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Supplemented feed with biological silage of fish-processing wastes improved health parameters and weight gain of mice

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ABSTRACT

Wastes from *Merluccius hubbsi* processing were used for biological silage elaborated with *Lactobacillus arizonensis* and for chemical silage performed with 0.18M sulfuric acid and 0.22M formic acid. Mice BALB/c were fed with isoenergetic diets, EFBS and EFCS, containing 36.3% (wt/wt) biological fish silage and 36.3% (wt/wt) chemical fish silage respectively. Promisingly, after 30 day consumption both additives did not provoke lesions in the gut, thinner wall, distension or abnormal vascularization. The higher concentration of lactic acid bacteria (LAB) in the gut of mice fed with EFBS (2.51×10^4 cfu LAB/g EFBS vs. 3.98×10^3 cfu LAB/g EFCS), together with the weight gain (23.8 ± 3.8 g vs. 16.7 ± 3.7 g), feed conversion ratio (4.12 vs. 6.71), protein efficiency rate (0.69 vs. 0.63), villi height (455 μm vs. 418 μm) for EFBS and EFCS respectively, support the probiotic effect of *L. arizonensis*. Nevertheless, both preparations are interesting options to envisage a promising outcome for recycling fish wastes.

1. Introduction

Harbor wastes generate larger amounts of effluents and in the absence of suitable systems for wastes management and treatment, a negative environmental impact is expected. As an example, in Patagonia (south Argentina), fish-processing wastes (e.g. fish heads, frames, and offal) are opencasts deposited, storm-water running off and leachate flows from opencast deposits creating a risk to receiving contaminated surface waters, groundwater or soil. The anaerobic conditions cause undesirable odor mainly generated by gases (e.g. methane and hydrogen sulphide) and volatile fatty acids (Groch, 2001). The negative impact also have touristic implications, since these big deposits promoted the overpopulation of seagulls (birds family *Laridae*), and these birds bite the whale calves provoking blooding animals and dead animals (Yorio and Giaccardi, 2002). Fishmeal production would be the main option to overcome the problem, since it is a valuable source of protein for livestock. However, harbors are situated in areas where the basic infrastructure is lacking; the introduction of sophisticated systems for wastes treatment may not be a viable option due to the costs involved. Therefore, fish silage represents an important option and a source of protein that can be used in large scale for replacing fishmeal.

The biological fish silage for animal feed has been mainly evaluated as a stable substitute of proteins, with low consideration to the benefits derived from the probiotic effect of properly selected LAB. This supplement could be added into the feed to provide additional advantages from the nutritional and sanitary aspects (e.g. increment of digestibility, contribution of vitamins, and protective activity against pathogenic bacteria) (Castellano et al., 2008). In animal production, the preventive use of antibiotics provokes lower yields and it is mainly related to intestinal illnesses. On the other hand, the antibiotics-resistant pathogenic microorganisms and the residual effects of antibiotic in humans have encouraged alternatives, as the probiotics and probiotic-prebiotics combinations (Ndaw et al., 2008). The strain L. *arizonensis* was selected among several LAB as the more suitable for silage of wastes from the processing of *M. hubbsi*, considering the kinetics of acidification and the lower optimum temperature for the process (28 °C), this strain constitute a promising alternative for opencast fish fermentation at locations with temperate to cold climes (Góngora et al., 2012). On the other hand, chemical silage would offer another option for the treatment of fish wastes; giving a stable product whose protein content is similar to that of the raw material, produced at lower cost and energy in comparison to the production process of fishmeal. Herein, we studied the performance of the biological fish silage and the chemical fish silage, performed with wastes from *M. hubbsi* processing, as feed additive on the diet of mice BALB/c.

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2. Materials and methods

2.1. Fish silages

Wastes of *M. hubbsi* (trimmings, heads, frames, fins, skin, and viscera) were purchased immediately after processing from an industrial plant located in Chubut, (Argentine). The samples were transported and kept at 4 °C, processed within 12 h. For biological fish silage (BFS), the material was supplemented with 25 g/L sucrose, sterilized (121 °C, 2 h) and inoculated with 25 mL/L of a 16 h old-culture of NRRL B-14768 *L. arizonensis* performed in Man Rogosa Sharpe (MRS) medium at 30 °C. The fermentation was carried out in a 10 L working volume stirred reactor for 24 h at 29 ± 1 °C. Unsterile material was acidified by adding 0.18 M sulfuric acid and 0.22 M formic acid for chemical fish silage (CFS).

3. Experimental feeds

Isoenergetic (12 MJ/kg) diets, containing constant protein concentration (23 % wt/wt), were designed according to National Research Council (1995). Control feed (CF) was performed with sunflower meal and soy meal as protein source. The compositions of the experimental feed with chemical fish silage (EFCS) and experimental feed with biological fish silage (EFBS) are shown in Table 1.

A mixer was used to include the silage to the dry ingredients and it was pelleted at 55 °C reaching 10% (wt/wt) moisture. The initial silage concentration was 10% (wt/wt) on the humid mixture and it reaches 36.3% w/w after the drying process.

For counting colony forming units of LAB (CFU/mL), the samples were centrifuged at 3000 rpm for 15 min and washed twice with 100 mM sterile phosphate-buffered saline (PBS) (pH 7.0). Dilutions of the suspension (0.1 mL) were inoculated in MRS agar plates and incubated 24-48 h at 37 °C (Kacem and Karam, 2006). The feed (CF, EFCS and EFBS) moisture, crude protein, ethereal extract, ash, crude fiber, calcium and phosphorus concentrations were analyzed according AOAC standard methods (Table 2).

4. Feeding trials

Three weeks old BALB/c mice were maintained between 18 and 20 °C and 60–80% (wt/wt) relative humidity, with a 12 h light–dark cycle. After feeding for 1 week on a basal diet, mice were randomly divided and kept in group cages (n = 5) with males or females and fed with the experimental diets (CF, EFCS and EFBS). Water and feed were administered *Ad libitum* and daily intake was gravimetrically controlled. The animals were weighed using an analytic scale and the cleaning and changed of sawdust bed was carried out every 3 days. After 4 weeks, each animal was placed in an aseptic chamber to collect stool samples (150 mg), suspended in 1.5 mL of sterile PBS (pH 7.2) and properly diluted in the same buffer for LAB counting (Dalloul et al., 2003).

Table 1
Feed composition: control (CF), feed containing chemical fish silage (EFCS) and feed containing biological fish silage (EFBS).

	CF	EFCS	EFBS
	Concentrations (%)		
Wheat middling	29.8	41.2	41.2
Sunflowers meal	35.3	19.7	19.7
NaCl	0.75	0.7	0.7
Sunflowers oil	0	1.35	1.35
Soybean meal (10% wt/wt moisture)	31.4	0	0
CFS (10% wt/wt moisture)	0	36.3	0
BFS (10% wt/wt moisture)	0	0	36.3
Dicalcium phosphate anhydrous	2	0	0
Polivitaminic preparation (Rosenbuch®)	0.75	0.75	0.75

Table 2
Composition of isoenergetic diets (12 MJ/Kg). CF: control feed, EFCS: experimental feed with chemical fish silage, and EFBS: experimental feed with biological fish silage. In brackets: number of the AOAC method used.

	CF	EFCS	EFBS
	Concentrations (%)		
Moisture (*934.01*)	10	10	10
Protein (*981.10*)	23	23	23
Ethereal extract (*922.06*)	14.7	13.7	14
Crude fiber (*962.09*)	9.0	6.7	6.5
Ash (*942.05*)	4.5	4.3	4.2
Free nitrogen extract	38.8	42.3	42.3
Calcium (*927.02*)	0.8	0.9	0.9
Phosphorous (*964.06*)	0.9	0.9	0.9
pH	7.2	4.6	4.8

5. Blood and organs evaluation

Blood samples were obtained by cardiac puncture of animals anesthetized with halothane and sacrificed by cervical dislocation, according to the international guide for the care and use of laboratory animals (National Research Council, 2011). Standard centrifugation procedure was used for hematocrit, while cholesterol (g/L) and uric acid (mg/dL) were measured by enzymatic spectrophotometric methods using commercially available enzymatic kits (Esterase Oxidase and Uricase for cholesterol and uric acid respectively Abbot Clinical Chemistry, USA). The measurements were performed using an automatic analyzer (Alcyon 300, Abbott, USA).

Distension degree of liver and kidneys, content (gas or mucus), serous layer vasodilatation of the stomach and gut were examined for lesions, excessive vasculature or inflammation. The assayed organs were classified in: 0 = without apparent lesions; 1 = slight lesions; 2 = moderate lesions and 3 = severe lesions according to Mann et al. (2012). Each section of the small intestine was weighed and measure.

For gut counting of LAB, 5 mm of duodenum at 2 cm of the pylorus, 5 mm of proximal jejunum at 2 cm of the beginning, 5 mm of distal ileum at 2 cm of the cecum, 5 mm colon at 2 cm of cecum, were cut in aseptic conditions. Each piece was weighed and homogenized following the procedure described above and the suspension inoculated in MRS agar plates (Dalloul et al., 2003). In this work, the villi tallness of any group did not presented differences ($p > 0.01$) among males and females.

6. Diet assessment

Protein efficiency rate (PER) defined as.

$$\mathrm{PER} = \frac{WI}{PI}$$

Where WI: weight increment and, PI: protein intake.

Feed conversion ratio (FC):

$$\mathrm{FC} = \frac{FI}{WI}$$

Where FI: feed intake.

7. Histological evaluation

Jejunum samples were conserved in 10% (vol/vol) formaldehyde. A section (1 cm gut) from each mouse, located at 10 cm of the ligament of Treitz, was extracted and subjected to microscopic assessment. Just about 400 tissue slices (7 μm) stained with haematoxylin and eosin were examined under a light microscope. Villi height was measured in

six points of each slide using a graticule device containing a 100 µm scale. The images of each observation were registered photographically and analyzed using the program TSView version 6.2.4.5 (Tucsen Imaging Technology Co. Limited, China).

8. Statistical analysis

The data of feed and water intake, weight gain, plasmatic uric acid and cholesterol concentration, weight and length of organs, concentration of LAB in each portion of the intestine and faeces and intestinal villus height was statistically analyzed by one factor variance analysis and Tukey test of multiple comparisons. Normal data distribution (Shapiro-Wilks amended test) and homogeneity of variance (Levene test) for each parameter, males and females of each group were assessed.

9.1. Results and discussion

The waste generated from the processing of *M. hubbsi* was used as feedstock to produce biological fish silage (BFS) and chemical fish silage (CFS). The first was performed with a previous selected strain of LAB (*L. arizonensis*) and the second by the addition of 0.18 M sulfuric acid and 0.22 M formic acid (Góngora et al., 2012). The compositions of both silages are shown in supplemented material (Table S1). These silages were highly similar regarding moisture, proteins, lipid, calcium and phosphorous levels. However, the applied process determined their appearance; in the case the CFS a darker color, likely due to Maillard-type reactions, while the BFS presented a creamy aspect and color (Fig. 1). The thermal-processes during feed pelleting may compromise the viability of bacteria and/or affect the biological activity of LAB (Kuo et al., 2013). However, *L. arizonensis* cultivated in a fish based medium tolerate temperatures of 60 °C for extensive time periods (D = 209 min) and this feature makes.

Feasible the formulation of EFBS containing viable LAB (Góngora et al., 2012). In this work, the BFS contained 2.9×10^7 cfu/g Lactobacilli and after processing (mixing and pelleting at 55 °C) it came to contain 1.3×10^7 cfu/g with 10–12% (wt/wt) moisture. It means that, cells count was 55% reduced in the final product, however, the fish.

Based medium was protective enough to maintain a final concentration of LAB between 10^6-10^7 cfu/g; a suitable concentration to exert the probiotic effect in mammalians (Nousiainen et al., 2004). Interestedly, the LAB concentration along the entire bowel was significantly higher ($p < 0.05$) for mice fed with EFBS than CF or EFCS groups. Fig. 2 shows that tendency at jejunum; while in the faeces, the same trend continued; being 2.5×10^4 CFU/g EFBS but 1.26×10^3 CFU/g and 4.0×10^3 CFU/g for CF and EFCS respectively. On the other hand, although the Tukey test for water intakes among the groups did not shown significant differences, a trend could be observed towards higher water consumption at the EFCS mice group in comparison with the EFBS group (Table 3). A likely explanation would be

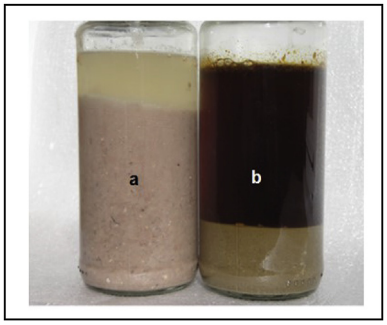

Fig. 1. Silage preparations. A. biological fish silage (BFS) and B. chemical fish silage (CFS).

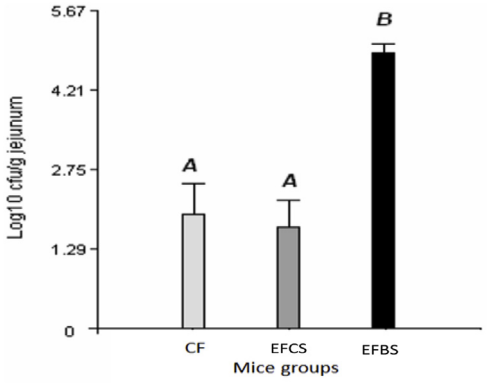

Fig. 2. LAB concentration at the jejunum of mice fed with control feed (CF), experimental feed with chemical fish silage (EFCS) and experimental feed with biological fish silage (EFBS). Differences among groups were evaluated by means of analysis of the variance and Tukey test. Different letters indicate significant differences ($p < 0.05$).

Table 3
Feed consumption (FC), water intake (WI) and weight (W) modifications during a 30-day trial on diets containing chemical or biological silage of *M. hubbsi* wastes. Differences among groups were evaluated by means of analysis of the variance and Tukey test. Different letters indicate significant differences within the row ($p < 0.05$).

	CF	EFCS	EFBS
WI (mL)	1857 [a]	2005 [a]	1563 [a]
FC (g)	1101 [a]	1121 [a]	980 [a]
Initial W female(g) male (g)	18.0 ± 3.6	19.6 ± 3.5	14.4 ± 1.1
	15.8 ± 2.4	15.6 ± 1.8	14.6 ± 2.9
Final W female (g) male (g)	33.8 ± 2.9	33.8 ± 1.9	30.4 ± 3.44
	32.4 ± 3.4	34.8 ± 3.9	34.4 ± 4.16
Increment of W (g)	16 ± 4 [a]	16.7 ± 3.7 [a]	23.8 ± 3.8 [b]
Feed conversion	6.8 [a]	6.71 [a]	4.12 [b]
Protein efficiency	0.63	0.63	0.69

the effect of acids in the diet (formic acid and sulfuric acid) that recently were related to a higher water consumption in piglets (Mesonero Escuredo et al., 2016), while the healthier gut of the mice of EFCS group would present a more efficient water absorption as was previously reported by Ma and Verkman (1999). Mice fed with CF, EFCS and EFBS did not shown also significant differences regarding feed intake after 30 days consumption. However, the weight gain was significantly higher (30%) for mice fed with EFBS. It could be due to the higher content of LAB, since it is possible to find several reports relating the increment of feed efficiency and the consumption of lactic acid bacteria (Nickolova and Penkov, 2004; Nousiainen et al., 2004; Mahdavi et al., 2005). The feed conversion and protein efficiency rate in both experimental feeds, EFCS and CF, were outperformed by the EFBS, suggesting a promising effects of *L. arizonensis* or the fermented product itself (Table 3). However, the concentration of plasmatic uric acid for the group fed with EFBS was significantly ($p < 0.05$) higher than those fed with EFCS and CF (Table 4) and that difference might be related with the increment in DNA content, supplied by the higher concentration of bacteria in the diet (El-Shafie et al., 2009; Raju et al., 2012). Regarding the plasmatic concentration of cholesterol for the mice group feed with CF was significantly lower in comparison with silages.

Fed groups ($p < 0.05$) (Table 4). Since lower concentrations of plasmatic cholesterol were associated to vegetarian and high fiber diets, the vegetables included in CF might be the reason for the reduced cholesterol concentration found in the control group (Mahdavi et al., 2005; Artiss et al., 2006). Treatments for elevated blood cholesterol in mammalians include dietary management and, several reports related

Table 4
Biochemical parameters of mice fed with control feed (CF), experimental feed with chemical fish silage (EFCS) and biological fish silage (EFBS). Differences among groups (n = 10/diet) were evaluated by means of analysis of the variance and Tukey test. Different letters indicate significant differences among the lines ($p < 0.05$).

Blood assay	CF	EFCS	EFBS
Hematocrit (%)	45.11 ± 0.8 [a]	45.1 ± 0.8 [a]	44.8 ± 1.23 [a]
Uric acid (mg/dl)	4.13 ± 0.9 [a]	4.34 ± 1.2 [a]	6.5 ± 0.9 [b]
Cholesterol (g/L)	1.19 ± 0.2 [a]	1.6 ± 0.2 [b]	1.87 ± 0.2 [b]

Table 5
Organs evaluation for mice fed with control feed (CF), experimental feed with chemical fish silage (EFCS) and experimental feed with biological fish silage (EFBS). Differences among groups were evaluated by means of analysis of the variance and Tukey test (n = 10). Different letters indicate significant differences among the lines ($p < 0.05$).

Organ	CF	EFCS	EFBS
	Weight (g)		
Kidney	0.47 ± 0.09[a]	0.54 ± 0.1 [a]	0.59 ± 0.28 [a]
Liver	1.46 ± 0.2[a]	1.6 ± 0.15[b]	1.9 ± 0.45[b]
Stomach	0.72 ± 0.22[a]	0.6 ± 0.18 [a]	0.8 ± 0.22 [a]
Duodenum	0.4 ± 0.08 [a]	0.4 ± 0.08 [a]	0.36 ± 0.05 [a]
Jejune ileum	1.5 ± 0.28 [a]	1.38 ± 0.19 [a]	1.6 ± 0.18 [a]
Cecum	0.57 ± 0.13[a]	0.34 ± 0.07 [b]	0.46 ± 0.12[ab]
Large intestine	0.67 ± 0.21[ab]	0.5 ± 0.09 [b]	0.7 ± 0.15 [a]

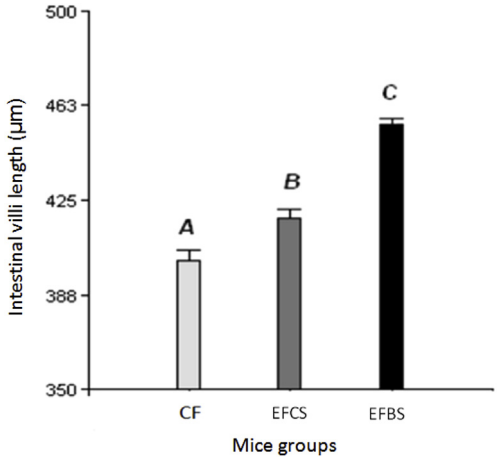

Fig. 3. Effect of experimental feed with chemical fish silage (EFCS) and experimental feed with biological fish silage (EFBS) on intestinal villi length after 30 days consumption. The rods represent the average intestinal villi length ± standard error for the diets assayed. Differences among mice groups were evaluated by means of analysis of the variance and Tukey test. Different letters indicate significant differences ($p < 0.05$).

the consumption of LAB with reduced levels of cholesterol by induction of bile salt hydrolases (Begley et al., 2006; Ooi and Liong, 2010; Guo and Yang, 2011). Although the cholesterol concentrations obtained in the EFBS-group was at levels considered normal for mice, the comparison with EFCS indicated that *L. arizonensis* was not efficient enough for reducing the plasmatic cholesterol concentration (Table 4).

The weight of liver for mice fed with EFBS was similar than those fed with EFCS, but significantly ($p < 0.05$) higher than CF fed mice (Table 5). Liver weight usually rises through adaptation to a new diets or toxic compounds (Fukushima and Nakano, 1995; Bakry, 2002; Ali et al., 2010; Kaware Mangesh, 2013). Although, the fact lacks of mechanistic explanations, mild inflammatory responses were considered positive to the liver as they favor the re-establishment of the tissue homeostasis (Brenner et al., 2013). A recent detailed review provide promising evidence that bile acids and microbiota jointly regulate nutrient absorption, hepatic metabolism, and inflammatory processes thus maintain the health of gut and liver (Liu et al., 2015). On the other hand, the length of intestinal villi is an important health parameter in mammalians; the micro-structure of the intestinal epithelium presented a significantly longer villus height for mice fed with EFBS and EFCS (Fig. 3). Previously, weight increment of the large intestine was related with high concentration of LAB in the rodents diet by El-Shafie et al. (2009) and a direct relationship between weight increment and higher villus longitude was described by Zambonino Infante et al. (1993), being in agreement with the results herein obtained for mice fed with EFBS. Since the sizes of the other organs evaluated did not exhibited significant differences among the diets and, gut lesions, like thickness wall reduction, distension or abnormal vascularization were not found, a classification = 0 was established for the feeds (Table 5). Although the high concentrations of LAB in the gut explained several mammalian health benefits, the probiotic effect of the strain *L. arizonensis* was not previously reported (Fuller R, 1989, Dalloul et al., 2003;

Daniel et al., 2006). Therefore, taking into consideration the higher concentration of LAB in the gut of mice fed with EFBS, together with the weight gain, the improvement of feed conversion, protein efficiency rate, larger villi, and absence of lesions in the entire bowel support the probiotic effect of *L. arizonensis*.

9. Summary and conclusions

Bio-economy relies on efficient fractionation of renewable resources trough out integrated bio-refineries. Considering availability and versatility, wastes generated in feed chain processing could evolve into an important feedstock for sustainable bio-based products via green chemical or biotechnological routes. Designing isoenergetic diets with silages of wastes from fish processing, we examined the effects of biological silage and chemical silage separately in BALB/c mice. Some of the differences found between the feeds would be related to the effect of *L. arizonensis*, however, sterilization of the feedstock precedes the main treatment for preparation of biological silage. Therefore, the chemical silage would also be an attractive option for reducing starting-costs of silage production. Even so, both products established interesting alternatives for recycling fish wastes, reducing environmental pollution and keep a sustainable animal feed chain supply.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.eaef.2018.04.001.

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