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Red beet and betaine as ingredients in rainbow trout (*Oncorhynchus mykiss*) diets: effects on growth metrics, nutrient retention and flesh quality.

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Red beet and betaine as ingredients in rainbow trout (*Oncorhynchus mykiss*) diets: effects on growth metrics, nutrient retention and flesh quality.

A control diet was compared to 4 experimental diets in which two red beet (14 and 28%) and betaine (0.9 and 1.63%) levels were incorporated on rainbow trout diets. The study was set up with an average weight of 69 ± 2.21 g and finished when fish reached commercial weight (175 to 250 g) after 105 days. The impact of the diets was studied based on the growth performance, biometric indexes, proximal composition, protein and fat retention efficiencies and apparent digestibility of fish reared on a recirculation system. Moreover, it was studied the effect of red beet and betaine on the flesh proximate composition and quality (water activity, colour, texture, TBARS and sensory characteristics) of the final product. Results showed that inclusions of 14% of red beet and 0.9% of betaine did not produce an effect on growth, nutritive or biometric parameters, nor nutrient retentions compared to control diet, however, higher concentrations had a negative effect on growth and nutritive parameters. These ingredients enhanced quality parameters regardless of the concentration used. Fish flesh enriched with the new ingredients showed lower water activity and better textural and colour properties than control diet and also had a dose-dependent effect on lipid oxidation.

Keywords: red beet; betaine; growth; rainbow trout; diet; fish product; quality; sensory scores.

1: Introduction

Carnivorous fish species, including salmonids, the incorporation of digestible carbohydrates (CHO) should not exceed 20% of the diet. Cereals (wheat, barley, oat, corn) have been traditionally the most utilized CHO sources in commercial salmonid diets (Sealey et al. 2008, Gaylord et al. 2009, Pinedo-Gil et al. 2016). However, those ingredients generally contain high fibre and starch content and these, together with the presence of some antinutritional components, produce limitations to the inclusion of plant ingredients on carnivorous fish diets (Oliva-Teles et al. 2015). Also, some CHO

sources produce a reduction of feed palatability, which leads to reduce fish intake and growth (Lim et al., 2016). On the other hand, plant ingredients can be an important source of antioxidant and other bioactive components (Ganessan et al. 2011). Red beet (*Beta vulgaris* L.) is a source rich in natural betaine and also rich in important nutrients including magnesium, sodium, potassium, vitamin C and betalains (Han et al. 2014). In aquaculture, betaine is widely used as a common additive due to its bioactive properties as osmoprotector and enhancing feed palatability. Its incorporation could also enhance the quality of the final product, especially on the colour of fish flesh. However, to the best of our knowledge, the use of red beet as a source of betaine in fish nutrition has been scarce studied. For this reasons, natural sources, such as red beet, as an alternative CHO ingredient in fish diets should be taken into account from a health concern point of view and its effect on the quality parameters of rainbow trout flesh. The objective of this work was to evaluate the impact of red beet and betaine incorporation at different concentrations on a controlled population of rainbow trout diets on their growth performance and final fish flesh quality parameters.

2: Material and Methods

2.1: Diets

Five extruded isoproteic (40% crude protein (CP) and isolipidic diets (18% crude lipid (CL) diets were formulated. A control diet was compared to four experimental diets using two red beet (14 and 28%) and betaine (0.9 and 1.63%) levels. Betaine was of natural origin obtained from red beet betaine. Both ingredients were combined in a factorial design. The five diets were identified as: Control diet (0% red beet; 0% betaine), diet A (14% red beet; 0.9% betaine), diet B (14% red beet; 1.63% betaine), diet C (28% red beet; 0.9% betaine) and diet D (28% red beet; 1.63% betaine). The

formulation and the composition of the diets are given in Table 1. Control diet was prepared using same ingredients as experimental diets but without red beet and betaine on the formulation. The control diet was not a commercial diet, was produced in the same conditions than modified diets. There were five feeding treatment groups, each in three replicates (n=3).

- TABLE 1 -

Diets were prepared using the cooking extrusion process with a semi-industrial twin-screw extruder (CLEXTRAL BC-45. St. Etienne, France). Processing conditions were the following: a screw speed at 0.63 x g, a temperature of 110 ºC and a pressure of 40-50 atm. Experimental diets were assayed in triplicate groups (n=3). Fish were fed by hand twice a day (8:00 am and 15:30 pm) until apparent satiation, six days per week during the whole experimental period. Pellets were distributed slowly to allow all fish to eat. The uneaten diet was collected and dried to determine feed intake (FI).

2.2: Rearing markers

2.2.1: Growth trial and fish sampling

A total of 900 rainbow trouts were provided by a local fish farm (Cien Fuentes Fishfarm, 19420 Cifuentes, Guadalajara, Spain) and transported alive to the Aquaculture Research Centre of the Agro-Technological Institute of Castilla y León, Spain. Prior to the feeding trial, all fish were acclimated to the indoor rearing conditions for two weeks and fish were fed once a day (8:00 am) up to apparent satiation using exclusively the control diet. Groups of 60 fish (average initial weight of 69 ± 2.21 g (mean ± SD)) were housed in 15 cylindrical fiberglass tanks (three per treatment, n=3). The capacity of each tank was 500 L (initial stocking density 8.4 ± 0.5 kg/m^3).

The trial was conducted in a recirculating freshwater system (RAS). Water temperature was 14.67 ± 0.57 ºC (mean ± SD). Level of dissolved oxygen in water was 7.97 ± 0.87 mg/l. All tanks were equipped with aeration and an oxygen probe. Water pH was 7.93 ± 0.12 and ammonia and nitrites concentration in water were 0.16 ± 0.14 and 0.19 ± 0.17 mg/l respectively. Water flow was 10.30 ± 0.98 l/h. The photoperiod consisted on 12 h of light and 12 h of dark intervals, having all tanks identical lightning conditions.

Fish were weighed and length measured at approximately 35-day intervals to study all rearing parameters (growth, final weight, biomass increment (BI), survival, thermal growth coefficient (TGC), specific growth rate (SGR) and nutritional parameters, FI and feed conversion ratio (FCR). Prior to weighing, all fish were starved for 24 h and anesthetized with MS222®; 200 mg/l. At the end of the growth trial, all fish were individually weighed and measured. Three fish were randomly sampled from each tank (n=3) and used for the determination of biometric indexes (condition factor (CF), viscerosomatic index (VSI) and heptosomatic index (HIS) and final whole fish proximate composition. The duration of the trial was 105 days.

2.2.1.1: Calculations of rearing markers.

Different indexes were evaluated in order to assess rearing parameters.

BI was evaluated as an indicator of fish biomass increment from day one to day 105 (1).

$$BI\ [g] = Bf - Bi \quad (1)$$

Where Bi and Bf are the initial and final biomasses of fish at the beginning and end of the feeding trial, respectively [g].

To determine the impact of stress response to the fish survival, mortality was registered during the whole experimental period. Knowing the initial number of fish and

dead fish allowed calculating **mortality** (2) and once determined, **survival** was calculated as follows (3):

$$\text{Mortality [\%]} = (\text{Number of fish died} / \text{Initial fish number}) \cdot 100 \quad (2)$$

$$\text{Survival [\%]} = 100 - \text{Mortality} \quad (3)$$

An accurate prediction of growth potential for fish under husbandry conditions is a prerequisite to estimate energy or feed requirements. The most commonly used formula is the **SGR**, which is based on the natural logarithm of body weight (4), but also **TGC** was calculated (5)

$$SGR = 100 \cdot ((\ln Wf - \ln Wi) / t) \quad (4)$$

$$TGC = (Wf^{(1/3)} - Wi^{(1/3)}) / [days \cdot \Sigma (T - 4)] \quad (5)$$

Where Wi and Wf are the initial and final body weights of fish at the beginning and end of the feeding trial, respectively [g], t is the experimental duration [d] and T is the temperature in ºC.

FCR measures animal efficiency in converting nutriment into muscle or weight gained overtime (6).

$$FCR = (F / (Bf - Bi)) \quad (6)$$

Where Bi and Bf are the initial and final biomasses of fish at the beginning and end of the feeding trial, respectively [g] and F is the weight of feed supplied to fish in the feeding trial.

In order to avoid an excessive amount of feed given, **FI** [g per 100 g fish and day] was calculated (7). Protein is the main nutrient in fish diets and to evaluate the weight gained per unit of protein fed protein efficiency ration (**PER)** was determined as shown in (8).

$$FI = 100 \cdot (\text{Feed consumption [g]} / (\text{average biomass} \cdot t)) \quad (7)$$

Where t is the experimental duration [d].

PER = wet weight gain / protein intake (8)

Calculated biometric indexes were the **CF** based on the weight-length data to evaluate fish population fitness (9); and **HSI** (10) and **VSI** (11) were used to evaluate the nutritional status.

$$CF = 100 \cdot (Wf / L^3) \quad (9)$$

Where Wf is the final body weight of fish at the end of the feeding trial [g] and L is the average body length of fish [cm].

$$HSI = 100 \cdot (\text{wet weight of the liver} / Wf) \quad (10)$$

Where Wf is the final body weight of fish at the end of the feeding trial [g].

$$VSI = 100 \cdot (\text{wet visceral weight} / Wf) \quad (11)$$

Where Wf is the final body weight of fish at the end of the feeding trial [g].

2.2.2: Apparent digestibility coefficients (ADCs)

Digestibility studies were conducted simultaneously to the feeding trial. After fish were fed for a second time, tanks were completely cleaned and faeces were collected in a settling column (Cho et al. 1982), which was emptied in the following morning at 8:00 h. Wet faecal content was then collected and dried at 60 ºC for 48 h prior to analysis (CP, CL, and ash-insoluble ashes (AIA). Over the whole experimental period, samples of faeces were collected from each tank (n=3).

The ADCs of protein, fat and carbohydrates in the diets tested were calculated according to the following formula (12):

$$ADC\ [\%] = 100 \cdot [100 - ((\text{marker in diet} / \text{marker in faeces}) \cdot (\text{PN in faeces} / \text{PN in diet})] \quad (12)$$

Where PN is the percentage of nutrient.

2.3: Proximate composition analysis

Compositional analyses were performed to the raw material (red beet), the ingredient (betaine), the diet, the fish and faeces obtained during the assay, and the final fish product (flesh). These analyses were performed in accordance with AOAC (1990) procedures: Dry matter (60 ºC to constant weight), ash (incinerated at 550 ºC to constant weight), crude protein (N · 6.25 and nitrogen was analysed by Dumas principle, TruSpec CN; Leco Corporation, St. Joseph, MI, USA) and crude lipid content using the Soxhlet extraction method. AIA was used as an indicator for the ADC, and was analyzed according to the method described by Atkinson et al. (1984) with some modifications. Briefly, 5 g of sample were ashed for 5 h at 550 ºC to ensure complete combustion of the organic material in the sample. The resulting ash was boiled until dryness in 75 ml of HCl (2 N) and boiled in other 75 ml HCl for 15 min. Samples were filtered hot through ashless filter paper and washed in boiling distilled water until the samples were neutralized. Finally, following Atkinson et al. (1984) method, samples were ashed for 5 h at 550 ºC. Betaine content on diets, faeces and fish flesh were analysed. Briefly, betaine and esters were extracted from the sample in a mixture of methanol and water. For total betaine determination, a part of the extract was saponified with a 2 M KOH solution, hydrolysing the betaine ester to free the betaine, which is then quantified. The extract was further diluted and analysed on LC/MS ESI + ionization in which the quantification was based on the known isotopic marker internal standard. The betaine content was expressed as mg/kg.

2.4: Quality markers of fish flesh and fish sampling

Every 35-d intervals three fish per tank (n=3) were randomly taken for the determination of quality parameters (water activity, colour, texture and sensory

188 analysis) until fish reached commercial weight (times of sampling: 0, 35, 70 and 105 d).

189 *2.4.1: Water activity (a_w)*

190 A_w was measured using an Aqualab 4TE (Decagon Devices inc., Pullman, WA, USA).
191 Six measurements were carried out in each flesh at three different locations (front,
192 central and tail). The study was evaluated in three independent fish flesh (n=3).

193 *2.4.2: Colour*

194 The colour was measured using a colorimeter (Minolta CM-2002, Osaka, Japan) for the
195 evaluation of CIELAB parameters. The L* value represents lightness and +a*, -a* and
196 b* values represent redness, greenness and yellowness, respectively. Six measurements
197 were taken directly over the muscle, randomly over skinless fish flesh. The study was
198 evaluated in three independent fish flesh (n=3). Hue (13) and Chroma (14) were
199 calculated using the following formulas for all experimental points:

200 $$\text{Hue} = \arctan(b^* / a^*) \quad (13)$$

201 $$\text{Chroma} = (a^{*2} + b^{*2})^{1/2} \quad (14)$$

202 *2.4.3: Texture analysis*

203 Texture was determined using a texture analyzer TA-XT2i (ANAME, Stable Micro
204 System, Vienna Court, Lammas Road, Godalming, Surrey, UK). A texture profile
205 analysis (TPA) was carried out using a penetration probe of 4 mm of diameter at speed
206 of 1 mm/s with a 5 mm distance; the instrument was equipped with a 25 kg load cell.
207 The time delay between cycles was 5 s. Previous to analysis, samples were peeled
208 manually and texture was analysed in the front, middle and tail parts. Fish flesh was
209 evaluated in the same position, with the muscle fibres perpendicular to the test probe.

The study was evaluated by triplicate in three independent samples of fish flesh per treatment (n=3).

TPA curves were used to evaluate the **hardness** [g] (maximum force required to compress the sample), **cohesiveness** (capacity of the sample to deform before rupture (A2 / A1, where A1 is the total energy required for the first compression and A2 is the total energy required for the second compression)), **elasticity** [mm] (capacity of the sample to recover its original shape after deformation force ends) and **gumminess** [g] (strength to disintegrate a sample to a constant state of swallowing (hardness × cohesiveness)).

2.4.4: Thiobarbituric Acid Reactive Substances (TBARS)

TBARS as an indicator of lipid oxidation was evaluated using the methodology described by Vyncke (1975). Briefly, ten grams of samples were mixed with 30 ml of 7.5% TCA. The mixture was homogenized and centrifuged for 5 min at 4 ºC and 5570 x g, and then filtered with Whatman nº 1 filters (Prat Dumas, France). Five ml of the filtrate were mixed with 5 ml 0.02 M TBA, incubated at 90ºC in a water bath during 40 min; the reaction was measured at 530 nm (Fluostar® Omega, BMG labtech, Germany). Two fish were analysed per treatment during the entire experiment (n=6) and the results were expressed as µmol malonaldehide (MDA) per kg of fresh flesh produced.

2.4.5: Sensory analysis

All sensory analysis were performed according to ISO standards (ISO 2001, 2008) in a sensory room compliant with ISO 2007 by a panel of eight people (four male and four female aged between 25 and 50) with previous experience in sensory analysis of food products. Nonetheless, in order to train the panel with the sensory assessment of fish products and optimise the tables used for sensory evaluation, the panel were trained in

the main characteristics necessary for the study.

Sensory analysis comprised fresh whole fish and fish meat samples. Whole fish was evaluated using the quality index method (QIM) and fish flesh was analysed using a quality descriptive method (QDM). Panellists were trained to perform both analyses. QIM was assessed following the guideline of QIM Eurofish (Martinsdóttir et al., 2001). Freshness was evaluated by giving demerit points according to certain aspects associated with general appearance such as skin, stiffness, odour, gill pots colour and odour, belly, and eyes brightness and shape. The trained judges scored ranked from 0-3 for each attribute. The maximum score of 3 corresponded to the fish with the worst quality parameters values.

For the QDM, panellists were trained to discriminate colour, texture, odour and acceptability of fish flesh. A continuous non-structured scale (1-10) was used for evaluation. The left side of the scale corresponded to the lowest intensity (value 1: white, soft, fresh odour and acceptable sample) whereas the right side corresponded to the highest intensity (value 10: dark, hard, rancid odour and non-acceptable sample).

Panellists evaluated one fish per treatment every 28 d during the experiment (n=2). Five samples, in pairs of whole fish and flesh of each treatment, were individually presented in porcelain dishes to each panellist. Samples were coded with random numbers and maintained at room temperature (RT) during evaluation.

2.5: Statistical analysis

The feeding trial was designed according to a factorial design with two red beet levels and two betaine levels. All data (rearing and quality parameters) were subjected to one-way ANOVA to determine the significance due to effects of dietary treatments, and two-way ANOVA to determine the significance due to levels of red beet, betaine or their interaction. Post Hoc was analysed by Tukey´s HSD test with statistical

259 significance determined at $p > 0.05$. All statistical analysis were carried out using
260 software SAS (SAS version 9, SAS Institute Inc., Cary, North Carolina, USA).

261 ***2.6: Ethical statement***

262 The rainbow trout study complied with the European Union Council Directive
263 2010/63/UE, which provides the minimum standards for animal protection, and was
264 also in accordance with the Spanish national legislation (Spanish Royal Decree
265 53/2013) based on animal protection in experimentation and other scientific practices
266 and approved by the Animal Ethics Committee of Agro-Technological Institute of
267 Castilla y León (Spain).

268 Fish in tanks were checked on a daily basis. Every four weeks, fish were
269 weighed individually and their health status was assessed by observation, after sedation
270 with MS222 dissolved in water (MS222®; 200 mg/l) to minimize animal suffering.

271 Animals were euthanized by excess of MS222 (300 mg/l) or with ice (when
272 quality samples were taken) and then fish were dissected.

273 **3: Results**

274 ***3.1: Diets***

275 Table 1 shows the proximate composition of the different experimental diets. Diets were
276 fish standard formulas in which the percentage of wheat was replaced by red beet. The
277 whole-wheat portion substituted the highest red beet concentration; the other
278 compounds were not modified.

3.2: Rearing markers

3.2.1: Growth performance, biometric parameters, body composition and nutrient retention efficiencies

The experiment started with an initial average fish weight of 69 ± 2.21 g (mean ± SD) and finished when fish reached commercial weight (175.27-250.72 g). Growth performance of rainbow trout fed with experimental diets is shown in Table 2. Results show that, at the end of 105 d, fish fed with red beet (RB1 and RB2) and betaine (B1 and B2) had a significant decrease ($p < 0.05$) on Wf and also on the SGR and TGC compared to control diets. When the interaction effect was studied, diet A did not show significant differences on growth performance in terms of Wf, SGR and TGC ($p > 0.05$) with the control group, whereas diet C significantly reduced ($p < 0.05$) those parameters compared to the other treatments. No significant differences were observed on FI. Besides, compared to control diet showed a significant decrease on PER and changes in the FCR were only affected by the inclusion of red beet, following an opposite tendency from PER. Fish fed with diet A did not show significant differences with control diet, while diet C showed the worst values from a productive point of view for PER and FCR.

- TABLE 2 -

CF, VSI and HSI were significantly affected by red beet (RB1 and RB2) and betaine (B1 and B2) concentration (Table 3). CF decreased significantly ($p < 0.05$) with the inclusion of both ingredients. On the contrary, the interactive effect (experimental diets) showed that fish fed with control and D diets had significantly higher CF values than the other dietary treatments. VSI increased significantly ($p < 0.05$) with the inclusion of red beet (RB1 and RB2) and betaine (B1 and B2). Increasing levels of red beet and betaine on the diet increased significantly ($p < 0.05$) VSI. Fish fed with diet D

did not show significant differences with control. On the other hand, HSI increased significantly with the inclusion of red beet, although this increase was only observed on fish fed with diet B1 and not in diets with higher betaine concentrations. The same effect was observed analysing the interactive effect (experimental diets).

In the present study, whole body composition was not significantly affected by the diet (Table 3).

Feed retention efficiencies are shown in Table 3. A significant decrease ($p < 0.05$) on the protein retention efficiency (PIR , % digested) was observed with increasing levels of red beet (RB1 and RB2) and betaine (B1 and B2) on the diet. Fat retention efficiency (FIR, % intake and % digested) was not significantly affected by the inclusion of red beet and betaine individually, only an insignificant tendency of decreasing the values was observed. Compared to control diet, when the interaction was studied, it was observed a significant decrease ($p < 0.05$) on PIR and FIR (%intake and %digested) with increasing red beet and betaine concentrations on the diet.

- TABLE 3 -

3.2.2: Apparent digestibility coefficients (ADC)

The red beet and betaine concentration did not have any significant effect on the ADCprotein and ADCCHO. However, ADClipid was significantly affected by red beet concentration (RB1 and RB2). Increasing red beet levels on diets produced a decrease on ADClipid finding values ranging from 87.64% in RB2 diets to 92.36 % in control diets (Table 4).

- TABLE 4 -

3.3: Fish flesh proximate composition

Results showed that red beet (RB1 and RB2) and betaine (B1 and B2) incorporated on

diets did not affect water and protein content of fish flesh. However, fat and ash contents were significantly affected by the diets (Table 5). Fat content was significantly affected by red beet (RB1 and RB2) and by the experimental diets. The increase of red beet levels decreased significantly ($p < 0.05$) the content of fat in fish flesh, while, the incorporation of betaine produce a significant increment. The combination of both ingredients produced a decrease on fat content with increasing levels of red beet and betaine, showing the highest fat content in fish fed with diet A (6.36%). Ash content decreased significantly ($p < 0.05$) with increasing levels of red beet and betaine.

- TABLE 5 -

Regarding the betaine content in fish flesh, results showed that fish fed with diets containing higher betaine concentration (B and D) presented higher values of betaine on flesh than those with lower concentration or control (Figure 1).

- FIGURE 1 -

3.4: Fish flesh quality markers

3.4.1: Water activity (a_w)

Figure 2 shows the a_w of fish fed with different experimental diets. The inclusion of the ingredients individually and collectively produced a significant decrease on the a_w of fish flesh compared to control diet.

- FIGURE 2 -

3.4.2: Colour

The inclusion of red beet and betaine on diets was studied for CIELAB parameters. The study showed L* modification by the ingredients but those differences were attributed to fish variability of the product rather than a diet effect. As it was expected, fish flesh

from fish fed with diets with the highest red beet and betaine concentration (D) showed higher redness values than samples from fish fed with lower red beet and betaine concentration and control (Figure 3). B*, hue and chroma values did not show significant effects between diets.

- FIGURE 3 -

3.4.3: Texture

Red beet and betaine concentration did not have a significant effect on textural parameters. Elasticity was the only parameter affected by the diets (Figure 4). Compared to control diet, a significant lower elasticity was observed in flesh from the fish that were fed with lower betaine concentrations (diets A and C).

- FIGURE 4 -

3.4.4: Thiobarbituric Acid Reactive Substances (TBARS)

At the end of the experimental growth period, fish fed with control diets and the highest red beet and betaine concentrations (separately or together) had similar TBARS values (Figure 5), although the differences were not significant. It was observed a decrease when red beet and or betaine were included on the diet.

- FIGURE 5 -

3.4.5: Sensory analysis

QIM was used for evaluating the sensory analysis of the whole fish. In all the parameters studied, at the end of the experimental growth period, only significant differences were found on odour and gills colour. Fish fed with the highest red beet and betaine concentration (D diets) showed higher rancid odour than the fish from the other experimental diets (data not shown). Fish fed with control and D diets had similar

values on gills colour, with the characteristic red colour, while fish fed with B and C diets presented pale gills (data not shown).

On the other hand, QDM was evaluated in fish flesh. Only significant differences were observed on meat colour. The study showed an effect on colour modification by the ingredients, but those differences found were rather due to fish variability than a diet effect (data not shown).

4: Discussion

The inclusion of 14% of red beet and 0.9% of betaine did not affect growth, nutritive or biometric parameters, nor nutrient retentions compared to control, while higher red beet and betaine concentrations had a negative effect on growth and nutritive parameters. At the end of the experimental assay, the level of red beet and betaine separately, produced a significant decrease on Wf, SGR AND TGC, whereas fish fed with diet with 28% red beet and 0.9% betaine significantly reduced those parameters compared to the other dietary treatments. Betaine has been reported as a feeding stimulant to fish, inducing an increase of FI, and consequently, improving growth rate (Normandes et al. 2006, Tiril et al. 2008). However, in this study, the inclusion of betaine on rainbow trout diets did not show significant differences on FI and did not improve rainbow trout growth. Similar results were reported with other fish species such as Atlantic salmon (Duston 1993), sea bass and sea bream (García-Alcázar et al. 1994) or piauçu (Normandes et al. 2006) when they were fed with betaine on their diets. Additionally, compared to control diet, there was a significant decrease on PER and changes in FCR were only affected by the inclusion of red beet, following an opposite tendency from PER. These results may be due, in part, to the influence of some antinutritional components in red beet such as tannins or oxalates that reduced the growth and could lead to a poor FCR and PER (Shyamala and Jamuna 2010, Lawal et al. 2012, Focken et al. 2015, Nyonge 2015).

399 However, for lower red beet and betaine concentrations, it seems to appear a positive
400 interaction, presenting no differences with control diet.

401 ADCs obtained on the present study indicated an adequate quality and efficiency
402 for the different experimental diets. Digestibility values in carnivorous fish normally
403 range 75-95% for protein and 85-95% for lipid (NRC, 1993); the obtained values were
404 between those ranges (84.42-89.21% for protein and 87.53-92.49% for lipid). Red beet
405 and betaine concentrations (separately and together) did not have significant effects on
406 ADCprotein and ADCCHO. However, ADClipid was significantly modified by the red
407 beet concentration: the inclusion of red beet on rainbow trout diets significantly
408 decreased ADClipid. This decrease might be associated to the modification on the lipid
409 and/or carbohydrate metabolism pathways; also could be attributed to the presence of
410 oxalate and its ability to bind minerals in the intestine, reducing the digestibility of fat
411 (Francis et al. 2001). Also, this effect could be related to the higher VSI and HSI found
412 on those diets higher in red beet. It seems that the inclusion of red beet and betaine on
413 rainbow trout diets increase visceral adipose tissue mass and decrease growth, as it has
414 been observed on the growth performance parameters. Similar results were reported in
415 other studies with other carbohydrate sources and fish species (Tan et al. 2006, Wu et
416 al. 2007, Cui et al. 2010). These authors indicated that CHO not absorbed, those not
417 used as an energy source, can be accumulated in the liver and transformed into lipids
418 and glycogen which lead on a higher HSI. More studies should be carried out to clarify
419 if the negative effects on HSI and VSI are attributable to the synthesis of lipids from the
420 structure of polysaccharides in red beet.

421 Whole body proximal composition was not significantly affected by the CHO
422 source, which its in accordance with previous studies on sea bass (Enes et al. 2006),
423 white sturgeon and hybrid tilapia (Lin et al. 1997) and for rainbow trout (Tekinay and

Davies 2001). However, other authors have reported a significant effect of the CHO source on the whole body proximal composition (Tan et al. 2006, Wu et al. 2007).

The inclusion of red beet and betaine on rainbow trout diets produced a significant decrease on PIR (% digested). These results obtained were in agreement with PER values, but were not in accordance with ADCprotein, in which there were no significant differences between diets. Compared to control diet, PIR and FIR (% intake and digested) significantly decreased with higher red beet and betaine concentrations. A low PIR and PER are explained by an inappropriate protein metabolism into muscle. This effect can be associated to several reasons, one of them is because of an incorrect CHO and lipid metabolism, which produces an accumulation of lipids on visceral pack and liver, while the protein is used as an energy source (Hemre et al. 2002, Cui et al. 2010, Kamalan et al. 2012).

As it was expected, the inclusion of red beet and betaine in fish diets, increased betaine concentration in fish flesh compared to control diets. This is important from a bioactivity point of view of the product. Due to the high residual levels found on flesh from fish fed with red beet and betaine, the authors of the present study considered interesting to investigate the antioxidant properties that betaine can provide to the final product.

With regard to red beet and betaine effects on flesh quality, it has been observed that the inclusion of these ingredients produced a reduction of a_w compared to control diet. a_w plays an important role on spoilage of fish (Ježek and Buchtová 2014). This is in agreement to the observed with the inclusion of other CHO sources, such as barley (Pinedo et al. 2016). The reduction on a_w values would help to reduce lipid oxidation and microbial growth, with advantages in shelflife.

When fish flesh colour was determined instrumentally, significant differences were observed on a* values, regarding the diet. As it was expected, redness (a* values) of fish flesh, increased significantly with the inclusion of red beet and betaine, and fish fed with diets with 28% red beet and 1.63% betaine showed the reddest meat. The increase of redness at higher red beet and betaine concentrations can be associated to betaine pigment and betalains content (Stintzing et al. 2002, Zhong et al. 2005). Flesh from fish fed with this diet also presented the highest flesh betaine content, which could explain the increase of redness. These results were not consistent with the observations of panellist on the QDM analysis that were not able to perceive a flesh colour change.

Lipid oxidation was evaluated as one of the most important indicators of quality. TBARS values did not show significant differences between flesh from fish fed with control diet and fish fed with red beet and betaine. However, although no significant differences were observed, the inclusion of both ingredients seems to reduce TBARS values (dose-dependent effect).

Experimental diets did not have a significant effect on acceptability of fish flesh, but, surprisingly, during QIM analysis panellists detected that fish fed with diets with 28% red beet and 1.63% betaine presented a more rancid odour than fish fed with the other rest diets. These results were correlated with a loss of freshness in these fish.

5: Conclusions

The inclusion of 14% of red beet and 0.9% of betaine on rainbow trout diets had not a negative effect on rearing parameters compared to control diet, however, it enhanced the quality of the final product. In addition, it was expected a potential beneficial effect associated with betaine, which was present on red beet. Betaine content on flesh from fish fed control diet was < 2 mg/kg and it increased to values ranging from 3240 to 5310 mg/kg when red beet and betaine were present on the diet. For this reason, further

studies would be necessary to verify if this ingredient enhances the nutritional and healthy (antioxidant) value of rainbow trout flesh.

6: Acknowledgements

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584 Table 1. Formulation and proximate composition of the experimental diets.

	Diets*				
	CONTROL	**A**	**B**	**C**	**D**
Ingredients [g/kg] - international feed number					
Fish meal	222	222	222	222	222
Wheat	338	168	160	0	0
Red Beet	0	140	140	280	280
Natural betain	0	23	48	20	45
Wheat gluten	170	175	160	201	189
Meat meal	103	103	101	105	92
Soybean oil	91	93	93	96	96
Fish oil	45	45	45	45	45
Maltodextrin	11	11	11	11	11
Multivitamin and mineral mix¶	20	20	20	20	20
Analyzed composition [% dry matter]					
Dry matter	95.00	96.50	96.10	94.70	94.40
Crude Protein (% CP)	38.30	40.60	41.10	39.90	41.20
Crude Fat (% CF)	17.60	17.40	19.50	17.30	16.80
Ash (%)	8.20	8.60	8.20	7.90	8.20
Betain (%)	0.00	0.90	1.63	0.90	1.63

[*] Different experimental diets: CONTROL (0% red beet, 0% betaine), A (14% red beet, 0.9% betaine), B (14% red beet, 1.63% betaine), C (28% red beet, 0.9% betaine) and D (28% red beet, 1.63% betaine); [¶] Contains: Choline, 10 g; DL-α-tocopherol, 5 g; ascorbic acid, 5 g; $Ca_3(PO_4)_2$, 5 g and a premix: 25 g. This premix contains per kg: retinol acetate, 20000 IU; calciferol, 10 IU; DL-α-tocopherol, 0.2 g; menadione sodium bisulfite, 0.016 g; thiamine hydrochloride, 0.05 g; riboflavin, 0.05 g; pyridoxine hydrochloride, 0.3 g; cyanocobalamine, 0.5 mg; nicotinamide, 0.3 g; pantothenic acid, 0.12 g; folic acid, 13 mg; biotin, 1.4 mg; ascorbic acid, 1.5 g; inositol, 0.3 g; betaine, 2 g; polypeptides, 0.24 g; Zn, 0.1 g; Se, 0.4 mg; I, 10 mg; Fe, 4 mg; CuO, 0.3 g; Mg 0.115 g; Co, 0.4 mg; methionine, 0.024 g; cysteine, 0.016 g; lysine, 0.026 g; arginine, 0.012 g; phenylalanine, 8 mg; tryptophan, 0.014 g (Dibaq Diproted S.A., Spain).

Table 2. Effect of red beet and total betaine level on growth and nutritive parameters of rainbow trout (values are least-squares means ± SEM, n=3).

	Red beet Level [¶] [%]			**Betaine Level [†] [%]**			**Interaction (Diets [*])**					**Two-way ANOVA (*p*-value)**		
	CONTROL	**RB1**	**RB2**	**CONTROL**	**B1**	**B2**	**CONTROL**	**A**	**B**	**C**	**D**	**SEM**	**Red beet**	**Betaine**	**Red beet •Betaine**
Growth parameters															
Initial weight [g]	69.82	71.02	69.09	69.82	70.85	69.24	69.82	73,54	68.51	68.17	69.78	1.628	0.555	0.664	0.057
Final weight [g]	250.72^{b}	214.06^{ab}	198.27^{a}	250.72^{b}	208.37^{ab}	203.15^{a}	250.72^{d}	241.47^{cd}	186.66^{ab}	175.27^{a}	215.52^{bc}	7.481	0.019	0.033	< 0.05
SGR [% / day] [≠]	1.22^{b}	1.04^{a}	1.00_{a}	1.22^{b}	1.02^{a}	1.02_{a}	1.22^{d}	1.13^{c}_{d}	0.95^{a}_{b}	0.90_{a}	1.07^{b}_{c}	0.031	0.012	0.016	< 0.05
TGC · 10^{-3} [§]	0.22^{b}	0.19^{a}	0.17_{a}	0.22^{b}	0.18^{a}	0.18_{a}	0.22^{d}	0.21^{c}_{d}	0.17^{a}_{b}	0.15_{a}	0.19^{b}_{c}	0.007	0.013	0.019	< 0.05
FI [g / 100 g fish / day] [#]	1.05	1.03	1.02	1.05	1.04	1.01	1.05	1.06	1.00	1.01	1.02	0.035	0.617	0.528	0.224
FCR°	1.18^{a}	1.26^{a}_{b}	1.37_{b}	1.18	1.34	1.30	1.18^{a}	1.19^{a}	1.32^{b}	1.50_{c}	1.28^{a}_{b}	0.024	0.022	0.115	< 0.05
PER⊙	2.19^{b}	1.95^{a}	1.80_{a}	2.19^{b}	1.87^{a}	1.87_{a}	2.19^{d}	2.07^{c}_{d}	1.84^{a}_{b}	1.68_{a}	1.90^{b}_{c}	0.037	0.001	0.007	< 0.05

[*] Diets explanation as in Table 1; [¶] Red beet concentration: RB1, diets with 14% of red beet and RB2, diets with 28% of red beet; [†] Betaine concentration: B1, diets with 0.9% of betaine and B2, diets with 1.64% of betaine; [a—c] Means with different superscripts in each row differ significantly ($p < 0.05$).

[≠] Specific growht rate [%/day] SGR = 100 · ln (final weight / initial weight) / days.

[§] Thermal growth coefficient TGC = (final weight $^{(1/3)}$ – initial weight $^{(1/3)}$) / [days · Σ (ºC – 4)]

[#] Feed Intake ratio [g/100 g fish/day]. FI = 100 · feed consumption [g] / biomass [g] · days.

[°] Feed Conversion Ratio FCR = feed intake [g] / weight gain [g].

[⊙] Protein Efficiency Ratio PER =Weight gain [g] / Protein intake [g].

Table 3. Effects of red beet and total betaine level on biometric parameters, body composition and nutrient retention of rainbow trout (values are least-squares means ± SEM, n=3).

	Red beet Level [¶] [%]	**Betaine Level [¶]**	**Interaction (Diets[*])**	**SEM**	**Two-way ANOVA (*p*-value)**

	[%]													
	CONTROL	**RB1**	**RB2**	**CONTROL**	**B1**	**B2**	**CONTROL**	**A**	**B**	**C**	**D**	**Red beet**	**Betaine**	**Red beet •Betaine**
Biometric indexes														
CF †	0.887^{b}	0.842^{a}	0.839^{a}	0.887^{b}	0.837^{a}	0.844^{a}	0.887^{b}	0.859^{ab}	0.823^{a}	0.803^{a}	0.861^{b}	0.010	< 0.05	< 0.05	< 0.05
VSI ≠	8.64^{a}	9.40^{ab}	10.00^{b}	8.64^{a}	9.80^{b}	9.60^{ab}	8.64^{a}	9.04^{a}	9.75^{ab}	10.56^{b}	9.44^{ab}	0.299	< 0.05	< 0.05	0.032
HSI #	1.16^{a}	1.36^{b}	1.37^{b}	1.16^{a}	1.45^{b}	1.28^{a}	1.16^{a}	1.46^{b}	1.26^{a}	1.45^{b}	1.29^{ab}	0.053	< 0.05	< 0.05	0.040
Proximal composition [% dry matter]														
Moisture [%]	72.33	74.60	73.13	72.33	73.57	74.01	72.33	73.23	75.97	73.90	72.55	1.133	0.299	0.595	0.133
Crude Protein [%]	14.01	13.14	14.19	14.01	13.40	13.97	14.01	12.99	13.28	13.80	14.49	0.612	0.167	0.493	0.774
Crude Lipid [%]	11.27	9.18	9.70	11.27	9.95	9.03	11.27	10.53	7.82	9.36	9.95	0.882	0.636	0.302	0.119
Ash [%]	2.45	2.47	2.47	2.45	2.35	2.57	2.45	2.24	2.69	2.46	2.49	0.094	0.957	0.060	0.065
Feed Retention efficiency [%]														
Protein														
PIR (% intake) ■	35.21	26.16	27.68	35.21	25.50	28.24	35.21	27.47	24.85	23.53	30.79	3.393	0.768	0.494	0.159
PIR (% digested) ⭘	41.06^{b}	29.88^{a}	31.34^{ab}	41.06^{b}	29.55^{a}	31.62^{ab}	41.06^{b}	32.41^{ab}	27.34^{ab}	26.69^{a}	34.83^{ab}	3.383	0.038	0.035	0.047
Fat														
FIR (% intake) ⊙	74.68	50.35	55.43	74.68	56.09	50.51	74.68^{b}	65.43^{ab}	35.27^{a}	46.74^{ab}	61.95^{ab}	8.252	0.627	0.368	0.015
FIR (% digested) §	80.92	54.51	63.91	80.92	63.04	56.60	80.92^{b}	71.51^{ab}	37.52^{a}	54.57^{ab}	70.91^{ab}	9.367	0.382	0.349	0.017

* Diets explanation as in Table 1; ¶ Red beet and betaine concentration on diets as explained in Table 2; [a—c] Means with different superscripts in each row differ significantly ($p < 0.05$).

† Condition factor [g/cm^3] CF = 100 · final weight / length^3

≠ Viscerosomatic Index [%] VSI = 100 · wet visceral weight / final weight.

Hepatosomatic Index [%] HSI = 100 · wet liver weight / final weight.

■ PIR (% intake) = 100 · (protein fish gain [g] / protein intake [g])

⭘ PIR (% digested) = 100 · (protein fish gain [g] / protein digested [g])

⊙ FIR (% intake) = 100 · (fat fish gain [g] / fat intake [g])

§ FIR (% digested) = 100 · (fat fish gain [g] / fat digested [g])

Table 4. Apparent digestibility coefficients (ADCs) of protein (ADCprotein), lipid (ADClipid) and carbohydrates (ADCCHO) in rainbow trout fed the experimental diets differing on the source of carbohydrate (wheat and barley) (values are least-squares means ± SEM, n=3).

	Red beet Level ¶ [%]			Betaine Level ¶ [%]			Interaction (Diets *)					SEM	Two-way ANOVA (*p*-value)		
	CONTROL	RB1	RB2	CONTROL	B1	B2	CONTROL	A	B	C	D		Red beet	Betaine	Red beet •Betaine
Apparent digestibility Coefficient (ADCs)															
$ADC_{protein}$	85.56	86.62	88.78	85.56	86.81	88.60	85.56	84.42	88.83	89.21	88.36	1.948	0.285	0.374	0.197
ADC_{lipid}	92.36[b]	91.51[b]	87.64[a]	92.36	89.02	90.12	92.36	90.52	92.49	87.53	87.74	1.818	0.050	0.558	0.637
ADC_{HCO}	42.82	41.98	42.48	42.82	47.82	38.83	42.82	40.00	42.48	49.77	35.19	7.876	0.907	0.574	0.432

* Diets explanation as in Table 1; ¶ Red beet and betaine concentration on diets as explained in Table 2; [a—c] Means with different superscripts in each row differ significantly ($p < 0.05$).

Table 5. Proximate composition of rainbow trout flesh fed with increasing red beet and betaine levels at the end of the experimental growth period (data are expressed as % of dry matter) (values are least-squares means ± SEM, n=3).

	Initial	Red beet Level ¶ [%]			Betaine Level ¶ [%]			Interaction (Diet *)					SEM	Two-way ANOVA (*p*-value)		
		CONTROL	RB1	RB2	CONTROL	B1	B2	CONTROL	A	B	C	D		Red beet	Betaine	Red beet •Betaine
Proximate composition [% dry matter]																
Moisture	78.80	77.65	78.05	78.60	77.65	77.85	78.80	77.65	77.35	78.75	78.35	78.85	1.201	0.740	0.064	0.825
Prote	14.	15.79	15.	14.	15.78	15.	14.	15.78	15.	14.	15.	14.	0.4	0.261	0.08	0.075

in	80		30	98		34	93		61	98	08	88	94		6	
Fat	4.50	5.68^{ab}	5.84^{b}	4.80^{a}	5.69	5.56	5.08	5.69^{ab}	6.36^{b}	5.33^{ab}	4.77^{a}	4.83^{a}	0.303	0.004	0.258	0.041
Ash	1.80	2.09^{b}	1.87^{a}	1.97^{ab}	2.09^{b}	1.98^{ab}	1.87^{a}	2.09	1.86	1.89	2.09	1.85	0.054	0.021	< 0.05	0.134

[*] Diets explanation as in Table 1; [¶] Red beet and betaine concentration on diets as explained in Table 2; [a—c] Means with different superscripts in each row differ significantly ($p < 0.05$).

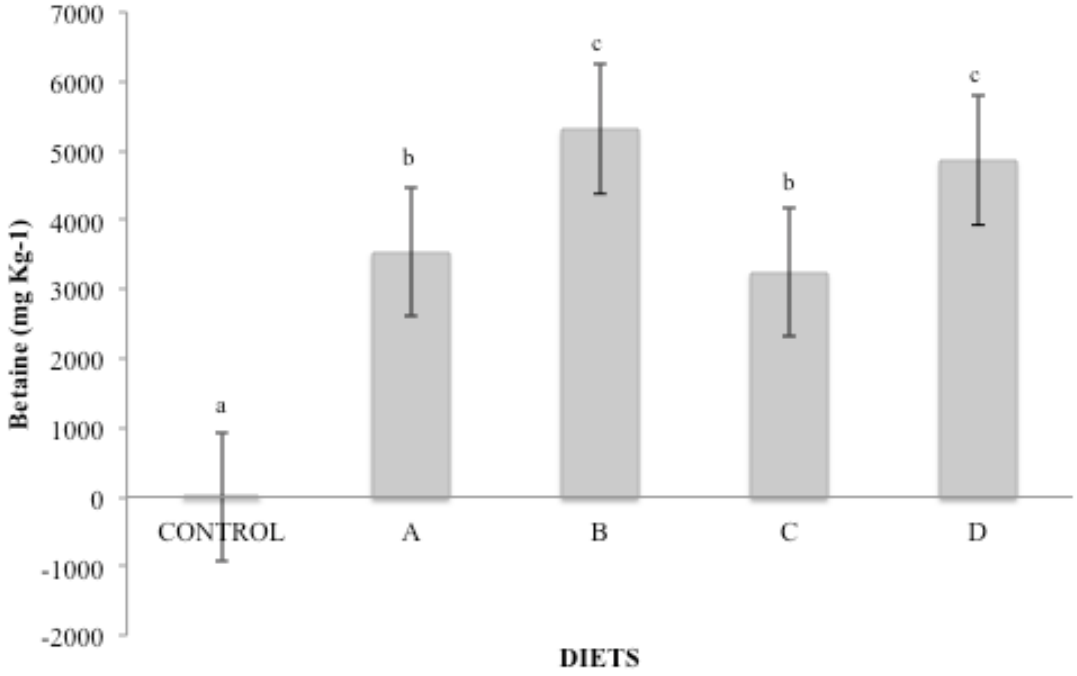
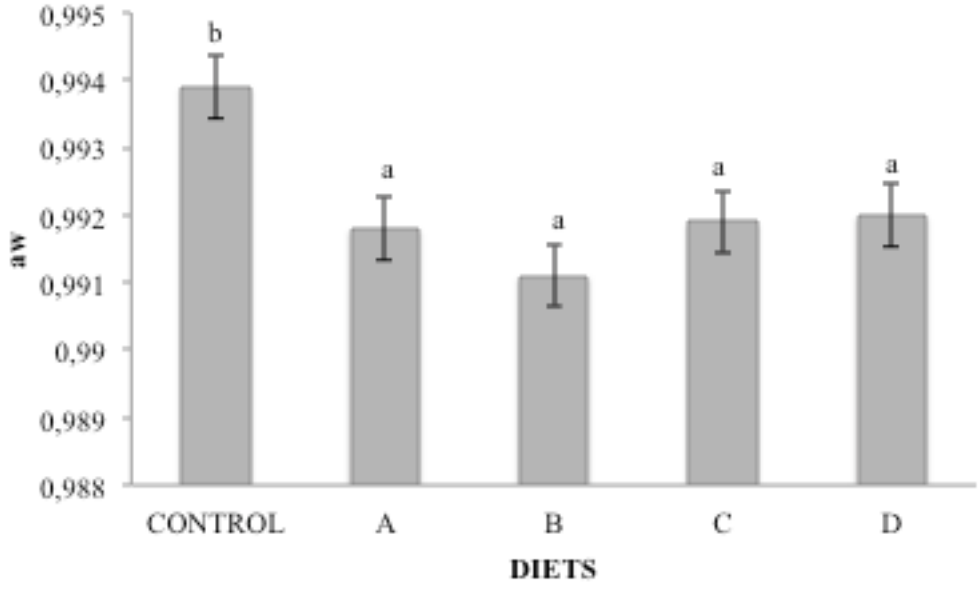


Figure 1. Effect of increasing levels of red beet and betaine on fish flesh betaine content. Data are presented as least-squares means ± standard error of the mean (n=3); significant differences ($p < 0.05$) are indicated with different letters above the column. CONTROL (0% red beet, 0% betaine), A (14% red beet, 0.9% betaine), B (14% red beet, 1.63% betaine), C (28% red beet, 0.9% betaine) and D (28% red beet, 1.63% betaine) are the different experimental diets.



674

675 Figure 2. Effect of red beet and betaine concentration on water activity (aw) of fish
676 meat at the end of the experimental growth period. Data are presented as least-squares
677 means ± standard error of the mean (n=3); significant differences ($p < 0.05$) are
678 indicated with different letters above the column. CONTROL (0% red beet, 0%
679 betaine), A (14% red beet, 0.9% betaine), B (14% red beet, 1.63% betaine), C (28% red
680 beet, 0.9% betaine) and D (28% red beet, 1.63% betaine) are the different experimental
681 diets.

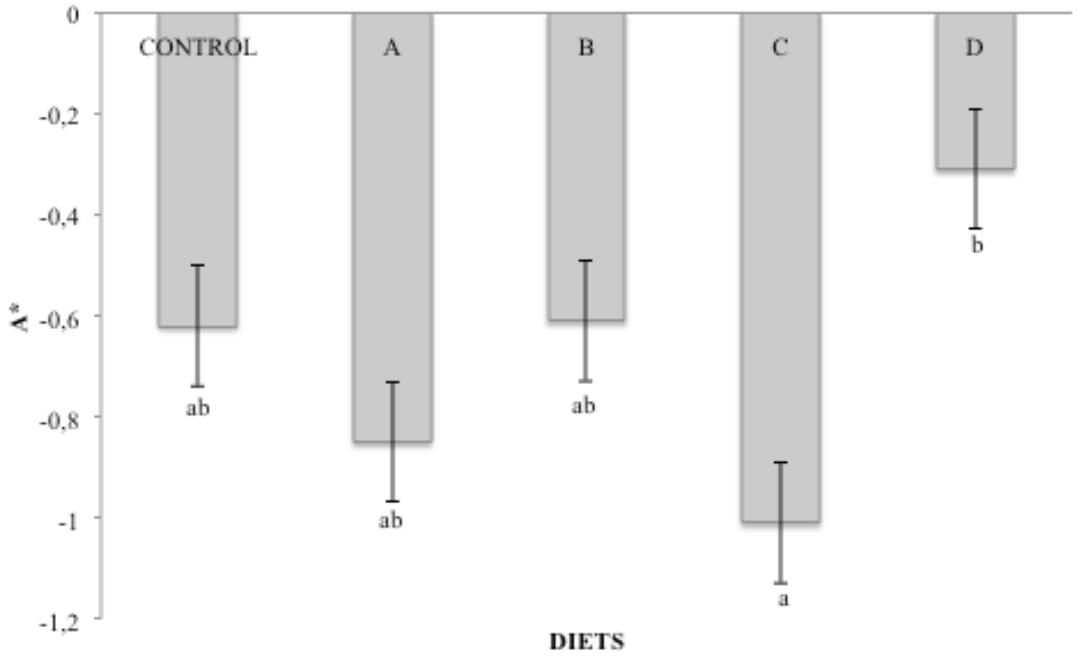


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Figure 3. Effect of red beet and betaine on fish flesh redness (A* velaues) at the end of the experimental growth period. Data are presented as least-squares means ± standard error of the mean (n=3); significant differences ($p < 0.05$) are indicated with different letters above the column. CONTROL (0% red beet, 0% betaine), A (14% red beet, 0.9% betaine), B (14% red beet, 1.63% betaine), C (28% red beet, 0.9% betaine) and D (28% red beet, 1.63% betaine) are the different experimental diets.

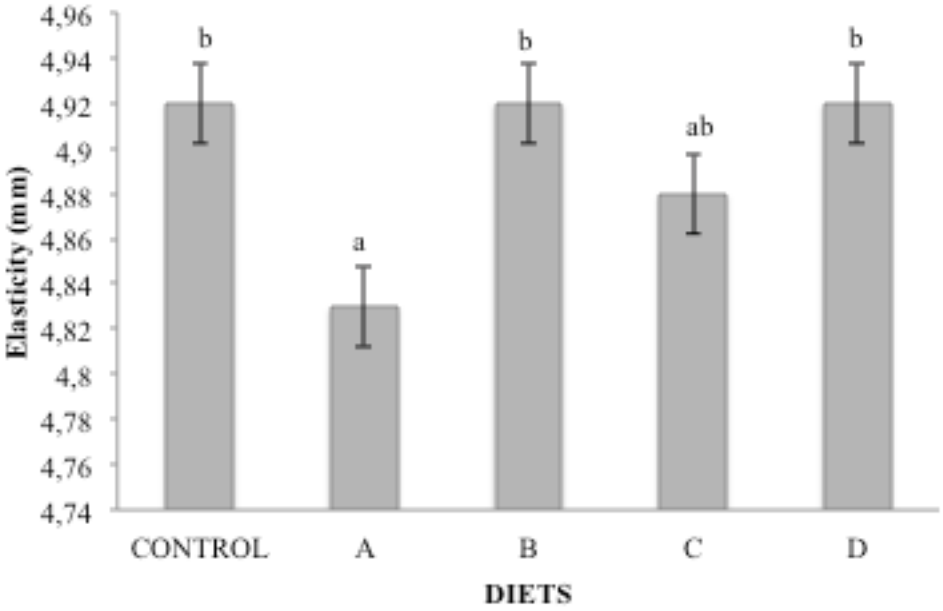

Figure 4. Effect of red beet and betaine on fish flesh elasticity at the end of the experimental growth period. Data are presented as least-squares means ± standard error of the mean (n=3); significant differences ($p < 0.05$) are indicated with different letters above the column. CONTROL (0% red beet, 0% betaine), A (14% red beet, 0.9% betaine), B (14% red beet, 1.63% betaine), C (28% red beet, 0.9% betaine) and D (28% red beet, 1.63% betaine) are the different experimental diets.

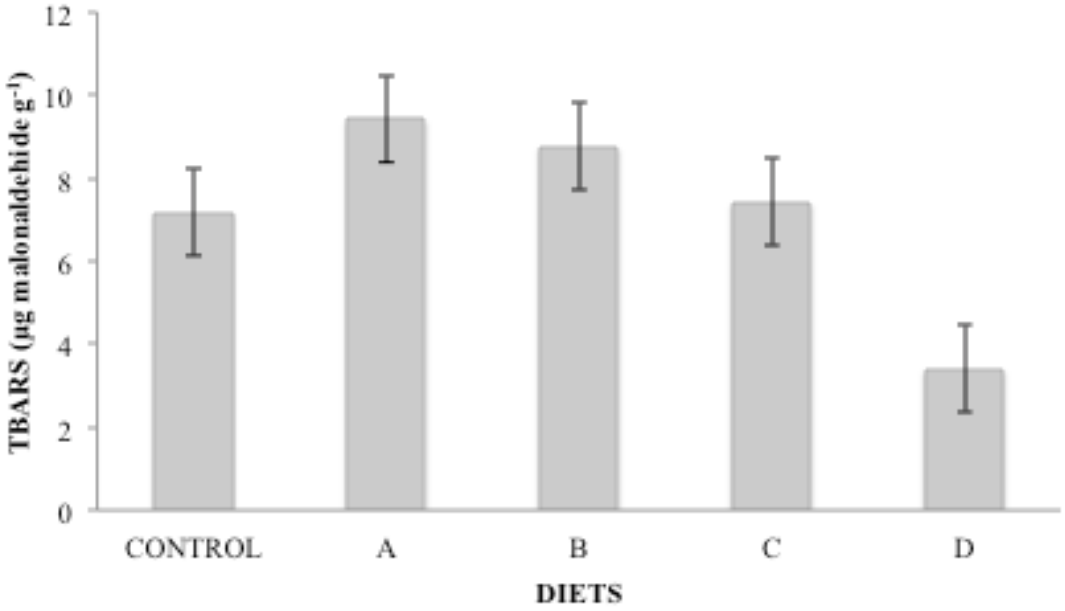

Figure 5. Effect of red beet and betaine concentration on lipid oxidation (TBARS) measured as µg malonaldehide g-1 of fish meat at the end of the experimental growth period. Data are presented as least-squares means ± standard error of the mean (n=6); absence of different letters above the column indicates no significant differences ($p > 0.05$) between treatments. CONTROL (0% red beet, 0% betaine), A (14% red beet, 0.9% betaine), B (14% red beet, 1.63% betaine), C (28% red beet, 0.9% betaine) and D (28% red beet, 1.63% betaine) are the different experimental diets.