J Food Sci Technol (April 2018) 55(4):1416–1425
https://doi.org/10.1007/s13197-018-3056-7

ORIGINAL ARTICLE

Chemical, microbiological, and sensory parameters during the refrigerated storage of silver catfish (*Rhamdia quelen*) exposed in vivo to the essential oil of *Lippia alba*

Ana Paula de Lima Veeck[1,2,3] · Ana Paula Daniel[1,2] · Bruna Klein[1] · Andréia Quatrin[1] · Ana Paula de Souza Rezer[2] · Liana Guidolin Milani[2] · Carla Cristina Zeppenfeld[5] · Mauro Alves da Cunha[5] · Clarissa Giesel Heldwein[4] · Berta Maria Heinzmann[4] · Thaylise Vey Parodi[5] · Bernardo Baldisserotto[5] · Tatiana Emanuelli[1,2]

Accepted: 26 January 2018 / Published online: 3 February 2018

Abstract This study evaluated whether the essential oil of *Lippia alba* (EO) used as a sedative for fish transport would increase the stability of silver catfish during ice storage. Fish were transported (6 h) with water alone (control), 30 or 40 µL/L of EO in water. After transport, fish were slaughtered and stored in ice. Data on mesophilic and psychrotrophic bacteria counts during storage did not support the evidence for the antimicrobial activity of EO. However, fish treated with EO (30 and 40 µL/L) had delayed onset of *rigor mortis*, delayed increase of pH after 34 days of storage, and delayed peak of hypoxanthine formation and its degradation. In addition, the demerit sensory score of EO-treated fish (30 and 40 µL/L) was lower than that of controls along the storage. Thus, the use of EO as a sedative in the water used to transport silver catfish can delay the loss of freshness and the deterioration of whole fish stored in ice.

Keywords Fish deterioration · Anesthetics · Refrigerated storage · Shelf life

Introduction

After slaughter, the freshness of refrigerated fish degrades due to various biochemical reactions, which include protein and ATP degradation, pH changes, and the production of undesirable volatile basic nitrogen compounds due to bacterial action (Ocaño-Higuera et al. 2011; Binsi et al. 2015). Thus, numerous methods have been developed to assess the quality of fish stored in ice, including the determination of total volatile basic nitrogen (TVB-N), trimethylamine, pH, hypoxanthine (Hx), and K value as well as sensory and microbiological analyses (Scherer et al. 2005, 2006; Ocaño-Higuera et al. 2009, 2011).

These *post mortem* biochemical changes are strongly influenced by the pre-slaughter procedures (Ribas et al. 2007; Nathanailides et al. 2011). The increased muscle activity and stress that occur in fish during transport, capture, and management can anticipate the onset of *rigor mortis*, which affects final product quality (Ribas et al. 2007; Nathanailides et al. 2011). Synthetic anesthetics have been used to minimize this stress damage, but these compounds are difficult to obtain and are expensive (Berka 1986). Nowadays, tricaine methanesulfonate (MS-222) is the only FDA-approved anesthetic for use in fish intended for human consumption, but it requires a 21-day withdrawal period prior to human consumption (Popovic et al. 2012). For this reason, the interest in alternative anesthetics, such as those from natural sources, has increased.

✉ Tatiana Emanuelli
tatiana.emanuelli@ufsm.br; tatiemanuelli@gmail.com

[1] Núcleo Integrado de Desenvolvimento em Análises Laboratoriais (NIDAL), Departamento de Tecnologia e Ciência dos Alimentos, Centro de Ciências Rurais, Universidade Federal de Santa Maria, Camobi, Santa Maria, RS 97105-900, Brazil

[2] Programa de Pós-graduação em Ciência e Tecnologia de Alimentos, Centro de Ciências Rurais, Universidade Federal de Santa Maria, Camobi, Santa Maria, RS 97105-900, Brazil

[3] Instituto Federal de Santa Catarina/IFSC, Lages, SC 88506-400, Brazil

[4] Departamento de Farmácia Industrial, Centro de Ciências da Saúde, Universidade Federal de Santa Maria, Camobi, Santa Maria, RS 97105-900, Brazil

[5] Departamento de Fisiologia e Farmacologia, Centro de Ciências da Saúde, Universidade Federal de Santa Maria, Camobi, Santa Maria, RS 97105-900, Brazil

Eugenol (clove oil active compound)- and isoeugenol (AQUI-S^{TM} active compound)-based anesthetics (Javahery and Moradlu 2012) have been used in fish production in Australia, Chile, New Zealand, South Korea, and Costa Rica (Zahl et al. 2012). Although no withdrawal period is required prior to human consumption, eugenol seems to be inappropriate for fish that will be slaughtered for human consumption in the near future because it adversely affects the flavor of fish fillets (Cunha et al. 2010a). In contrast, the essential oil of *Lippia alba* (EO) (Verbenaceae) was recently demonstrated to induce fish anesthesia, to inhibit the increase of plasma cortisol levels caused by handling, and to reduce transport stress (Becker et al. 2012) without changing the odor or flavor of the fish fillets (Cunha et al. 2010b). Moreover, EO used as a sedative during fish transport can delay the lipid oxidation of fillets during frozen storage (Veeck et al. 2013).

Lippia alba is a shrub native to Central and South America (Barros et al. 2009) that is widely used in tea beverages and in popular medicine for its sedative properties (Hennebelle et al. 2008). In addition, it was also shown that EO has antimicrobial activity in vitro (Oliveira et al. 2006). Therefore, we hypothesized that the use of EO as a sedative to reduce the stress of fish transport would promote the extension of fish shelf life during refrigerated storage.

Hence, the present study was undertaken to evaluate whether the use of EO as a sedative for silver catfish (*Rhamdia quelen*) transport would improve the chemical, microbiological, or sensory stability of whole fish during ice storage. Silver catfish is a freshwater fish found in a widespread area that ranges from southern Mexico to central Argentina (Gomes et al. 2000). It has attracted the attention of fish producers and consumers because of its resistance, good feed efficiency (Meyer and Fracalossi 2004) and the tasters' preference for its flesh in sensory tests, which was similar compared to other species as carps and Nile tilapia (Barcellos et al. 2012).

Materials and methods

This study was approved by the Institutional Ethics on Animal Use Committee.

Experimental procedure

One hundred thirty-five silver catfish (*R. quelen*; 187.9 ± 9.1 g, 27.5 ± 0.8 cm) specimens were obtained from the Department of Animal Husbandry, Federal University of Santa Maria, Brazil. Fish were transported for 6 h on paved road in plastic bags with 7 L of water and 8 L of pure oxygen and at a loading density between 188.9 and 213.3 g/L (average: 200.4 g/L). Three different treatments were applied to the water used during transport: 0 (control), 30, or 40 μL/L of EO (equivalent to 0, 24, or 32 mg/L, respectively, because the density of this essential oil is about 0.80). Each plastic bag contained 15 fish and was considered as 1 replicate. Three independent replicates were conducted for each treatment. Before transportation, the fish from EO treatments were exposed to 200 μL/L of EO for 3 min to induce sedation. This initial sedation was used to avoid the increase in ventilation rate that silver catfish were reported to present in the first hour of transport with EO added to the water (Becker et al. 2012). The EO was diluted in ethanol (1:10 v/v) and added to the water for transport. Cunha et al. (2010b) demonstrated that ethanol at the highest concentration used in the present study (360 μL/L) did not induce any level of sedation in silver catfish. The transport time, loading density, and concentrations of EO were chosen based on previous studies of our group (Cunha et al. 2010b; Becker et al. 2012).

The water parameters were monitored before and after transport according to Becker et al. (2012).

After transport, fish were slaughtered by hypothermia and washed with tap water several times to remove adhering blood and slime. Immediately after the slaughter (time 0), 6 fish from each treatment (2 fish from each different replicate) were used to analyze the proximate composition, pH, nucleotides, and TVB-N and to perform microbiological and sensory analysis. The remainder of the fish were stored in high density polyethylene boxes (100 kg capacity), uniformly packed in ice flakes (1 kg ice per kg of fish). An individual box was used for each treatment, and boxes were stored at (2 ± 1 °C) for up to 34 days. The water was drained, and the ice was replaced daily over the whole storage period. To assess the shelf life of whole fish stored in ice, the analyses were performed 2, 6, 9, 16, 27, and 34 days after the beginning of the storage. At each time point, 6 fish were taken from each treatment (2 fish from each different replicate) to assess pH, nucleotides, and TVB-N and perform microbiological and sensory analyses.

Three fish from each treatment were used to evaluate the index of *rigor mortis*. The *rigor mortis* index was determined in the whole fish (n = 3 per treatment) stored at 2 ± 1 °C during the first 144 h after slaughter.

Essential oil of *L. alba*

Lippia alba was cultivated in São Pedro do Butiá (28°07′25.1″S, 54°53′35.7″W), Rio Grande do Sul State, Brazil. The plant material was identified by botanist Dr. Gilberto Dolejal Zanetti (Department of Industrial Pharmacy, UFSM), and a voucher specimen (SMDB No. 10050) was deposited in the herbarium of the Department

of Biology, UFSM. EO was obtained from fresh leaves of the plant by hydrodistillation for 2 h using a Clevenger type apparatus. In this method, the distillate is collected in a graduated glass tube, and the aqueous phase is automatically reused in the distillation flask (European Pharmacopoeia 2007). Two separate distillations were performed, and the samples obtained were pooled to yield EO, which was stored at − 4 °C in amber glass bottles until use.

The composition of the EO was analyzed by gas chromatography-mass spectrometry (GC/MS) in a hyphenated Agilent 6890 system equipped with a 5973 series mass selective detector and fitted with a fused silica capillary column HP5-MS (Hewlett Packard, 5% phenylmethylsiloxane, 30 m × 0.25 mm × 0.25 µm). The analysis parameters were: 1 µL injection volume at 100:1 split ratio, He (1 mL/min) as the carrier gas, injector temperature of 220 °C, oven temperature set at 40 °C for 4 min and then increased to 260 °C at 4 °C/min, interface temperature of 250 °C, ionization energy of 70 eV, and bank data was from NIST (2002). The EO components were identified based on their retention indices determined using a calibration curve of a homologous series of n-alkanes (C8–C32), which were injected under the same chromatographic conditions as the samples and fragmentation models of the mass spectra, and both were compared with data from the literature (Adams 2001). The concentration of the constituents was calculated using the area of their respective peaks, related to the total area of all constituents of the sample, and obtained by analysis using gas chromatography.

Fish analysis

Rigor mortis index

The measurement of the onset and resolution of r*igor mortis* was based on the curvature of the tail (Bito et al. 1983). Fish was placed on a flat table so that the body behind the posterior end of the dorsal fin was hanging over the edge. The *rigor* index (I_R) was determined by the following formula: $I_R = [(Lo - Lt)/Lo] \times 100$, where L represents the vertical distance between the base of the caudal fin and the table surface measured immediately after death (Lo) and during storage (Lt). Fish were stored at 2 ± 1 °C between *rigor* measurements. Pre-rigor was defined as the state at which the I_R was less than 10%, in-rigor was the state at which I_R ranged between 80–100%, and post-rigor was the state at which I_R fell below 10%.

Proximate composition

Fish were filleted, and the proximate composition of fillets was determined immediately after slaughtering as follows. The moisture was determined as the weight loss after 4 h at 60 °C in an assisted-air circulation oven, followed by 8 h at 105 °C. The ash content was determined at 550 °C according to AOAC (1996). The crude protein (N × 6.25) was determined by the microKjeldahl procedure (AOAC 1996). The fat content was gravimetrically determined after extraction using chloroform and methanol (Bligh and Dyer 1959).

pH measurement

The pH analysis was carried out in fish muscle (anterior portion) using an insertion glass electrode, and measurements were made with a digital pH-meter at room temperature.

Nucleotide analysis

The ATP and its breakdown products were extracted according to the method of Ryder (1985). Briefly, 5 g of muscle from the anterior dorsal region of the fish were homogenized with 25 mL of 0.6 M perchloric acid at 0 °C for 1 min in an IKA Ultra Turrax (T18 basic) homogenizer. The homogenate was centrifuged at 2000×*g* for 10 min, and 10 mL of the supernatant was neutralized to pH 6.5–6.8 with 1 M potassium hydroxide. Potassium perchlorate was removed by centrifugation, and the supernatant was stored at − 20 °C for subsequent analysis. A Shimadzu Prominence liquid chromatograph equipped with a model LC-20AT pump, a model DGU-20A5 online degasser, and a model SPD-M20A diode array detector (set at 254 nm), operated by the LC Solution version 1.25 software, were used for all analyses. Separation was achieved in a C18 reversed-phase Shim-pack CLC ODS (M) column (250 × 4.6 mm, 5 µm) with a guard column (Shim-pack GODS, 10 × 4 mm) (Shimadzu, Tokyo, Japan) using 0.04 M potassium dihydrogen orthophosphate (KH_2PO_4) and 0.06 M dipotassium hydrogen orthophosphate (K_2HPO_4) (pH = 7.0) as mobile phase A and acetonitrile as mobile phase B. The buffer solutions were filtered through a 0.45-µm Millipore filter before use. The mobile phase was eluted at a flow rate of 0.8 mL/min following the gradient proposed by Vallé et al. (1998). A standard curve was prepared for each compound evaluated (ATP, ADP, AMP, IMP, Hx, and HxR; ICN Biomedicals Inc., Aurora, Ohio) in the range of 0.01–0.4 mg/mL. The samples were filtered through a 0.22-µm Millipore filter before injection because additional potassium perchlorate

precipitates during storage at − 20 °C. All reagents used were of HPLC grade.

Total volatile basic nitrogen (TVB-N) analysis

TVB-N in the flesh was determined by distillation of the sample alkalified with MgO (2 g) as described by Furuichi et al. (1997), except that before distillation, the protein fraction was separated by homogenizing the fish muscle with 5% trichloroacetic acid at a ratio of 1:2 (w/v).

Microbiological analyses

For all analyses, flesh samples (25 g) were aseptically obtained by cutting slices from the dorsal, ventral, and tail area and then blending in 0.1% (w/v) peptone for 2 min in a Stomacher. The appropriate serial dilutions were plated onto plate count agar (Difco, Detroit, MI). The total count was determined using the pour plate method after incubation at 35 to 37 °C for 48 h (Downes and Ito 2001). The psychrotrophic count was determined with the spread plate method after incubation at 7 to 10 °C for 10 days (Downes and Ito 2001).

Sensory analysis

The sensory assessment of whole fish was conducted using the Tasmanian Food Research Unit (TFRU) freshness assessment scheme, a system developed at CSIRO Division of Food Research (Branch and Vail 1985), with minor modifications for silver catfish (Table 1). On each day of analysis, 2 fish from each treatment were assessed by an expert panel (n = 15–19). Panelists were selected using a questionnaire to determine their interest in participating in the research and possible exclusion factors such as allergies, smoking, etc. The panelists were trained using the TFRU assessment scheme (Table 1) and fish at different degrees of deterioration and then a preliminary test using the TFRU assessment scheme (Table 1) was applied for exclusion of non-sensitive panelists. Each panelist was given up to 4 simple descriptors and scored demerit points from 0 to 3, where 0 represented the highest quality, and any higher score indicated poorer quality. The scores for the separate characteristics were summed to give an overall sensory score. The panelists were also asked to state whether the fish were acceptable or unacceptable for consumption, and this judgment was used to determine fish shelf life.

Statistical analysis

The results of the proximate composition were analyzed using one-way analysis of variance (ANOVA), and the other results were analyzed using two-way factorial ANOVA (3 treatments × 2 time points for the physicochemical parameters of water, 3 treatments × 11 time points for *rigor mortis*, and 3 treatments × 7 time points for the other results). The differences between the averages were post hoc evaluated using Duncan's test. Differences were considered to be significant when $p < 0.05$.

Table 1 Modified Tasmanian Food Research Unit (TFRU) sensory assessment scheme for silver catfish

Parameters assessed	Demerit points[a]			
	0	1	2	3
General				
Firmness	Firm	Slightly firm	Soft	Very soft
Stiffness	Pre-rigor	Rigor	Post-rigor	–
Odor	Fresh	Strong but not spoiled	Spoiled	–
Eyes				
Clarity	Clear	Slightly cloudy	Cloudy	Very cloudy
Iris	Visible	Slightly visible	Not visible	–
Gills				
Colour	Dark red	Red	Slightly brown	Dark brown or grey
Mucus	Absent	Slight	Moderate	Excessive
Odor	Fresh	Strong	Spoiled	Very spoiled
Belly				
Firmness	Firm and elastic	Firm and not elastic	Soft	Very soft
Color	Bright white	Greyish	Brownish-yellow	–
Vent				
Condition	Normal	Dilated	Excessive	–

[a]Total demerit points (0–28)

Results and discussion

The possibility of linking sedation or anesthesia during fish handling to the extension of fish shelf life seems compelling. Therefore, this research was undertaken to monitor the changes in chemical, microbiological, and sensory parameters during the ice storage of whole fish that was sedated with EO before slaughter.

Composition of EO

In the present study, 33 compounds were identified in the EO, mainly mono- and sesquiterpenoids. EO contained (g/100 g of oil) 56.4 linalool, 6.5 germacrene-D, 6.1 1,8-cineole, 4.1 Z-carveol, 4.0 β-caryophyllene, 3.0 germacrene-B, 1.3 α-humulene, 1.3 β-elemene, 1.1 caryophyllene oxide, and 1.1 sabinene, whereas the other compounds were found at concentrations lower than 1 g/100 g. The present study found a composition of EO similar to previous studies that also found linalool to be the major constituent followed by 1,8-cineole or eucalyptol and germacrene-D (Barros et al. 2009; Veeck et al. 2013).

Water parameters

The transport of fish for 6 h increased the water temperature (18.8 ± 0.2 vs. 17.2 ± 0.1 °C before transport, $p < 0.05$) and reduced the dissolved oxygen levels (4.8 ± 0.6 vs. 7.8 ± 0.2 mg/L before transport, $p < 0.05$) but did not change the pH (6.5 ± 0.1 vs. 6.3 ± 0.1 before transport, $p > 0.05$). The addition of EO did not change the physico-chemical parameters of water during transport (data not shown). In a previous study on the use of EO to sedate silver catfish, the dissolved oxygen levels also decreased in the water after 4 h of transport, however the temperature did not change (Becker et al. 2012). The increase of temperature observed in the present study may have occurred because the transport was made under sun exposure.

Proximate composition

The use of EO as a sedative to reduce the stress of fish transport did not affect the proximate composition of fish fillets (data not shown, $p > 0.05$), which is in agreement with a previous study (Veeck et al. 2013). The average proximate composition (g/100 g of wet weight) was 78.1 ± 0.6 moisture, 1.2 ± 0.1 ash, 12.9 ± 1.1 protein, and 4.2 ± 1.0 fat.

Physico-chemical analysis

ANOVA revealed a significant treatment x time interaction on the *rigor mortis* ($p < 0.05$). The fish treated with 30 and 40 µL/L EO showed a delayed onset of *rigor mortis* (lower *rigor* index values than controls at 4 h after slaughter), whereas those treated with 30 µL/L EO had a faster resolution of *rigor mortis*, as indicated by the lower *rigor* index values compared with controls at 72–96 h after slaughter ($p < 0.05$; Fig. 1). Fish deterioration usually occurs in four sequential stages: *rigor mortis*, dissolution of *rigor*, autolysis (loss of freshness), and bacterial spoilage (Ocaño-Higuera et al. 2009, 2011). *Rigor mortis* is associated with the acidification process caused by the production of lactic acid in muscle tissue. Pre-slaughter stress induces greater physical activity prior to death, which leads to the consumption of the glycogen energy reserve and production of lactic acid in muscle (Nathanailides et al. 2011). The use of less stressful procedures before slaughtering may delay the onset of *rigor* (Nathanailides et al. 2011). In fact, Senegal sole (*Solea senegalensis*) stunned with clove oil showed a delayed onset of rigor and improved freshness features when compared with fish stunned by hypothermia and slaughtered by asphyxia (Ribas et al. 2007). Thus, the delayed onset of *rigor mortis* in EO-treated fish observed in the present study may be associated with the decrease in transport stress caused by EO (Becker et al. 2012).

The muscle pH values increased in all groups during storage in ice (Fig. 2). This increase was significant after 27 days for the controls and 40 µL/L EO-treated fish and after 34 days of storage for the 30 µL/L EO-treated fish when compared with the initial time ($p < 0.05$). The in vivo exposure to EO affected the muscle pH of silver catfish. The fish exposed to 30 µL/L EO initially had higher pH than controls (day 0), which is consistent with the lower lactic acid production due to less stressful

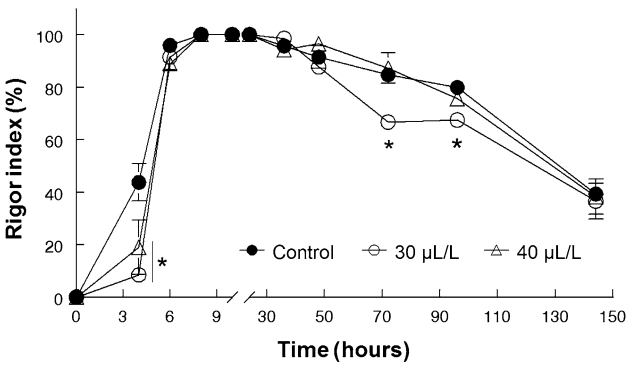

Fig. 1 Evaluation of *rigor mortis* in silver catfish exposed in vivo to different concentrations of the essential oil of *L. alba*. Values are the mean ± standard error (n = 3). ANOVA revealed a significant time x treatment interaction on the rigor mortis index. *Significantly different from control at the same time point ($p < 0.05$)

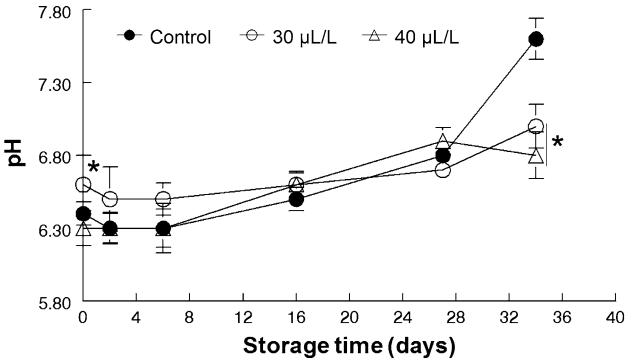

Fig. 2 Effect of in vivo exposure to different concentrations of the essential oil of *L. alba* on the muscle pH of silver catfish during storage in ice. Values are the mean ± standard error (n = 3). ANOVA revealed a significant time × treatment interaction on the pH value. *Significantly different from controls at the same day of storage ($p < 0.05$). For clarity purposes only the statistical differences between control and treatments are indicated in the figure, whereas the differences observed in the same group along the storage are presented in the results section

conditions before slaughter. Conversely, after 34 days of storage, the fish with either EO treatment had lower muscle pH values than control fish ($p < 0.05$; Fig. 2). The increase in muscle pH at the end of storage is related to the proteolysis that occurs due to microbial growth and is related to fish spoilage (Ocaño-Higuera et al. 2009). Thus, the lower pH of the EO-treated fish at the end of storage indicated a delay of fish spoilage. Brazilian law established 6.8 as the maximum acceptable pH of fish for human consumption (Scherer et al. 2006). Thus, after 27 days, only the fish treated with 30 µL/L EO were within this limit, whereas after 34 days of storage, only the fish treated with 40 µL/L EO were within this limit.

The degradation of ATP in *post mortem* muscle correlates well with the loss of freshness in a wide range of fish (Ocaño-Higuera et al. 2011) including silver catfish (Daniel et al. 2014). In fish muscle, ATP degradation follows the following sequence: adenosine 5′-triphosphate (ATP) → adenosine 5′-diphosphate (ADP) → adenosine 5′-monophosphate (AMP) → inosine 5′-monophosphate (IMP) → HxR (inosine) → Hx (hypoxantine) → xanthine (Howgate 2006). In our study, IMP was the predominant nucleotide in fish muscle after slaughtering, with an initial value of 8.8 ± 0.4 µmol/g (Fig. 3a). In addition, we did not detect ATP and found very low levels of ADP and AMP (Fig. 3a). These results indicated a rapid degradation of ATP into IMP. The ADP levels remained very low and almost unchanged during storage and were not affected by EO ($p > 0.05$). ANOVA revealed an effect of storage time on the values of AMP, IMP, and HxR ($p < 0.05$), without an effect of the EO treatment. For this reason, the results of all treatments were pooled (Fig. 3a). The AMP values decreased slightly (0.5 µmol/g at day 0 vs. 0.3 µmol/g at

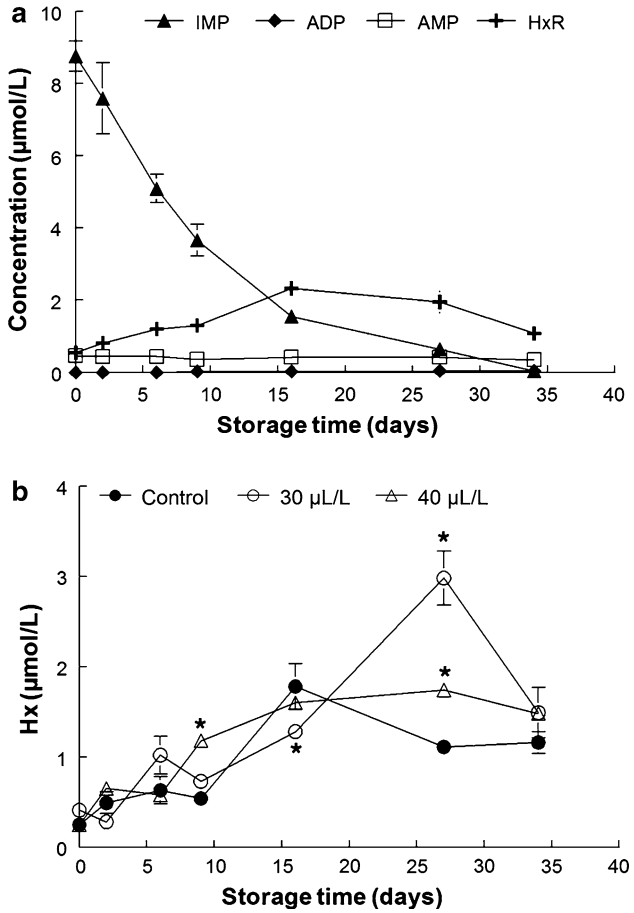

Fig. 3 Effect of in vivo exposure to different concentrations of the essential oil of *L. alba* on the degradation of nucleotides in the muscle of silver catfish during storage in ice. Values are mean ± standard error. In **a** ANOVA revealed a significant effect of refrigerated storage time but no effect of treatments on the levels of IMP, ADP, AMP, and HxR. For this reason, results are the average of all treatments at each time point (n = 9). In **b** ANOVA revealed a significant time × treatment interaction on the levels of Hx (n = 3 for each treatment, at each time point). *Significantly different from controls at the same day of storage ($p < 0.05$). For clarity purposes only the statistical differences between control and treatments are indicated in the figure, whereas the differences observed in the same group along the storage are presented in the results section

day 34, $p < 0.05$) during storage. The HxR values increased from day 6 of storage up to day 16 when compared with the initial value (1.2 and 2.3 vs. 0.6 µmol/g at day 0; $p < 0.05$) and then decreased on day 34 of storage (1.1 µmol/g; $p < 0.05$). The IMP values decreased from day 6 of storage onwards (8.8 µmol/g at day 0 vs. 0.04 µmol/g at day 34), which indicated a reduction in freshness. Furthermore, the loss of IMP is related to the progressive loss of desirable fresh fish flavor during chilled storage (Howgate 2006). This decrease in the IMP levels was due to the degradation of IMP into HxR and Hx, which increased up to days 15–25 (Fig. 3). This behavior was

similar to that found by Özoğul et al. (2007) for sardine and by Daniel et al. (2014) for silver catfish.

There was a significant treatment x time interaction on the values of Hx. The Hx values increased for all groups during storage, but this behavior was different depending on the treatment (Fig. 3b). For control fish the peak of Hx formation occurred at day 16 of storage (higher than controls at all other time-points, $p < 0.05$). In contrast, the fish exposed to 30 µL/L EO had lower Hx concentration than controls at day 16 of storage and higher Hx concentration than controls at day 27 of storage at the time of peak formation of Hx in this EO-treated group (higher than 30 µL/L EO at all other time-points, $p < 0.05$). The Hx levels in fish treated with 40 µL/L EO started to increase at 9 days of storage and thereafter increased slightly up to day 27 of storage ($p < 0.05$). Differing from the other groups, the 40 µL/L EO-treated fish showed no decrease in Hx levels. In addition, this group had higher Hx values than controls at 9 and 27 days of storage ($p < 0.05$). At the end of the storage period, the levels of Hx decreased in the controls and 30 µL/L EO-treated fish, probably due to its degradation into xanthine, which in turn can be oxidized to uric acid (Venugopal 2002; Howgate 2006). Thus, we suggest that the higher concentration of EO may delay Hx degradation because we did not observe a decrease in Hx levels in this group at the end of storage. Whereas the initial steps of ATP degradation up to the formation of HxR are a result of endogenous enzymes and are mainly related to the loss of freshness, the degradation of HxR to uric acid is due to enzymes from spoilage microorganisms. Because of the rate-limiting step between xanthine and uric acid, HxR and Hx accumulate in the muscle (Venugopal 2002). Thus, the delayed Hx degradation in the EO-treated fish (higher Hx values than controls at 27 days of storage) suggests a delay in fish deterioration. *Post mortem* nucleotide degradation has also been shown to be delayed in fish sedated with the essential oil of *Aloysia triphylla* but this oil has delayed IMP degradation into HxR (Daniel et al. 2014).

The values of TVB-N underwent minimal changes during the storage (2.7–7.5 mg N%) and were not affected by the treatment with EO (data not shown, $p > 0.05$). TVB-N are nitrogenous compounds, such as ammonia and trimethylamine, which are formed primarily by the action of microorganisms and are indicators of the deterioration of refrigerated fish. In our study, although microbial counts increased over time (Fig. 4), the TVB-N values remained well below 30 mg N%, which is the maximum acceptable limit for human consumption in various countries, including Brazil (Daniel et al. 2014). In fact, TVB-N is widely used to assess the quality of marine fish, but its utility in freshwater fish has been disputed because these fish contain minimal amounts of trimethylamine oxide and urea, which form trimethylamine and ammonia by

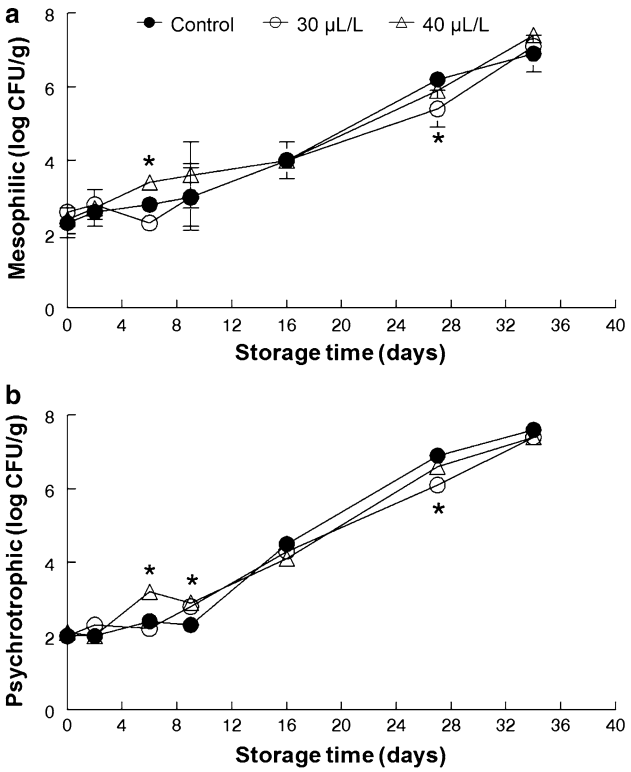

Fig. 4 Effect of in vivo exposure to different concentrations of the essential oil of *L. alba* on the mesophilic (**a**) and psychrotrophic (**b**) bacteria counts of silver catfish during storage in ice. Values are the mean ± standard error (n = 3). In **a** ANOVA revealed a significant effect of storage time and treatment but no interaction between these two variables on the mesophilic counts. In **b** ANOVA revealed a significant time × treatment interaction on the psychrotrophic counts. *Significantly different from controls at the same day of storage ($p < 0.05$). For clarity purposes only the statistical differences between control and treatments are indicated in the figure, whereas the differences observed in the same group along the storage are presented in the results section

microbial action (Scherer et al. 2006). Thus, the TVB-N value seems inappropriate for the evaluation of silver catfish spoilage, as previously demonstrated by Daniel et al. (2014).

Microbiological analysis

The initial quality of fish used in the study was good, as indicated by the low initial bacterial counts. For fresh fish, the microbiological limit for human consumption proposed by ICMSF (1986) is 10^7 CFU/g in aerobic plate count analysis. The mesophilic counts increased during ice storage with significant differences from the initial counts, observed from day 6 of storage onwards ($p < 0.05$; Fig. 4a). However, counts exceeded the limit only at day 34 of storage. The fish treated with 40 µL/L EO had a higher mesophilic count than controls at day 6 of storage, whereas the fish treated with 30 µL/L EO had a lower

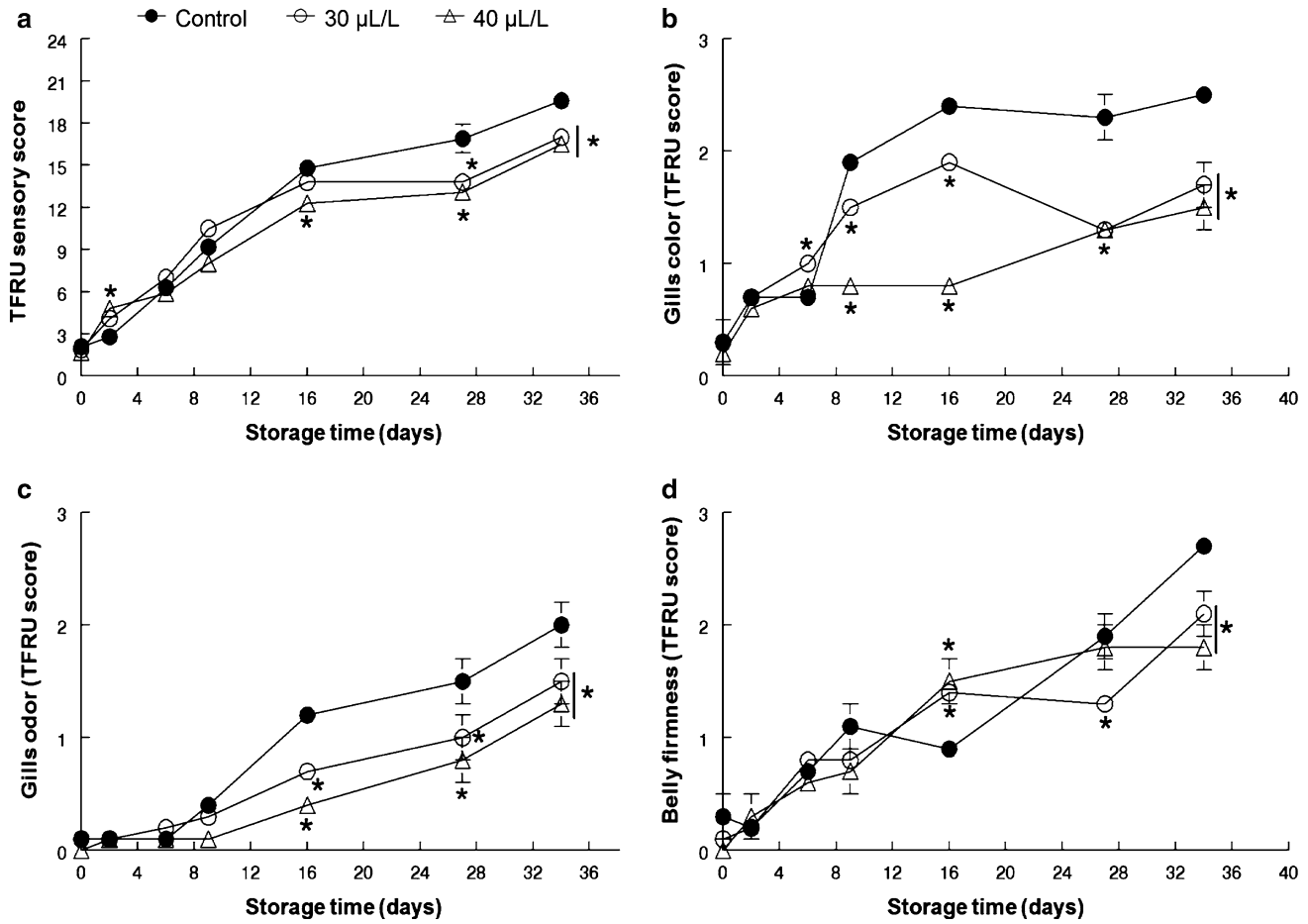

Fig. 5 Effect of in vivo exposure to different concentrations of the essential oil of *L. alba* on the sensory quality of silver catfish during storage in ice: TFRU sensory score (**a**), gills color (**b**), gills odor (**c**), and belly firmness (**d**). Scores are the demerit points obtained in the TFRU scheme; higher scores indicate poorer quality. Values are the mean ± standard error (n = 15–19). ANOVA revealed a significant time × treatment interaction on the sensory scores. *Significantly different from controls at the same day of storage ($p < 0.05$). For clarity purposes only the statistical differences between control and treatments are indicated in the figure, whereas the differences observed in the same group along the storage are presented in the results section

mesophilic count than controls at day 27 of storage ($p < 0.05$; Fig. 4a).

The psychrotrophic counts also increased during ice storage with significant differences from the initial counts observed after days 16, 9, and 6 of storage onwards for the control fish, 30 µL/L EO-treated fish, and 40 µL/L EO-treated fish, respectively ($p < 0.05$; Fig. 4b). Similar to the mesophilic counts, the psychrotrophic counts exceeded 10^7 CFU/g only after 34 days of storage. The fish treated with 40 µL/L EO had higher psychrotrophic counts than controls at day 6 and 9 of storage, whereas the fish treated with 30 µL/L EO had higher counts than control at day 9, but no difference was observed between these groups afterwards. In contrast, the fish treated with 30 µL/L EO had lower psychrotrophic counts than controls only at day 27 of storage (Fig. 4b).

Thus, the overall results of the mesophilic and psychrotrophic bacteria counts did not support the evidence for the antimicrobial activity of EO because the observed inhibitory effect was small and did not persist during storage. EO treated fish also showed higher bacterial counts than control at sporadic time points but this behavior occurred when counts were within the acceptable values for human consumption and therefore would not contribute to limit fish shelf life.

Sensory analysis

The demerit sensory score of silver catfish assessed by the modified TFRU scheme increased linearly during the storage in ice (Fig. 5a). Although the fish treated with 40 µL/L EO had a slightly higher demerit score than controls at 2 days of storage ($p < 0.05$), these fish had lower demerit score than controls from day 16 of storage onwards, whereas the fish treated with 30 µL/L EO had lower values than controls from 27 days onwards

($p < 0.05$, Fig. 5a). The panelists indicated that the control fish were unacceptable for consumption at day 27 of storage ($p < 0.05$, data not shown). Thus, the shelf life of control fish estimated by sensory evaluation was slightly shorter than the shelf life estimated by microbiological counts (32–34 days). In contrast, the fish exposed to EO were considered unacceptable only at day 34 of storage (data not shown), which is in good agreement with mesophilic counts, which exceeded the limit between days 32 and 34 of storage (Fig. 4). At the limit of acceptability, control fish had a sensory score of 16.9, similar to that observed for the EO-treated fish that had sensory scores of 17.0 and 16.5 (Fig. 5a). The demerit points of all parameters evaluated in the TFRU sensory assessment scheme changed during storage ($p < 0.05$, data not shown). Similar to recent data on silver catfish shelf life (Daniel et al. 2014), we found that some parameters, such as gills (color, mucus, and odor), eyes (clarity and iris), and belly firmness and color were good indicators of silver catfish freshness because they showed the greatest changes in demerit points during the storage. The treatment with EO significantly affected the gills color and odor and belly firmness (Fig. 5b–d), which were the major factors responsible for the lower TFRU demerit score of the EO-treated fish (Fig. 5a). Compared with control fish, the fish treated with EO had lower demerit points for gills color from day 9 of storage onwards and for gills odor from day 16 of storage onwards (Fig. 5b, c). The demerit points for belly firmness of the 30 μL/L EO-treated fish were lower than controls from day 27 of storage onwards, whereas those of the 40 μL/L EO-treated fish were lower than controls at day 34 of storage (Fig. 5d). According to the sensory evaluation, silver catfish freshness was indicated by fresh dark red gills, fresh fish odor, clear eyes with visible iris, and firm bright white belly, whereas poor quality was indicated by gills slightly brown color and spoiled smell, moderately gill mucus, cloudy to very cloudy eyes with no visible iris, and soft and grayish- to brownish-yellow belly.

The delay in the onset of *rigor mortis* was associated with higher pH after slaughter (day 0), which combined with the delay in muscle pH increase over time and in the peak of Hx, possibly contributed to the lowest demerit score of the EO-treated fish observed from day 16 onwards.

Although the microbiological analysis did not indicate an antimicrobial activity of EO, the biochemical and sensory analyses pointed to a consistent protective action of EO against fish deterioration. Thus, we cannot rule out that some specific bacteria responsible for fish deterioration could be inhibited in the EO-treated fish. In fact, although widely used, total bacteria counts were shown to be a poor measure of freshness quality because major deteriorating bacteria, such as hydrogen sulphide-producing bacteria, constitute only a small proportion of the total aerobic flora (Serio et al. 2014). In addition, EO has been shown to have antimicrobial activity in vitro (Oliveira et al. 2006).

Because *L. alba* leaves are eaten as a spice, its infusion is widely used as tea and in popular medicine, and toxic effects from EO were reported only after exposure to high doses (equal or greater than 1000 mg/kg in mice) (Olivero-Verbel et al. 2010), it is assumed to be safe for use as an anesthetic in fish for human consumption.

Conclusion

The overall results of this study indicate that the use of EO as sedative in water for silver catfish transport can delay the loss of freshness and the deterioration of whole fish during refrigerated storage. Thus, *L. alba* may be a promising source of natural active compounds for use in aquaculture and food industry.

Acknowledgements T.E. and B.B. are the recipients of National Council of Technological and Scientific Development (CNPq) research fellowships. A.P.L.V., B.K and A.Q. were the recipients of CNPq scholarships. The work was supported by Brazilian calls PRONEX (Research Support Foundation of the State of Rio Grande do Sul (FAPERGS)/CNPq) and Casadinho/PROCAD (The Ministry of Science Technology and Innovation/CNPq/The Ministry of Education/The *Coordination for the Improvement* of Higher Education Personnel). The authors are grateful to agronomist Carmo J. Heinzmann for cultivating the plant species and to Prof. C. A. Mallmann for GC-MS analysis.

Compliance with ethical standards

Conflict of interest Authors T. V. Parodi, B. M. Heinzmann and B. Baldisserotto obtained a patent for "A process for obtaining an anesthetic composition from *Lippia alba*, the obtained composition and its use as anesthetic" in Brazil (PI 1103966-3 A2).

References

Adams RP (2001) Identification of essential oil components by gas chromatography/quadrupole mass spectrometry. Allured Publishing Corporation, Carol Stream

AOAC (1996) Official methods of analysis of the association of official analytical chemists, 16th edn. Association of Official Analytical Chemists, Arlington

Barcellos LJG, Quevedo RM, Kreutz LC, Ritter F, Pandolfo A, Hemkemeier M, Colla L (2012) Comparative analysis of different fish polyculture systems. J World Aquac Soc 43:778–789

Barros FMC, Zambarda EO, Heinzmann BM, Mallmann CA (2009) Seasonal variability and terpenoid biosynthesis of the essential oil of *Lippia alba* (Mill.) N. E. Brown (Verbenaceae). Quím Nova 32:861–867

Becker AG, Parodi TV, Heldwein CG, Zeppenfeld CC, Heinzmann BM, Baldisserotto B (2012) Transportation of silver catfish, *Rhamdia quelen*, in water with eugenol and the essential oil of *Lippia alba*. Fish Physiol Biochem 38:789–796

Berka R (1986) The transport of live fish: a review. FAO Report. EIFAC Technical Paper 48. United Nations, Rome

Binsi PK, Viji P, Visnuvinayagam S, Ninan G, Sangeeta G, Triveni A, Ravishankar CN (2015) Microbiological and shelf life characteristics of eviscerated and vacuum packed freshwater catfish (*Ompok pabda*) during chill storage. J Food Sci Technol 52:1424–1433

Bito M, Yamada K, Mikumo Y, Amano K (1983) Studies on rigor mortis of fish. Bull Tokai Reg Fish Res Lab 109:89–96

Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Physiol Pharmacol 37:911–917

Branch AC, Vail AMA (1985) Bringing fish inspection into computer age. Food Aust 37:352–355

da Cunha MA, Zeppenfeld CC, Garcia LO, Loro VL, da Fonseca MB, Emanuelli T, Veeck APL, Copatti CE, Baldisserotto B (2010a) Anesthesia of silver catfish with eugenol: time of induction, cortisol response and sensory analysis of fillet. Cienc Rural 40:2107–2114

da Cunha MA, de Barros FMC, Garcia LO, Veeck APL, Heinzmann BM, Loro VL, Emanuelli T, Baldisserotto B (2010b) Essential oil of *Lippia alba*: a new anesthetic for silver catfish, *Rhamdia quelen*. Aquaculture 306:403–406

Daniel AP, Veeck APL, Klein B, Ferreira LF, Cunha MA, Parodi TV, Zeppenfeld CC, Schmidt D, Caron BO, Heinzmann BM, Baldisserotto B, Emanuelli T (2014) Using the essential oil of *Aloysia triphylla* (L'Her.) Britton to sedate silver catfish (*Rhamdia quelen*) during transport improved the chemical and sensory qualities of fish during storage in ice. J Food Sci 79:S1205–S1211

Downes FP, Ito K (2001) Compendium of methods for the microbiological examination of foods, 4th edn. American Public Health Association, Washington, D.C

European Pharmacopoeia (2007) European Directorate for the Quality of Medicines, 6th edn. Council of Europe, Strassbourg

Furuichi Y, Taniguchi J, Murabayashi J (1997) A rapid and convenient method for the determination of amide nitrogen in food proteins. Nippon Nogeikagaku Kaishi 71:395–401

Gomes LC, Golombieski JI, Gomes ARC, Baldisserotto B (2000) Biology of *Rhamdia quelen* (Teleostei, Pimelodidae). Cienc Rural 30:179–185

Hennebelle T, Sahpaz S, Gressier B, Joseph H, Bailleul F (2008) Antioxidant and neurosedative properties of polyphenols and iridoids from *Lippia alba*. Phytother Res 22:256–258

Howgate PA (2006) Review of the kinetics of degradation of inosine monophosphate in some species of fish during chilled storage. Int J Food Sci Technol 41:341–353

ICMSF (International Commission on Microbiological Specifications for Foods) (1986) Microorganisms in foods. 2. Sampling for microbiological analysis: Principles and specific applications, 2nd edn. Blackwell Scientific Publications, London

Javahery S, Moradlu AH (2012) AQUI-S, a new anesthetic for use in fish propagation. Glob Vet 9:205–210

Meyer G, Fracalossi DM (2004) Protein requirement of jundia fingerlings, *Rhamdia quelen*, at two dietary energy concentrations. Aquaculture 240:331–343

Nathanailides C, Panopoulos S, Kakali F, Karipoglou C, Lenas D (2011) Antemortem and postmortem biochemistry, drip loss and lipid oxidation of European sea bass muscle tissue. Procedia Food Sci 1:1099–1104

Ocaño-Higuera VM, Marquez-Ríos E, Canizales-Dávila M, Castillo-Yáñez FJ, Pacheco-Aguilar R, Lugo-Sánchez ME, García-Orozco KD, Graciano-Verdugo AZ (2009) Postmortem changes in cazon fish muscle stored on ice. Food Chem 116:933–938

Ocaño-Higuera VM, Maeda-Martínez AN, Marquez-Ríos E, Canizales-Rodríguez DF, Castillo-Yáñez FJ, Ruíz-Bustos E, Graciano-Verdugo AZ, Plascencia-Jatomea M (2011) Freshness assessment of ray fish stored in ice by biochemical, chemical and physical methods. Food Chem 125:49–54

Oliveira DR, Leitão GG, Santos SS, Bizzo HR, Lopes D, Alviano CS, Alviano DS, Leitão SG (2006) Ethnopharmacological study of two *Lippia* species from Oriximiná, Brazil. J Ethnopharmacol 108:103–108

Olivero-Verbel J, Guerrero-Castilla A, Stashenko E (2010) Toxicity of the essential oil of the cytral chemotype of *Lippia alba* (Mill.) N. E. Brown. Acta Toxicol Argent 18:21–27

Özoğul F, Özoğul Y, Kuley E (2007) Nucleotide degradation in sardine (*Sardina pilchardus*) stored in different storage condition at 4°C. J Fishscicom 1:13–19

Popovic NT, Strunjak-Perovic I, Coz-Rakovac R, Barisic J, Jadan M, Persin AB, Klobucar RS (2012) Tricaine methane-sulfonate (MS-222) application in fish anaesthesia. J Appl Ichthyol 28:553–564

Ribas L, Flos R, Reig L, MacKenzie S, Barton BA, Tort L (2007) Comparison of methods for anaesthetizing Senegal sole (*Solea senegalensis*) before slaughter: stress responses and final product quality. Aquaculture 269:250–258

Ryder JM (1985) Determination of adenosine triphosphate and its breakdown products in fish products by high performance liquid chromatography. J Agric Food Chem 33:678–680

Scherer R, Augusti PR, Steffens C, Bochi VC, Heckttheuer LH, Lazzari R, Radünz-Neto J, Pomblum SCG, Emanuelli T (2005) Effect of slaughter method on postmortem changes of grass carp (*Ctenopharyngodon idella*) stored in ice. J Food Sci 70:348–353

Scherer R, Augusti PR, Bochi VC, Steffens C, Fries LLM, Daniel AP, Kubota EH, Radünz-Neto J, Emanuelli T (2006) Chemical and microbiological quality of grass carp (*Ctenopharyngodon idella*) slaughtered by different methods. Food Chem 99:136–142

Serio A, Fusella GC, López CC, Sacchetti G, Paparella A (2014) A survey on bacteria isolated as hydrogen sulfide-producers from marine fish. Food Control 39:111–118

Vallé M, Malle P, Bouquelet S (1998) Evaluation of fish decomposition by liquid chromatographic assay of ATP degradation products. J AOAC Int 81:571–575

Veeck APL, Klein B, Ferreira LF, Becker AG, Heldwein CG, Heinzmann BM, Baldisserotto B, Emanuelli T (2013) Lipid stability during the frozen storage of fillets from silver catfish exposed in vivo to the essential oil of *Lippia alba* (Mill.) N.E. Brown. J Sci Food Agric 93:955–960

Venugopal V (2002) Biosensors in fish production and quality control. Biosens Bioelectron 17:147–157

Zahl IH, Samuelsen O, Kiessling A (2012) Anesthesia of farmed fish: implications for welfare. Fish Physiol Biochem 38:201–218