Chapter 1
Pitangueira Leaf Extracts as Alternative to Traditional Additives in Fresh Pork Sausage

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1.1 Introduction

The use of natural additives to extend food shelf-life has been the subject of several research papers due to the new food conception adopted by consumers around the world, which aims at acquiring health and wellness from food products. Although alternatives to chemical additives is extremely desired by consumers, the use of plant preservatives is not a current reality yet. Besides that, natural antioxidants are less efficient and more expensive than synthetic ones (Fasseas et al. 2008).

Research about the effectiveness of natural additives is necessary due to the wide variety of bioactive compounds, and the lack of knowledge regarding extraction conditions, ways to maintain their stability, behavior and application in different food matrixes, quantities, economic viability, and sensory acceptance.

Meat is very susceptible to alterations during storage, which affect its color, odor, texture, and nutritional composition, mainly due to lipid and protein oxidation

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and spoilage (Pham et al. 2014). The utilization of plant-derived additives as alternatives to synthetic ones in meat products has been reported and reviewed recently (Falowo et al. 2014; Hygreeva et al. 2014; Karre et al. 2013; Pham et al. 2014; Qi et al. 2015). Extracts from less conventional plants, such as Pitangueira (Eugenia uniflora L) has attracted attention due to its bioactive properties (Vargas et al. 2016, 2019; Lorenzo et al. 2018).

In this chapter, the utilization of pitangueira leaf extracts as an alternative to traditional additives in fresh pork sausage is described. Two main experiments are reported: (1) several procedures to improve the amounts of bioactive compounds in extracts are described, and (2) fresh pork sausages with natural and/or traditional synthetic additives were assessed for their physical-chemical properties during 12 day-cold storage.

1.2 Quality Parameters of Pork Meat

1.2.1 pH, Oxidation and Microbiology

In the conversion of muscle into meat, pH falls from 7 to 5.5, approximately, in normal conditions (Greaser 2001; Trindade and Gressoni-Júnior 2008), reaching the isoelectric point of most meat proteins (approx. 5.5) and, therefore, lowering muscle water holding capacity (WHC) (Lawrie 2006). This water loss (around 1%) exudes myoglobin, causing meat to become lighter, softer and with moist aspect. These events promote desirable meat quality characteristics (color, texture, appearance, and yield). In this sense, final pH has a direct effect on water loss in meat and on quality parameters.

Lipid oxidation is the most significant reaction in all stored living tissues, negatively contributing to meat quality attributes (discoloration, off-flavor and others) (Pearson et al. 1983). Self-oxidation is the main lipid oxidation pathway (Berger and Hamilton 1995; Ramalho and Jorge 2006), which is influenced by light, pH, temperature, myoglobin concentration, presence of ions and unsaturated fatty acids (Chaijan 2008; Monahan 2000; Silva et al. 1999).

Meat color depends on the heme-globular myoglobin protein (Renerre 2000), that when exposed to oxygen for long periods oxidizes to metmyoglobin becoming brownish, which is associated with the lack of freshness by consumers (Gill 1996; Renerre 2000). Variations in myoglobin color are determined by the oxidative state and the type of molecule attached to the iron atom from the prosthetic heme group in this protein.

According to Faustman and Wang (2000), the interaction between lipid and myoglobin oxidation cannot be ignored since their products can act as pro-oxidants in muscle tissues (Baron and Andersen 2002; Chaijan 2008).

Meat quality can be altered by two groups of microorganisms: pathogenic, which can cause enteric or systemic diseases and fatal infections; and spoilage, which

alters the overall quality of meat (Marshall and Bal'a 2001; Nychas et al. 2008). The contamination of meat products by one of these groups can lead to major economic losses and/or serious risks to public health. Meat spoilage, together with protein and lipid oxidation are the main problems affecting the quality of meat products during shelf life.

1.3 Chemical Additives Used in Meat Products

Salt was the first food additive used to prevent meat spoilage and keep the product viable for later consumption (Pegg and Shahidi 2006). Another important additive in sausage formulation is nitrite (when allowed), which is involved in reactions that promote color, flavor and stability of cured meat products (Martin 2001; Pegg and Shahidi 2006). However, its use has been a cause of concern since the early twentieth century (Martin 2001), because of its toxicity in high concentrations, and its participation in the formation of carcinogenic compounds called nitrosamines (Pegg and Shahidi 2006). In 2015, this issue became intensified due to the classification of processed meat (e.g., sausages) containing nitrite, as 'carcinogenic to humans' by the International Agency for Research on Cancer (IARC), from the World Health Organization (WHO). The findings were on the bases of evidence of colorectal cancer and by positive association with stomach cancer (Bouvard et al. 2015).

Among the traditional food additives for meat products, the synthetic antioxidants butyl hydroxyanisole (BHA), butyl hydroxytoluene (BHT) and propyl gallate (PG) are commonly used (USDA 2015). Nevertheless, their use has also been questioned concerning safety, as they are suspect of having some toxic or carcinogenic effect (Soares 2002). Besides the mentioned synthetic antioxidants, other additives such as acidulants, acidity regulators, flavorings, colorants, color stabilizers, stabilizers, thickeners, flavor enhancers and humectants are also widely used in the preparation of meat products.

1.4 Plant Bioactive Compounds

Among plant bioactive compounds, the secondary metabolites are the most targeted by the food industry, as they usually contain phytochemicals of interest. Plant bioactive compounds can be divided into terpenes and terpenoids, alkaloids and phenolic compounds (Brielmann et al. 2006; Croteau et al. 2000), and the extraction of each type of compound is mainly dependent on its polarity. In this sense, not only the choice of solvent (Azmir et al. 2013), but also the determination of the extraction technique should be considered to obtain the desired bioactive compounds. Among the unconventional extraction techniques found in the literature, the ultrasound technique stands out for its speed, efficiency and low cost. This technique has been widely studied to obtain polyphenols and has shown positive results in increasing

carotenoid extraction from plant by-products (Wijngaard et al. 2012). In this type of extraction, the phenomenon called cavitation occurs occasioning disruption of plant cells, allowing better mass transfer and solvent penetration (Cavalheiro 2013; Sharmila et al. 2016).

1.4.1   Bioactive Compounds in Pitangueira Leaves

Due to the easy adaptation of pitangueira trees (*Eugenia uniflora* Linneus), this species is widely distributed in South American countries, and in several states in Brazil (Sobral et al. 2010). The Myrtaceae family comprises more than 5000 species, of which some has been characterized for antioxidant activity (Consolini and Sararubbio 2002; Nair et al. 1999; Sobeh et al. 2016).

The chemical profile of Myrtaceae, especially the species *Eugenia uniflora*, points to a range of phytochemicals such as flavonoids, quercetin, quercitrin, myricetin and myricitrin, as well as mono and sesquiterpenes (Amorim et al. 2009; Ogunwande et al. 2005; Schmeda-Hirschmann et al. 1987; Victoria et al. 2012).

Besides studies on pitangueira pulp and fruits, it seems that scientific interest on the properties of its leaves is increasing. According to Canabarro et al. (2019), pitangueira leaves are recognized as an important source of bioactive compounds of pharmaceutical and cosmetic interest. Some studies about the bioactive compounds from its leaves point to the possible existence of different chemotypes, which seems to be related to its fruit color biotypes (Mesquita et al. 2017; Costa et al. 2016). Mesquita et al. (2017) determined the profile of volatile compounds of fresh leaves from orange, red and purple fruit-biotype pitangueira trees, and suggested the existence of two varieties of pitangueira. On the other hand, Costa et al. (2016) identified three different types of compound profiles from pitangueira leaves without the existence of different varieties, but a high polymorphism instead.

Regarding phenolics, three main compounds have been identified in pitangueira leaf extract (Vargas et al. 2019), which are: myricitrin and quercetin 3-α-fucopiranoside as the major compounds in intermediate polar fraction (ethyl acetate) of the extracts; and quinic acid as the main compound in the polar fraction (Fig. 1.1).

1.4.2   Properties of Pitangueira Leaf Extracts

Because of its therapeutic properties, pitangueira leaves (Fig. 1.2) have been traditionally used in folk medicine in several tropical and subtropical countries to heal many health disorders and control biochemical blood parameters (Auricchio and Bacchi 2003; Ogunwande et al. 2005; Schumacher et al. 2015). This species is so popular that its use as a medicinal herb is provided by the Brazilian legislation (Anvisa 2005).
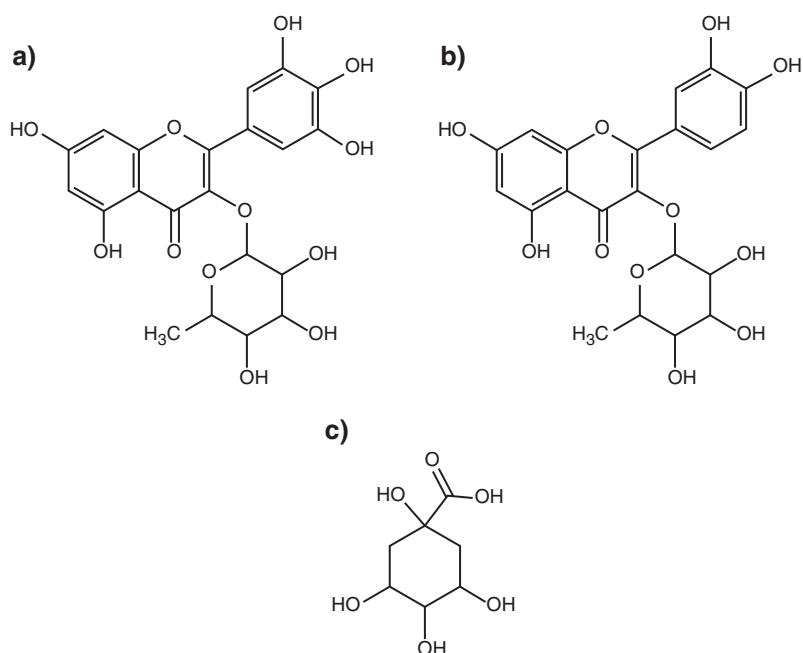
Fig. 1.1 Chemical formulae of phenolic compounds found by Vargas et al. (2019) in pitangueira leaf extracts: myricitrin (**a**), quercetin 3-α-fucopyranoside (**b**) and quinic acid (**c**) (Source: http://www.chemspider.com)

Fig. 1.2 Pitangueira leaves used to prepare the hydroethanolic extracts

The use of pitangueira in folk medicine inspired different studies on the properties of its leaves: it has been reported that pitangueira leaf extracts can inhibit the increase of plasma glucose and triglyceride (Matsumura et al. 2000) and its infusions presented anti-inflammatory effect in rats (Schapoval et al. 1994). Inhibitory

effect against enzyme xanthine oxidase has also been attributed to hydroethanolic extracts (7:3 EtOH: H$_2$O) of pitangueira leaves with no oral toxicity in mice (Schmeda-Hirschmann et al. 1987).

Also, aqueous extracts made of dried leaves from pitangueira have demonstrated higher in vitro antioxidant capacity when compared to rosemary extracts, considered a standard among the studied plants with antioxidant activity (Vargas et al. 2016). Besides that, *in vitro* antibacterial activity for negative and positive Gram bacteria has been reported by these authors. Moreover, extracts (60:40 EtOH: H$_2$O) from pitangueira leaves showed *in vitro* antioxidant and antimicrobial activity (Lorenzo et al. 2018).

Studies also reported that pitangueira leaf essential oil is a source of phenolic compounds with antioxidant (Garmus et al. 2014), antimicrobial and antifungal activity (Auricchio and Bacchi 2003; Ogunwande et al. 2005; Schapoval et al. 1994; Victoria et al. 2012).

To the best of our knowledge, very little information is found concerning the use of pitangueira leaf extracts to delay food spoilage and deterioration. It has been reported, though, that aqueous extracts were not able to improve lipid oxidation stability when applied in high concentration (1 mL/10 g) in refrigerated ground beef (Vargas et al. 2016). On the contrary, powder hydroethanolic extracts (60:40 EtOH: H$_2$O) at concentrations 250, 500 and 1000 mg/kg were as efficient as BHT to prevent lipid oxidation in pork burgers (Lorenzo et al. 2018). Besides meat products, pitangueira leaf extracts were tested in canola oil, and at 200 ppm it allowed canola oil stability by the inhibition of primary and secondary lipid oxidation processes (Vargas et al. 2019).

The use of pitangueira leaf extracts as an alternative to traditional additives to preserve fresh pork sausages during cold storage is reported below.

1.5 Pitangueira Leaf Extracts

1.5.1 Extraction Process of Pitangueira Leaves

The extraction process of pitangueira dried leaves followed several steps, in which different hydroethanolic proportions (water: ethanol—100:0, 20:80, 40:60, 60:40, 80:20, 0:100), ultrasound bath periods (15, 30 and 45 minutes), and temperatures (30, 60 and 80 °C) were assessed. Previous tests showed that the best plant material: solvent ratio for extract preparation was 1 g freeze-dried plant material to 10 ml solvent. These different extraction conditions allowed the understanding that higher ethanolic levels decreased all color parameters (L*, a* and b*) and occasioned higher Brix degree in extracts (Fig. 1.3).

The extraction process from pitangueira dried leaves that allowed the highest total phenolic was achieved with hydroethanolic proportion 40:60 (water: ethanol), 45 min in an ultrasound bath and magnetic stirring extraction at 80 °C. Table 1.1
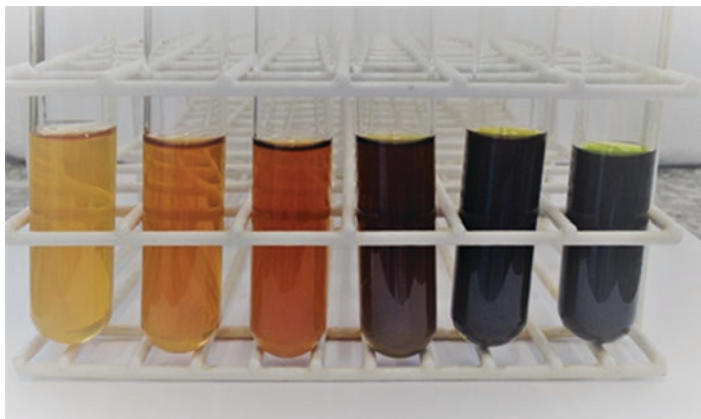
Fig. 1.3 Pitangueira leaf extracts prepared with different hydroethanolic proportions (water:ethanol—100:0, 20:80, 40:60, 60:40, 80:20, 0:100, from the left to the right), at 30 °C

Table 1.1 Extraction condition, total phenolic compounds as gallic acid equivalent (GAE), color parameters (CIE Lab), pH and total soluble solid content (°Brix) of the obtained pitangueira leaf extract*

Extraction conditions	Total phenolic Compounds**	L*	a*	b*	pH	°Brix
45' US 40:60 80 °C	0.29 ± 0.60	0.7 ± 0.4	1.5 ± 0.2	1.3 ± 0.7	4.87 ± 0.04	20.8 ± 0.3

*Means ± standard error. **g GAE/g dry matter

contains the main physical-chemical characteristics of the hydroethanolic extract chosen to be incorporated into fresh pork sausages.

1.5.2 Cytotoxicity of Pitangueira Leaf Extracts

Human Dermal Fibroblasts adult (HDFa) cells were exposed to different concentrations of PLE, and an effect on their viability after 48 h was found. From the concentration 1 mg/mL, a marked decrease in cell viability was noted. Through these results, it was possible to estimate the IC50, that is, the concentration of PLE that inhibited 50% of cell viability, which was 0.451 mg/mL.

Microscopic analysis (Fig. 1.4) of the plates showed a smaller number of cells after 24 h and 48 h culture at concentration 0.2 mg/mL, which corroborates the spectrophotometric analysis of cell viability performed by staining with yellow tetrazolium MTT (3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyltetrazolium bromide). The presence of granules in the culture medium increased proportionally to the pitangueira extract concentration, starting from concentration 0.2 mg/mL.
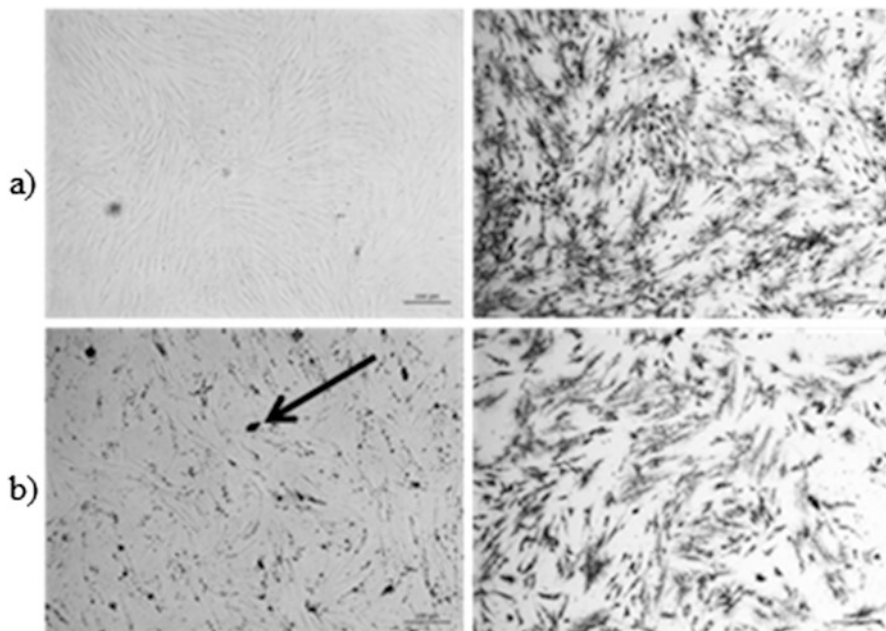
Fig. 1.4 Optical micrography of HDFa cells exposed or not to pitangueira leaf extracts after 24 h (left) and 48 h (right), stained with MTT (the arrow shows the presence of granules, resulting from the extract concentration): (**a**) control, (**b**) 0.2 mg/mL

Thus, the use of PLE is considered safe at moderate concentrations, since it did not present any alteration in cell viability at concentrations below 1 mg/mL. Likewise, the cytotoxic effect of pitangueira leaf ethanolic extract was studied by Cunha et al. (2016) in human leukocytes, where cell viability was determined microscopically in cell suspensions containing extract concentrations from 0.001 to 0.48 mg/mL. Nevertheless, these authors did not observe an alteration in cell viability in human leukocytes exposed to pitangueira leaf ethanolic extract. Similarly, pitangueira leaf methanolic extracts used by Braga et al. (2007) did not show cytotoxicity to J774 murine macrophage cells at a concentration of 0.25 mg/mL. This difference was possibly due to differences in the type of cell, type of extraction and experimental conditions.

1.5.3 *Antioxidant Activity of Pitangueira Leaf Extracts*

PLE showed high antioxidant activity by radical scavenging methods, namely DPPH⋅ (2,2-diphenyl-1-picryl-hydrazyl) assay, described by Brand-Williams et al. (1995), and the ABTS⋅+ (2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)) assay, described by Re et al. (1999). These extracts were able to inhibit DPPH

radical absorbance significantly, demonstrating a high percentage of scavenging ability ($81.5 \pm 2.6\%$) when used at concentration 0.4 mg/mL.

As a matter of comparison, Schumacher et al. (2015) also evaluated the antioxidant activity by assessing the percentage of radical sequestration of pitangueira leaf extracts and reported radical inhibition of 39.9 ± 1.9, 27.6 ± 2, and $34.2 \pm 1.5\%$ at concentration 40 mg/mL for aqueous, methanol/acetone and ethanol extracts, respectively. Besides that, Lorenzo et al. (2018) also reported high antioxidant activity of pitangueira leaf extracts by the DPPH radical scavenging assay expressed as EC50 (0.242 ± 0.014 mg/mL). Likewise, Victoria et al. (2012) reported the antioxidant activity of pitangueira leaf essential oil as EC50 of 0.83 mg/mL.

Concerning the ability of PLE in scavenging the ABTS$^{•+}$ radical expressed as TEAC (mg Trolox equivalent/g DM extract), the antioxidant activity achieved in the current study was 93.73 ± 7.9 mg Trolox/g DM. Other authors also reported the antioxidant activity of pitangueira leaf extracts using the ABTS$^{•+}$ radical assay: Lorenzo et al. (2018) also assessed pitangueira leaf extracts and obtained 570.97 mg Trolox/g DM. In turn, Schumacher et al. (2015) found a statistical difference between the aqueous (8,9 mg Trolox/g sample) and ethanol (6,2 mg Trolox/g sample) extracts ($p < 0.01$).

The results on the determination of the antioxidant activity obtained in this study allowed the understanding that the use of ultrasound-assisted extraction, followed by magnetic stirring using higher temperatures (80 instead of 30 or 60 °C), occasioned better phenolic compound extraction and thus, increased antioxidant power of PLE.

For all the revised studies about pitangueira leaves, it is considered common sense that little is known about the bioactive compounds responsible for the antioxidant activity of the species. In general, this property is attributed to the synergic effect of major and minor compounds present in extracts of the species' leaves. Nevertheless, some of the substances present in this kind of extract may not have the desired bioactivity able to prevent food oxidation, and in this sense, the hydroethanolic extracts were fractionated in an attempt to identify which fractions have better antioxidant activity.

Assays to assess the antioxidant power of fractions (nonpolar, intermediate and polar) of PLE were carried by the capture of the radical ABTS$^{•+}$ and as a result, only the polar and intermediate fractions (obtained by the use of ethyl acetate) showed significant antioxidant power (Table 1.2).

Table 1.2 Antioxidant activity of fractions from pitangueira leaf extracts by the ABTS$^{•+}$ radical scavenging assay

Fractions	TEAC* (mg Trolox/mg MS extract)
Nonpolar	0.4[b]
Intermediate	79.4[a]
Polar	112.3[a]

*TEAC—Trolox equivalent. [a–b]Means followed by equal letters in the same row do not differ statistically by Tukey test at 5%

As a follow-up to the antioxidant assay from the fractions of the pitangueira extracts, the identification of the main compound from polar and intermediate fractions was carried out by HPLC-DAD-MS, as reported in Vargas et al. (2019), whose results revealed that main compounds found in the intermediate fraction were quinic acid in polar fraction and myricitrin and quercetin 3-α-fucopiranoside in intermediate fraction.

1.5.4   Antibacterial Activity of Pitangueira Leaf Extracts

PLE was also submitted to antibacterial activity analysis and presented very satisfactory results for the sensitivity tests (inhibition zones—IZs), minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). Results from these tests were reported by Lorenzo et al. (2018), and according to them, all strains were equally inhibited (IZ) by the extract ($p > 0.05$). Except for *E. coli*, the growth of all bacterial strains (*Bacillus cereus* ATCC 14579; *Escherichia coli* ATCC 25922; *Pseudomonas aeruginosa* ATCC 15442; Salmonella spp. ATCC 13076, and *Staphylococcus aureus* ATCC 25923) was inhibited (MIC) by the use of different concentrations of PLE. Likewise, Souza et al. (2004) reported an inhibitory effect of pitangueira leaf methanolic extract for *Staphylococcus aureus* and *Bacillus subitilis* strains, and whereas no antibacterial action was found for *E. coli*.

1.6   Shelf Life of Fresh Pork Sausages with Pitangueira Leaf Extracts

Our experiment regarding the use of pitangueira leaf extracts as an alternative to traditional additives in fresh pork sausages is described as follows. In this study 4 sausage formulations have been prepared: Positive Control, with sodium nitrite and sodium erythorbate; Erythorbate Control, with sodium erythorbate and reduced-sodium nitrite; Negative Control, with reduced-sodium nitrite; and Extract, with reduced sodium nitrite and pitangueira leaf extract in levels defined by antibacterial assays. To assess the use of pitangueira leaf extract as an alternative additive, the shelf life of fresh pork sausages was conducted for 12 days in cold (4 ± 1 °C) storage in which instrumental color, pH, water activity, lipid oxidation, microbiology, and sensory acceptance were monitored throughout time.

1.6.1 Instrumental Evaluation of Color, pH, and Water Activity of Sausages with Pitangueira Leaf Extract

Instrumental color parameters L*, a* and b* of the studied sausages were analyzed to verify the effect of the treatments and the storage time, or even the interaction between these factors, in the referred parameters.

For parameter L* (luminosity), no significant interaction was observed between the factors under study, but there was a significant effect of the treatment factors and storage time in isolation. During the cooling period, the sausage luminosity, regardless of the treatment, decreased during the first 8 days, from 63.15 to 58.36, increasing slightly on the 12th day of storage, reaching 61.74. This variation in the luminosity of sausage samples may be due to the different physicochemical reactions that occur during storage. Similar results were obtained by Baldin et al. (2016) in fresh pork sausages added, or not, with microencapsulated jabuticaba peel extract. These authors did not observe an interaction between the factors and treatment time, but there were changes over time, reported to decrease L* parameter for all treatments ($p < 0.05$). Among the tested treatments, the lowest average luminosity was obtained for the samples of the extract treatment (58.04 ± 2.2), indicating the change of this parameter occasioned by the color of the plant extract.

In parameter a*, which measures the intensity of the colors green to red, a significant interaction was observed between treatment factors and storage time. In other words, the factors acted together in determining the intensity of a* in the sausages under study. From what can be seen in Fig. 1.5, the red intensity of fresh sausage samples from all treatments showed, in general, a growing trend over the storage
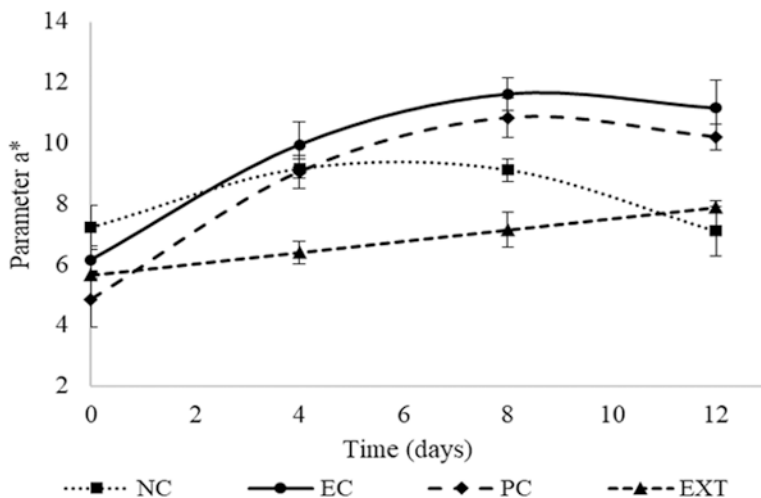

Fig. 1.5 Evolution of color parameter a* from fresh pork sausage samples from different treatments during refrigerated (4 °C) storage (*PC* positive control, *EC* erythorbate control, *NC* negative control, *EXT* extract)

period, which was expected by the presence of nitrite in all formulations. The inclusion of pitangueira leaf extracts in fresh sausages caused lower values of parameter a*, which is probably due to the green coloration of the plant extract, as mentioned above.

The other treatments presented similar behavior, with a slight decrease in this shade after the 8th day of storage, most probably due to the beginning of the myoglobin oxidation process. However, there was a more pronounced decrease in the red intensity of the negative control treatment samples, also from the 8th day of the sampling period. This behavior was already expected since the negative control treatment did not contain antioxidant substances, containing only a reduced-sodium nitrite content. Thus, protein oxidation was more accelerated in this treatment.

Regarding parameter b*, which measures the intensity of the colors blue to yellow, no effect of time or treatment was observed, nor the interaction of these factors, indicating that the intensity of yellow of fresh sausages was unchanged during the experiment. Although statistical analysis showed treatment effect and time for sausage samples regarding water activity, little change was generally observed, so that it remained around 0.97 for samples from all treatments throughout the entire period. These values are as expected, as it is a fresh meat product. Concerning pH, a significant interaction between treatment and factors storage time was observed, and in general, there was a tendency for this variable to increase (Fig. 1.6).

It was observed that the samples of the erythorbate control and negative control treatments showed both values and variations very similar over the period, which was expected since neither of them has any additives with antibacterial action. The evolution of the mean pH values of the extract treatment samples was also very
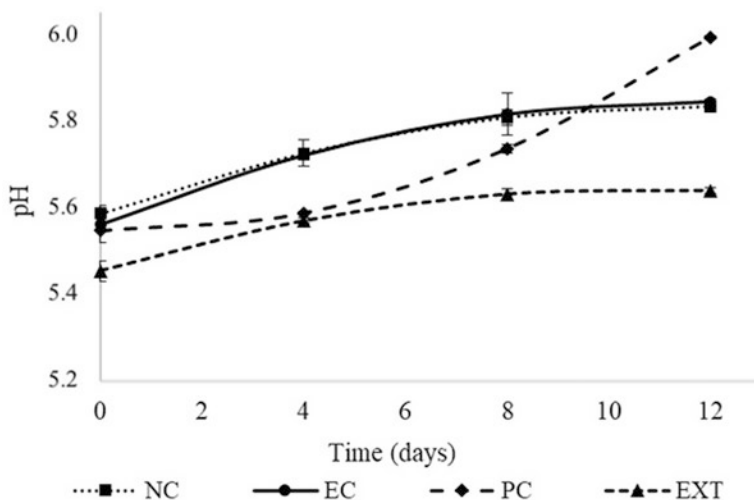

Fig. 1.6 Evolution of pH of fresh pork sausage samples from different treatments during the refrigerated (4 °C) storage (*PC* positive control, *EC* erythorbate control, *NC* negative control, *EXT* extract)

similar to the latter, but with lower values, probably caused by the acidity of the plant extract itself, whose pH was 4.87. In turn, the positive control sausages kept their pH below that of the negative and erythorbate control samples for most of the shelf time, as expected. Lower pH values were also observed by Lorenzo et al. (2014), in ground pork with natural extracts of green tea and grape seed, a fact that the authors attributed to the properties of bioactive compounds formed by acid groups present in the extracts. Thus, it is also considered that the presence of bioactive compounds such as chemical acid, identified in the phenolic compounds of plant extracts (Vargas et al. 2019), may have influenced the low pH values of the extract treatment sausages.

Generally, the pH of the treatment samples all showed an increase over the storage period, which according to Nychas et al. (1998) can be due to the action of many species of bacteria that produce ammonia by metabolizing amino acids, promoting the pH increase of deteriorating meat products.

The lower pH values of the sausages in the extract treatment presents a great advantage both in the control of microbiological growth, as in some meat product quality attributes as greater water retention capacity with a consequent increase in juiciness and yield, a decrease of cooking and freezing weight losses and improved texture.

1.6.2 Lipid Oxidation of Fresh Pork Sausages with Pitangueira Leaf Extract

Lipid oxidation, measured by TBARS method, in fresh pork sausage samples, showed significant interaction ($p < 0.05$) between factors, which means that responses to this variable were dependent both on time and treatment (Fig. 1.7). As can be seen, except for extract treatment, there was a growing trend for TBARS values in fresh sausage samples with an overall average ranging from 0.65 to 1.2 mg MDA/kg sample, namely, lipid oxidation of the mentioned treatments increased during shelf life.

In the raw material used to prepare the sausage formulations, some degree of lipid oxidation was detected by the presence of secondary compounds from the oxidative lipid process (malondialdehyde-MDA) in relatively high quantities on day 0. However, the increase of such compounds and thus MDA values throughout storage time was expected since chemical reactions as oxidation still occur in meat samples under refrigeration.

Among the tested sausage formulations, extract treatment was the only one capable of maintaining lipid oxidation values at initial levels (Table 1.3), showing minimal propagation of this chemical reaction, which is promoting better protection of the lipids present in the fresh pork sausages with pitangueira leaf extract. This is a very important finding because it demonstrates the high antioxidant capacity of pitangueira leaf extracts when used in this type of meat product stored under
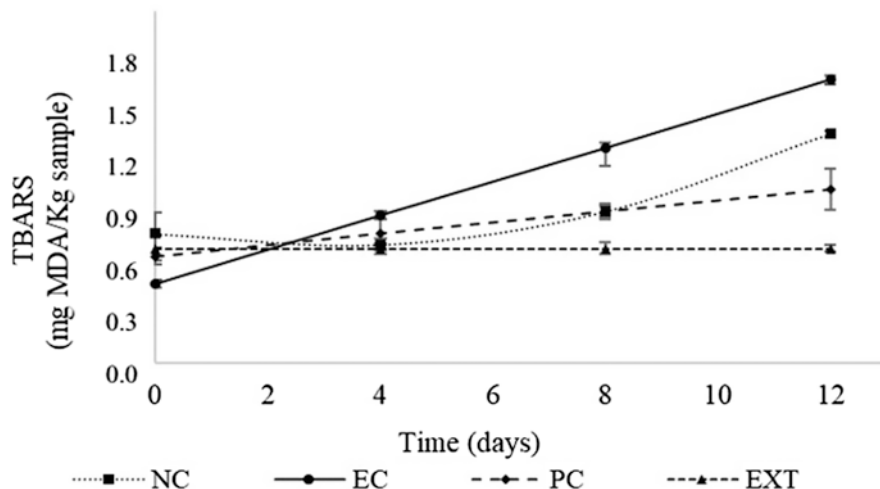
Fig. 1.7 Evolution of lipid oxidation by TBARS in fresh pork sausage samples from different treatments during the refrigerated (4 °C) storage (*PC* positive control, *EC* erythorbate control, *NC* negative control, *EXT* extract)

Table 1.3 Average values of lipid oxidation (TBARS) of fresh pork sausages from different treatments under cold storage of 12 days

Sausage treatment	TBARS (mg MDA/kg sample)*
Negative control	0.94 ± 0.31[ab]
Erythorbate control	1.10 ± 0.60[a]
Positive control	0.84 ± 0.19[bc]
Extract	0.68 ± 0.13[c]

*Means ± standard error. [a–c]Means followed by equal letters in the same row do not differ statistically by Tukey test at 5%

refrigeration. Besides that, the fact that the extracts protected sausages from lipid oxidation corroborates with results from the antioxidant activity analysis performed in these extracts (see Sect. 1.3).

Also, it should be noted that since samples were already oxidized at the beginning of the storage period, the antioxidant activity of extracts showed an ability to act mainly during the propagation phase of lipid oxidation (Ramalho and Jorge 2006). This capacity PLE was probably due to the presence of phenolic acids, such as quinic acid, which have not only the ability to chelate metals that participate in the generation of reactive species and hence initiate lipid oxidation, but also the ability to control the propagation phase of the oxidative process by donating electrons to reactive species already found in the environment (Shahidi et al. 1992; Soares 2002). These results demonstrate the effectiveness of PLE as natural antioxidants in chilled pork products.

The evolution of TBARS in sausages from treatment positive control was similar to treatment extract, showing a slight increase over time, as expected. Treatments

erythorbate and negative control had prominent growth at the end of the storage period, demonstrating little protection from lipid oxidation of fresh pork sausages during the 12-day cold storage.

1.6.3 Microbiology of Fresh Pork Sausages

In these assays, microbiological plates from day 12 were dismissed since the amount of colony-forming units (CFU) was too high, hindering counts for all the tested microorganisms. Nevertheless, little variation between treatments was observed for *Pseudomonas* spp., where the increasing tendency of colony amount has been observed for this bacteria species (Fig. 1.8). To some extent the increase in *Pseudomonas* spp. counts were expected, as this is one of the major spoilage bacteria in refrigerated meat (Marshall and Bal'a 2001). Therefore, no antibacterial effect was observed for *Pseudomonas* spp. growth with the use of additives from the different treatments in the current experiment.

Counts of psychrotrophic also showed an increasing trend for all treatments (Fig. 1.9). Nevertheless, sausages from treatment positive control had lower counts throughout the storage period, as expected for a synthetic additive. Although sausage samples already had high initial counts (day 0), the antibacterial action of sodium nitrite additive in sausages from positive control seemed sufficient to contain the increase of psychrotrophic during the storage period, thus evidencing its efficacy in refrigerated meat products. On the other hand, treatments erythorbate and extract were not efficient to control this species' growth.

Total coliform counts in sausage samples (Fig. 1.10), presented, in general, a decreasing tendency during cold storage. This type of behavior can be expected in food products during cold storage due to nutrient competition caused by the increase
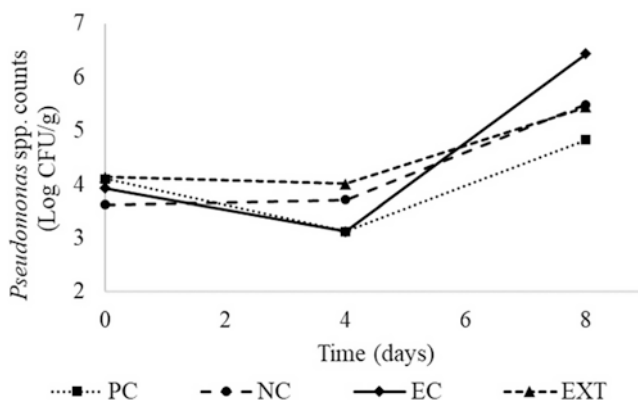

Fig. 1.8 Evolution of *Pseudomonas* spp. counts in fresh pork sausage samples from different treatments during the refrigerated (4 °C) storage (*PC* positive control, *EC* erythorbate control, *NC* negative control, *EXT* extract)

Fig. 1.9 Evolution of psychrotrophic counts in fresh pork sausage samples from different treatments during the refrigerated (4 °C) storage (*PC* positive control, *EC* erythorbate control, *NC* negative control, *EXT* extract)
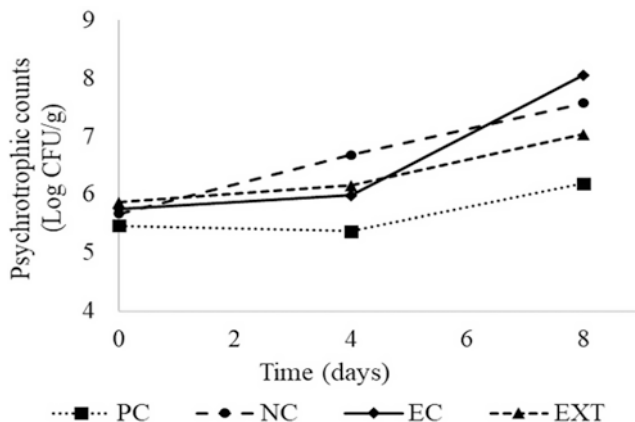

Fig. 1.10 Evolution of total coliform counts in fresh pork sausage samples from different treatments during the refrigerated (4 °C) storage (*PC* positive control, *EC* erythorbate control, *NC* negative control, *EXT* extract)
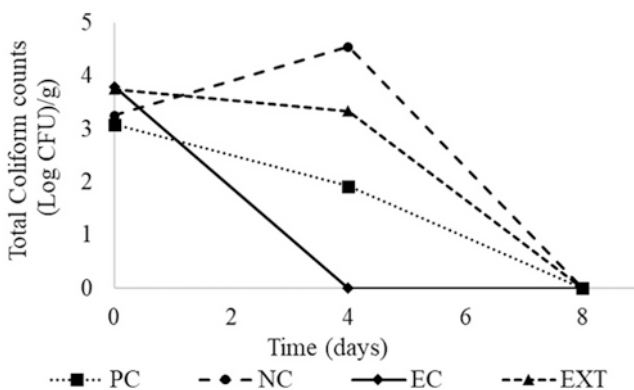
of spoilage microorganisms, such as *Pseudomonas* and psychrotrophic, which has been confirmed by the evolution of such species (Fig. 1.8 and 1.9).

A variation in total coliform counts between samples from the different treatments during the storage period was observed, especially on day 4, when CFU from negative control samples increased, probably because of the lack of substances capable of inhibiting this species' growth. By that time, the competition for nutrients was probably not very high, considering the population of *Pseudomonas* and psychrotrophic bacteria.

Although it is known that the initial microbiological load is related to the physiological state of the animal and sanitary conditions during slaughter (Marshall and

Bal'a 2001; Nychas et al. 2008), the contamination during processing of meat products directly influences its microbiological status, especially considering total coliform population. In the present experiment, the use of additives (traditional or natural) has influenced the counts of the total coliform population from treatments positive control, erythorbate control, and extract.

1.6.4 Sensory Acceptance of Pork Sausages with Pitangueira Leaf Extracts

Sensory acceptance of cooked pork sausages was assessed to verify consumers' preference, duly approved by the Ethics Committee of Faculdade de Zootecnia e Engenharia de Alimentos from Universidade de São Paulo (FZEA/USP CAAE 74595917.8.0000.5422, approval 2.247.424). Consumers (n = 106) used a 9-points hedonic scale (1—dislike extremely and 9—like extremely) to evaluate attributes taste, aroma, color, texture and global acceptance from sausages.

For all the tested attributes significant differences were found (Table 1.4), in which sausages from treatment extract received lower grades, in general. Grades for attribute taste were around 7 (like moderately) for all the tested treatments, which means that consumers approved this attribute. Sausages from treatments positive control received better grades than treatment extract, while sausages from erythorbate and negative control had intermediate values.

For the aroma attribute, sausages from negative control and extract had similar grades, while samples from positive control and erythorbate control received higher grades than treatment extract. This indicates that the presence of extracts into sausages affected sausages' aroma negatively. In some cases, a smell of green grass was reported by consumers, which may have influenced the lower means from this treatment.

Sausages from treatment extract received the lowest mean grades for color attribute. Consumers complained that color was too dark, which has also been detected by the laboratory team during the process and storage of raw sausages from this treatment. This difference in sausages' color is due to extracts' dark green appearance (Fig. 1.3) and can be understood as an issue that needs to be solved when using

Table 1.4 Sensory acceptance notes of fresh sausages*

Treatment**	Taste	Aroma	Color	Texture	Global acceptance
PC	7.4 ± 1.2[a]	7.0 ± 1.4[a]	7.2 ± 1.4[a]	6.8 ± 1.3[ab]	7.1 ± 1.2[a]
NC	7.1 ± 1.4[ab]	6.7 ± 1.4[ab]	6.5 ± 1.5[b]	6.7 ± 1.4[b]	6.7 ± 1.4[ab]
EC	7.2 ± 1.3[ab]	6.9 ± 1.3[a]	6.8 ± 1.7[ab]	7.1 ± 1.5[a]	7.0 ± 1.3[a]
EXT	7.0 ± 1.4[b]	6.5 ± 1.5[b]	5.5 ± 1.9[c]	6.6 ± 1.6[b]	6.5 ± 1.4[b]

*Means ± standard error. **PC positive control, NC negative control, EC erythorbate control, EXT extract. [a-c]Means followed by equal letters in the same row do not differ statistically by Tukey test at 5%

this kind of natural additive. On the other hand, considering the new trend in food consumption, in which natural-based products are well accepted by consumers even when traditional appearance is altered, it is believed that by justifying the use of plant extracts in sausage formulations this matter will no longer exist.

The texture of pork sausages seemed not to be affected by the use of extracts since no difference was found among samples from negative control, positive control and extracts. About global acceptance, sausages from treatment extract were less accepted than the ones from treatments positive and erythorbate control, which has been attributed mainly to the influence of aroma and color in sausages containing pitangueira leaf extracts, as samples were darker and with a grass-like smell.

The findings from the current experiment were very important to better understand how consumers react when a plant-based additive is applied to a meat product, and thus support meat industry decisions towards the shift from chemical to natural additives in meat products.

1.7   Final Remarks

Among the tested procedures to obtain phenolic compounds from PLE, the extraction method developed in this study, using 1 g freeze-dried plant material/10 mL solvent (40:60, EtOH: H_2O), assisted by ultrasonic treatment for 45 minutes, followed by magnetic stirring at 80 °C, provided the best results not only for the in vitro antioxidant capacity of these extracts, but also to its antibacterial activity.

The presence of PLE in formulations of fresh pork sausages decreased product's luminosity and redness, nevertheless, it promoted excellent lipid protection, evidencing the remarkable antioxidant capacity of this extract. PLE did not influence the microbial growth of fresh pork sausage in the tested proportions. Less global acceptance of sausages containing PLE seems to be related to the dark-green color and grass-like odor from the extract.

Considering that the yellowish polar and intermediate fractions of PLE showed antioxidant activity and that the dark green non-polar fraction showed almost none antioxidant activity, the use of only active fractions (polar and intermediate) in meat products to promote better brightness, red intensity and consumer acceptance is suggested. Another suggestion is to use an activated charcoal layer to remove pigments from PLE before its use in meat products.

The utilization of PLE in fresh pork sausage has been described thoroughly in this chapter, and according to results obtained and the consulted literature, this extract showed a huge potential to be used as a suitable natural alternative to chemical additives in meat products under cold storage.

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