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Analysis of the inhibition of proteolytic microorganisms in red tilapia (*Oreochromis spp*) fillets preserved with propolis (*Apis mellifera Linnaeus*)

Análisis de la inhibición de microorganismos proteolíticos en filetes de tilapia roja (*Oreochromis spp*) conservados con propóleo (*Apis mellifera Linnaeus*)

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Abstract

The food industry is focused on replacing chemical preservatives with organic alternatives for food preservation and safety. The present study seeks to analyze the use of propolis in the conservation of red tilapia fillets. Propolis was applied in two concentrations (15% and 30%) and two impregnation times (1.5 and 3 hours) to red tilapia fillets stored at 4-5°C for 30 days. Several parameters, including pH, water-holding capacity (WRC), and basic volatile nitrogen concentration (N-BVT), were evaluated at 10-day intervals. In addition, a microbiological analysis of mesophilic microorganisms and *E. coli* present was carried out. From day 20, significant differences were observed in the color of the fillet according to the chromatic coordinates L^*, a^* and b^*. The sensory analysis showed that the sensory properties were maintained when the acceptance values were higher than 6.5. The most effective treatment was propolis in a concentration of 15% with a soaking time of 1.5 hours and preserved for 20 days. This approach showed that propolis effectively extends the shelf life of fillets by preventing proteolytic damage. In addition, it inhibits the proliferation of microorganisms by maintaining the load of mesophiles and *E. coli*, as well as the physicochemical parameters (pH, CRA and N-BVT) according to the NTE-INEN 183-2013 standard. In conclusion, propolis is a promising organic preservative for the food industry.

Keywords: Bacteria, fillet, inhibition, normative, proteolytic.

Resumen

La industria alimentaria se centra en reemplazar los conservantes químicos con alternativas orgánicas para la conservación y seguridad de los alimentos. El presente estudio, busca analizar el uso de propóleo en la conservación de filetes de tilapia roja. Se aplicó propóleo en dos concentraciones (15% y 30%) y dos tiempos de impregnación (1,5 y 3 horas) a filetes de tilapia roja almacenados a 4-5 °C durante 30 días. Se evaluaron varios parámetros, incluidos el pH, la capacidad de retención de agua (CRA) y la concentración básica de nitrógeno volátil (N-BVT), a intervalos de 10 días. Además, se realizó un análisis microbiológico de microorganismos mesófilos y *E. coli* presentes. A partir del día 20 se observaron diferencias significativas en el color del filete según las coordenadas cromáticas L^*, a^* y b^*. El análisis sensorial mostró que las propiedades sensoriales se mantuvieron cuando los valores de aceptación fueron superiores a 6,5. El tratamiento más efectivo fue el propóleo en concentración del 15% con un tiempo de impregnación de 1,5 horas y conservado durante 20 días. Este enfoque mostró que el propóleo extiende efectivamente la vida útil de los filetes al prevenir el daño proteolítico. Además, inhibe la proliferación de microorganismos al mantener la carga de mesófilos y *E. coli*, así como los parámetros fisicoquímicos (pH, CRA y N-BVT) según la norma NTE-INEN 183-2013. En conclusión, el propóleo es un conservante orgánico prometedor para la industria alimentaria.

Palabras clave: Bacteria, filete, inhibición, normativa, proteolítica.

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1 Introduction

The shelf life of fish is very important in the industrial and commercial field involving fish farming, hence, the use of biopreservation agents for whole fish or in vacuum-packed filet is an alternative to reduce the use of chemical preservatives that affect the health of the consumer, and avoid the proliferation of microorganisms that cause their deterioration, maintaining the quality and safety traits established by the control bodies (Ahmad et al., 2017; Rodríguez-Pérez et al., 2020).

Tilapia stands out for being a very desired fish because of its great taste, texture, color and great versatility when making various preparations; thus, its cultivation has spread potentially in recent years, and this new business opportunity has given rise to new ideas that contribute to improvements in exports and different methods of consumption and marketing of the product (Jácome et al., 2019).

Likewise, there is an increase interest on the part of consumers, industrialists, and researchers to resort to natural sources of food additives that can be used to preserve food without affecting human health (Vargas-Sánchez et al., 2013). In this way, a natural product such as propolis has been successfully introduced into the food industry, which can offer these properties (Rodríguez-Pérez et al., 2020; Farag et al., 2021).

Propolis is a resinous substance produced by bees whose characteristics vary depending on the weather season, geographical area, type of bees and existing vegetation; it contains between 50 to 60% resins and balms, 30 to 40% wax, 5 to 10% pollen, and 8 to 10% essential oils; it is composed of around 180 substances, mainly flavonoids and phenolic acids or esters in 50% (Rodríguez-Pérez et al., 2020; Sarıkahya et al., 2021; Farag et al., 2021; Salleh et al., 2021). Propolis is used by bees to maintain the optimal conditions of the hive and its honey, avoiding the growth of microorganisms that alter it, so that its properties can be studied as a natural food additive. Considering the relationship of its flavonoid content and its biological effect, propolis is a bioactive product that stands out for its antimicrobial and antioxidant activity, strong flavor, and typical aroma, capable of establishing multiple synergistic combinations with other components (Viloria et al., 2012; Rodríguez-Pérez et al., 2020; Peixoto et al., 2021; Salleh et al., 2021).

Propolis has useful characteristics for the food industry such as its antioxidant, antimicrobial, antifungal and antiparasitic (antiprotozoal) activity (Peixoto et al., 2021; Vică et al., 2021; Afata et al., 2022), reason for which it can be used in meat products (beef, chicken, pork, fish or shellfish), vegetable oils, unpasteurized dairy products, fruits and fruit juices. The antibacterial activity of propolis has been studied in different microorganisms, including Gram-positive and Gram-negative bacteria. The Gram-positive bacteria evaluated included *Staphylococcus aureus, Streptococcus sp., Micrococcus sp., Bacillus sp. Listeria monocytogenes*; as for the Gram negative bacteria evaluated are *Salmonella typhi, Salmonella typhimuriu, Pseudomona aeruginosa, Acinetobacter baumannii, Klebsiella pneumoniae, Escherichia coli, Helicobacter pylori, Shigella* spp. For propolis, the minimum inhibitory concentrations (MIC) for these microorganisms vary depending on the way they are applied and the chemical composition of the geographical area of origin.

According to the information collected by Przybyłek and Karpiński (2019), the MIC of the ethanolic extract of propolis for Gram positive bacteria (*S. aureus* as representative) is within 8-1500 μg mL^{-1}, while the MIC for Gram negative bacteria (*E. coli* as representative) is within 116-5000 μg mL^{-1}. Thus, higher efficacy on Gram positive bacteria and to a lesser extent on Gram positive bacteria has been established as Gram-negative due to the action of bioactive compounds present in propolis and that directly affect the cell wall of these bacteria, making them susceptible to cell breakdown and subsequent lysis (Nedji and Loucif-Ayad, 2014; Santos et al., 2017; Zhang et al., 2017; Torres et al., 2018; Afata et al., 2022).

This study aims to investigate the effect of propolis (*Apis mellifera Linnaeus*) on the inhibition of proteolytic microorganisms in vacuum-packed red tilapia (*Oreochromis* spp.) filets by measuring their physicochemical and microbiological parameters, as well as their sensory attributes during the storage period. This study responds the need to address the challenge faced by the fishing industry to preserve the properties of packaged products, without increasing the use of synthetic chemical preservati-

ves.

2 Materials and Methods

This research was carried out in the food analysis laboratories of the Faculty of Zootechnical Sciences of the Technical University of Manabí, located at kilometer 2 $^1/_2$ of Ánima via Chone-Boyacá, in Chone, Manabí province, area with a potential evapotranspiration of 107.04 mm, average annual temperature of 25.2 °C and average annual precipitation of 54.63 mm (Cabrera-Estupiñán et al., 2017).

The propolis used in the study was obtained from a poultry farm located in the city of Flavio Alfaro, Manabí province. The red tilapia (*Oreochromis* sp.) was acquired in the local market of Chone, taking specimens of 525 g of average weight. Then the tilapias were eviscerated, and cut into filets of an average weight of 370 g. Each filet maintained its skin to maintain the stability of the muscle. In each filet parallel cuts were made of 5 mm with the aim of getting the propolis through these cuts and impregnate the fillets.

To evaluate the preservative action of propolis in vacuum sealed red tilapia filets ,an experimental design was established applying a cubic factorial model attenuated with the factors A) storage time (10, 20 and 30 days), B) propolis concentration in relation to the weight of the filet (0, 15 and 30%), and C) impregnation time (1.5 and 3 h). Each filet of the experimental design was packed in 2/60 gage transparent polyester (PET) bags, U seal, Zipack, Avidditi®brand, ISO 9001:2008 certified (Manuli Fitasa, Brazil) using a vacuum atmosphere generated by a chamber with high-capacity vacuum generator pump (20 m3 h^{-1}), VM400TE/B model 440 x 420 x 75 mm and double seal bar 400 x 100 mm, from Fin brand Teck S.A. Subsequently, the packaged filets were stored at refrigeration temperatures of 4.5 $\pm$ 0,5 °C for 30 days. The analyzes were performed in triplicate both at the physicochemical and sensory levels.

2.1 Physicochemical parameters

2.1.1 pH analysis

For determining this parameter, a potentiometer Orion A:211 was used (Thermo Scientific™, United States) which has a 6mm electrode, which is inserted directly into tilapia filets.

2.1.2 Percentage of water holding capacity

A sample of 2 g of raw filet was placed on circular filter paper. Then, it was placed between two glass plates with a weight of 5 kg, for five minutes. Water retention capacity (WRC) was determined by the difference in both initial and final weights (Rebouças et al., 2020).

$$\%WRC = \frac{P_i - P_f}{P_i} \times 100 \quad (1)$$

Where P_i is the initial weight of the filet, and P_f is the final weight of the filet.

2.1.3 Stability to proteolytic degradation

The amount of total volatile nitrogen bases (N-BVT) was used for Valencia-Junca et al. (2019) method with modifications. A sample of 10 g of tilapia filet was ground with 50 mL of distilled water into an Oster®food processor; the mixture formed was placed in a 500 mL Erlenmeyer with 200 mL of distilled water. Then, it was distilled incorporating 2 g of MgO and a drop of silicone was added to inhibit the formation of foams. The distilled product was placed in a 250 mL flask with a 3% boric acid solution and 0.04 mL of methyl red and methylene blue as an indicator of the presence of ammonium. The titration of the distillate was performed with 0.1N HCl until obtaining a turn from green to pink. The following equation was used to calculate N-BVT in mg $100g^{-1}$ of fish filet.

$$\%mg\ N-BVT = \frac{(V \cdot C \cdot 14 \cdot 100)}{10} \quad (2)$$

Where, V = Volume of added hydrochloric acid;
C = Normal concentration of hydrochloric acid;
14 = Atomic weight of N;
10 = Weight of sample

2.1.4 Instrument color determination

Color determination was performed using a colorimeter (Kónica, Minolta Chroma Meter CR400, Japan), with illuminant D65 and observer of 2° (calibrated equipment with a standard plate with reference values Y = 89.5 x = 0.3176 y = 0.3340). The measurements were expressed in terms of luminosity L^* and chromaticity parameters a^* and b^*.

2.2 Microbiological parameters

For the microbiological analysis, the standard solution was prepared using 10 g of tilapia filet homogenized with 90 mL of peptone water to conduct serial dilutions of each of the bacterial groups to be quantified.

2.2.1 Count of aerobic mesophilic micro-organisms

For determining aerobic mesophilic microorganisms, planting in stretch marks was performed using the Agar Plate Count (APC), then incubation at 35°C was carried out for 48 hours. After the incubation period, the colony forming units (log CFU g^{-1}) were counted.

2.2.2 *Escherichia coli* count

This analysis was carried out using the Most Probable Number (MPN g^{-1}) methodology, for which test tubes were taken inside containing Durham bells and were subsequently incubated at 35°C for 48 hours. Test tubes showing turbidity and gas presence were taken as presumptively positive. Then, the presence of coliforms was confirmed using the Kovacs reagent. The positive tubes were inoculated in Bright Green Lactose Bile broth and incubated at 35°C for 48 hours. In addition, the test tubes that showed turbidity and gas production that were classified as positive were planted in Methylene Blue Eosine Agar (MBE) and incubated for 24 hours to confirm the presence of *Escherichia coli*.

2.3 Sensory analysis

The sensory panel was composed of twenty semi-trained judges, who evaluated the organoleptic attributes such as color, aroma, flavor and texture, for which a 9-point hedonic scale valued was used (Jonaidi-Jafari et al., 2018), where 1 is "extremely disliked" and 9 "extremely liked". The value of 4 was considered as a minimum acceptability benchmark. For the sensory evaluation, the filets were cut into small pieces, were covered with breadcrumb, and then were fried. The portions of filet were placed in containers labeled with the code of the treatments analyzed. A glass of water at room temperature was provided to each judge to allow tasting the different treatments.

2.4 Statistical Analysis

For data analysis, the statistical software InfoStat version 2020 was used. A multifactorial ANOVA was performed to analyze the physicochemical and microbiological variables, in order to evaluate the effect of storage time, propolis concentration, impregnation time and their interactions.

For the sensory analysis, a simple ANOVA was performed to evaluate the existence of significant differences in each of the attributes evaluated. The post hoc analysis of Tukey ($p < 0{,}05$) was applied to check the existing differences between treatments.

3 Results and Discussion

3.1 Physicochemical parameters of red tilapia filets (*Oreochromis* sp.)

3.1.1 pH analysis

The pH values in the red tilapia filets impregnated with propolis determined that this parameter ranged from 6.12 to 7.53, with a mean of 6.85 (Figure 1). The pH tended to decrease as the storage days progressed, existing a similar behavior considering both impregnation times of propolis design. Until the end of the storage period, the control treatment obtained pH values of 5.91 and 5.96 for the two impregnation times; the pH for treatments with 15% propolis with impregnation time of 1.5 hours was 6.46 and 3 hours 6.27; in contrast, treatments with 30% propolis and impregnation times of 1.5 hours and 3 hours had pH of 6.24 and 6.18, respectively.

The analysis of variance determined a significant difference in the pH value in factor A (storage time) and factor B (propolis concentration), even in the interactions AC (storage time - impregnation time) and BC (propolis concentration - impregnation time) (Table 1). The pH values reported are related to those determined by Montoya Camacho et al. (2021) in studies conducted with black tilapia to evaluate the biochemical changes undergone by the muscle of this fish when stored in a temperature between 0 and 5°C.

The pH values recorded are within the ranges established by the Ecuadorian technical regulation NTE-INEN 183-2013 (INEN, 2014), which establis-

hes that the pH during its commercialization is 6.5 in the internal part of the muscle and 6.8 in the external part of the organism.

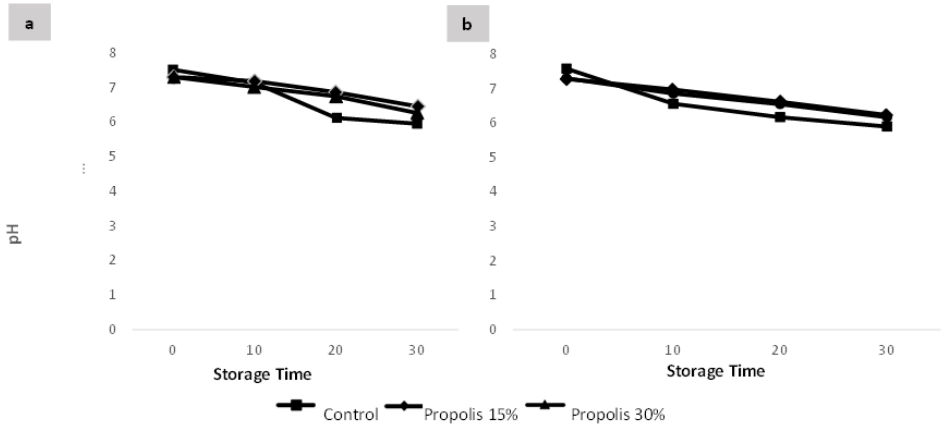

Figure 1. pH behavior in red tilapia filets (*Oreochromis* sp.) impregnated with propolis, a) impregnation of propolis at 1.5 hours, b) impregnation of propolis at 3 hours.

Table 1. Analysis of variance of the treatments employed in this study for pH.

Source	SC	gl	CM	p - value
A-Storage time	6.05	1	6.05	<0.0001
B-Propolis concentration	0.4413	1	0.4413	<0.0001
C-Time impregnation	0.0702	1	0.0702	0.0558
AB	0.0137	1	0.0137	0.3928
AC	0.2491	1	0.2491	0.0005
BC	0.4052	1	0.4052	<0.0001
ABC	0.0026	1	0.0026	0.7085
Residual	1.05	57	0.0184	
Total Cor	11.39	71		

Equation 3 determines the multiple regression of the pH parameter and allows predicting the response of each factor by identifying and comparing the coefficients thereof.

$$\begin{aligned} pH = {} & 6,52 + (-1,49 \cdot A) + (-0,1535 \cdot B) + \\ & (-0,0667 \cdot C) + (-0,0226 \cdot AB) + \\ & (-0,0789 \cdot AC) + (-0,0919 \cdot BC) + \\ & (-0,0099 \cdot ABC) \end{aligned} \quad (3)$$

The pH values vary between 6.81 and 6.91 in the treatments using propolis at 15% and 30% at an impregnation time of 1.5 hours, while the values were 6.68 and 6.70 respectively at impregnation times of 3 hours. Hence, conserving tilapia filets with propolis is an organic and feasible alternative.

3.1.2 Water Retention Capacity (WRC)

WRC results of propolis-preserved filets range from 46.43% to 86.99% (Figure 2). The results indicate a decrease in the WRC as the storage time progressed. The control treatment obtained WRC values of 51.85% at 1.5 h of impregnation and 46.45% at 3 h of impregnation. Treatments with 15% propolis with impregnation times of 1.5 and 3 hours had WRC values of 62.92% and 58.19%, respectively, while treatments with 30% propolis and impregnation times of 1.5 hours and 3 hours had WRC values of 60.92% and 55.95%, respectively.

The statistical analysis of variance for the WRC (Table 2) determines that the main effects of the

model were A (storage time), B (propolis concentration), and interactions AB (storage time- propolis concentration) and AC (storage time - impregnation time) with p values of <0.0001 in all detailed cases, there being significant differences in the values of WRC.

According to Campus et al. (2010) WRC is a parameter that measures the ability of muscle to retain free water by capillarity and stress forces that have been subjected to the sample, in this case the red tilapia filets; thus, WRC ranges between 70-80 % are considered optimal values to estimate the freshness of fish.

For this study, the WRC values were 71.88, 77.34 and 75.96 % in red tilapia filets with an impregnation time of 1.5 hours and up to 10 days of storage time and with 3 concentrations of propolis; meanwhile, the WRC values were 73.12, 78.37 and 75.84 % for the impregnation time of 3 hours, up to the 10th day of storage and 3 concentrations of propolis. After day 10, the WRC reduced to values between 53.53 and 58.56 % in both concentrations in impregnation times of 3 and 1.5 hours respectively.

$$\begin{aligned} WRC = {} & 72,15 + (-15,02 \cdot A) + 1,32 \cdot B + \\ & (-0,3919 \cdot C) + 1,93 \cdot AB + \\ & (-2,33 \cdot AC) + (-0,1328 \cdot BC) + \\ & (-0,0237 \cdot ABC) \end{aligned} \tag{4}$$

Equation 4 determines the multiple regression of the WRC parameter. In terms of real factors, this equation can be used to make predictions about the response for given levels of each factor, thus identifying the relative impact of the factors by comparing their coefficients.

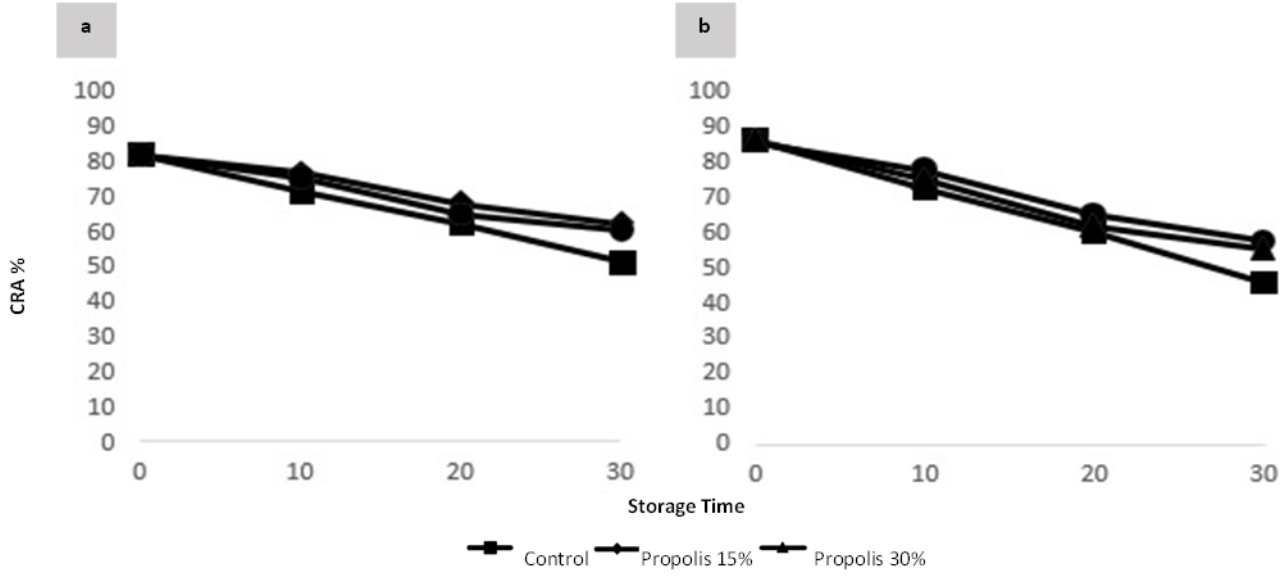

Figure 2. WRC values in red tilapia filets (*Oreochromis sp.*) preserved with propolis, a) impregnation of propolis at 1.5 hours, b) impregnation of propolis at 3 hours.

The values of WRC are favorable until the day of storage in the treatments performed with propolis at 15 % and 30 % for the impregnation time of 1.5 hours and 3 hours, because the filets lose juiciness after these days, affecting unfavorably the sensory parameter taste. According to Melody et al. (2004), the space for water to be retained in myofibrils is reduced as rigor progresses, and the fluid can be forced into extramiofibrillary spaces where it is more easily lost as a drip as a result of the lateral contraction of myofibrils that occurs during rigor, which can be transmitted to the entire cell if proteins that

Table 2. Analysis of variance of the treatments considered in the study for the WRC.

Source	SC	gl	CM	p-value
A-Storage time	600.75	1	600.75	<0.0001
B-Propolis concentration	31.13	1	31.13	<0.0001
C-Time impregnation	2.41	1	2.41	0.0488
AB	99.34	1	99.34	<0.0001
AC	215.75	1	215.75	<0.0001
BC	0.8262	1	0.8262	0.2433
ABC	0.0149	1	0.0149	0.8747
Residual	33.28	56	594	
Total Cor	9171.03	70		

bind myofibrils to each other and myofibrils to the cell membrane are not degraded.

3.1.3 Analysis of total volatile nitrogen bases (N-BVT)

According to Cicero et al. (2014), fish has high protein index and hence it is a highly recommended product mainly in balanced and healthy diets, but these organisms suffer internal alterations, where the degradation of nitrogen compounds occurs mainly by the action of bacteria, which mainly form trimethylamine (TMA) and ammonium, increasing the pH at the end of the rigor mortis phase. The measurement of the amount of N-BVT in a fish filet sample is used to determine the state of the deterioration process, this being an indicator of its freshness (Howgate, 2010).

As for the N-BVT values for red tilapia filets, significant differences were observed between treatments from day 20 of storage (Figure 3). The behavior of N-BVT in red tilapia filets preserved under vacuum determined a value of 22.58 mg N-BVT $100g^{-1}$ for the control treatment, while treatments with propolis showed a decreasing behavior in this parameter according to the impregnation time and concentration of propolis; thus, the treatment at 15% resulted in 19.69 mg N-BVT $100g^{-1}$ (1.5 hour impregnation) and 14.68mg N-BVT $100g^{-1}$ (3 hours impregnation).

From day 20, the control treatments showed increments in the N-BVT value, exceeding the permissible limit value of 30 mg N-BVT $100g^{-1}$ to be considered fresh fish filet acceptable for consumption, established in the regulations issued by the Comunidad Europea (2008) and stated in the Ecuadorian standard NTE-INEN 183-2013. The treatments with 15% and 30% propolis did not exceed the established normal value, presenting values between 10.77 and 36.22 mg $100g^{-1}$. The values recorded within the permissible limit determined over the storage time of red tilapia filets could be influenced by the antibacterial activity of propolis, which prevents proteolytic degradation in the filet, as mentioned by Basiri et al. (2015) on a seafood conservation study.

The statistical analysis of variance for the effect (Table 3) determined that the variation sources that were significant were A (storage time), B (propolis concentration), C (impregnation time), interactions AB, AC and ABC, with significant differences in the values of the N-BVT.

Equation 5 determines the multiple regression of the N-BVT parameter and can be used to predict about the response for given levels of each factor, thus identifying the relative impact of the factors by comparing the coefficients thereof.

$$\begin{aligned} N-BVT = {} & 17,09+6,12\cdot A+(-2,16\cdot B)+ \\ & 3,57\cdot C+(-1,99\cdot AB)+ \\ & 0,7075\cdot AC+0,0865\cdot BC+ \\ & 0,5514\cdot ABC \end{aligned} \tag{5}$$

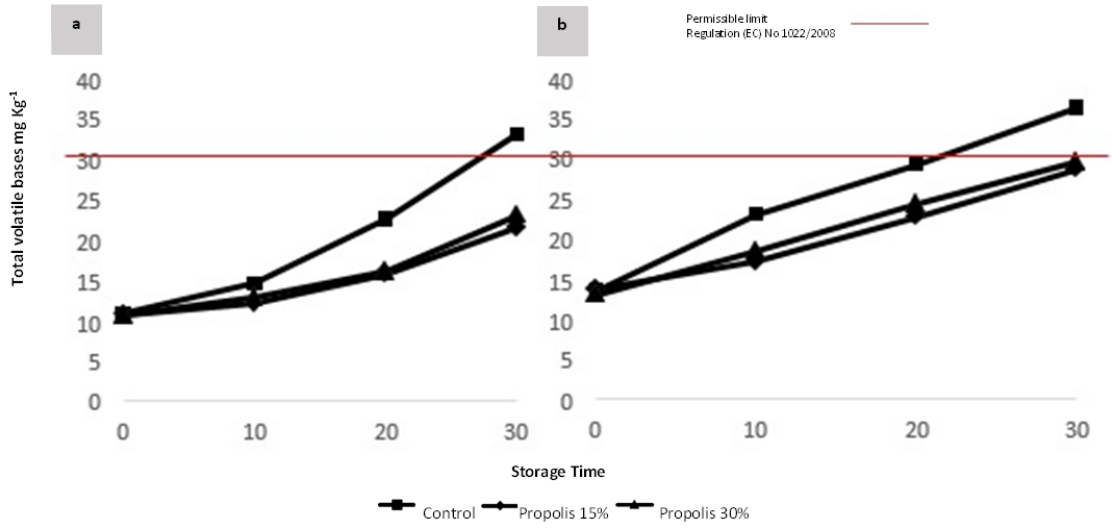

Figure 3. N-BVT values in red tilapia filets (*Oreochromis sp.*) preserved with propolis and with its permissible regulation a) Impregnation of propolis at 1.5 hours b) Impregnation of propolis at 3 hours.

Table 3. Analysis of variance of the treatments considered in the study for N-BVT.

Source	SC	gl	CM	p-value
A-Storage time	102.08	1	102.08	<0.0001
B-Propolis concentration	87.71	1	87.71	<0.0001
C-Time impregnation	201.50	1	201.50	<0.0001
AB	105.11	1	105.11	<0.0001
AC	20.02	1	20.02	<0.0001
BC	0.3588	1	0.3588	0.2934
ABC	8.11	1	8.11	<0.0001
Residual	18.18	57	0.3190	
Total Cor	3847.46	71		

3.1.4 Instrument color determination

Regarding the values of the chromatic coordinates L*, a* and b* for red tilapia filets, significant differences were observed between treatments from day 20 of storage. The chromatic coordinate L* (luminosity) decreases as the days of storage increase. In any case, the impregnation time of 1.5 hours reduced these values in contrast to the impregnation time of 3 hours. This behavior is similar to that reported by Magalhães et al. (2019), who determined the quality of snacks from mechanically separated red tilapia filets. Likewise, Zapata and De la Pava (2018) determined that the red tilapia filets used in the preparation of sausages lose their brightness as the storage time.

The analysis of variance for the chromatic parameters in this study (Table 4), shows that for L the significant terms of model are A, AB, BC and ABC. For the parameter a* (red), the significant terms of the model are A, B, AB and AC. The a* values increased steadily as the storage time and the concentration of propolis employed in the filets increased. In parameter b* (yellow), the significant terms were A, B, C, AB, AC and BC.

Parameters a and b (red and yellow) increase significantly and progressively during the 30 days of refrigerated storage and independently of the factors analyzed in this research.

$$\begin{aligned} L^* = {} & 19,56 + (-1,84 \cdot A) + 0,0213 \cdot B + \\ & (-0,0120 \cdot C) + 0,9873 \cdot AB + \\ & (-0,1229 \cdot AC) + (-0,1829 \cdot BC) + \\ & (-0,2893 \cdot ABC) \end{aligned} \tag{6}$$

$$a^* = 7,42 + 1,70 \cdot A + (-1,45 \cdot B) + (-0,0802 \cdot C) + (-1,21 \cdot AB) + (-0,1909 \cdot AC) + (-0,0756 \cdot BC) + 0,0001 \cdot ABC \quad (7)$$

$$b^* = (-0,5200) + 2,12 \cdot A + (-0,2152 \cdot B) + (-0,6360 \cdot C) + (-0,3244 \cdot AB) + (-0,1975 \cdot AC) + (-0,0594 \cdot BC) + 0,0334 \cdot ABC \quad (8)$$

Equations 6, 7 and 8 are presented in terms of codified factors and can be used to make predictions about the response for each factor, and allow to identify the relative impact of the factors by comparing their coefficients.

Table 4. Analysis of p-values of treatments considered in the study for the CieLab color scale.

Source	p-value		
	L*	a*	b*
A-Storage time	<0.0001	<0.0001	<0.0001
B-Propolis concentration	0.8830	<0.0001	<0.0001
C-Time impregnation	0.9389	0.2731	<0.0001
AB	<0.0001	<0.0001	<0.0001
AC	0.2164	<0.0001	<0.0001
BC	0.0462	0.0739	0.0020
ABC	0.0196	0.9982	0.1798
Residual	6.20	4.72	0.9182
Total Cor	852.82	379.10	117.25

3.2 Microbiological analysis

Figure 4 shows the microbial growth results for mesophilic bacteria in red tilapia filets for each factor during storage days. The count of mesophiles in filets without propolis showed continuous growth during storage, while the microbial load of mesophiles tended to decrease in treatments with propolis at 15 and 30%. The control treatments showed an increase until day 30 of mesophilic bacteria of 11.74 and 11.92 log CFU g^{-1} for the impregnation times of 1.5 and 3 hours, respectively.

The mesophilic count at the end of the storage days for the concentration of 15 and 30% of propolis at 3 hours of impregnation had values of 6.03 and 6.27 log CFU g^{-1}. The tilapia filets impregnated with propolis during a 1.5 h impregnation time obtained values of 4.39 and 4.13 log CFU g^{-1} for the concentrations of propolis at 15 and 30%, respectively. The results at 1.5 h of impregnation are within the ranges determined by the NTE-INEN 183-2013 and the International Commission on Microbiological Specifications of Food (ICMSF) (Roberts et al., 2005).

The inhibition effect of mesophilic microorganisms in the concentrations of propolis at 15 and 30% in an impregnation time of 1.5 h during the 30 days of storage was superior to other investigations that use propolis as a preservative; thus the count of mesophilic in these conditions were lower than those reported by Suarez et al. (2014) and Duman and Özpolat (2015), who obtained a count of mesophilic higher than 5.4 log CFU g^{-1} during 20 days storage, using propolis solutions on fish filets in a concentration of 1.2 mg mL^{-1} and 0,5%, respectively, under similar storage conditions as in the present study.

The results determined a development of *E. coli* at 10 days of storage time of the red tilapia filets, both in the impregnation times of propolis of 1.5 and 3 hours and with all concentrations of propolis evaluated; however, some values obtained in this study are below the highest limit allowed by the NTE INEN 183:2013, which establishes a maximum of 2.69 log UFC g^{-1} of *E. coli* concentration.

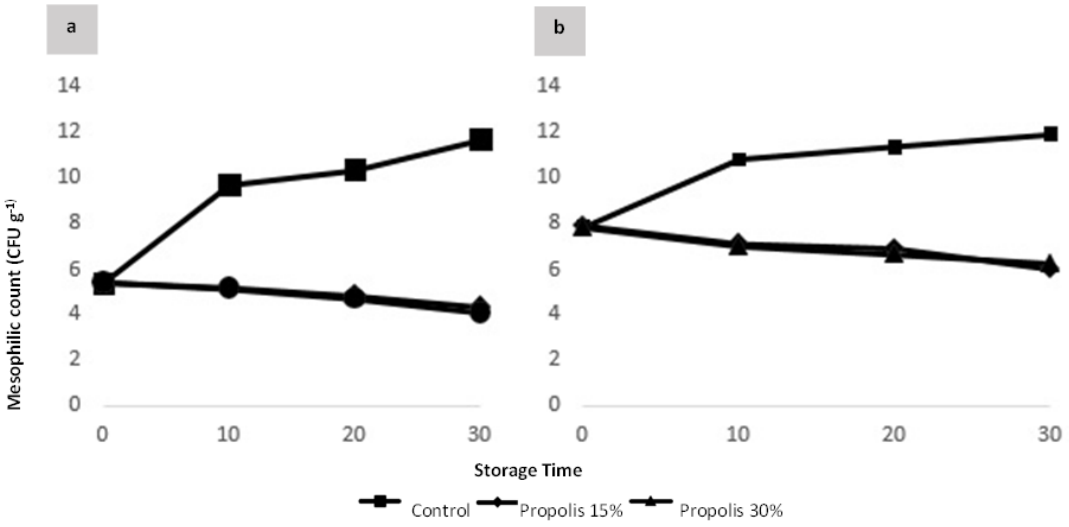

Figure 4. Concentration of mesophiles in red tilapia filets (*Oreochromis sp.*) preserved with propolis and with its permissible regulation a) Impregnation of propolis at 1.5 hours b) Impregnation of propolis at 3 hours.

The values obtained during the 30 days of storage for the impregnation time of 1.5 h are between 1.73 and 6.61 log CFU g^{-1} for the 0% of propolis concentration (control), from 1.71 to 3.57 log CFU g^{-1} with the 15% of propolis concentration and from 1.74 to 3.27 log CFU g^{-1}, for 30% of propolis concentration; likewise, the values during the 30 days of storage for the impregnation time of 3 hours were 2.13 to 6.87 log CFU g^{-1} for 0% propolis concentration and 2.08 to 5.11 and 2.14 to 5.15 log CFU g^{-1} for 15 and 30% propolis concentration (Figure 5). These values are lower than those mentioned by Talledo-Solórzano et al. (2020) who obtained presence of these bacteria between 3.32 and 5.17 log CFU g^{-1} in tilapia filets treated with lactic acid bacteria.

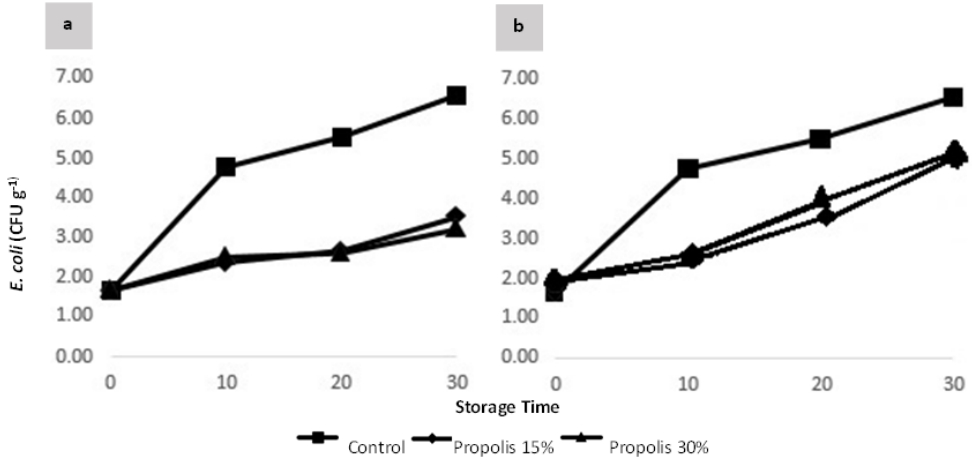

Figure 5. Count of *E. coli* in red tilapia filets (*Oreochromis sp.*) preserved with propolis and with its permissible regulations. a) Impregnation of propolis at 1.5 hours; b) Impregnation of propolis at 3 hours.

The results of the microbiological analysis indicate that the concentrations of propolis at 15 and 30% with an impregnation time of 1.5 h are adequate to maintain the mesophilic count within the limits allowed in the different regulations of quality in force during the storage of 30 days, and also these conditions are adequate to maintain the levels of *E. coli* in acceptable quality levels for up to 20 days in the storage conditions evaluated.

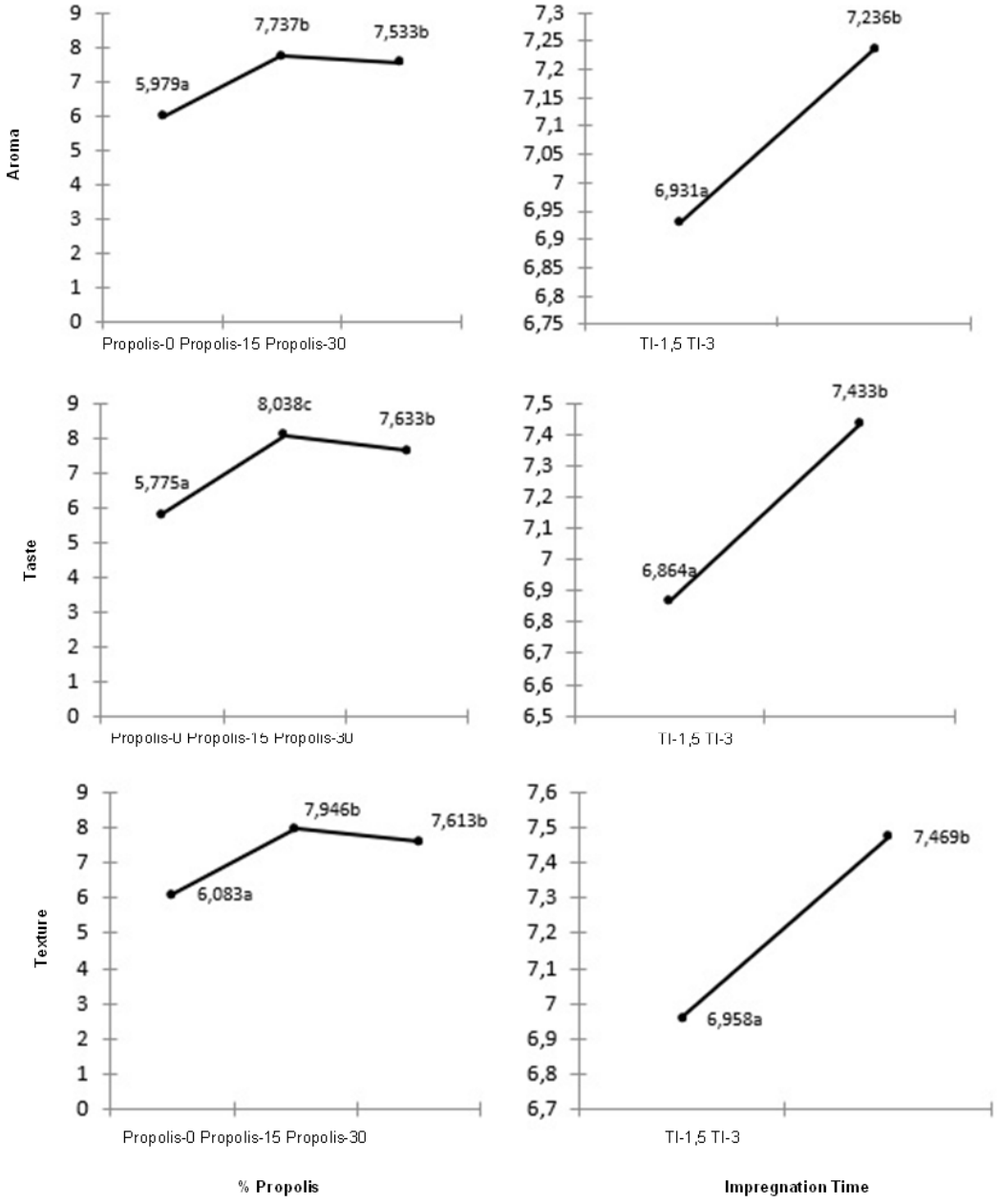

Figure 6. Analysis of the organoleptic parameters of red tilapia filets (*Oreochromis sp.*) impregnated with two doses of propolis (15 and 30%), and two impregnation times (1.5 and 3 h) to 10 days of storage for the sensory characteristics of aroma, flavor and texture

On the other hand, both concentrations of propolis with 3 h of impregnation do not allow to maintain the concentration of allowed mesophiles during the days of storage, and they maintain the concentration of *E. coli* in acceptable quality levels until 10 days of storage; for this reason, the impregnation time of 3 h is inadequate to avoid the proteolytic deterioration of the filets by the proliferation of microorganisms evidenced.

Thus, the most appropriate treatment to maintain the microbiological quality within the limits allowed by the standard NTE-INEN 183-2013 is 15% propolis with an impregnation time of 1.5h, for a storage time of 20 days at 4-5°C vacuum-packed.

3.3 Sensory analysis

Figure 6 shows the sensory analysis results of propolis-impregnated red tilapia filets stored at 4-5°C. The exposed data show the results of the sensory analysis applied to tilapia filets with ten days of storage, time in which all treatments comply with the physicochemical and microbiological quality values established in the current quality regulation.

The treatments of the present study with both concentrations of propolis (15 and 30%), two impregnation times (1.5 and 3 h) and with a storage time of 10 days obtained an acceptability level higher than 6.5 in the attributes of aroma, flavor and texture.

Regarding the propolis concentration, this analysis determined that in the attributes of aroma, flavor and texture tilapia filets with 15% propolis with acceptance levels between 7.7 and 8 have greater acceptance. Regarding the impregnation time, the sensory panel preferred the treatments with impregnation time of propolis at 3 hours, with acceptance values between 7.2 and 7.4. Considering the storage time of ten days, the treatments had acceptance values between 6.5 and 6.7 and there was no difference with respect to the control treatment, demonstrating the conservation of the organoleptic characteristics within this storage time at 4-5 °C (4-11°F).

Overall, the acceptance was greater than 80% for all the sensory characteristics highlighted in the present study. Likewise, studies carried out by Talledo-Solórzano et al. (2020) demonstrated a great acceptance by the sensory panel for red tilapia filets preserved with lactic acid bacteria, where the average acceptance value per attribute was higher than 4.

4 Conclusions

By evaluating the effect of propolis at concentrations of 15 and 30% as a preservative agent in the preservation of red tilapia filets (*Oreochromis sp.*) vacuum-packed and stored at 4-5°C, it is possible to affirm that propolis can maintain the physicochemical and microbiological properties of these filets. Under these conditions, the treatment of 15% propolis with an impregnation time of 1.5 h for a storage time of 20 days maintains acceptable levels of quality within the limits allowed by the standard NTE-INEN 183-2013 and European Community Regulation (EC) N° 1022/2008.

Propolis is also useful as a preservative in the preservation of red tilapia filets (*Oreochromis sp.*), since it is directly involved in the inhibition of proteolytic deterioration of fish fibers, maintaining low concentration levels of mesophilic microorganisms, *E. coli* and the physicochemical parameters immersed in the deterioration process and analyzed in the present study (pH, N-BVT, CRA and color). Finally, sensory analysis of red tilapia (*Oreochromis sp.*) filets showed that the use of propolis as a preservative allows maintaining the organoleptic quality during the storage time.

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