive bacteria *S. aureus*, and two Gram-negative bacteria, *E. coli* and *Salmonella* Typhimurium, were evaluated (Table 3). The BPB extract showed MIC and MBC, respectively of 6.5 mg/mL and 12.5 mg/mL against *S. aureus,* which is characteristic of a great *in vitro* activity, probably due to the presence of benzenepropanoic acid, ethyl ester, vanillin and other compounds in the propolis by-product. Bittencourt et al. (2015) cleared that the bioactivity of benzenepropanoic acid, ethyl ester (ethyl hydrocinnamate) from propolis has an authentic relationship with the inhibitory power against *S. aureus*, and synergistic effects happen among the phenolic compounds present in the Brazilian green and brown propolis extracts. Several authors have revealed that various classes of chemicals can be associated with the extracts' antimicrobial activity and are subservient on microbial species used in the analysis (Bittencourt et al., 2015; Tomaszewski et al., 2019).

The BPBF and CF, however, showed MIC and MBC >12.5 mg/mL against all bacteria tested (Table 3). According to Probst, Sforcin, Rall, Fernandes, & Fernandes (2011), Brazilian propolis extract showed the best antimicrobial activity in a concentration of 1.54 mg/mL and 19.24 mg/mL for *S. aureus* and *E. coli*, respectively. Regarding these findings, the concentration of the by-product in the BPB film need to be increased to inhibit all the bacteria, as well as *E. coli*. In fact, several authors have reported that propolis has greater antibacterial activity against gram-positive bacteria than gram-negative bacteria (Ristivojević et al., 2018). And for this reason, higher concentrations of propolis extract are necessary to stop the growth of this bacteria. According to Hames-Kocabas, Demirci, Uzel, and Demirci (2013), gram-negative bacteria have a chemically more complex cell wall and a higher lipid content, which explains a better resistance.

Based on our findings, the content of bioactive compounds in the film containing by-products may vary since the extraction of bioactive compounds from propolis has already gone through an industrial production process. Besides, the technological process used to make biodegradable films can interfere with the stability of bioactive compounds since the films were produced by extrusion-blow processing technique that after to second extrusion process the temperature reached 125 °C (Carpes et al., 2021). Thus, the degradation of bioactive compounds is expected and probably there is a reduction of the antioxidant and antimicrobial activities. Nonetheless, the extrusion process is an industrial technology, being the same used by the industry to produce plastics with low-density polymers (Correa-Pacheco et al., 2020).

Table 3
Antibacterial activity of Brazilian propolis by-product (BPB), Brazilian propolis by-product film (BPBF) and control film (CF).

Tested samples	*Staphylococcus aureus* (ATCC 25923)		*Salmonella* Typhimurium (ATCC 14028)		*Escherichia coli* (ATCC 25922)	
	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)
BPB	6.25	12.5	12.5	>12.5	12.5	>12.5
BPBF	12.5	>12.5	12.5	>12.5	12.5	>12.5
CF	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5
Chloramphenicol	1.2	1.2	1.2	1.2	1.2	1.2

All analyses were performed using three replicates. MIC: minimum inhibitory concentration.
MBC: Minimal bactericidal concentration.

3.3. Mechanical properties of the films

Tensile strength at break, elongation at break and elasticity modulus were measured in order to study of the effect of Brazilian propolis by-product incorporation on the mechanical properties of the biodegradable plastic films with cassava starch (Table 4). Significant reduction in the tensile strength at break and elongation at break in the films with BPB were observed. In fact, the incorporating propolis by-product in the biodegradable plastic film developed by extrusion process affect some mechanical properties. This effect manifested as a 13% reduction in both properties tensile strength and in the elongation at break in the films. Similar results were reported by Toledo, Bavato, Rosseto, Cortesi, and Bruschi (2015) in pharmaceutical films with gelatin or ethylcellulose and propolis by-product produced by a solvent casting method.

In this study, there were also statistically significant differences between the elasticity modulus of films with and without BPB. In this case, it was observed an 11% increase in the elasticity modulus with the addition of 4% propolis by-product. This increase in the elasticity of BPBF is in accordance with Suriyatem, Auras, Rachtanapun, and Rachtanapun (2018), who used propolis extract in active films from rice starch, carboxymethyl and chitosan by casting methods.

According Liu et al. (2016), the mechanical properties of the films changed, probably due to the high temperature used in extrusion processing. The increase in the elasticity modulus and a decrease in the elongation at break is related to the induced orientation in the polymer chains during processing and suggest that there is an induced crystallization during heating (Correa-Pacheco et al., 2020; Liu et al., 2016). Elongation at break is a deal of the film stretchability prior to breakage and the films with high values (above of 100%) of this parameter are interesting for several applications, such as plastic films for use in food, agricultural or pharmaceutical areas (Assis, Lopes, Costa, Flôres, & Rios, 2017). Additionally, the increase in elasticity modulus implied a more flexible film than the control film and according to Correa-Pacheco et al. (2020), it can be indicative of a higher resistance to deformation of the films. Thus, with the assertive BPBF formulation and the extrusion process condition used in this study, the increase in the modulus of elasticity of the polymeric matrix of the film can be attributed to the high rigidity of the propolis residue incorporated in the polymeric matrix.

Table 4
Mechanical properties of the Brazilian propolis by-product film (BPBF) and control film (CF).

Parameters/ samples	Tensile strength at break (MPa)	Elongation at break point (%)	Elasticity modulus (MPa)
CF	2.54 ± 0.13^{a}	383.66 ± 27.66^{a}	10.78 ± 1.53^{b}
BPBF	2.21 ± 0.16^{b}	332.17 ± 49.71^{b}	11.96 ± 0.14^{a}

All data are the mean ± SD of fifteen replicates. Mean followed by different letters in the same column differs significantly *t*-test ($p < 0.05$).

4. Conclusion

This is the first report in the development of a biodegradable cassava starch plastic extruded with the addition of a Brazilian propolis by-product. In this study, the BPB showed the potential to be employed as a protective ingredient in extrusion packaging systems, since the BPBF displayed interesting mechanical, antioxidant, and antibacterial properties. The propolis by-product film developed appeared to be resistant, flexible, and was capable of scavenging free radicals. In addition, inhibiting the growth of known foodborne pathogens like *Staphylococcus aureus* and *Salmonella* Typhimurium. Many of the compounds reported in this research can act as antioxidants and thus limit the extent of lipid oxidation in food matrixes. However, new studies will be needed, principally in the use of this film in foods susceptible to lipid oxidation. Besides, a sensory evaluation to determine whether a propolis by-product affects the food's flavor. Thus, a better understanding of the potential use of this Brazilian propolis by-product as a functional ingredient in biodegradable packaging will be required. Nevertheless, the use of agro-industrial by-products with biological properties in biodegradable films already follows the current trends in environmental care to minimize the use of traditional plastics.

CRediT authorship contribution statement

Carlize Bertotto: Formal analysis, Writing – original draft. **Ana Paula Bilck:** Resources, Methodology. **Fabio Yamashita:** Funding acquisition, Supervision. **Ofélia Anjos:** Data curation, Writing – review & editing. **Md Abu Bakar Siddique:** Formal analysis. **Sabine Martina Harrison:** Investigation, Methodology, Supervision. **Nigel Patrick Brunton:** Funding acquisition, Writing – review & editing. **Solange Teresinha Carpes:** Conceptualization, Project administration, Supervision, Funding acquisition, Writing – review & editing.

Declaration of competing interest

Authors have no conflict of interest to declare.

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