Aquaculture International
https://doi.org/10.1007/s10499-018-0286-7

Wheat grains fermented by fungal mycelia (*Pleurotus ostreatus* or *Lentinus edodes*) as alternative feed ingredients for juvenile rainbow trout (*Oncorhynchus mykiss*)

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Received: 2 November 2017 / Accepted: 23 July 2018/Published online: 01 August 2018

Abstract

We investigated the effects of replacing non-fermented wheat grains with wheat grains fermented by fungal mycelia in the diet of juvenile rainbow trout (*Oncorhynchus mykiss*). We assessed growth performance, feeding parameters, and body composition in three experimental groups (0.33 ± 0.01 g, in triplicates of 50 individuals each). The diets for all the groups contained ca. 43% protein and 19% lipids. Experimental diets were made by replacing the 100 g kg^{-1} of wheat grains present in the basal diet (CTRL) with the same proportion of wheat grains fermented by *Pleurotus ostreatus* (PWD) or *Lentinus edodes* (LWD) mycelium. Fish were fed to apparent satiation twice a day for 56 days. Both, PWD and LWD, significantly increased fish body weight from day 28 onwards. Final body weight was 2.37 ± 0.04 g (CTRL), 4.29 ± 0.02 g (PWD), and 3.50 ± 0.05 g (LWD), and feeding efficiency (%) was increased from 64.5 ± 0.7 (CTRL) to 92.5 ± 0.5 (PWD) and 84.8 ± 1.5 (LWD). The experimental diets also improved nutrient retention efficiency (%): 30.0 ± 0.5 (PWD), 27.7 ± 1.1 (LWD), and 21.0 ± 0.1 (CTRL), for crude protein; 40.3 ± 0.6 (PWD), 31.0 ± 1.8 (LWD), and 16.1 ± 0.7 (CTRL), for ether extract; and 16.1 ± 0.1 (PWD), 14.0 ± 0.3 (LWD), and 11.6 ± 0.6 (CTRL), for phosphorus. Body lipid content was highest for PWD followed by LWD and CTRL (81.4 ± 1.4, 63.2 ± 2.5, 42.3 ± 2.6 g kg^{-1}, respectively), while viscerosomatic index was lowest for PWD ($p < 0.05$). Liver glycogen in LWD and PWD fish (0.62 ± 0.10 and 0.21 ± 0.08% liver weight) was significantly higher than in CTRL fish (0.05 ± 0.01% liver weight). Wheat-mycelium meals appear to be suitable dietary ingredients for improving juvenile rainbow trout growth and nutritional performance. These benefits vary according to the mushroom species used.

Keywords Feed formulation · Growth metrics · Nutrient retention · Proximate composition · Salmonids · Solid substrate fermentation

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Abbreviations

CP	Crude protein
EE	Ether extract
FBW	Final body weight
FD	Feed delivered
FE	Feed efficiency
IBW	Initial body weight
LAB	Lactic acid bacteria
LW	*L. edodes* wheat-mycelium meal
LWD	*L. edodes* wheat-mycelium diet
P	Phosphorus
PW	*P. ostreatus* wheat-mycelium meal
PWD	*P. ostreatus* wheat-mycelium diet
SSF	Solid state fermentation
TGC	Thermal growth coefficient
VSI	Viscerosomatic index
WW	Whole wheat grains

Introduction

The nutritional properties of edible mushrooms including oyster mushroom (*Pleurotus ostreatus*) and shiitake (*Lentinus edodes*) have been confirmed through intensive research (see reviews by Bisen et al. 2010; Deepalakshmi and Mirunalini 2014). In humans, the consumption of mushrooms leads to significant nutritional improvements associated with their high biological value proteins, polysaccharides, dietary fiber, vitamins, and minerals (Wani et al. 2010; Schneider et al. 2011; Cohen et al. 2014). In addition, mushrooms contain diverse biologically active compounds with medicinal properties (Aida et al. 2009; Hearst et al. 2009; Wasser 2014).

Feeds supplemented with whole fruiting bodies, extracts, or purified β-glucans from *P. ostreatus* (Kamilya et al. 2006; Dobsíková et al. 2013; Bilen et al. 2016), *L. edodes* (Nikl et al. 1991; Djordjevic et al. 2009; Baba et al. 2015), and *Ganoderma lucidum* (Yin et al. 2009; Chang et al. 2013; Liu et al. 2015) have been shown to improve the health condition of economically important fish species by reinforcing antioxidant defenses, immune response, and/or disease resistance. However, there are scarce reports on the effects of mushrooms on fish growth performance and feeding parameters. In a recent study, Pascual et al. (2017) have found that a diet supplemented with fruiting bodies of *Grifola gargal* enhances growth, lipid content, and nutrient retention in juvenile rainbow trout (*Oncorhynchus mykiss*). Incorporation of *Pleurotus* spp. extract or fruiting bodies in the diet slightly improves growth parameters in *O. mykiss* and other fishes (Ahmed et al. 2015; Bilen et al. 2016; Van Doan et al. 2016), while there are no successful antecedents about *L. edodes* effects on fish growth (Djordjevic et al. 2009). A deeper knowledge of mushroom effects on cultured fish would contribute to the development of quality dietary supplements to be incorporated into the formulation of a well-balanced diet.

Biotransformation of vegetable substrates by microorganisms is often used to increase nutritional and functional properties, such as flavor, aroma, texture, and nutrient bioavailability in the original food (Steinkraus 1994; Nout and Kiers 2005). Since the mid-1970s, solid-state

fermentation (SSF) has been used for the development of industrial bioprocesses (Rodríguez Couto and Sanromán 2006; Singhania et al. 2009; Thomas et al. 2013), including the biotransformation of crops and crop residues for detoxification and nutritional enrichment (Cohen et al. 2002; da Luz et al. 2013; Nasehi et al. 2017). SSF is defined as any fermentation process on a non-soluble material that acts both as a physical support and as a source of nutrients, in absence of free flowing liquid (Pandey 1992). Although yeast and some bacteria are suitable for SSF, filamentous fungi are normally used for this process since their mycelia can grow over particle surfaces in a low moisture environment, secreting enzymes, and specific bioactive compounds to ferment those solid substrates (dos Santos et al. 2004; Singh nee' Nigam 2009; Hole et al. 2012). Cereals, legumes, and oil seeds are particularly suitable as substrates for fungal SSF (reviewed by Gowthaman et al. 2001 and Gan et al. 2017). Particularly, wheat grains' (*Triticum* spp.) antioxidant properties and total phenolic content are improved by SSF with filamentous fungi, such as molds (Hyphomycetes) (Bhanja Dey et al. 2009; Bhanja Dey and Kuhad 2014; Sandhu et al. 2016) and mushrooms (Basidiomycetes and Ascomycetes) (Zhang et al. 2012; Postemsky and Curvetto 2014; Subramaniam et al. 2014). In addition, Skrede et al. (2002) have reported that the incorporation of whole wheat flour fermented by lactic acid bacteria (LAB) to salmonid diets improves nutrient digestibility. However, to our knowledge, there are no reports about the use of wheat grains fermented by edible mushrooms in the formulation of animal feeds. In this study, we analyzed the effects of the inclusion of wheat-mycelium mixture obtained by SSF with *P. ostreatus* or *L. edodes* in the diet of juvenile rainbow trout. We replaced the wheat grains, which are included at 100 g kg^{-1} in the basal diet routinely used in the hatchery, with wheat grains fermented by either *P. ostreatus* or *L. edodes* (experimental diets) and studied growth performance, nutrient retention, and viscerosomatic index in juvenile rainbow trout.

Materials and methods

Preparation of wheat-mycelium meal

Whole wheat grain (WW) inoculated with the basidiomycetes *Pleurotus ostreatus* var. *florida* (strain A01) or *Lentinus edodes* (strain B01) was purchased from CISPHoCoMe (*Centro de Investigación y Servicios para la Producción de Hongos Comestibles y Medicinales*, Argentina). Mycelium development and wheat biotransformation (SSF) were performed in closed bags, during 40 days at 20–24 °C in a dark room. After this period, the *P. ostreatus* or *L. edodes* wheat-mycelium mixtures (PW or LW, respectively) were dried at 60 °C, grounded to powder, and stored at − 18 °C. The proximate compositions of both products were analyzed as described below and are detailed in Table 1.

Experimental diet formulation

The basal diet was formulated in our laboratory, according to Hardy (2002), to meet the nutrient requirements of rainbow trout. White fish meal (crude protein 596 g kg^{-1}) and fish oil were supplied by AGUSTINIER S.A. (Argentina). Blood meal and hydrolyzed feather meal were purchased from POLLOLIN S.A. (Argentina). Experimental diets (PWD and LWD) were prepared by replacing 100 g kg^{-1} of whole wheat in the basal diet (CTRL) for equal amounts of PW or LW meal. Control and experimental feeds were prepared by mixing the

Table 1 Proximate analysis of whole wheat grains (WW) biotransformed by *P. ostreatus* (PW) or *L. edodes* (LW) mycelia on dry weight basis

Proximate composition (g kg^{-1})	WW	PW	LW
Crude protein (CP)[1]	140.5	119.4	117.2
Ether extract (EE)	18.2	19.5	19.0
Ash	17.3	25.0	23.6
Nitrogen-free extract (NFE)[2]	824.0	836.1	840.1
Total nitrogen (TN)	24.1	20.5	20.1

[1] CP was estimated using the wheat grain conversion factor (5.83), according to Merrill and Watt (1973)

[2] NFE = total − moisture − CP − EE − ash

ingredients and adding water to form a paste, which was then pelletized and dried in a hot air column. The resulting pellets were crumbled, sieved to obtain 0.7–1.2-mm fragments, and stored at 4 °C. All diets were designed to be isonitrogenous, isolipidic, and isoenergetic. The feed ingredients and proximate composition are summarized in Table 2.

Table 2 Formulate and proximate composition of the experimental diets

Diets	CTRL	PWM	LWM
Ingredients (g kg^{-1})			
Fishmeal, white	570	570	570
Fish oil	160	160	160
Whole wheat	149	49	49
P. ostreatus wheat mycelium	0	100	0
L. edodes wheat mycelium	0	0	100
Blood meal	50	50	50
Feather meal	50	50	50
Vitamin and mineral premix[1]	10	10	10
Choline chloride	5.0	5.0	5.0
L-Lysine	4.0	4.0	4.0
L-Methionine	1.0	1.0	1.0
Ascorbic acid	1.0	1.0	1.0
Carboxymethyl cellulose	20	20	20
Proximate composition (g kg^{-1})			
Moisture	58.5	55.4	59.3
Crude protein (CP)[2]	423	431	436
Ether extract (EE)	180	198	184
Ash	164	161	162
Phosphorus (P)	26.0	25.5	26.2
Nitrogen-free extract (NFE)[3]	175	155	158
Gross energy (kJ g^{-1})[4]	20.1	20.6	20.3

[1] Vitamin and mineral premix contains (as g kg^{-1} premix) vitamin A, 2400000 IU; vitamin D_3, 480,000 IU; vitamin E, 50000 IU; thiamine, 2; riboflavin, 4; pyridoxine, 3; pantothenic acid, 8; biotin, 0.2; niacin, 30; folic acid, 1.2; cyanocobalamin, 0.006; ascorbic acid, 30; menadione, 1.6; sodium selenite, 0.06; potassium iodide, 0.35; cupric sulfate pentahydrate, 1; zinc sulfate heptahydrate, 14; manganese sulfate monohydrate, 10; ferrous sulfate, 20; cobalt chloride, 0.4

[2] Crude proteins (CP) were estimated using 6.25 as conversion factor

[3] NFE = total − moisture − CP − EE − ash

[4] Gross energy was calculated as 23.6, 39.5, and 17.2 kJ g^{-1} of CP, EE, and NFE, respectively (NRC 1993)

Chemical analysis

Chemical analysis of WW, PW, and LW, basal and experimental diets, and fish whole body were performed by standard procedures according to AOAC (1990). Total nitrogen (TN) was determined using the semi-micro Kjeldahl method and crude protein (CP) was estimated using 6.25 × TN. For WW, PW, and LW analysis, a conversion factor of 5.83 was used according to Merrill and Watt (1973). Ether extract (EE) was measured gravimetrically following sample extraction of 1 g in petroleum ether, using a Soxhlet apparatus. Moisture and ash were measured gravimetrically, the former after drying in an oven at 105 °C for 3 h and the latter by combustion in a muffle at 550 °C for 6 h. Nitrogen-free extract (NFE) was calculated by difference (total − CP − EE − ash − moisture content). To estimate the gross energy of each diet, the corresponding CP, EE, and NFE were multiplied by 23.6, 39.5, and 17.2 kJ g^{-1}, respectively (NRC 1993). Total phosphorus was assayed by wet digestion of the samples with HNO_3 + $HClO_4$, and detection was made with the ascorbic acid method (Takeuchi 1988).

Fish and experimental design

O. mykiss fry were obtained from the hatchery of *Centro de Ecología Aplicada del Neuquén* (CEAN). The trials were carried out in 50-L PVC containers supplied with a constant freshwater flow from the Chimehuín River and maintained in a 14:10 light:dark cycle. During the experiment, water quality conditions were as follows: daily mean temperature 11.3 ± 1.7 °C (min. 8.1, max. 14.4 °C), pH 6.8 ± 0.2, conductivity 76 ± 19 μs cm^{-1}, and dissolved oxygen above 94%. A total of 450 fry were randomly distributed into 9 groups of 50 individuals (16.4 ± 0.2 g total biomass per container). The initial body weight (IBW) was 0.33 ± 0.01 g. At the beginning of the experiment, an extra sample of 50 fry (day 0—initial sample) was euthanized by benzocaine overdose, stored at − 20 °C, chopped, and used for whole-body proximate composition chemical analysis. Three diets were used for the experiment (CTRL, PWD, and LWD), following a randomized design of triplicates. Fish were hand-fed to apparent satiation twice a day (except on Sundays), at 10:00 and 17:00 h for 56 days (austral spring season, October to December). Special care was taken to ensure that fish had eaten all the feed. Throughout the experiment, besides food deprivation every Sunday, once every 2 weeks, all the fish in each container were starved from Saturday 17 h to Monday 10 h, anesthetized with 100 ppm benzocaine, counted, and weighted to calculate total biomass. Dead fish were counted daily at feeding times in order to calculate percentage survival by container as (100 × final fish number / initial fish number). The experimental protocols were approved by the Bioethics Committee, School of Biochemical and Pharmaceutical Sciences, National University of Rosario, Argentina (6060/116).

Growth estimation, feeding parameters, and nutrient retention

At the end of the experiment (16 h after the last meal), all fish in each container ($n = 3$) were weighed and counted to calculate the following parameters: Final body weight (FBW) was estimated by dividing total fish biomass by the number of fish; thermal-unit growth coefficient (TGC) was calculated as $1000 \times (FBW^{0.209} - IBW^{0.209}) / (T \times D)$, where T is the mean daily temperature in °C and D is the number of days between measurements (Dumas et al. 2007). Feeding efficiency (FE) was calculated as $100 \times (\text{fish biomass}_{\text{day } 56} + M - \text{fish biomass}_{\text{day } 0}) /$

FD, where FD is the feed delivered between measurements (g) and M is the body weight (g) of the dead fish.

Nutrient retention efficiency for CP, EE, and phosphorus (in %) was calculated for each container ($n = 3$) as $100 \times [((\text{fish biomass}_{\text{day 56}}\ (\text{g}) \times \text{fish nutrient content}_{\text{day 56}}\ (\text{g kg}^{-1})) - (\text{fish biomass}_{\text{day 0}}\ (\text{g}) - \text{fish nutrient content}_{\text{day 0}}\ (\text{g kg}^{-1}))) / (\text{total feed delivered}\ (\text{g}) \times \text{diet nutrient content}\ (\text{g kg}^{-1}))]$ (Glencross et al. 2004).

Sample collection and liver glycogen

At the end of the experiment, a sample of 20 fish per container ($n = 3$) was euthanized by benzocaine overdose, chopped, and used for whole-body proximate composition chemical analysis. Another five fish per container ($n = 3$) were randomly selected and euthanized by benzocaine overdose. Then, these were individually weighed (g) and their abdominal cavity was opened. The whole digestive tracts and livers were dissected and weighed (g) to calculate viscerosomatic index (VSI) as 100 × (viscera weight (including liver) + perivisceral adipose tissue weight) / body weight.

Glycogen content in liver was determined according to Van Handel (1965). The livers of two individuals per tank ($n = 3$) were excised, weighted, and immediately frozen at − 20 °C. Tissues were then homogenized in PBS, and glycogen was extracted and precipitated with ethanol 96% in an electrolyte solution. Spectrophotometric measurements were carried out at 620 nm, after hydrolysis in acid anthrone reactive (0.2% in H_2SO_4) for 10 min. Glycogen content (mg) was calculated using a glucose standard curve, and results were expressed as percentage of fresh liver weight.

Middle intestine histology

To assess possible alterations in the mid intestine tissue caused by the diets, a histological examination was performed at the end of the feeding trial. Three fish from each container ($n = 3$) were randomly selected and euthanized by benzocaine overdose. Then, approximately 1 cm of mid intestine was immediately removed from each fish, rinsed in ice-cold Cortland saline (pH 7.4, plus 5 mM HCO_3Na and 5.55 mM glucose), and fixed overnight in Bouin's solution. Samples were stored in 70% ethanol until dehydrated and embedded in paraffin according to standard histological techniques. Cross sections of 3–5 μm thickness were obtained and stained with Masson's trichrome, periodic acid-Schiff (PAS), and Alcian blue (pH 2.5). Stained sections (2–3 by fish) were observed and photographed with a light microscope (Nikon Eclipse E600).

Integrity of microvilli and infiltrated cells into the mucosal layer were evaluated. Lamina propria and submucosal connective tissue thickness were assessed based on the criteria adopted by Barnes et al. (2014) for histological sections of distal intestine of rainbow trout.

The PAS and Alcian blue stained sections were used for counting goblet cell with neutral or acid mucopolysaccharides in the tunica mucosa layer at × 200 magnification using the ImageJ 1.49v software. Quantification was carried out in areas where the cutting plane of villi allows distinguishing the simple epithelium as a single layer of tall columnar cells (enterocytes) over a clear lamina propria. The quantitative data were reported as mean number of goblet cells per linear millimeter of mucosal surface. For each section, a distance between 2.2 and 5.7 mm was used.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM), $n = 3$. Normal distribution and homogeneity of variance were checked by Kolmogorov-Smirnov's and Bartlett's tests, respectively. Diet effects on growth at different times were analyzed by repeated measures two-way ANOVA and Bonferroni's post hoc comparisons. One-way ANOVA and Tukey's post hoc comparisons were applied to identify differences among diets for all the other variables. Proportion and percentage data were normalized by the arcsine square root transformation. We considered a value of $p < 0.05$ as statistically significant (Zar 1999).

Results

Growth performance

Two-way ANOVA showed a statistically significant interaction between diet and time factors in the fish's average body weight ($p < 0.0001$). As shown in Fig. 1, from day 28 onwards, fish fed with both experimental diets showed higher growth rates than CTRL fish ($p < 0.001$ and $p < 0.05$, for PWD and LWD, respectively). Besides, average body weight was higher in fish fed with PWD than in those fed with LWD ($p < 0.001$). At the end of the trial (day 56), the observed differences in growth performance were reflected in FBW ($p < 0.001$, Table 3). Accordingly, the TGCs calculated were 0.64 ± 0.01 (CTRL), 0.89 ± 0.01 (PWD), and 0.80 ± 0.01 (LWD) and were significantly different among all diets ($p < 0.001$). No significant differences among diets were detected in fish percentage survival (Table 3).

Estimations of VSI showed a significantly lower value in fish fed with PWD (14.3 ± 0.5%) when compared to those fed with CTRL (16.9 ± 0.6%, $p < 0.05$, Table 3). Liver glycogen in LWD fed fish was significantly higher than in CTRL ($p < 0.001$) and PWD fed fish ($p < 0.05$). Glycogen content for PWD fish was also significantly higher than that for the CTRL ($p < 0.01$, Table 3).

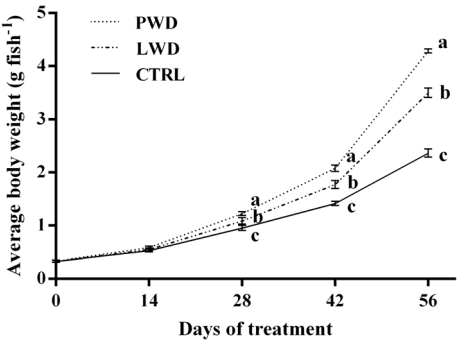

Fig. 1 Growth curve of *Oncorhynchus mykiss* fed with wheat mycelium supplemented (PWM - LWM) or basal (CTRL) diets during 56 days. The average body weight was calculated dividing total biomass per fish number in each container ($n = 3$). Values are expressed as mean ± SD. Different letters indicate significant differences among diets on the same day (Bonferroni's post-test, $p < 0.05$)

Table 3 Growth parameters, feeding performance, and liver glycogen analysis of *O. mykiss* fed with supplemented (PWM and LWM) or basal (CTRL) diets during 56 days

		CTRL	PWM	LWM	ANOVA *p*
IBW	g fish^{-1}	0.33 ± 0.01	0.33 ± 0.01	0.33 ± 0.01	0.963
FBW	g fish^{-1}	2.37 ± 0.04^{a}	4.29 ± 0.02^{b}	3.50 ± 0.05^{c}	< 0.001
Survival	%	98.7 ± 0.7	98.0 ± 1.2	96.7 ± 0.7	0.420
FD	g	158 ± 3^{a}	209 ± 1^{b}	184 ± 3^{c}	< 0.001
FE	%	64.5 ± 0.7^{a}	92.5 ± 0.5^{b}	84.8 ± 1.5^{c}	< 0.001
Glycogen[1]	%	0.05 ± 0.01^{a}	0.21 ± 0.08^{b}	0.62 ± 0.10^{c}	< 0.01

Data are presented as mean values ± SEM of three replicates. Different superscript letters in the same row represent significant differences among groups by Tukey test ($p < 0.05$)

IBW initial body weight, *FBW* final body weight, *FD* feed delivered, *FE* feeding efficiency

[1] % of fresh liver weight

Feeding performance, proximate analysis, and nutrient retention ratio

FD was highest in the group fed with PWD and lowest in the CTRL group with significant differences among the three groups ($p < 0.001$). The FE value obtained for fish fed with CTRL was significantly improved by both experimental diets ($p < 0.001$), and FE of PWD was higher than that of LWD ($p < 0.01$, Table 3).

The whole-body proximate composition of the studied groups is shown in Table 4. There were no significant differences in CP, ash, and phosphorus contents among groups. However, EE was significantly increased ($p < 0.001$) in fish fed with both PWD and LWD, when compared with CTRL (Table 4). EE was also significantly higher in fish fed with PWD than in those fed with LWD ($p < 0.01$). Moisture content of fish receiving PWD decreased significantly ($p < 0.01$) when compared with those receiving CTRL and LWD (Table 4).

The nutrient retention ratios for CP, EE, and phosphorus were significantly improved ($p < 0.01$) by both experimental diets with respect to CTRL. As shown in Table 4, CP retention was improved by PWD and LWD (30.0 ± 0.5 and 27.7 ± 1.1%, respectively) with respect to the CTRL (21.0 ± 0.1%). EE retention in fish fed with PWD (40.3 ± 0.6%) was significantly higher ($p < 0.01$) than that for fish fed with LWM (31.0 ± 1.8%), and both were higher than that obtained for the CTRL (16.1 ± 0.7%). In the same way, phosphorus retention was highest

Table 4 Whole-body proximate composition analysis and nutrient retention rates of *O. mykiss* fed with supplemented (PWD and LWD) or basal (CTRL) diets during 56 days

		Initial	CTRL	PWD	LWD	ANOVA *p*
Moisture	g kg^{-1}	840.0	793.0 ± 8.0^{a}	748.0 ± 1.0^{b}	763.7 ± 2.7^{a}	< 0.01
CP	g kg^{-1}	110.0	133.9 ± 2.5	137.6 ± 2.9	139.3 ± 3.0	0.521
EE	g kg^{-1}	25.9	42.3 ± 2.6^{a}	81.4 ± 1.4 ^{b}	63.2 ± 2.5^{c}	< 0.001
Ash	g kg^{-1}	14.1	24.1 ± 1.3	22.9 ± 0.1	22.7 ± 0.1	0.887
P	g kg^{-1}	2.37	4.15 ± 0.15	4.27 ± 0.03	4.17 ± 0.03	0.454
CP retention (%)			21.0 ± 0.1^{a}	30.0 ± 0.5^{b}	27.7 ± 1.1^{b}	< 0.005
EE retention (%)			16.1 ± 0.7^{a}	40.3 ± 0.6^{b}	31.0 ± 1.8^{c}	< 0.001
P retention (%)			11.6 ± 0.6^{a}	16.1 ± 0.1^{b}	14.0 ± 0.3^{c}	< 0.001

Data are presented as mean values ± SEM of three replicates. Different superscript letters in the same row represent significant differences among groups by Tukey test ($p < 0.05$)

CP crude protein, *EE* ether extract, *P* phosphorus

in PWD fed fish (16.1 ± 0.1%), followed by LWD (14.0 ± 0.3%), and both were significantly higher ($p < 0.01$) than that in CTRL fish (11.6 ± 0.6%, Table 4).

Middle intestine histology

Histological examination of trout mid intestine did not reveal differences between experimental diets. In the tunica mucosa layer, normal enterocytes with basal nuclei were observed and microvilli were visible as a PAS positive continuous border without disruptions. Infiltrated cells were equally observed in all the studied sections (Fig. 2a).

The evaluation of goblet cell number showed no differences among diets, neither with PAS nor with Alcian blue stains ($p = 0.942$ and 0.972, respectively). The values obtained (in cells/linear mm) for PAS were 21.2 ± 4.5 (CTRL), 20.8 ± 3.7 (PWD), and 19.6 ± 1.7 (LWD) and for Alcian blue were 21.4 ± 1.7 (CTRL), 20.9 ± 1.0 (PWD), and 20.8 ± 2.7 (LWD). There were no evident differences in lamina propria and submucosal connective tissue thickness among diets. Representative micrographs are shown in Fig. 2.

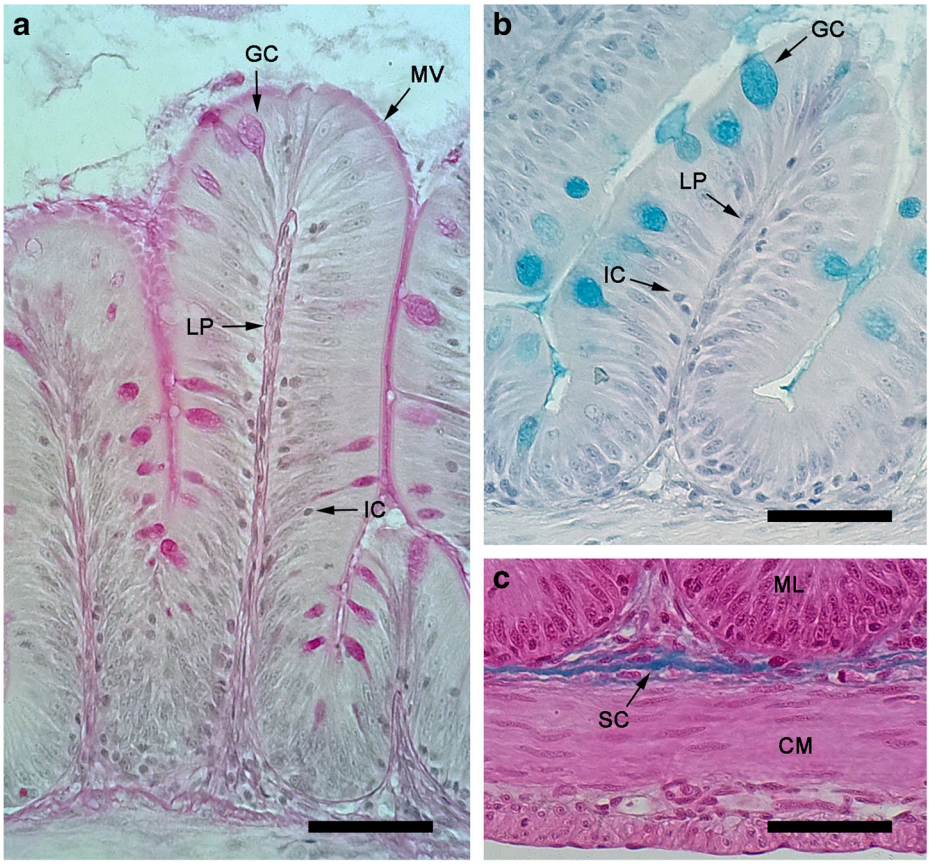

Fig. 2 Cross sections of *Oncorhynchus mykiss* mid intestine. No histological differences were observed between diets. Representative micrographs of mucosal and submucosal layers stained with periodic acid-Schiff's (PAS) reaction (**a**), Alcian blue (pH 2.5) (**b**), or Masson's trichrome (**c**). CM, circular muscle; GC, goblet cell; IC, infiltrated blood cell; LP, lamina propria; ML, mucosal layer; MV, microvilli; SC, stratum compactum. Scale bar 50 μm

Discussion

In this study, we modified the diet of juvenile rainbow trout by replacing 2/3 of the whole wheat used in the basal diet (10% of the total) with wheat grains biotransformed by mycelia of the edible mushrooms *P. ostreatus* and *L. edodes*. We show, for the first time, that the inclusion of wheat biotransformed by *P. ostreatus* and *L. edodes* significantly improves growth performance. There are only few previous studies dealing with the effects of these mushroom species on fish growth, without promising results. *P. ostreatus* byproducts fermented by LAB included as new dietary ingredient to replace fish meal produced negative effects on fish growth performance (Katya et al. 2014). In contrast, Bilen et al. (2016) obtained slightly positive effects on rainbow trout growth and feed conversion ratio using methanolic extracts of *P. ostreatus* fruit body. For *L. edodes*, Djordevic et al. (Djordjevic et al. 2009) found no significant effects on *O. mykiss* growth rate upon dietary administration of purified β-glucan (Lentinan).

The FE value obtained for the CTRL diet was lower than expected (Woynarovich et al. 2011), while TGC was within the confidence interval calculated from the data published by Dumas et al. (2007) for *O. mykiss* smaller than 20 g, although near the lower limit. This relative underperformance in the control group could be related to the low CP content of our control and experimental diets (c.a. 43% compared with the optimum range of 45–50% for *O. mykiss* of the same stanza (Hardy 2002)). LWD and PWD diets increased both FD and FE with respect to CTRL, which supports the important improvements registered in growth performance (25–38% in TGC and 47–81% in FBW for LWD and PWD, respectively). Besides, fish receiving wheat-mycelium diets showed higher macronutrient retention rates than CTRL fish, which suggest an improvement in nutrient utilization. These fish were more effective to convert feed's protein into body weight, as suggested by the increased protein retention efficiency (CP retention rate), and had higher lipid retention rates than CTRL. Accordingly, Skrede et al. (2002) showed that LAB fermentation of whole wheat improves the digestibility of lipids and energy in salmon diets. However, nutrient digestibility of the new ingredients proposed in this study should be assessed in order to determine their nutritional value and appropriate inclusion levels in formulated diets (Glencross et al. 2007).

In addition, both wheat-mycelium diets increased the lipid content of trout's whole-body composition (assessed as EE) without a significant effect on the protein content when compared with fish receiving CTRL diet. Lipid content of fish fed with PWD and LWD was 90 and 50% higher than that of CTRL fish. This could be explained by the observed reduction in moisture in fish fed with both experimental diets. However, it is interesting to notice that PWD significantly reduced VSI with respect to the CTRL diet, suggesting that the increase in lipid content produced by this supplement occurred in the carcass and not in the perivisceral adipose tissue. The reduction of visceral percentage in fish is encouraged since the viscera are discarded during slaughter, and this process directly influences economic returns in trout production (Kause et al. 2016).

The comparison between both experimental diets showed that the benefits associated to dietary inclusion of wheat mycelia were dependent on the mushroom species used for fermentation. Besides the inherent differences in the nutrient composition and antioxidant properties of fruit bodies and mycelia of both species (Lobanok et al. 2003; Reis et al. 2012; Cohen et al. 2014), we observed no differences in proximal composition between both wheat-mycelium meals. Although we measured a c.a. 16% lower CP content in the wheat-mycelium meals than in the whole wheat meal, which is probably an error due to poor homogenization,

the final CP contents of the wheat-mycelium and control feeds were almost identical (Tables 1 and 2). However, a more detailed characterization of wheat-mycelium meals is needed since differences in bioactive compounds, e.g., antioxidant activity, GABA, and vitamin levels, have been reported for wheat fermented with different mushroom species (Postemsky and Curvetto 2014; Subramaniam et al. 2014; Gan et al. 2017). In our study, incorporation of PW-fermented wheat produced a superior performance than LW-fermented wheat in all measured parameters, with the exception of liver glycogen. We observed that both diets promoted the deposition of liver glycogen reserves, with increases of 12-fold for LWD and 4-fold for PWD with respect to the CTRL. These results are difficult to compare with the literature, since the antecedents on liver glycogen content in *O. mykiss* fry are scarce. For farmed juvenile trout (14 g body weight), Boujard and Leatherland (1992) have reported a range between 3 and 7% fresh liver weight, which is about 100- and 8–24-fold higher than our results for CTRL and experimental diets, respectively. This difference could be explained by differences in fish size/developmental stage (2–4 g body weight in this study) (Gilmour et al. 2012) or in diet formulation (Hilton and Dixon 1982). Nevertheless, the differences among diets in our experiment cannot be explained by size difference, since fish fed with LWD have lower final weight than those fed with PWD and have 3-fold higher liver glycogen content. This effect is more probably explained by the presence of different bioactive compounds in these two mushroom species. On the other hand, the method for processing starch in fish feed elaboration has been reported to affect glycogen deposition in liver by modifying their digestible carbohydrate levels (Hilton and Slinger 1981; Kim and Kaushik 1992; Hemre et al. 2002). In this regard, Skrede et al. (2002) have shown that LAB fermentation of wheat whole flour improves the digestibility and utilization of starch in salmon (*Salmo salar* L.) although these authors have not reported glycogen values. In this context, our results suggest that dietary replacement of wheat grains with biotransformed wheat grains increases the digestible carbohydrate levels of the diet and/or modulate metabolic processes which lead to enhanced growth performance and liver glycogen reserves. Furthermore, these metabolic effects depend on the mushroom species used for biotransformation.

In this study, no negative effects on survival were observed in *O. mykiss* fed with CTRL or experimental diets. In addition, there were no histological alterations in mid intestine preparations from fish receiving experimental or CTRL diets. Accordingly, Uluköy et al. (2016) did not find histological changes in the intestine of *O. mykiss* receiving diets with *L. edodes* water extracts for 6 weeks.

In conclusion, the inclusion of wheat grains biotransformed by *P. ostreatus* and *L. edodes* in fish feed promotes growth, feeding performance, and liver glycogen reserves of juvenile rainbow trout. The in vivo benefits observed in this study support the idea that mycelium-fermented wheat would be a suitable option to incorporate edible mushroom properties into fish diet. In addition, the improvement of feeding performance and nutrient retention would imply environmental benefits such as decreased nutrient loads in aquaculture effluents.

Acknowledgements The authors wish to thank Pablo Morzenti and Antonio Miloro for their contributions to this work. We acknowledge the helpful suggestions of two anonymous reviewers.

Funding information This study was supported by the grants CONICET PIP 11220130100529 and ANPCYT PICT 2013-1415 to CML. This study also received funding from *Ministerio de Seguridad and Trabajo y Ambiente de la Provincia de Neuquén*.

Compliance with ethical standards The experimental protocols were approved by the Bioethics Committee, School of Biochemical and Pharmaceutical Sciences, National University of Rosario, Argentina (6060/116).

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