Research Article

Antioxidant and antimicrobial effects of stevia (*Stevia rebaudiana* Bert.) extracts during preservation of refrigerated salmon paste

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Stevia (*Stevia rebaudiana* Bert.) is a relevant source of natural phenolic compounds with antioxidant and antimicrobial properties. The aim of this study was to evaluate the potential protective effect of crude stevia extracts on the quality and shelf-life of salmon (*Salmo salar*) paste. For this, polyphenol extracts obtained by water extraction, ethanol/water extraction and supercritical CO_2 with ethanol extraction were evaluated in preserving salmon paste. Salmon paste was stored under refrigerated conditions (5°C) for 21 days, being primary, secondary, and total lipid oxidations monitored along storage by means of peroxide, p-anisidine, and TOTOX indices, respectively. In addition, ω3/ω6 ratio, polyene index, and α-tocopherol were monitored. Microbiological analysis comprised the investigation of aerobic mesophiles and psychrotrophes. Salmon paste samples treated with ethanol/water and supercritical CO_2-ethanol stevia extracts exhibited the highest ($p < 0.05$) ω3/ω6 ratio and α-tocopherol content. Besides, partial inhibition of both primary and secondary lipid oxidation events and aerobes and psychrotroph growth was also observed in both samples. These results correlated with the fact that ethanol/water and supercritical CO_2-ethanol extracts provided the highest DPPH and FRAP values. These results open the way to the utilization of bioactive compounds from stevia leaves for the preservation of foods derived from salmon.

Practical applications: The results obtained in this research show the possibility of using stevia and/or its derivatives of the sweetener industry as an alternative source of natural antioxidants in refrigerated fatty fish paste. The results indicate that it is possible to obtain advantages in the refrigerated salmon paste, based on the use of some extracts of stevia, which can help to inhibit lipid oxidation and development of pathogenic microorganisms. Further studies on the use of stevia and its derivatives should focus on the application of clean separation technologies such as supercritical fluid extraction.

Keywords: Antimicrobial / Lipid oxidation / Salmon / Stevia / Supercritical fluid

Received: November 11, 2016 / Revised: April 25, 2017 / Accepted: June 1, 2017

DOI: 10.1002/ejlt.201600467

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Abbreviations: AV, p-anisidine value; **E/W,** hydroalcoholic extract (water: ethanol: 50:50 v/v); **FRAP,** ferric reducing/antioxidant power; **IC_{50},** concentration of polyphenols in extract needed to achieve 50% discoloration of DPPH; **Ip,** inhibition power; **PV,** peroxide value; **$scCO_2$,** supercritical carbon dioxide; **SCE,** $scCO_2$-ethanol extract (95:5 v/v); **TOTOX,** total oxidation.; **TP,** total phenolic; **W,** aqueous extract

1 Introduction

Currently, changes in consumers' life style and their concerns to eat safer and healthier foods have prompted the seafood industry and researchers to undertake the development of novel preservation methods able to minimize lipid oxidation and microbial spoilage [1, 2]. Studies have shown that the quality and shelf life of fish may be improved by the addition of natural antioxidants before or after fish slaughtering [3, 4]. Such strategies have been reported to delay lipid oxidation rate and the microbial growth in seafood [5, 3].

A considerable amount of by-products of the food industry are usually rich in natural antioxidants such as polyphenols, tocopherols, and carotenoids, among others, which may find application in the food, medical, and cosmetic industries [6]. In fact, several reports have linked the use of natural antioxidants with improvements of quality and shelf life extension of seafood products [7, 8].

One such vegetable, well known for its application in the sweetener industry, is *Stevia rebaudiana* Bertoni. The *Stevia* genus belongs to the plant Asteraceae, which is indigenous to the tropical regions of South America currently cultivated due to its sweetener properties and its very low caloric content [9]. In addition to steviosides and rebaudiosides with sweetener properties, *S. rebuadiana* leaves also contain phenolic compounds; including tannins and flavonoids, among other bioactive compounds [10]. Thus, several reviews on *Stevia* spp. have reported anti-hipertensive, anti-hyperglycaemic and antiviral activities [11]. In this sense, recent studies have focused on the investigation of the physiological role of phenolic and flavonoid compounds with potential antioxidant activity [12–16]. Likewise, recent studies have accounted for the antimicrobial activity of stevia extracts [16–18]. Such studies generally considered conventional solvents such as water, ethanol, acetone, etc. [19] while only a few have considered emerging extraction methods based on microwaves, ultrasounds, pressurized water and supercritical fluids [7]. Currently, a significant fraction of the bioactive compounds from stevia leaves is discarded as a residue of the steviosides sweetener industry. In this respect, little attention has been paid to the recovery of novel bioactive compounds with potential antioxidant and antimicrobial activity for the food industry. To the best of our knowledge, there is only one report of the application of supercritical carbon dioxide ($scCO_2$) for the extraction of steviosides from stevia [20], but little information is available concerning the application of $scCO_2$ to polyphenols extraction [21, 22]. Accordingly, the aim of this study was to evaluate the antioxidant and antimicrobial activity of crude stevia extracts obtained by three different extraction methods: supercritical carbon dioxide extraction with 5% ethanol (SCE), water extraction (W), and ethanol/water extraction (E/W), to the preservation of a seafood product of remarkable value (i.e., salmon paste).

2 Materials and methods

2.1 Raw materials

Stevia (*Stevia rebaudiana* Bert.*)* leaves provided by Manto Verde S. A. (Quillota, Chile) were dried at 40 ± 1.0°C to a moisture content of 5.4 ± 0.5%, and then ground to a particle size of 0.8 ± 0.1 mm and stored at 25 ± 1.0°C in sealed polyethylene bags.

Salmon (*Salmo salar*) employed as lipid matter provided from the firm Nova Austral (Puerto Montt, Chile).

Salmon (*Salmo salar*) employed as lipid matter was obtained as frozen fillets (−20°C) from Nova Austral (Puerto Montt, Chile) and kept at the same temperature in our laboratory for further analysis.

2.2 Preparation of phenol extracts

Phenol extracts were prepared by three different methods using solvents that are considered food grade (GRAS), i.e., solid–liquid extraction with water (W), with an ethanol/water mixture (E/W: 80/20, v/v) or with supercritical CO_2-ethanol 5% (SCE) extraction.

Five grams of ground stevia leaves were mechanically stirred (Shaker Polyscience Dual Action, 042901, MI, USA) with 1 L of distilled water for 3 h at room temperature (25 ± 1.0°C). Then the mixture was allowed to stand in the dark in the absence of light for 24 h at room temperature (25 ± 1.0°C). After that, the solid–liquid mixtures were vacuum filtered, with the steviosides discarded of filtered solution by crystallization. "Steviosides removal was confirmed using reversed-phase HPLC" at the end please add (data not published) [23, 24]. Finally, the extracts were dried at 40°C in a rotary evaporator and re-dissolved to 1 L with distilled water and immediately analyzed and applied to salmon paste.

Similarly, five ground stevia was extracted with E/W mixture, finally eliminating the ethanol by rotary evaporation at 40°C and re-dissolved extract to 1 L with distilled water. Stevia extraction was also achieved by SCE condition at laboratory level. For this, a supercritical device (Applied separations, SFE Speed 7071, CA, USA), provided with refrigeration equipment (Julabo F200, CA, USA), a high-pressure pump for the CO_2, and a positive shift pump for introducing the ethanol (HPLC-Pumpe, K-501, CA, USA) was employed.

For supercritical extraction, 0.24 mL/min of the mixture on 20 g SCE ground stevia was applied directly to the flow. The process was conducted at constant temperature (35°C) and pressure (40 MPa) conditions [25]. The extraction was kept in a static state for 30 min to allow contact between the sample and CO_2, and to maintain the equilibrium temperature and pressure conditions applied evenly across the sample. The period of dynamic extraction was extended to 15 min, until the equipment outlet valve obtained no more

extract. The extraction yield was determined concentrating an aliquot of 50 mL of extract at 45°C, being then weighted in an analytical balance.

2.3 Chemical analyses on dried stevia

The total phenolic (TP) content was determined by the Folin–Ciocalteu colorimetric method [26]. Absorbance was spectrophotometrically measured at 765 nm (ATI Unicam UV/vis, model UV3-200, London, England) by employing a gallic acid calibration curve. Results were expressed as mg gallic acid equivalents (GAE)/g extract.

The antiradical capacity of the stevia leaves was measured by the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay [27] and calculated as inhibition power (% Ip):

$$\%\mathrm{Ip} = [(A_0 - A_1)\backslash(A_0 * 100)]$$

where A_1: absorbance of the test sample and A_0: absorbance of control.

Measurements of antiradical capacity of different dilution of extracts were carried out at 517 nm, results were expressed as the concentration of extract (μg/mL) at which a 50% inhibition was attained (IC_{50}).

The antioxidant power of the stevia leaves extracts were assessed by the FRAP (ferric reducing/antioxidant power) assay [28]. Results were expressed as mmol Fe^{+2}/100 g dry weight sample.

2.4 Effect of stevia leaves extract addition on the quality of refrigerated salmon paste

Salmon fillets (1.6 kg) freshly harvested and kept for two days in refrigeration at 5°C, were homogenized (Moulinex mixer, AD 6011, Paris, France) in the form of fish paste. Based on previous studies of the antioxidant capacity of stevia extracts [29], the fish paste obtained was divided into four portions of 450 g. To each salmon paste was added the required amount of stevia leaf extract so that the polyphenol concentration was about 400 mg GAE/kg. In this way, the following samples were obtained; Salmon paste with 2.7 g of stevia aqueous extract (S + W); Salmon paste with 4.4 g of dry ethanol/water extract (S + E/W); Salmon paste with 2.0 g extract obtained by $scCO_2$/ethanol mixture 5% (S + SCE) and salmon control paste without added extract (S). The homogenization of each extract in the pasta was aided by adding a minimum amount of water (30 mL). Subsequently, each paste was divided into three pieces of 150 g each and stored hermetically in polyethylene bags under refrigeration conditions (5°C) for 21 days, being sampled after 0, 7, 14, and 21 days. Sensory analysis performed previously indicated that the extracts applied showed no alteration of the characteristic flavor of the fish paste [29].

2.4.1 Assessment of lipid quality indices in refrigerated salmon paste

Fatty acid profile was determined by GLC method [30] and expressed as percentage of methyl esters. Finally, the polyene index (PI) was calculated as the (C 20:5ω3 + C 22:6ω3)/C 16:0 ratio.

The peroxide value (PV) was determined on the lipid extract by the Cd 8-53 iodometric method [31]. The results are expressed as mEq active oxygen/kg lipid. The *p*-anisidine value (AV) was determined in fish muscle according to the Cd 18-90 method, based on the reaction between α- and β-unsaturated aldehydes (primarily 2-alkenals) and *p*-anisidine reagent [31]. Results are expressed as 100 times the absorbance measured at 350 nm in a 1 cm path length cuvette from a solution containing 10 g lipid/L reaction medium. TOTOX index was determined according to the following calculation: $\mathrm{TOTOX} = 2 \times \mathrm{PV} + \mathrm{AV}$, [32].

2.4.2 Tocopherols content in refrigerated salmon paste

The effect of stevia extracts on loss of endogenous antioxidants known as tocopherols during refrigerated storage of samples was analyzed by HPLC and fluorescence detection employed, according to the Ce 8-89 (1993) standard method [31]. Results were expressed as μg tocopherol/g lipids.

2.4.3 Microbiological analyses of refrigerated salmon paste

To determine whether stevia extracts controlled the growth of microorganisms within the health regulations accepted by the Chilean government, samples of 10 g of salmon paste with and without stevia extract were taken aseptically. The samples were mixed with 90 mL of 0.1% peptone water (Merck, Darmstadt, Germany) and homogenized in sterilized stomacher bags (AES, Combourg, France), as previously described [5]. Serial dilutions from the microbial extracts were prepared in 0.1% peptone water. Total aerobes were investigated by surface inoculation on plate count agar (PCA, Oxoid Ltd., London, UK) after incubation at 30°C for 48 h. In the same way pychrotrophic were also investigated, serial dilutions from the microbial extract were seeded in PCA and incubated for 7 days at a temperature of 7–8°C [5].

2.5 Statistical analysis

Data from the different chemical and microbiological analyses were subjected to the ANOVA method to explore differences resulting from the effects of the presence of salmon pastes of the different stevia extracts. Comparison of means was performed using the least-squares difference

(LSD) method. Analyses were carried out by means of the Statgraphics Plus 5.1. program; differences among batches were considered significant for a confidence interval at the 95% level ($p < 0.05$) in all cases.

3 Results and discussion

3.1 In vitro antioxidant properties of stevia extracts

Table 1 shows the extraction yields obtained by aqueous (W), hydro alcoholic (E/W) and supercritical carbon dioxide extraction with ethanol (SCE). The yields of the crude extracts obtained expressed as g of extract/100 g of stevia. The yield of E/W extract was higher than that of W and SCE (17.81%, compared to 9.74 and 11.82%, respectively). However, SCE extraction provided higher ($p < 0.05$) phenolic content (2397 mg GAE/100 g wd) than the other extraction methods (1465 and 1633 mg GAE/100 g wd for W and SCE extracts, respectively). Phenolic compounds have shown a higher affinity for polar organic solvents (i.e., ethanol) than for water [33]. However, SCE extraction (only 5% ethanol) has provided the higher TP content. These results are in agreement with other studies [15] reporting 2085 mg GAE/100 g for aqueous extracts and 2525 mg GAE/100 g for ethanol/water stevia extracts due to the more efficient extraction of phenolic compounds. On the other hand, the ethanol extraction of stevia phenolic compounds (TP) has been reported [7] with yields of 8013 and 8647 mg GAE/100 g, when combined with microwave or ultrasounds treatments, respectively. Shukla et al., [34], also found a higher TP yield with ethanol extraction (6150 mg GAE/100 g) that with water extraction (5674 mg GAE/100 g). Although the TP yields reported in this work are moderate, SCE extraction can be considered a valuable strategy to recover the phenolic compounds from the residual by-products generated by the sweetener industry focusing on steviosides extraction.

Results obtained from DPPH assay (Table 1) indicate that the %Ip for extracts obtained for W, E/W, and SCE was 87.42, 77.31, and 49.57, respectively. On the other hand, scavenging ability of free radicals of SCE was higher than E/W and W, with values of IC_{50} of 49.57, 13.02, and 21.18 μg GAE/mL, respectively. These results are lower that those reported by others authors [35] with an IC_{50} value between 54 and 68 μg/mL in methanolic extracts. Likewise, it has also been reported previously an IC_{50} value of 83.45 μg/mL in water extracts [34].

FRAP assay is based on the reduction of Fe^{3+} to Fe^{2+} and is used as an indicator of electron donation, which is a relevant mechanism of the antioxidant activity of phenolic compounds. The results of the FRAP analysis obtained in this study are shown in Table 1. Thus, SCE and E/W extracts exhibited the best results (17.53 and 14.61 mmol Fe^{2+}/100 g sample, respectively). On the other hand, Ivanovic et al. [36] reported higher FRAP values (27.76 mmol Fe^{2+}/100 g) for supercritical extracts of a 50/50 mixture of clove (*Eugenia caryophyllata*) and oregano (*Origanum vulgare*), under extraction conditions of 10 MPa and 40°C.

3.2 Results of chemical analysis of the quality of refrigerated salmon pastes

3.2.1 Lipid oxidation in salmon paste

Results obtained for the primary lipid oxidation (namely, PV assessment; Fig. 1) indicated that all samples showed significant differences ($p < 0.05$) depending on the storage time. Samples from S + E/W and S + SCE (Abbreviations were noted in material and method.) showed the propagation of lipid oxidation until day 14 and a final decrease at later storage times. In contrast, the control (S) and W batches exhibited steady increases of PV during the whole storage time. Moreover, S + W, S + E/W, and S + SCE batches provided PV values always below 4 meq active oxygen/kg lipids, these being lower than the control "S" in the 14–21 days period. This result indicated a protective effect of stevia extracts on lipid oxidation regardless of the extraction method used. A decreasing PV was observed, being the order of effectiveness of extracts according to the sequence: W < W/E < SCE.

On the other hand, AV, which is representative of secondary lipid oxidation (Fig. 2), revealed clear and

Table 1. Extraction yield, total phenol (TP) content, and antioxidant capacity assessment by the DPPH (IC_{50}) and the FRAP assays[a] for the different kinds of stevia leaves extracts[b]

Extract	Yield (g extract/100 g wd)	TP Ip (mg GAE/100 g wd)	Ip (%)	IC_{50} (μg GAE/mL)	FRAP (mmol Fe^{+2}/100 g)
W	9.74 ± 0.06x	1465 ± 41x	87.42 ± 2.13z	49.45 ± 6.11z	11.83 ± 0.20x
E/W	17.81 ± 1.0z	1633 ± 158x	77.31 ± 5.11y	13.02 ± 1.31y	14.61 ± 0.38y
SCE	11.82 ± 0.12y	2397 ± 177y	49.57 ± 1.89x	21.18 ± 3.42x	17.53 ± 0.20z

[a]Mean values of three replicates ($n = 3$) and standard deviations. For each column, mean values followed by different letters (x, y, z) indicate significant differences ($p < 0.05$).
[b]Abbreviations employed for the different kinds of stevia leaves extracts: W (aqueous), E/W (ethanol/water; 80/20 v/v), and SCE (supercritical CO_2 conditions and ethanol; 95/5 v/v).

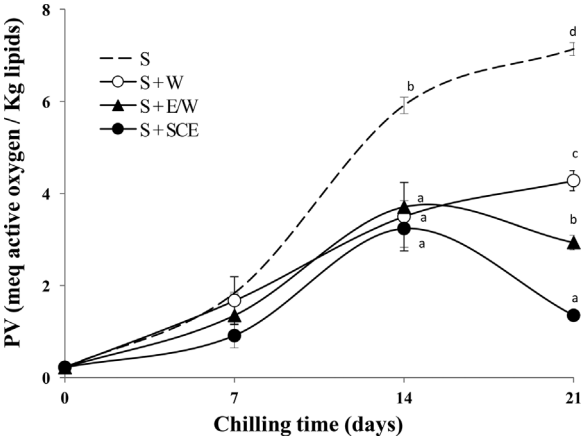

Figure 1. Effect of different stevia extracts addition on the peroxide value (PV) in refrigerated salmon paste. Mean values of three replicates ($n = 3$); standard deviations are indicated by bars. At each sampling time, mean values accompanied by different letters indicate significant differences ($p < 0.05$). Abbreviations employed for the different kinds of samples as expressed in Table 2.

significant ($p < 0.05$) differences among samples depending on the storage time and batch. In this case all batches displayed a progressive increase of AV until day 14, when secondary oxidation reached its maximum; then, a general decrease was observed at the end of the study. An inhibitory effect could be observed for all kinds of stevia extract for the 7–14-day period; this effect was still present at the end of the experiment in samples corresponding to E/W and SCE extracts. Values obtained in this study are in agreement with previous reports of secondary lipid oxidation on frozen salmon [37]. Other authors considered [38] that an AV value of 10 was acceptable for fish oil. Such a value was not reached by any of the stevia batches considered in this study. Remarkably, the SCE batch exhibited the lowest mean level of secondary lipid oxidation in salmon paste.

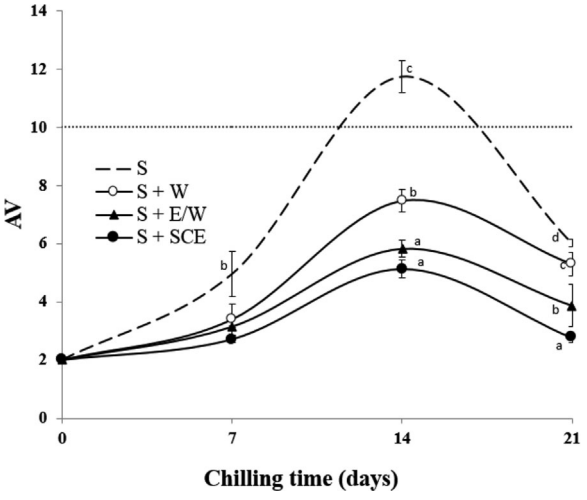

Figure 2. Effect of different stevia extracts addition on the p-anisidine value (AV) in refrigerated salmon paste. Mean values of three replicates ($n = 3$); standard deviations are indicated by bars. At each sampling time, mean values accompanied by different letters indicate significant differences ($p < 0.05$). Abbreviations employed for the different kinds of samples as expressed in Table 2. Acceptability limit value (i.e., 10 score) proposed by Masson (1994) [38] is expressed.

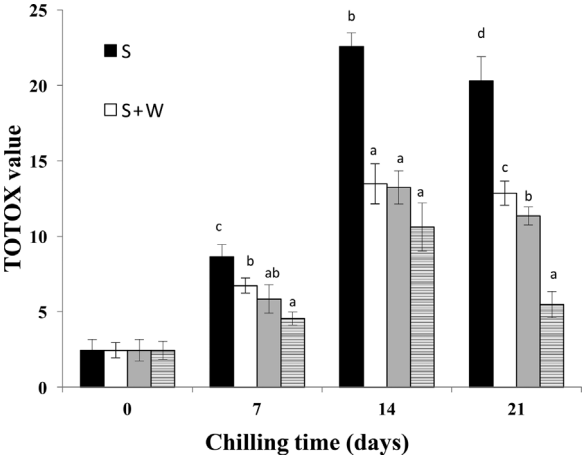

Figure 3. Effect of different stevia extracts addition on the total oxidation (TOTOX) value in refrigerated salmon paste. Mean values of three replicates ($n = 3$); standard deviations are indicated by bars. At each sampling time, mean values accompanied by different letters indicate significant differences ($p < 0.05$). Abbreviations employed for the different kinds of samples as expressed in Table 2.

TOTOX index, which links PV and AV parameters providing an idea of the total oxidation of the samples, exhibited significant ($p < 0.05$) differences depending on the storage time and sample type (Fig. 3). All samples displayed a maximum score on day 14, the control "S" had the highest value and the SCE batch the lowest value.

The effect of polyphenolic extracts of stevia on the evolution of primary and secondary lipid oxidation products in salmon paste was similar to that reported [3] in either chilled or frozen minced horse mackerel including polyphenols from grape pomace and other plant by-products.

These authors also reported that the antioxidant activity of polyphenols was based on the degree of polymerization and galloylation. Thus, a moderate degree of polymerization (namely, monomeric units) and a low level of galloylation (0.15–0.25 gallate/ molecule) showed to be the most effective for the inhibition of lipid oxidation in pelagic fish muscle [3]. Additionally, the presence of galloyl groups in the polyphenols structure showed to increase in their activity either in the protection of hemoglobin from oxidation and in the prevention of lipid oxidation in fish muscle [3].

Table 2. Assessment[a] of the ω3/ω6 and the polyene index (PI) ratios in salmon paste including different kinds of stevia extracts[b] that were stored at 5°C for 21 days

	ω3/ω6 ratio chilling time (days)				PI chilling time (days)			
Sample	0	7	14	21	0	7	14	21
S	2.77 ± 0.12	2.31 ± 0.51x	2.16 ± 0.03x	2.19 ± 0.02x	1.52 ± 0.02	1.48 ± 0.02	1.41 ± 0.03x	1.35 ± 0.01x
S + W	2.71 ± 0.06	2.81±0.05 y	2.29 ± 0.04 x	2.31 ± 0.04x	1.53 ± 0.03	1.50 ± 0.16	1.48 ± 0.03 y	1.36 ± 0.03x
S + E/W	2.68 ± 0.07	2.76 ± 0.08 y	2.69 ± 0.01y	2.60 ± 0.02y	1.51 ± 0.02	1.52 ± 0.12	1.48 ± 0.01y	1.51 ± 0.07y
S + SCE	2.64 ± 0.08	2.68 ± 0.06 y	2.75 ± 0.12y	2.72 ± 0.08y	1.53 ± 0.06	1.50 ± 0.08	1.51 ± 0.02y	1.52 ± 0.02 y

[a]Mean values of three replicates ($n = 3$) and standard deviations. For each column, mean values followed by different letters (x, y) indicate significant differences ($p < 0.05$) as a result of the stevia extract added. No letters are included when differences were not found ($p > 0.05$).
[b]Abbreviations employed for the different kinds of samples: S (salmon paste, control), S + W (salmon paste including stevia water extract), S + E/W (salmon paste including stevia 80/20 v/v ethanol/water extract), and S + SCE (salmon paste including stevia supercritical CO_2 extract), in agreement with conditions expressed in Table 1.

3.2.2 ω3/ω6 ratio and polyene index

Table 2 shows the ω3/ω6 ratio and polyene index (PI) in salmon paste after the addition of stevia extract, and then stored at 5°C for 21 days. The results indicated significant ($p < 0.05$) differences for the ω3/ω6 ratio depending on the batch and storage time. Thus, a ω3/ω6 ratio decrease at day 7 was observed in control samples when compared with any batch including a stevia extract; during the 14–21-days period, a higher ω3/ω6 ratio was obtained in samples containing E/W or SCE stevia extracts. On the other hand, the significant differences ($p < 0.05$) in the PI value observed during the study reflected the changes in total EPA and DHA content during storage time and the characteristics of the fish paste type (with and without extract of stevia). Thus, a higher PI was obtained in S + E/W and S + SCE samples at days 14 and 21 when compared with control and S + W samples; additionally, a protective effect was also observed at day 14 in samples corresponding to the W extract. These results are in agreement with those reported for salmon fillets in the presence of added polyphenols obtained from seeds, algae, etc. [3, 5]. The protective action of the polyphenols was also reported in frozen salmon fillets by the application of an external polyphenol-based film from barley shell [39]. In the present study, the lipid damage in salmon paste was found to be partially inhibited by the application of stevia extracts.

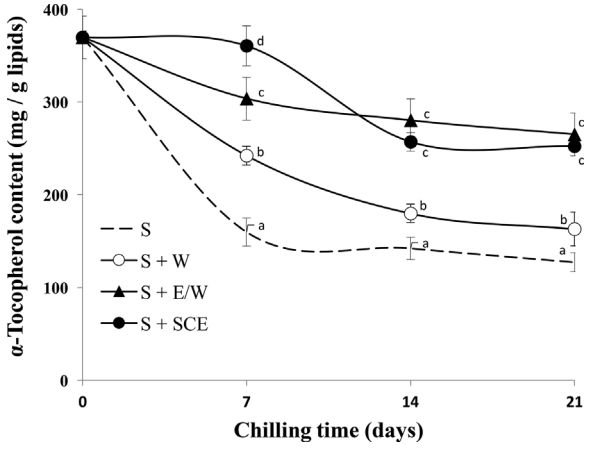

Figure 4. Effect of different stevia extracts addition on alpha-tocopherol content in refrigerated salmon paste. Mean values of three replicates ($n = 3$); standard deviations are indicated by bars. At each sampling time, mean values accompanied by different letters indicate significant differences ($p < 0.05$). Abbreviations employed for the different kinds of samples as expressed in Table 2.

3.2.3 α-Tocopherol content

The tocopherol content in the lipid fraction of salmon paste is shown in Fig. 4. The results indicated significant ($p < 0.05$) differences depending on the batch and storage time. A progressive decrease was observed for this parameter in all samples as storage time progressed. The loss of α-tocopherol was significantly ($p < 0.05$) higher for the control batch as compared to batches including stevia extracts. Remarkably, tocopherol remained relatively constant in the S + E/W sample during the first week, a result that was not observed in the S + SCE sample.

The results of this study are in agreement with previous reports on chilled salmon, in which the tocopherol content decreased as fish deterioration and storage time progressed [40]. Similar results were also reported for frozen muscle in Atlantic salmon (*S. salar*) [25], Coho salmon (*Oncorhynchus kisutch*) [41], and horse mackerel (*Trachurus trachurus*) [42]. Such α-tocoferol losses have been explained in terms of their protective effect on lipids with respect to oxidation during frozen storage. Moreover, the presence of stevia polyphenols might have enhanced α-tocopherol antioxidant activity either alone or in combination with

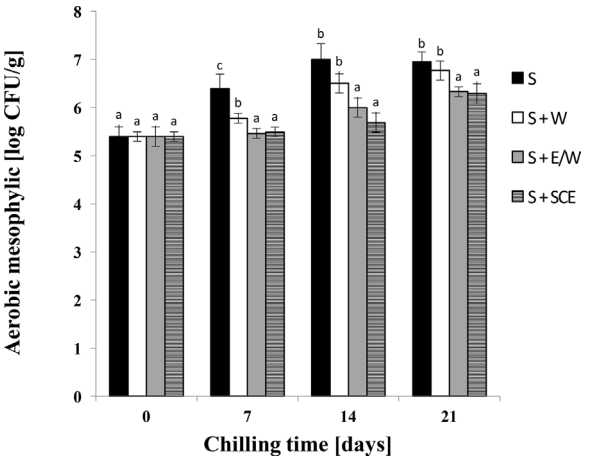

Figure 5. Effect of different stevia extract addition on the mesophylic bacteria counts in refrigerated salmon paste. Mean values of three replicates ($n = 3$); standard deviations are indicated by bars. At each sampling time, mean values accompanied by different letters indicate significant differences ($p < 0.05$). Abbreviations employed for the different kinds of samples as expressed in Table 2.

other natural antioxidants from fish muscle (i.e., ascorbate, ubiquinol, glutathione, astaxanthine, etc.), these increasing the efficiency of the conversion of tocopheroxil radicals to tocopherol, thus ensuring the protection of the paste against lipid oxidation. These combined mechanisms between endogenous antioxidants and added polyphenols have previously been reported [3].

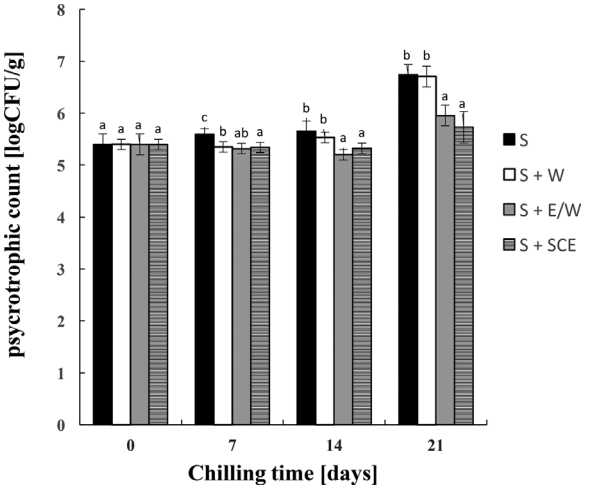

Figure 6. Effect of different stevia extracts addition on the psychrotrophic bacteria count in refrigerated salmon paste. Mean values of three replicates ($n = 3$); standard deviations are indicated by bars. At each sampling time, mean values accompanied by different letters indicate significant differences ($p < 0.05$). Abbreviations employed for the different kinds of samples as expressed in Table 2.

3.3 Microbiological content of refrigerated salmon pastes

Figure 5 shows the results for mesophylic aerobes count in the different salmon paste batches during refrigerated storage. The results indicate a slower evolution of microbial growth in S + W, S + E/W, and S + SCE samples as compared with control paste "S". After day 7, such differences were found to be significant ($p < 0.05$). This could suggests a moderate antimicrobial effect of stevia extracts. The most relevant inhibition of microbial growth, near to 1 log unit, was observed on day 14 on E/W and SCE extracts, as compared with the control "S". Moreover, all samples except for the control sample displayed aerobic counts below 6 log units, meeting the limit established by Chilean regulations (RSA, 2015) [43].

These results are in agreement with finding of others authors [44, 45, 18], who reported on the inhibitory effect of aqueous stevia extracts on the growth of *Escherichia coli*, *Staphylococcus aureus*, and *Penicillium chrysogenum*. Besides, it has been reported previously [13] a higher antimicrobial effect at temperatures close to food refrigeration for extracts with higher phenolic compound concentration, results that also support those obtained in the present study. Likewise, the antimicrobial effect of crude stevia extracts has also been combined with other technologies such as high pressure processing, this provides a reduction of five log cycles (5D) of the content of *Listeria monocytogenes*, and very relevant effects on polyphenol oxidase and peroxidase enzymes [14].

With respect to the psychrotrophic bacteria count (Fig. 6), they follow a similar pattern as aerobic count, with significantly ($p < 0.05$) lower counts being determined in all samples as compared with the control batch until day 7. Afterwards (14–21-days period), this result was only observed in E/W and SCE batches. Similar results have been reported for hydroalcoholic polyphenolic extracts from the Bifurcaria bifurcata alga [5]. Thus, although the inhibition of psychrotrophic growth did not reach 1 log unit, the results obtained in the present study allowed to conclude a remarkable inhibitory effect of crude polyphenol stevia extracts (namely, E/W and SCE extracts) on aerobic and psychrotrophic bacteria in salmon paste.

4 Conclusions

This study reports, to the best of our knowledge and for the first time, the inhibitory effect of stevia phenolic compounds on lipid oxidation and microbial activity in refrigerated salmon paste. The results indicated a better stability against lipid rancidity together with a moderate inhibition of microbial growth. Such results were obtained after a detailed comparison of stevia extracts prepared with water, ethanol/water, and supercritical carbon dioxide, the latter two methods providing the most promising results in terms of

polyphenols extractability and yield. Thus, SCE and E/W extracts provided a better control of primary and secondary lipid oxidation compounds and moderate inhibition of both aerobes and psychrotrophs in salmon paste. Besides, both extracts provided a more stable ω3/ω6 ratio and PI value, together with a higher residual concentration of α-tocopherol for the 21-day refrigerated storage as compared with the control batch. These results open the way to the potential utilization of bioactive compounds and extracts from stevia leaves, a by-product of the sweetener industry, for the preservation of nutritional and microbial qualities of salmon paste and derivatives of seafood.

This study was financially supported by grants: Innova Project 11CEII-9568 and Fondecyt Regular 1120312.

The authors have declared no conflict of interest.

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