**REPLACEMENT OF FISH MEAL BY SOLID STATE FERMENTED LUPIN
(*LUPINUS ALBUS*) MEAL WITH *LACTOBACILLUS PLANTARUM* 299V:
EFFECT ON GROWTH AND IMMUNE STATUS OF JUVENILE
ATLANTIC SALMON (*SALMO SALAR*)**

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Abstract

The aim of this study was to assess quality of solid state fermented (SSF) lupin with *Lactobacillus plantarum* 299v, and its effects (on growth, feed utilization, digestibility and immunity) of juvenile Atlantic salmon (*Salmo salar*), when used as fish meal replacer. Five experimental diets were formulated to provide 40% crude protein and 21% dietary lipid (dry matter basis) with the raw or fermented lupin meal-based protein source replacing fish meal at 15% and 30%. Triplicate groups of fish (averaging 3.53 ± 0.05 g) were fed with experimental diets for 8 weeks. Fermentation process modified nutrient profile of lupin meal and enriched it with lactic, citric and acetic acids. Fish in the FL15% group showed a higher ($P<0.05$) final body weight, weight gain, FCR, SGR, and PER compared to those of C group. Apparent digestibility coefficient (ADC) of protein and nitrogen-free extract showed significantly higher values in FL15% experimental group, compared to those shown in C group. Fish in the FL15% group showed a higher ($P<0.05$) lysozyme activity and leucocyte respiratory burst compared to that shown by fish samples in the C experimental group; phagocytic activity did not record differences among experimental groups. In conclusion, replacement of fish meal by raw or fermented lupin meal did not compromise growth, apparent digestibility coefficients and immune status of juvenile Atlantic salmon and even improved fish performance when supplemented at 15%.

Key words: Atlantic salmon, digestibility, growth, immunity, lupin meal

Fish meal (FM) has been widely used for manufacturing aquafeeds because of its long-chain omega-3 fatty acids, vitamins and minerals, and balanced amino acids essential for cultured fish growth (Al-Thobaiti et al., 2017; Tacon and Metian, 2015; Zhang et al., 2018). However, decreases in fisheries stocks and high price of fish meal have made it hard to meet the aquaculture consumption demands of this industry. In consequence, a global priority for finding alternative protein sources to replace fish meal in aquafeeds is essential (Sun et al., 2015). As a response, plant protein sources to replace FM have attracted scientific and industrial attention. Lupin is a promising plant protein source due to its mass supply, price and nutrient contents (Tabrett et al., 2012). Studies have been conducted in order to determine the effects of lupin in fish (Glencross et al., 2003; Salini and Adams, 2014), observing neither no negative effects nor a decrease in growth of fish (Acar et al., 2018).

Fermentation or bioprocessing of plant proteins is an alternative for improving nutrients profile and enriching fermented substrate with a series of secondary metabolites (Ray, 2001). Solid state fermentation (SSF) is a method in which inoculation is done on a solid substrate, such as a plant meal, with microorganisms and fermented under controlled conditions of temperature, and humidity (Pandey, 2003). Many microorganisms have been used in SSF processes. Lactic acid bacteria (LAB) is one of them (Zhang et al., 2014). There are many studies demonstrating the beneficial effects of LAB in growth, digestibility and nutrient utilization (Giri et al., 2013; Van-Doan et al., 2014; Yu et al., 2017) and immunity (Gao et al., 2018; Xia et al., 2018) of aquatic animals. *Lactobacillus plantarum* 299v is a LAB that has been demonstrated to produce abundant organic acids (OA) such as lactic, citric and acetic acids (Vandenbergh et al., 2018) during fermentation process. OA are short-chain fatty acids or carboxylic acids which have antimicrobial properties and the capacity to improve growth, digestibility and immunity of aquatic animals (Sarker et al., 2011; Vielma and Lall, 2006). It has been previously demonstrated that SSF processes with *Lactobacillus* produce organic acids blends (OAB) which are a mixture of OA potentiating their capacity to improve fish performance (Ng and Koh, 2016).

Fish meal replacement by SSF plant meals in fish diets has been widely studied (Sharawy et al., 2016; Sun et al., 2015). However, studies using fermented lupin meal (with *L. plantarum*) as replacer of fish meal in Atlantic salmon diets, are scarce. Therefore, the aim of this study was to assess quality of SSF lupin with *L. plantarum* 299v, and its effects on growth, feed utilization, digestibility and immunity of juvenile Atlantic salmon (*S. salar*), when used as fish meal replacer.

Material and methods

Preparation of fermented lupin meal

The bacterium *L. plantarum* 299v was isolated from a commercial probiotic product. By 16S rDNA sequencing, the strain was identified as *L. plantarum* (sequence homology 99%). The inoculum to the SSF was prepared in sterile defatted milk (10% p w/v) and incubated at 37°C for 24 h. The meal of dehulled lupin was

obtained from the local market. Before using the product, a thermo-sanitation process (90°C, 3 h) was applied to the meal for blocking the microbial activity. After cooling down to room temperature, lupin meal was homogeneously spread in two previously sterilized steel trays (86 cm × 46 cm × 2 cm). The grown bacterial culture (90 mL) was mixed with a sterile water in the amount needed to achieve 60% of humidity of total substrate; the trays were covered with plastic wrap. SSF was conducted at 37°C until the pH of liquid retained by the solid (2 g of moistened substrate in 15 mL of water) reached a value of 4 (within 48 h approximately). At this point, temperature of fermentation machine was elevated to 65°C for drying until obtaining a constant weight. During fermentation process, humidity/moisture and bacterial content (CFU) of solid substrate were monitored at 0 h, 36 h and after drying. Humidity was calculated using a moisture balance (A&D Weighing, MS70, IL, USA). Counting of CFU was conducted according to method described in Panigrahi et al. (2004). Fermented lupin was minced and kept at 4°C until use.

Content of organic acids

The content of organic acids (lactic, citric and acetic acid) of raw and fermented lupin meal, and experimental diets was determined by high performance liquid chromatography according to methodology described in Cunha et al. (2001) with some modifications. In brief, the chromatographic separation was achieved using reverse-phase column C18 (ODS) (Hypersil BDS, ThermoFisher Scientific, MA, USA). The mobile phase used was a gradient of water and acetonitrile at a flow rate of 1 mL/min, column temperature 60°C, injection volume 10 µL. The effluent was monitored using a UV detector (EM-1 Econo UV monitor, Bio-Rad, CA, USA) at 265 nm. Identification and quantification were based on internal standard methods.

Experimental diets

Five experimental diets were formulated to be isonitrogenous (42% crude protein) and isolipidic (22% crude lipid). Diets were supplemented with 1% of chromium (III) oxide (Sigma-Aldrich, Darmstadt, Germany) as marker for digestibility test. Experimental design included a basal diet (C) formulated with a high fish meal protein source and four basal diets replacing fish meal by either raw or fermented lupin: RL15% (basal diet + 15% raw lupin), FL15% (basal diet + 15% fermented lupin), RL30% (basal diet + 30% raw lupin), FL30% (basal diet + 30% fermented lupin) (Table 1).

Diets were manufactured at the Animal Feed Pilot Plant of the Aquaculture School, Catholic University of Temuco, Chile. Prior to mixing, major meal ingredients were finely ground through a 200-µm mesh using an Ultra Centrifugal Mill (Restch ZM 200, Haan, Germany). Ingredients were weighed and thoroughly mixed prior to the extrusion process. Extruded feed was obtained by a cooking-extrusion process with a laboratory twin-screw extruder (Cletral BC-21; Cletral, Firminy, France) with a 3-mm diameter die. Resultant moist pellets were oven-dried at 60°C for approximately 15 h. Oil was added, according to the formulation of each diet, by means of a laboratory vacuum coater (Dinnissen model VC10, Sevenum, the Netherlands). Diets were stored at -20°C until use.

Table 1. Formulation and proximal composition (g kg⁻¹ diet) of experimental diets for Atlantic salmon (*Salmo salar*)

Ingredients (g kg ⁻¹ diet)	Diets				
	C	RL15%	FL15%	RL30%	FL30%
Fish meal ¹	562	480	475	397	387
Lupin meal ²	0	150	0	300	0
LAB fermented lupin meal	0	0	150	0	300
Starch gel ³	130	130	130	130	130
Fish oil ⁴	80	80	80	80	80
Raps oil ⁵	70	66	66	63	62
Vitamin premix ⁶	5	5	5	5	5
Mineral premix ⁷	15	15	15	15	15
Chromium III oxide ⁸	10	10	10	10	10
Alpha-cellulose ⁹	128	64	69	0	11
Total	1000	1000	1000	1000	1000
Proximate composition (g kg ⁻¹ diet)					
Dry matter	955	962	970	965	972
Protein	400	403	402	409	405
Lipid	211	207	208	204	210
Fibre	101	70	65	44	20
Ash	117	109	110	101	95
Nitrogen-free extract	185	215	222	250	265
Gross energy (MJ kg diet)	21	22	22	22	22
Chromium III oxide	9	9	9	9	9

¹ Supplied by Alimentos marinos (ALIMAR S.A.) Jack Mackerel meal. Super Prime (protein 68%, lipids 9.9%, ashes 14.5%).

² Supplied by Graneles de Chile S.A. Rancagua, Chile.

³ Supplied by Productos Químicos de Chile, Santiago, Chile.

⁴ Supplied by Biomar Chile S.A., Puerto Montt, Chile.

⁵ Supplied by Molinera Gorbea, Gorbea, Chile.

⁶ Supplied by DSM Nutritional Products S.A., Puerto Varas, Chile. (IU/kg or g/kg of premix): vitamin A, 1.0 MIU; vitamin D₃, 0.5 MIU; vitamin E, 0.04 MIU; vitamin K, 3.4 g; vitamin B₁, 4 g; vitamin B₂, 6 g; vitamin B₅, 10 g; vitamin B₆, 2 g; vitamin B₉, 1.6 g; vitamin B₁₂, 0.004 g; niacin, 40 g; biotin, 0.1 g; vitamin C, 100 g; choline, 200 g; inositol, 50 g.

⁷ Supplied by BioMar Chile S.A. (per g mixture: mg; Cu: 8.3 mg; Mn: 67 mg; Co: 1.7 mg; Y: 1.7; Zn: 200 mg)

⁸ Supplied by Sigma Aldrich Química Limitada, Santiago, Chile.

⁹ Supplied by Sigma Aldrich Química Limitada, Santiago, Chile.

Experimental fish and feeding trial

The feeding trial was carried out at the wetlab of the aquaculture school, Catholic University of Temuco, Chile. Juvenile salmon were transported from a local fish hatchery to the experimental facilities and once there, health status of fish was checked by visual observation, according to indications proposed by Johansen et

al. (2006). Fish were fed with a commercial diet during the two weeks of acclimation period. At the beginning of the experiment, 750 fish (averaging 3.53 ± 0.05 g) were equally distributed in 15 tanks (60 L, designed with a modified feces collector-Guelph system) of an open system. Average temperature was $14^\circ\text{C} \pm 1.5$. pH values recorded a range between 6.8 and 7.2. Supplemental aeration was provided to maintain the dissolved oxygen near saturation ($> 85\%$). Each tank was then randomly assigned to one of three replicates of the five dietary treatments. The photoperiod was maintained on a 12-h light: 12-h dark schedule. Fish were fed to apparent satiation two times daily (08:00 am and 04:00 pm). Feeding trial lasted 8 weeks.

Growth sampling and analysis

Before starting experimental feeding period, twenty fish (from stocking group), and at the end of trial, twelve fish per experimental group, were sampled and stored frozen (-20°C) for analysis of whole body composition. Sampling was conducted at regular intervals of 0, 30 and 60 days for measuring weight and total length. Overnight fasting was done before each sampling, and the following calculations were done:

FBW (final body weight); WG (weight gain = final body weight – initial body weight); FCR (feed conversion rate = dry weight of consumed diet/gained body weight); SGR (specific growth rate = $[\ln(\text{FBW}) - \ln(\text{IBW})]/\text{Number of rearing days} \times 100$); thermal growth coefficient ($\text{TGC} = (\text{Final body weight} - \text{Initial body weight}) / (\text{IBW} \times \text{Sum of daily water temperature}) \times 100$); condition factor ($K = (\text{weight (g)} / \text{length}^3 (\text{cm})) \times 100$); and PER (protein efficiency ratio = $\text{Net weight gain (g)} / \text{administered protein (g)}$). Feed consumption (FC) was calculated on a daily basis. Mortality was monitored throughout the experimental period.

Nutrient apparent digestibility

Two weeks before the end of feeding trial, feces were obtained on a daily basis, by setting feces collectors in each experimental tank, after last daily-feeding and syphoning uneaten feed in tanks. Feces were collected for ten consecutive days until reaching 80 g of pooled sample for each tank. Samples were freeze-dried (lyophilizer ALPHA 1-4 LD, Christ), powdered and kept at -20°C until analysis. Chromium III oxide determination of diets and feces was conducted by atomic absorption spectrophotometry (AA-6800 spectrophotometer Shimadzu, Tokyo, Japan), according to method proposed by Furukawa and Tsukahara (1996). Data obtained was used to calculate nutrient apparent digestibility coefficients: $\text{ADC} = 100 - (100 \times (\text{Feces nutrient \%} / \text{Diet nutrient \%}) \times (\text{Cr}_2\text{O}_3\% \text{ diet} / \text{Cr}_2\text{O}_3\% \text{ feces}))$.

Proximate composition analysis

Proximate composition and gross energy of raw/fermented lupin meals, experimental diets, whole body and feces samples were analyzed using standard methods (AOAC, 1995).

Immunity sampling and analysis

After completion of the feeding trial, fish were starved for 24 h and then the final sampling was carried out. Six fish per tank ($n=18$ per treatment) were anesthetized with 20% w/v benzocaine (BZ-20; Veterquímica SA, Santiago, Chile) and bled by

puncturing the caudal vein with heparinized 1-mL syringes. Fresh plasma was frozen at -80°C until analyzed.

Lysozyme activity was measured by turbidimetric assay (Parry et al., 1965) using a 96-well plate using a microplate reader (Elx808 Bio Tek, USA) (absorbance 520 nm). Following the method proposed by Sakai et al. (1996), respiratory burst and phagocytic activity were determined using leucocytes of anterior portion of head kidney (HK). This tissue was conserved in L-15 medium (Sigma, St. Louis, MO, USA). HK was macerated with 34/51% (resultant cellular suspension) of Percoll gradient (Pharmacia, Uppsala, Sweden). Hanks medium was used for washing isolated interphase cells. Viable phagocytic cells were quantified by trypan blue exclusion (viability $>95\%$).

Leucocyte respiratory burst activity was assessed by measuring intracellular superoxide anion production (Yin et al., 2006). A 96-well plate was used to analyze reduction of nitroblue tetrazolium (NBT). After works, optical density was measured with a multiscan spectrophotometer (Spectra Count, Packard, USA) at 630 nm. A direct counting method was used for assessing phagocytic activity of leucocytes. Percentage of leucocytes with phagocytosed cells determined the level of immune stimulation of analyzed cells.

Statistical analysis

Data analysis was done using STATISTICA software (version 12.0, StatSoft, OK, USA). Effects of treatments were determined by ANOVA analysis. Obtained data was submitted to sigma-restricted parameterization and effective hypothesis decomposition methods. In order to discriminate homogeneous groups, differences between means were analyzed by Duncan's multiple range test (5% level of significance).

Results

Solid state fermentation (SSF) and organic acids (OA)

During all SSF process, humidity and CFU were measured (Table 2). The color of raw lupin was yellow, while after the initial heat treatment, fermentation and drying processes, the color of the meal changed to a dark-yellow. Dry matter and protein showed slightly higher values in fermented lupin meal compared to the contents of the raw lupin meal. In contrast, a lower trend was exhibited by the contents of fibre, ash and nitrogen-free extract in the fermented lupin meal, compared to those values recorded in raw lupin meal. Lactic acid was not detected in raw lupin meal, however it recorded a notably high amount in the fermented lupin meal. Citric and acetic acids increased in fermented lupin meal, compared to those shown in raw lupin meal (Table 3).

Lactic, citric and acetic acids showed higher values in diets supplemented with any level of the fermented lupin meal than those shown in diets partially supplemented with any level of raw lupin meal. Lactic and citric acids were only detected in diets supplemented with fermented lupin meal. Compared to C, pH was lower in diets supplemented with any level of fermented lupin (Table 4).

Table 2. Humidity (%) and cell concentration (colony forming CFU) of the fermented lupin (*Lupinus albus*) meal by *L. plantarum* 299v at the inoculation time (0 h), after 36 h and after drying

Time (h)	Humidity (%)	CFU/g wet basis
0	70	7×10^3
36	65	10×10^7
After drying	6.9	15×10^3

Table 3. Proximal composition (g kg⁻¹) and organic acid (mg g⁻¹ dry matter basis) content of raw and fermented lupin (*Lupinus albus*) meal with *Lactobacillus plantarum* 299v

	Raw lupin meal	Fermented lupin meal
Proximal composition		
Dry matter	910	940
Protein	380	410
Lipid	80	80
Fibre	30	20
Ash	30	25
Nitrogen-free extract	470	450
Gross energy (MJ/kg)	20	20
Organic acids		
Lactic acid	0	62
Citric acid	6	13
Acetic acid	4	14

Table 4. Organic acids (g kg⁻¹ diet) and pH levels of experimental diets for Atlantic salmon (*Salmo salar*)

Organic acids (g kg ⁻¹) and pH	Ingredients				
	C	RL15%	FL15%	RL30%	FL30%
Lactic acid	0.00	0.00	12.89	0.00	75.55
Citric acid	0.00	1.01	2.18	2.30	4.92
Acetic acid	0.00	2.52	6.08	4.14	10.44
pH	5.94	5.88	5.65	5.87	5.40

Experimental diets proximate composition

Dry matter content of experimental diets slightly increased as either raw or fermented lupin meal supplementation level increased. Fibre and ash content of experimental diets decreased as either raw or fermented lupin meal supplementation level increased. The content of nitrogen-free extract increased as either raw or the fermented lupin meal supplementation level increased. Protein, lipid, gross energy and chromium III oxide content did not show important differences among all experimental diets (Table 5).

Whole-body proximate composition

The whole-body proximate composition of juvenile Atlantic salmon fed the experimental diets for 8 weeks is presented in Table 5. The results showed that there were no significant differences ($P>0.05$) in whole-body composition among fish fed different diets.

Table 5. Whole-body proximate composition (g kg), after an 8-week feeding test, of juvenile Atlantic salmon (*Salmo salar*) fed experimental diets formulated with 15% and 30% replacement of fish meal by either raw or fermented lupin meal

	C	RL15%	FL15%	RL30%	FL30%
Dry matter	250±10.84	253±14.54	245±16.51	249±2.63	247±7.54
Protein	125±7.00	128±10.74	125±1.02	122±3.86	123±2.14
Lipid	76±3.40	76±3.59	72±5.41	79±1.49	74±3.13
Ash	82±4.54	83±5.71	80±5.96	70±4.42	73±3.23

Growth and survival

All experimental diets were well accepted by the fish during the 8-week experimental period. At the end of feeding trial, final body weight, weight gain, SGR and PER were significantly ($P<0.05$) higher in FL15% group compared to C group. However, FCR was significantly lower ($P<0.05$) in FL15% compared to that of C. A significantly ($P<0.05$) higher final body weight, weight gain, SGR and PER were recorded in FL15% compared to those shown in RL15%. FCR recorded a significantly ($P<0.05$) lower value in FL15%, compared to that shown in RL15% group. Final body weight, weight gain, SGR, TGC and PER were significantly ($P<0.05$) lower in FL30% compared to those shown in RL30%. In contrast, FCR recorded a significantly ($P<0.05$) higher value in FL30% compared to that shown in RL30%. SGR was significantly ($P<0.05$) lower in FL30% compared to that shown in FL15% (Table 6).

Table 6. Growth, feed utilization and survival, after an 8-week feeding test, of juvenile Atlantic salmon (*Salmo salar*) fed experimental diets formulated with 15% and 30% replacement of fish meal by either raw or fermented lupin meal

	C	RL15%	FL15%	RL30%	FL30%
Initial body weight (g fish ⁻¹)	3.53±0.07 a	3.46±0.04 a	3.56±0.06 a	3.53±0.03 a	3.57±0.09 a
Final body weight (g fish ⁻¹)	5.30±0.20 ab	5.20±0.26 ab	6.27±0.21 c	5.97±0.51 bc	4.67±0.25 a
Weight gain (g fish ⁻¹)	1.77±0.20 ab	1.74±0.26 ab	2.71±0.25 c	2.43±0.50 bc	1.10±0.17 a
Feed intake (g fish ⁻¹)	1.10±0.10 ab	0.86±0.16 a	1.15±0.08 ab	1.19±0.18 b	0.90±0.07 ab
FCR	1.60±0.20 bc	1.70±0.33 bc	1.04±0.09 a	1.34±0.15 ab	2.04±0.27 c
SGR	0.68±0.10 ab	0.68±0.08 b	0.94±0.08 c	0.87±0.14 bc	0.45±0.05 a
TGC	0.26±0.00 ab	0.26±0.03 ab	0.28±0.08 ab	0.34±0.06 b	0.17±0.02 a
PER	1.48±0.10 ab	1.48±0.26 ab	2.41±0.21 c	1.83±0.20 bc	1.23±0.15 a
K factor	1.42±0.10 ab	1.34±0.08 ab	1.60±0.18 b	1.45±0.01 ab	1.16±0.18 a
Survival	71.3±3.06 a	75.33±5.03 a	78.67±2.31 a	76.00±2.00 a	72.67±4.20 a

FCR, feed conversion ratio; SGR, specific growth rate; TGC, thermal growth coefficient; PER, protein efficiency ratio; K, condition factor.

a, b, c – different letters in a row denote significant ($P<0.05$) differences among experimental groups.

Nutrient apparent digestibility

ADC of protein and nitrogen free extract were significantly ($P<0.05$) higher in FL15% compared to those shown in C, RL15%, and FL30% experimental groups (Table 7).

Table 7. Apparent digestibility coefficients (ADC), after an 8-week feeding test, of juvenile Atlantic salmon (*Salmo salar*) fed experimental diets formulated with 15% and 30% replacement of fish meal by either raw or fermented lupin meal

	C	RL15%	FL15%	RL30%	FL30%
Dry matter	69.15±0.10 a	70.82±1.87 a	73.60±1.58 a	74.96±0.51 a	72.28±4.63 a
Protein	91.10±0.43 a	89.02±2.58 a	93.95±0.46 b	93.70±0.25 ab	88.93±3.84 a
Lipid	96.90±0.30 ab	95.54±1.13 ab	98.17±0.31 b	97.99±0.44 b	95.46±2.99 ab
Ash	46.50±1.44 a	46.69±2.68 a	49.27±0.57 a	49.64±1.71 a	48.26±2.80 a
Nitrogen-free extract	42.56±3.15 a	50.12±5.51 b	60.49±4.41 c	51.99±0.69 bc	46.64±0.90 ab
Gross energy (MJ kg diet)	80.83±0.02 a	80.32±1.17 a	87.85±8.73 a	85.52±1.33 a	82.28±4.09 a

a, b, c – different letters in a row denote significant ($P<0.05$) differences among experimental groups.

Immune response

Fish fed with a diet containing a lower supplementation of fermented lupin meal (FL15%) recorded a significantly higher ($P<0.05$) lysozyme activity compared to that shown by fish in C group (Figure 1). Fish fed the FL15% diet, showed a significantly ($P<0.05$) higher leucocyte respiratory burst compared to that recorded in fish fed the C diet (Figure 2). Phagocytic activity did not show significant differences ($P>0.05$) among experimental groups (Figure 3).

Figure 1. Lysozyme activity (mean ± SD, n = 8), after an 8-week feeding test, of juvenile Atlantic salmon (*Salmo salar*) fed experimental diets formulated with 15% and 30% replacement of fish meal by either raw or fermented lupin meal

Figure 2. Respiratory burst (mean $\pm$ SD, n = 8), after an 8-week feeding test, of juvenile Atlantic salmon (*Salmo salar*) fed experimental diets formulated with 15% and 30% replacement of fish meal by either raw or fermented lupin meal

Figure 3. Phagocytic activity (mean $\pm$ SD, n = 8), after an 8-week feeding test, of juvenile Atlantic salmon (*Salmo salar*) fed experimental diets formulated with 15% and 30% replacement of fish meal by either raw or fermented lupin meal

Discussion

L. plantarum has been widely researched in fermentation processes of plant meals. However, few investigations have been conducted with this species of bac-

teria to bioprocess plant meals used as fish meal replacers in fish diets. In present study, lupin meal was fermented by *L. plantarum* 299v under controlled conditions of temperature and humidity. After 36 h of fermentation, CFU increased notably. Similar observation was made by Pranoto et al. (2013) who found that fermenting sorghum flour with *L. plantarum* increased CFU by 5 log.

It is well documented that during fermentation of *L. plantarum* several reactions occur modifying some properties of fermented substrate such as moisture content (color variation of bioprocessed meal) and production of different metabolic products (e.g. secondary metabolites) (Smit et al., 2005). Our results agree with Vo et al. (2015) who observed a color change produced by an increased dry matter of fermented sweet lupin (*L. angustifolius*). Fermentation can result in variation of components like fibre and ash content in plant based ingredients. These variations can result in significant changes in color of fermented meal (Wang et al., 2016 a). In present study, content of fibre and ash in the fermented lupin meal were lower than the content of the raw meal. Previous studies have demonstrated that certain probiotic strains secrete various enzymes that worked synergistically to degrade fibres during the fermentation process, decreasing crude fibre content (Soccol et al., 2017). In this study, SSF increased protein (7.89%) and decreased nitrogen-free extract (4.26%) content in fermented lupin meal. Similar results were reported by Chi and Cho (2016) who observed that protein content in soybean meal increased 6.42%, 0.95%, 1.91% and 5.61%, in respect to control, after fermentation with *Bacillus amyloliquefaciens* U304, *L. acidophilus*, *L. plantarum*, and *S. cerevisiae* CJ1697, respectively. This increment of protein and reduction of nitrogen-free extract in fermented lupin meal, could be explained by the microbial capacity to decompose soluble polysaccharides into organic acids and carbon dioxide gas, reducing the total amount of carbohydrates (and thus the content of nitrogen-free extract) and increasing the level of protein content in fermented plant meals (Dai et al., 2017). Furthermore, another study showed that fermentation of lupin significantly reduced the amount of anti-nutritional factors (ANFs) and improved amino acid profiles of the lupin (*L. angustifolius*) fermented by *L. acidophilus*, *L. aporogenes*, and *L. kefir*, for 72 h (Vo et al., 2015). These authors suggested that a significant reduction of ANFs in plant meals increased the level and bioavailability of certain nutrients such as protein.

Several investigations have been conducted to study organic acid production during *Lactobacillus* fermentation, processing carbohydrates and protein substrates through several metabolic pathways (Cizekiene et al., 2018; Ho et al., 2018; Liong and Shah, 2005). In present research, lactic, citric and acetic acids were measured in *L. plantarum* 299v fermented lupin meal. Production of lactic acid by *Lactobacillus* sp. is well documented (Fu and Mathews, 1999). A significant production of lactic acid has been reported when fermenting molasses enriched potato stillage by *L. paracasei* (Mladenovic et al., 2018). In our study, citric acid was detected in a lower level (13 g kg⁻¹), compared to that recorded by lactic acid. Citric acid is a common metabolite and an intermediate in cellular metabolism of all aerobic organisms (Srisukchayakul et al., 2018) such as *Lactobacillus*. Microbial production of citric acid during SSF methods has been previously reported. Hang et al. (1987)

reported that fermentation of kiwi fruit peel by *Aspergillus niger* NRRL 567, can result in the production of 100 g of citric acid per kg of fermented substrate. In our study, we measured the acetic acid (14 g kg^{-1}) produced from fermented lupin. Acetic acid production by *Lactobacillus* bacteria, has been studied in some aspects (Oude-Elferink et al., 2001) such as production patterns, and anaerobic conversion. A study led by Li et al. (2016) measured production of acetic acid during fermentation of plant substrates when algal carcass was bioprocessed with *L. delbrueckii*.

Quality and quantity of ingredients significantly affect nutrient contents in diets. Therefore, an accurate diet formulation is essential when fish meal is replaced by plant meals (Al-Thobaiti et al., 2017). Diets evaluated in this study were formulated to contain a balanced nutritional profile (isonitrogenous and isolipidic) to satisfy minimum requirements of Atlantic salmon. There were not significant differences observed in proximate composition among experimental diets designed with either fish meal, raw or fermented lupin. A previous study replacing fish meal in Atlantic salmon diets, by dehulled lupin meal did not show significant differences in proximal composition of experimental diets (Bransden et al., 2001). Although no significant differences were detected among experimental diets for juvenile Atlantic salmon, a slight decrease of fibre and ash was recorded as raw or fermented lupin meal increased. A decreased fibre ratio (compared to that of control diet) was also observed in a solid-state fermented feed for sea cucumber (Wang et al., 2016 a). Other authors found that ash ratio decreased as inclusion level of fermented lupin (as fish meal replacer) increased, in barramundi diets (Vo et al., 2015). In present study, nitrogen-free extract increased as raw or fermented lupin increased, this fact may be explained by high carbohydrate content of lupin and by the organic acid produced during the metabolization of polysaccharides and carbohydrates (starch, free sugars and non-starch polysaccharides) (Smith et al., 2007).

Whole body proximate composition was not affected by consumption of experimental diets replacing raw or fermented lupin meal. Similar results were reported by Moniruzzaman et al. (2017), who observed that whole body proximate composition of final fish did not show any significant differences ($P>0.05$) among experimental groups of juvenile rainbow trout (*Oncorhynchus mykiss*) fed different types of fermented protein concentrates as fish meal replacer.

In present study, we evaluated two levels (15% and 30%) of either raw or fermented lupin meal with *Lactobacillus plantarum* 299v as fish meal replacer in diets for Atlantic salmon. Although growth performance (e.g. SGR) of juvenile Atlantic salmon showed certain differences among experimental groups, growth was low. This can be explained by small size of initial fish (averaging $3.53 \pm 0.05 \text{ g}$) and by environmental conditions (of the season) causing a significant variability of water temperature $14^\circ\text{C} \pm 1.5$ during all experimental period. No differences in weight gain were detected among groups fed fish meal-based diet (C) and groups fed diets supplemented with raw lupin meal (RL15% and RL30%). Similar results were found by Acar et al. (2018) who found that replacing fish meal by raw lupin (*L. albus*) meal at 15% and 30% in rainbow trout (*O. mykiss*) diets did not cause adverse effects regarding growth parameters. Furthermore, Saez et al. (2015) observed that fish meal replacement with raw dehulled lupin meal did not affect growth of Atlantic salmon.

This study demonstrated that replacing fish meal by 15% of fermented lupin meal, significantly improved growth performance (final body weight, weight gain, FCR, SGR and PER) of juvenile Atlantic salmon, compared to that of C experimental group and even to that of 15% supplementation of raw lupin meal. Our results agree with Vo et al. (2015) who observed that replacing fish meal by 45% and 60% of fermented sweet lupin, *Lupinus angustifolius*, significantly increased the final weight of barramundi compared to that shown in control group. Other authors reported an improvement in growth performance of sea cucumbers fed fermented artificial diets compared to that of cucumbers fed unfermented diets (Wang et al., 2016 a). Juvenile Atlantic salmon significantly enhanced growth when fed diets formulated with 15% of fermented lupin. This benefit can be explained by presence of beneficial bacterial residues (Shiu et al., 2013) and by the improved nutritional value of fermented meal (Vo et al., 2015) replacing fish meal in experimental diets. Although we did not assess amino acids content of raw and fermented lupin meal in fish diets, previous studies have demonstrated the evidence that SSF plant meals improve amino acids profiles hence favoring growth of fish when incorporated in aquafeeds (Dai et al., 2017; Vo et al., 2015).

SSF processes of plant meals apart from providing an improved nutrient profile to fish diets, enrich them with a blend of OA (namely OAB) and at the same time, acidifying obtained pellets (Ng and Koh, 2016). Our experimental diets containing fermented lupin meal in their formulation, also recorded a decreased pH value (-0.29 for the FL15% and -0.54 for the FL30% experimental diets) in relation to pH registered in control diet. The pH reduction is ascribed to microbial production of short-chain organic acids functioning as “acidifiers” (Luckstadt, 2008). Previous studies have demonstrated that OA contained in fish diets affects pH of pellets (Castillo et al., 2014). Acidic pH modifies gastrointestinal tract physiology and energy utilization, consequently increasing the availability of nutrients and reducing the pH in the digestive tract through H^+ deposition (Luckstadt, 2008; Pandey and Satoh, 2008; Ringo 1991). Hence, presence of an OAB in experimental diets, may favor juvenile Atlantic salmon growth as shown in present study, where fish fed diet formulated with 15% of lupin meal showed an improved growth compared to that shown in C, RL15% and FL30% experimental groups. It is well demonstrated that individual OA has the capacity to improve growth in different species. Lactic acid has demonstrated growth improvement capacity in different species (Gislason et al., 1996; Ringo et al., 1994). Numerous studies have demonstrated that citric acid can improve growth and feed utilization in various fish species (Khajepour and Hosseini, 2012; Baruah et al., 2007). Sugiura et al. (2006) reported that dietary acetic acid decreases stomach pH and slightly reduces that of gut, increasing nutrient utilization of rainbow trout. In this study, fish fed FL15% resulted in a significantly ($P<0.05$) higher growth performance compared to that of FL30%. This difference could have been caused by the total amount of organic acid blend (OAB) present in each diet: 26.80 g kg^{-1} (FL15%) and 96.31 g kg^{-1} (FL30%) (Table 4). OAB contained in fermented lupin produced a positive synergistic effect (Koh et al., 2016) on growth of juvenile Atlantic salmon when fish meal was replaced by the fermented lupin meal at 15% but not at 30%. Positive effects of OAB enormously rely on quantity present in diet as reported by

Katya et al. (2018) who observed that a higher OAB level presence in diet caused a null or detrimental effect in olive flounder, *Paralichthys olivaceus*.

Nutrient digestibility is an important indicator that reflects digestive physiology of fish. In this study, we evaluated ADC of dry matter, protein, lipid, ash, nitrogen-free extract and gross energy. ADC of nutrients in experimental juvenile Atlantic salmon were not compromised when fish meal was replaced by raw lupin meal (at 15% or 30% levels). Although earliest studies have demonstrated the negative influences in nutrient digestion of fish in response to dietary plant proteins, evidences also indicate that raw plant-based protein feeding had no adverse effects on apparent digestibility of nutrients in aquatic animals (Gatlin et al., 2007). Hansen et al. (2006) reported that Atlantic cod may be fed with untreated plant-based feeds up to 44% without any adverse impact to nutrient digestibility. A high inclusion of a mixture of raw materials (soybean, wheat gluten and corn gluten meal) was used in juveniles of turbot (*Psetta maxima*) diets without compromising digestibility and gut histology (Bonaldo et al., 2011).

Use of fermented plant meals has reported certain modifications in nutrient digestibility of fish (Vo et al., 2015). These effects significantly rely on supplementation level of fermented plant meal. In our study, although 30% replacement of fish meal by the fermented lupin meal did not improve ADC of nutrients compared to those shown in C diet, this supplementation level did not negatively compromise digestibility coefficients of nutrients. Neutral results were also found by Rahimnejad et al. (2019) who observed that SSF soybean did not negatively affect digestibility in Japanese seabass (*Lateolabrax japonicus*) fed diets replacing fish meal by fermented soybean meal by *Pseudozyma aphidis* ZR1. In contrast, other studies have found that ADC of nutrients has been improved when supplementing diets with plant meals treated by certain bio-thermal treatments. In our study, we used lupin meal previously submitted to a bio-thermal process. Compared to those values shown in C diet, ADC of protein and nitrogen-free extract was significantly higher in fish fed FL15%. This fact may be explained by the evidence that a combined activity of different enzymes (amylase, cellulose, alginic acid enzyme and protease) enhances in aquatic animals consuming feeds supplemented with fermented meals at certain levels (Wang et al., 2016 b).

In our study, we observed an improvement in the ADC of protein in fish fed diets replacing fish meal by fermented lupin meal at 15%. This benefit could be explained by an OAB present in experimental diet. Most of the OA have the function of stimulating secretion of gastric and digestive enzymes to improve protein digestion (Su et al., 2014). These physiological responses are exposed by previous results demonstrating an improvement in nutrient digestibility of fish fed diets containing organic acid blends (Koh et al., 2016; Ng et al., 2015). After our digestibility trial we observed an improvement in apparent digestibility coefficients of protein and nitrogen-free extract in fish fed diets containing 26.80 g of OAB per kg⁻¹ (FL15%) compared to fish fed a diet containing 96.31 g of the OAB per kg⁻¹ (FL30%) derived from a fermentation process. Koh et al. (2016) observed a higher digestibility of nutrients in red hybrid tilapia (*Oreochromis* sp.) fed diets containing 10 g kg⁻¹ OAB than that recorded in C diet and even to that shown in fish fed diets containing

5 g kg⁻¹. Another study demonstrated that nutrient digestibility was higher in white shrimp (*Litopenaeus vannamei*) fed diets containing 20 g kg⁻¹ of OAB compared to those individuals fed 0, 10 and 40 g of OAB per kg⁻¹ (Romano et al., 2015).

In this research we studied the immune response of fish fed diets formulated with raw and fermented lupin. Previous studies have reported results without negative effects on immunity of fish fed diets formulated with plant meals (Bransden et al., 2001). Fuentes-Quesada et al. (2018) reported no immunological inflammatory response when totoaba (*Totoaba macdonaldi*) was fed diets formulated with unprocessed soybean meal for eight weeks. Similarly, in our study, diets replacing fish meal by 15 or 30% of raw lupin meal, did not compromise immune status of fish. In contrast, our results also revealed that using SSF lupin at 15%, increased lysozyme activity and respiratory burst of juvenile Atlantic salmon. Likewise, Rahimnejad et al. (2019) found that Japanese seabass fed diets replacing fish meal by fermented soybean meal by *Bacillus pumillus* improved lysozyme and complement activity. In our study we observed an improved immunity of juvenile Atlantic salmon, when SSF lupin (fermented with *L. plantarum* 299v), replaced fish meal at 15%. A previous study, using *L. plantarum* P8 to ferment soybean meal supplemented in juvenile turbot (*Scophthalmus maximus* L.) diets reported a significant improved immune response (Wang et al., 2016 b) of this species. Using a mixture of SSF soybean meal and corn gluten meal with *Bacillus subtilis* as fish meal replacer at 30% (FPC-A diet), Moniruzzaman et al. (2017) reported an improvement in superoxide dismutase, lysozyme and myeloperoxidase activities of rainbow trout (*O. mykiss*). This immune stimulation may be attributed to the presence of OA (produced by lactic acid bacteria during fermentation processes), in diets supplemented with SSF plant meals (Ng and Koh, 2016). Immunomodulatory properties of OA in aquatic organisms have been previously demonstrated (Su et al., 2014; Abu-Elala and Ragaa, 2015; Liu et al., 2014). In our study, lysozyme activity of FL15% experimental fish, was significantly higher and respiratory burst presented a higher tendency compared to those values shown in FL30%. This difference could be attained to presence of OAB (26.80 g kg⁻¹ and 96.31 g kg⁻¹) in SSF lupin (15% and 30%, respectively). Immune response of fish, enormously relies on level of OAB present in diets. He et al. (2017) observed that an OAB (composed by 25% of citric acid and 16.7% of ascorbic acid) supplemented at 0.9 g kg⁻¹ and 1.2 g kg⁻¹, produced a significantly higher immune response in Pacific white shrimp (*L. vannamei*) compared to that shown in experimental groups fed supplemented diets with 0.0, 0.3 and 0.6 g kg⁻¹ of the same OAB.

In conclusion, replacement of fish meal by raw and fermented lupin did not compromise growth, nutrient digestibility or immune status of juvenile Atlantic salmon. In our study, it was demonstrated that when supplementing fermented lupin meal at a certain level (15%), growth performance, nutrient digestibility and immune response of juveniles of Atlantic salmon, was improved. While a 30% replacement of fish meal by fermented lupin did not show improvements. These differences may be attributed to different levels of OAB (lactic, citric and acetic acids) derived from fermented lupin meal (with *L. plantarum*) supplemented in each diet. Use of fermented lupin meal may represent a promising alternative for replacing fish meal in aquafeeds.

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