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# In vitro assays predicts mineral retention and apparent protein digestibility of different fish feed measured using a juvenile *P. mesopotamicus* model

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## Abstract

Feed chemical composition only provides information about nutrient content of a diet, but not its bioavailability. The aims were to determine mineral bioaccessibility and protein digestibility of three different fish feed, using in vitro methods and to correlate them with feed mineral retention and apparent protein digestibility measured by juvenile *Piaractus mesopotamicus* model, considering elliptical joint confidence region (EJCR) of weighted least-squares analysis. For in vivo assays, juvenile *P. mesopotamicus* were randomly stocked into conical tanks and fed during 38 days with a commercial diet, an experimental diet, or the same experimental diet added with phytase (0.2 g/kg). Total body mineral retention (mineral bioavailability indicator) and apparent protein digestibility of different diets were measured. For in vitro methods, mineral dialysability (mineral bioaccessibility indicator) and protein digestibility of the same diets were determined. No significant difference in morphometric and haematological markers were detected between diets. For iron, zinc and phosphorous bioavailability, and protein digestibility, weighted least-squares analysis showed the optimal point (1, 0) was included in EJCR, indicating in vivo and in vitro methods were comparable and correlated satisfactorily. In vitro methods were able to reproduce accurately the results obtained from traditional in vivo assays using juvenile *P. mesopotamicus* model.

**KEYWORDS**
fish feed, mineral dialysability, mineral retention, protein digestibility

## 1 | INTRODUCTION

A formulated fish feed can contain all dietary essential nutrients, but if nutrient bioavailability is low, growth and physiological state of fish will be affected (Fernandez Gimenez, Diaz, Velurtas, & Fenucci, 2009). Bioavailability can be defined as the proportion of a nutrient in a diet or dietary supplement that is absorbed and used for normal body functions. It involves nutrient absorption and transport to the relevant body tissues and its conversion to physiologically active compound, in such a way the nutrient can be used to maintain normal metabolic functions (Fairweather-Tait, 1992). The amount of nutrient available for absorption or bioaccessible depends on composition and physical characteristics of diet, the presence of enhancers or inhibitors of absorption, luminal gastrointestinal secretions and interactions that occur as a result of the interplay of these factors (Cao et al., 2007; Sugiura, Dong, Rathbone, & Hardy, 1998).

In vivo methods for determining mineral absorption and protein digestibility of fish feed involves measuring of apparent digestibility coefficients (Abimorad, Squassoni, & Carneiro, 2008). Besides the methodological difficulties involved with digestibility trials, related to the separation of fish faeces from the water without contamination of uneaten feed, the in vivo studies are time-consuming, expensive and tedious (Gomes, Teles, Gouveia, & Rema, 1998; Vandenberg & De La Noüe, 2001). In contrast, in vitro techniques are quick,

  

relatively simple and less expensive than in vivo assays, allowing better control of experimental variables (Gomes et al., 1998; Wienk, Marx, & Beynen, 1999).

Regarding mineral bioaccessibility, numerous in vitro methods have been developed to estimate the fraction of a mineral or trace element that is bioaccessible. These techniques evaluate solubility, dialysability and mineral uptake by cells (Drago, 2016). Mineral dialysability is one of the most frequently used assay. It involves pepsin digestion at acid pH followed by digestion with pancreatin and bile salts at higher pH. The proportion of the element that diffuses through a semipermeable membrane during intestinal digestion stage represents the dialysability of mineral and is used as an estimator of the element proportion available for absorption (Miller, Schricker, Rasmussen, & Van Campen, 1981). Although no in vitro method can reproduce the prevailing physiological conditions of in vivo studies, mineral dialysability showed good relationship with in vivo studies for iron and zinc (Bosscher, Van Caillie-Bertrand, et al., 2001, Bosscher, Zhengli, et al., 2001; Schricker, Miller, Rasmussen, & Van Campen, 1981).

The pH adjustment during gastrointestinal digestion and final pH at which the dialysis is performed can be a source of result variability (Bosscher, Zhengli, et al., 2001). To achieve accurate pH adjustment, mineral dialysability method was optimized by Wolfgor, Drago, Rodríguez, Pellegrino, and Valencia (2002). The authors defined the way to calculate the molarity of PIPES [piperazine- N,N′-bis(2-ethane-sulphonic acid)] buffer to assure a final pH independent of the food matrix. This method was widely used to estimate mineral bioaccessibility of different extruded food such as extruded whole grain red sorghum (Llopart, Drago, De Greef, Torres, & González, 2014), extruded cereals fortified with different iron sources (Cagnasso et al., 2013), extruded products based on whole grain with the addition of wild legumes (Pastor Cavada et al., 2013), etc.

Several in vitro methods have been developed to estimate protein digestibility of fish feed. Most studies have focused on salmonids (primarily rainbow trout) and to a lesser extent other species such as Gilthead seabream (*Sparus aurata*), bluefin tuna (*Tunnus thynnus*) common carp (*Cyprinus carpio*) and turbot (*Psetta maxima*) (Moyano, Saénz de Rodrigáñez, Díaz, & Tacon, 2015), but none of them on *P. mesopotamicus*. These methods usually use an enzymatic extract obtained from the gastrointestinal tract of fish (Dimes & Haard, 1994; Eid & Matty, 1989). While these methods provide results comparable to those obtained in vivo (Dimes & Haard, 1994), they require fish to obtain the enzymatic extracts. In addition, the use of enzymatic cocktails from fish makes the methods more expensive and less reproducible (Gomes et al., 1998), since enzyme activity can change with fish age, sexual maturation, specie and procedures using these cocktails need standardization (Dimes & Haard, 1994). Although, Yasumaru and Lemos (2014) standardized the hydrolytic capacity of the species-specific enzyme extracts on purified protein substrates, most of the studies using fish enzymes did not. Another point with in vitro methods used for assess protein digestibility as pH-stat or drop methods is the use of alkaline pH (>8.0), without gastric phase. Such an approach may be incorrect for many fish species with a functional stomach (Moyano et al., 2015).

Rudloff and Lönnerdal (1992) proposed an in vitro method that measures protein digestibility under controlled pH conditions after pepsin and pancreatin digestion. This method is more reproducible than those mentioned above, since it uses commercial enzymes whose activity is standardized. In addition, protein digestibility of different extruded food such as extruded maize products added with a red seaweed *Porphyra columbina* (Cian, Caballero, Sabbag, González, & Drago, 2014), extruded whole grain red sorghum (Llopart et al., 2014) and extruded whole rice grain (Albarracín, González, & Drago, 2015), was estimated by Rudloff and Lönnerdal (1992) method.

Morales and Moyano (2010) used a computer-controlled system that allow closer simulation of in vivo dynamic physiological processes occurring within the stomach and small intestine using rainbow trout digestive enzymes for determining nitrogen and phosphorus bioaccessibility and bioavailability. Even though this system might be closest to an in vivo situation, and could be used as a reference to design a similar model for studies in fish nutrition, it has the disadvantages of the high cost and the difficulty of carrying out experiments routinely (Moyano et al., 2015). In contrast, equilibrium-dialysability method to estimate mineral bioaccessibility is less expensive because it does not require sophisticated equipment and can be performed routinely (Drago, 2016). As far as we know, there is no report of the use of equilibrium-dialysability method to estimate mineral bioaccessibility (particularly, iron, zinc and calcium) as screening of fish feed. Even less is known about the correlation between the results obtained from these in vitro methods and those achieved with traditional in vivo methods using a fish model. The aims of this work were: (i) to determine mineral bioaccessibility and protein digestibility of three fish feed, using in vitro methods (ii) to correlate them with feed mineral retention and apparent protein digestibility measured by juvenile *Piaractus mesopotamicus* model, using weighted least-squares (WLS) and considering elliptical joint confidence region of weighted least-squares analysis.

## 2 | MATERIALS AND METHODS

### 2.1 | Reagents

All reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Pepsin (P7000), pancreatin (P1750) and bile extract (B8631) were porcine origin.

### 2.2 | Diets and chemical composition

Three different diets ($D_1$, $D_2$ and $D_3$) were evaluated by in vivo assays in juvenile *Piaractus mesopotamicus* model and by in vitro mineral bioaccessibility and protein digestibility. Diet 1 ($D_1$) was an extruded commercial diet, containing fish meal (Cian, Bacchetta, Cazenave, & Drago, 2017). The diets $D_2$ and $D_3$ were extruded experimental diets formulated with corn meal, soybean meal and bovine plasma proteins concentrate. In addition, $D_3$ had 0.2 g/kg of thermo-resistant microbial phytase (Ronozyme, NOVOZYME®). Diets were extruded using a Brabender 10 DN single-screw extruder,

using a 3:1 compression ratio screw, a 3/20 mm (diameter/length) die, a screw speed of 175 rpm, 16.0 g/100 g blend moisture, 180°C barrel temperature and 180°C die temperature. The feeding rate of the extruder was at full capacity.

Chemical composition and phosphorus of diets were determined using Association of Oficial Analytical Chemist (AOAC) (2000) approved methods. Phytic acid was determined according to AOAC anion-exchange method (AOAC, 2000) and AOAC phosphorus method (AOAC, 2000). Total starch was quantified according to Tovar, Bjoerck and Asp (1990).

Iron, zinc and calcium contents of diets were measured by atomic absorption spectroscopy after dry mineralization using an atomic absorption spectrophotometer analyst 300 Perkin-Elmer (Norwalk, CT, USA).

Phytase activity of diets was tested according to Hassaan, Soltan, Agouz, and Badr (2013). One phytase unit (PU) was defined as the enzyme activity that releases 1 μmol phosphorous/min under the given reaction conditions. Results were expressed as PU/kg diet.

All assays were performed in triplicate ($n = 3$).

## 2.3 | In vivo methods

### 2.3.1 | Fish and feeding trial

Juvenile *P. mesopotamicus* were obtained from a fish farm (Pez Campero Paraná, Argentina). The experiment was performed in the Aquaculture Laboratory at the Instituto Nacional de Limnología (CONICET, Argentina) in a recirculating water system supplied with dechlorinated city (tap) water, and equipped with an external quartz-anthracite filter (Multiválvula Vulcano Filtro VC10). The water flow to the tanks was at 15.1 L/min with artificial aeration and 12 hr light/12 hr dark photoperiod regime provided by artificial illumination. The physico-chemical parameters of the water remained within the values recommended by Urbinati, Goncalves, and Takahashi (2010) for *P. mesopotamicus* (temperature 25.0 ± 1°C, dissolved oxygen 6.67 ± 0.63 mg/L, pH 6.15 ± 0.32, electrical conductivity 189.40 ± 25.93 μs/cm and total ammonia nitrogen 0.24 ± 0.1 mg/L).

Prior to the feeding trial, all fish were acclimated to the indoor rearing conditions for 2 weeks. At the start of the feeding experiment, 36 juvenile *P. mesopotamicus* (initial body weight 10.8 ± 2.4 g) were randomly stocked into six conical tanks with six fish per tank. The three test diets were randomly assigned to duplicate tanks: Diet 1 ($D_1$, with fish meal), diet 2 ($D_2$, experimental diet without fish meal) and diet 3 ($D_3$, diet 2 with phytase enzyme). In all diets, 5 g/kg chromic oxide was used as an indigestible inert marker. Fish were fed twice a day, during 38 days. The feed delivered to fish was previously weighed. Left-over diet was collected after 1 hr to prevent nutrient leaching, dried and weighed. The conical geometry of tank was used to collect faeces by sedimentation following the modified Guelf method. Upon finishing the collections, the faeces were oven-dried at 65°C, ground and stored at −20°C until chemical analysis.

The experiment was conducted in accordance with national and institutional guidelines (CONICET 2005) for the protection of animal welfare.

### 2.3.2 | Sample collection and morphometric indexes

At the end of the feeding trial, fish from each diet treatment were fasted for 24 hr and then anaesthetized in benzocaine 100 mg/L as described Parma de Croux (1990). Body weight (g), total and standard length (cm) were recorded for each individual. Blood was collected immediately from the caudal vessel (Reichenbach-Klinke, 1980). Plasma was separated from whole blood by centrifugation at 1,409 × *g* for 5 min, and stored at −80°C. Fish were dissected, the gastrointestinal tract was exposed, and a small cut was made in both, stomach and intestine walls. An electrode was inserted into the cut and the pH of stomach and intestinal content was measured. The white muscle was dissected, quickly frozen in liquid nitrogen, and subsequently stored at −80°C until zinc content analysis.

### 2.3.3 | Haematological parameters

Red blood cell (RBC) counts were performed with a Neubauer chamber. Haematocrit (Ht) was determined by micro-method. Haemoglobin concentration (Hb) was measured by cyanomethemoglobin method (Houston, 1990). Mean cell volume (MCV), mean cell haemoglobin (MCH) and mean cell haemoglobin concentration (MCHC) were calculated from primary indices according to Cazenave, Wunderlin, Hued, and Bistoni (2005). Plasma iron concentration was measured by a commercial kit (Wiener Lab®).

The samples from each fish were collected individually and the results reported represents a mean of the values obtained from the duplicate tanks with 6 fish each ($n = 12$). All assays were performed by triplicate.

### 2.3.4 | Mineral bioavailability of diets

Mineral bioavailability of diets was estimated by total body mineral retention (Satoh et al., 2003), using juvenile *P. mesopotamicus* model. Whole fish were ground using a Moulinex AD5661AR meat mincer (Buenos Aires, Argentina) in order to obtain a homogenate. Phosphorus content of homogenates was determined following AOAC (2000) methods. Iron, zinc and calcium content were measured by atomic absorption spectroscopy after dry mineralization. All assays were performed at least in triplicate.

Total body mineral retention (TBMR) was calculated as the amount of mineral retained in whole fish expressed as a percentage of total amount of mineral consumed during the experiment:

$$\mathrm{TBMR}(\%) = \left[\frac{(\mathrm{ME} - \mathrm{MB})}{(\mathrm{MC})}\right] \times 100$$

Where, TBMR: total body mineral retention, ME: mineral content (mg) of fish at the end of experiment (accumulated final weight x

mineral content of fish at the end of experiment), MB: mineral content (mg) at the beginning of experiment (accumulated initial weight x mineral content of fish at the beginning of experiment) and MC: mineral (mg) consumed during experiment ((accumulative feed intake – total unconsumed feed) x mineral content of diet).

Also, other markers were evaluated as estimators of bioavailability. For iron, haemoglobin and plasma iron concentration were evaluated. For zinc, Zn content of white muscle was measured by atomic absorption spectroscopy after dry mineralization as mentioned before. Zinc content was expressed as mg of mineral per 100 g tissue.

The samples from each fish were collected individually and the results reported represents a mean of the values obtained from the duplicate tanks with 6 fish each ($n$ = 12). All assays were performed by triplicate.

### 2.3.5 | Apparent protein digestibility of diets

Apparent protein digestibility of diets was measured according to Abimorad et al. (2008), using the juvenile *P. mesopotamicus* model. Apparent protein digestibility was calculated as follow (Nose 1960):

$$\mathrm{APD}(\%) = 100 - 100 \times \left[\left(\frac{\%\mathrm{Cr_2O_{3\,D}}}{\%\mathrm{Cr_2O_{3\,F}}}\right) \times (\%\mathrm{Protein_F} \times \%\mathrm{Protein_D})\right]$$

Where, APD: apparent protein digestibility, $\%Cr_2O_{3\ D}$: chromic oxide content of diets, $\%Cr_2O_{3\ F}$: chromic oxide content of faeces, $\%Protein_{\ F}$: protein content of faeces and $\%Protein_{\ D}$: protein content of diets. Protein content of faeces was determined by semi micro Kjeldahl using Association of Oficial Analytical Chemist (AOAC) (2000) approved methods. $Cr_2O_3$ content of diets and faeces was measured by graphite furnace under STPGFAA conditions and flame atomization. Samples were digested by microwave, using a Milestone START D digester (Shelton, CT, USA). A Perkin Elmer Spectrometer, PinAAcle 900T (Norwalk, CT, USA) was used. The assays were performed for each tank by duplicate ($n$ = 12).

## 2.4 | In vitro methods

### 2.4.1 | Mineral bioaccessibility of diets

In vitro bioaccessibility of $D_1$, $D_2$ and $D_3$ diet was estimated using dialysability method modified by Wolfgor et al. (2002). Ground diets were prepared to 12 g solid /100 g dispersion, using deionized water. Aliquots (25 g) of homogenized samples were adjusted to pH 2.0 with 4 mol/L of HCl and after addition of 0.8 ml pepsin digestion mixture (16 g/100 ml pepsin solution in 0.1 mol/L HCl), were incubated at 37°C for 2 hr in a shaking water bath. In order to adjust the pH during the digestion and dialysis stage, and to obtain a uniform final pH in digest/dialysate systems, a PIPES buffer with molarity varying according to the matrix was used (0.22 mol/L PIPES buffer for $D_1$ and 0.13 mol/L for $D_2$ and $D_3$ diet). At the end of pepsin digestion, dialysis bags (cut off: 6–8 kDa) containing 20 ml PIPES buffer were placed in each flask and were incubated for 50 min in a shaking water bath at 37°C. Then, 6.25 ml of bile pancreatin solution (2.5 g/100 ml bile and 0.4 g/100 ml pancreatin in 0.1 mol/L $NaHCO_3$) were added to each flask and the incubation continued for another 2 hr. Then, the content of each dialysis bag (dialysates) was weighed. Its pH was measured and analysed regarding mineral content. Mineral dialysability was calculated as the amount of dialysate mineral expressed as a percentage of total mineral content in the sample:

$$\text{Mineral dialysability}\,(\%) = \left(\frac{\mathrm{MD}}{\mathrm{MS}}\right) \times 100$$

Where, MD: mineral dialysed (mg) and MS: mineral of sample (mg).

Iron, zinc, calcium and phosphorous contents of dialysates were measured as mentioned before. The assays were performed three times in quadruplicate ($n$ = 12).

### 2.4.2 | Protein digestibility of diets

Protein digestibility of diets was measured using the in vitro method described by Rudloff and Lönnerdal (1992). Ground diets were prepared to 23 g solid/100 g dispersion, using deionized water. Aliquots (10 g) of homogenized samples were adjusted to pH 2.0 with 6 mol/L of HCl. Thirty milligrams of pepsin for sample were added. Taking into account the protein content of diets, the enzyme/substrate ratio was 1/17.5. Then, the samples were incubated at 37°C for 30 min in a shaking water bath. After pepsin hydrolysis, the pH was gradually increased to 7.0 with 1 mol/L of $NaHCO_3$, during 10 min. Then, 2.5 ml of a pancreatin solution (4 g/100 ml in 0.1 mol/L $NaHCO_3$) was added and dispersion incubated for 1 hr at 37°C. Digested samples were immediately placed in boiling water for 4 min to inactivate the enzymes. Five millilitres of 20% w/v trichloroacetic acid was added to 5 ml of digested samples for non-digested protein precipitation. Then, the samples were centrifuged at 3,500 × $g$ for 30 min at room temperature. In addition, an enzyme blank without sample was performed to estimate the nitrogen content provided by the system (NB). Also, initial non-protein nitrogen soluble in 20% of trichloroacetic acid from diets (NPNi) was measured. Protein digestibility was defined as the increase in non-protein nitrogen (NPN) after digestion, in relation to total nitrogen (TN) of sample. In vitro protein digestibility (IPD) was calculated as:

$$\mathrm{IPD}\,(\%) = \left[\frac{(\mathrm{NPN} - (\mathrm{NPNi} + \mathrm{NB}))}{(\mathrm{TN} - \mathrm{NPNi})}\right] \times 100$$

Nitrogen content (NPN, TN, NPNi and NB) was measured using micro-Kjeldhal method (Association of Oficial Analytical Chemist (AOAC), 2000). The assays were performed three times in quadruplicate ($n$ = 12).

## 2.5 | Correlation between in vitro and in vivo methods

Correlation between in vitro and in vivo methods was performed using linear regression. Mineral dialysability of different diets ($y$) was

plotted vs. total body mineral retention in juvenile *P. mesopotamicus* model (*x*). On the other hand, IPD of different diets (*y*) was plotted vs. apparent protein digestibility obtained from juvenile *P. mesopotamicus* model (*x*). Since the variance in the variable *x* was significantly lower than that of variable *y*, the correlations were performed by weighted least-squares or WLS (Olivieri & Goicoechea, 2007), considering the elliptical joint confidence region (EJCR). EJCR test allows investigating whether the point (1, 0) is included in the joint confidence elliptical region of the slope and the intercept. In this situation, the slope may be considered to be zero and the intercept to be unity. Therefore, if optimal point (1, 0) is included in elliptical region, the methods are comparable and correlate satisfactorily (Olivieri & Goicoechea, 2007).

Moreover, iron bioaccessibility was correlated with plasma iron concentration and haemoglobin concentration. Also, zinc bioaccessibility was correlated with white muscle zinc content from *P. mesopotamicus* fed with different diets, using WLS method.

## 2.6 | Statistical analysis

Results were expressed as means with their standard errors. One-way analysis of variance (ANOVA) was performed and the statistical differences among samples were determined using LSD test (least significant difference). Significance was accepted at $p < .05$. All statistical analyses were performed with STATGRAPHICS Centurion XV 15.2.06 (Statpoint Technologies, Inc., Warrenton, Virginia, USA). The correlation between in vitro and in vivo methods by weighted least-squares (WLS) was performed with Matlab 7.6.0.324 (Math Works, Natick, MA, USA).

# 3 | RESULTS

## 3.1 | Chemical composition and phytase activity of diets

Chemical composition and phytase activity of diets are shown in Table 1. The chemical analysis confirmed diets supplied a similar amount of macronutrients. However, $D_1$ showed higher content of ash, calcium, phosphorous, iron and phytic acid than $D_2$ and $D_3$ diets. In agreement with diet formulation, phytase activity only was detected in $D_3$.

## 3.2 | Mineral bioavailability and apparent protein digestibility of diets

Fish promptly accepted all diets, and no mortality occurred during the feeding trial. It was observed no significant difference among diet treatment in final body weights (Table 2).

Except for iron content in plasma, which was significantly higher for fish consuming $D_3$ ($p < .05$), there were not significant differences in morphometric and haematological markers among diets ($p > .05$) (Table 2). Also, white muscle Zn content was different among fish feed with different diets, the values being higher for $D_3$ than $D_2$ and $D_1$ diets.

**TABLE 1** Chemical composition and phytase activity of $D_1$, $D_2$ and $D_3$ diets

| Components[a] | $D_1$ (g/kg)[b] | $D_2$ (g/kg)[b] | $D_3$ (g/kg)[b] |
|---|---|---|---|
| Dry matter | 899.9 ± 1.4* | 900.6 ± 5.3** | 895.3 ± 4.7** |
| Crude protein | 273.7 ± 5.6 | 285.5 ± 4.2 | 276.8 ± 4.7 |
| Crude lipid | 41.3 ± 1.9 | 34.4 ± 2.6 | 35.7 ± 1.9 |
| Total starch | 430.6 ± 7.2 | 444.2 ± 4.3 | 446.5 ± 0.3 |
| Ash | 81.9 ± 0.7** | 23.6 ± 0.9* | 25.6 ± 0.4* |
| Calcium | 18.7 ± 0.7** | 1.6 ± 0.1* | 1.8 ± 0.2* |
| Phosphorous | 10.5 ± 0.2** | 2.2 ± 0.2* | 2.4 ± 0.2* |
| Zinc | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.1 ± 0.0 |
| Iron | 0.3 ± 0.0** | 0.2 ± 0.0* | 0.2 ± 0.0** |
| Phytic acid | 10.5 ± 0.5** | 8.1 ± 0.2* | 7.9 ± 0.1* |
| Phytase activity (PU/kg)[c] | N.d | N.d | 4,004.4 ± 158.9 |

N.d: not detected.

[a]Chemical composition expressed as mean ± *SD* ($n = 3$).

bDifferent symbols in a row (*, **, etc.) mean significant differences between samples ($p < .05$).

[c]PU: μmol P/min·

Fish consuming $D_3$ had higher total body iron and zinc retention than those fed with $D_2$ and $D_1$ diets ($p < .05$) (Table 3). Regarding phosphorus retention, fish consuming $D_3$ showed higher values than those found for $D_2$ and $D_1$ diets ($p < .05$). However, there were not significant differences in calcium retention among diets.

Table 4 shows apparent protein digestibility (APD) of different diets. Fish consuming $D_3$ had higher APD than those fed with $D_2$ and $D_1$ diets ($p < .05$).

## 3.3 | In vitro mineral bioaccessibility and protein digestibility of diets

Stomach pH was 2.12 ± 0.19, 2.02 ± 0.16 and 1.99 ± 0.25 for $D_1$, $D_2$ and $D_3$ diets respectively. There were not significant differences between stomach pH of *P. mesopotamicus* consuming different diets ($p > .05$). Taking this into account, the pH of pepsin digestion mixtures were adjusted to 2.00 ± 0.02 for all diets.

Intestinal pH from *P. mesopotamicus* fed with $D_1$, $D_2$ and $D_3$ diet was 8.07 ± 0.09, 6.40 ± 0.05 and 6.50 ± 0.06 respectively. $D_1$ diet induced higher intestinal pH than $D_2$ and $D_3$ diets ($p < .05$). The mean value of the intestinal content pH was 6.99. In line with this, the pH of dialysates was adjusted to mean value of 7.00, using different PIPES molarity according to Wolfgor et al. (2002).

Iron bioaccessibility of $D_2$ and $D_3$ diets was higher than that obtained for $D_1$ ($p < .05$), but there was no significant difference between $D_2$ and $D_3$ diets ($p > .05$) (Table 3). However, zinc and phosphorus bioaccessibility of $D_3$ was significantly higher than that

**TABLE 2** Morphometric, haematological biomarkers and white muscle zinc content of *P. mesopotamicus* fed with $D_1$, $D_2$ or $D_3$ diet during 38 days

| | Diets[a] | | | |
|---|---|---|---|---|
| | $D_1$ | $D_2$ | $D_3$ | *p-value* |
| Final body weight (g) | 19.5 ± 5.9 | 22.0 ± 4.6 | 27.2 ± 8.6 | .1009 |
| CF | 3.5 ± 0.2 | 3.7 ± 0.2 | 3.5 ± 0.3 | .1219 |
| LSI | 0.5 ± 0.1 | 0.9 ± 0.1 | 0.9 ± 0.1 | .1057 |
| RBC ($\times 10^3$ per $mm^3$) | 2.0 ± 0.5 | 1.7 ± 0.2 | 1.8 ± 0.4 | .2941 |
| Haematocrit (%) | 28.1 ± 2.5 | 32.0 ± 3.7 | 28.4 ± 2.2 | .5944 |
| Haemoglobin (g/100 ml) | 6.9 ± 0.4 | 7.2 ± 0.5 | 8.1 ± 0.44 | .4658 |
| MCH (pg) | 35.9 ± 6.3 | 39.4 ± 5.0 | 47.7 ± 7.2 | .1041 |
| MCV (fL) | 143.6 ± 31.0 | 163.7 ± 14.8 | 187.9 ± 35.8 | .1144 |
| MCHC (%) | 24.9 ± 1.7 | 25.5 ± 3.2 | 24.3 ± 2.4 | .9634 |
| Plasma iron concentration (μg/dl) | 56.3 ± 8.1* | 74.0 ± 1.5** | 83.3 ± 1.7*** | .0001 |
| WBC (/μl) | 2,297.0 ± 126.2 | 2,398.3 ± 384.5 | 2,285.5 ± 273.9 | .4936 |
| White muscle zinc content (mg/100 g tissue) | 1.0 ± 0.1* | 1.3 ± 0.1** | 1.6 ± 0.0*** | .0123 |

CF: Condition factor; LSI: Liver somatic index; MCH: Mean corpuscular haemoglobin; MCHC: Mean corpuscular haemoglobin concentration; MCV: Mean corpuscular volume; RBC: Red blood cells count; WBC: White blood cells count.
aMean ± *SD* ($n$ = 12). Different symbols in a row (*, **, etc.) mean significant differences between samples ($p < .05$).

**TABLE 3** Bioaccessibility and bioavailability of iron, zinc, calcium and phosphorus of $D_1$, $D_2$ and $D_3$ diets

| | Bioaccessibility (%)[a,b] | Bioavailability (%)[a,c] | | | | | |
|---|---|---|---|---|---|---|---|
| Mineral | $D_1$ | $D_2$ | $D_3$ | $D_1$ | $D_2$ | $D_3$ | *p-value* |
| Iron | 5.3 ± 1.0* | 12.2 ± 0.8** | 13.2 ± 0.2*** | 6.1 ± 0.3* | 11.2 ± 1.0** | 13.1 ± 0.1*** | .0000 |
| Zinc | 7.4 ± 0.3* | 9.7 ± 0.1** | 13.6 ± 1.0*** | 7.1 ± 0.2* | 9.6 ± 0.2** | 12.5 ± 0.7*** | .0001 |
| Calcium | 14.8 ± 1.4* | 17.3 ± 1.8* | 18.2 ± 2.5* | 44.5 ± 1.8** | 43.3 ± 2.3** | 44.0 ± 1.7** | .0002 |
| Phosphorus | 11.2 ± 0.2* | 14.5 ± 0.1** | 19.4 ± 0.5*** | 33.7 ± 2.4**** | 52.8 ± 2.5***** | 62.3 ± 3.6****** | .0001 |

aMean ± *SD* ($n$ = 12). Different symbols in a row (*, **, etc.) mean significant differences between samples ($p < .05$).
[b]Bioaccessibility estimated by in vitro dialysability method.
[c]Bioavailability measured as total body mineral retention *in P. mesopotamicus*.

**TABLE 4** *In vitro* protein digestibility (IPD) and apparent protein digestibility (APC) of $D_1$, $D_2$ and $D_3$ diets

| Diets | IPD (%)[a] | APD (%)[a,b] |
|---|---|---|
| D1 | 86.4 ± 3.9* | 87.2 ± 1.6* |
| D2 | 95.6 ± 2.5** | 94.3 ± 1.4** |
| D3 | 97.4 ± 2.7*** | 98.3 ± 1.0*** |

aMean ± *SD* ($n$ = 12). Different symbols row (*, **, etc.) mean significant differences between samples ($p < .05$).
[b]Apparent protein digestibility measured in juvenile *P. mesopotamicus* model.

found for $D_1$ and $D_2$ diets ($p < .05$). Regarding calcium bioaccessibility, it ranged from 14.8% to 18.2%, but there was no significant difference among diets ($p > .05$).

In vitro protein digestibility of $D_3$ was higher than those obtained for $D_2$ and $D_1$ ($p < .05$). Moreover, $D_2$ showed higher protein digestibility than $D_1$ ($p < .05$) (Table 4).

## 3.4 | Correlation between in vitro and in vivo methods

Elliptical joint confidence region from correlation between in vitro and in vivo methods using WLS is shown in Figure 1. For iron, zinc and phosphorus, the optimal point (1, 0) was included in EJCR, indicating that in vitro mineral bioaccessibility and TBMR were comparable and correlated satisfactorily (Figure 1a, 1b, and 1c respectively). Moreover, for iron and zinc there were not significant differences in bioaccessibility and total body mineral retention for each diet (Table 3).

In contrast, there was no correlation between calcium bioaccessibility and total calcium retention. Thus, the optimal point (1, 0) was not included in EJCR (data not shown). Moreover, the values of total calcium retention were significantly higher than those obtained for each diet with the in vitro method (Table 3).

For IPD and APD, the optimal point (1, 0) was included in EJCR (Figure 1d), indicating that both methods were comparable and

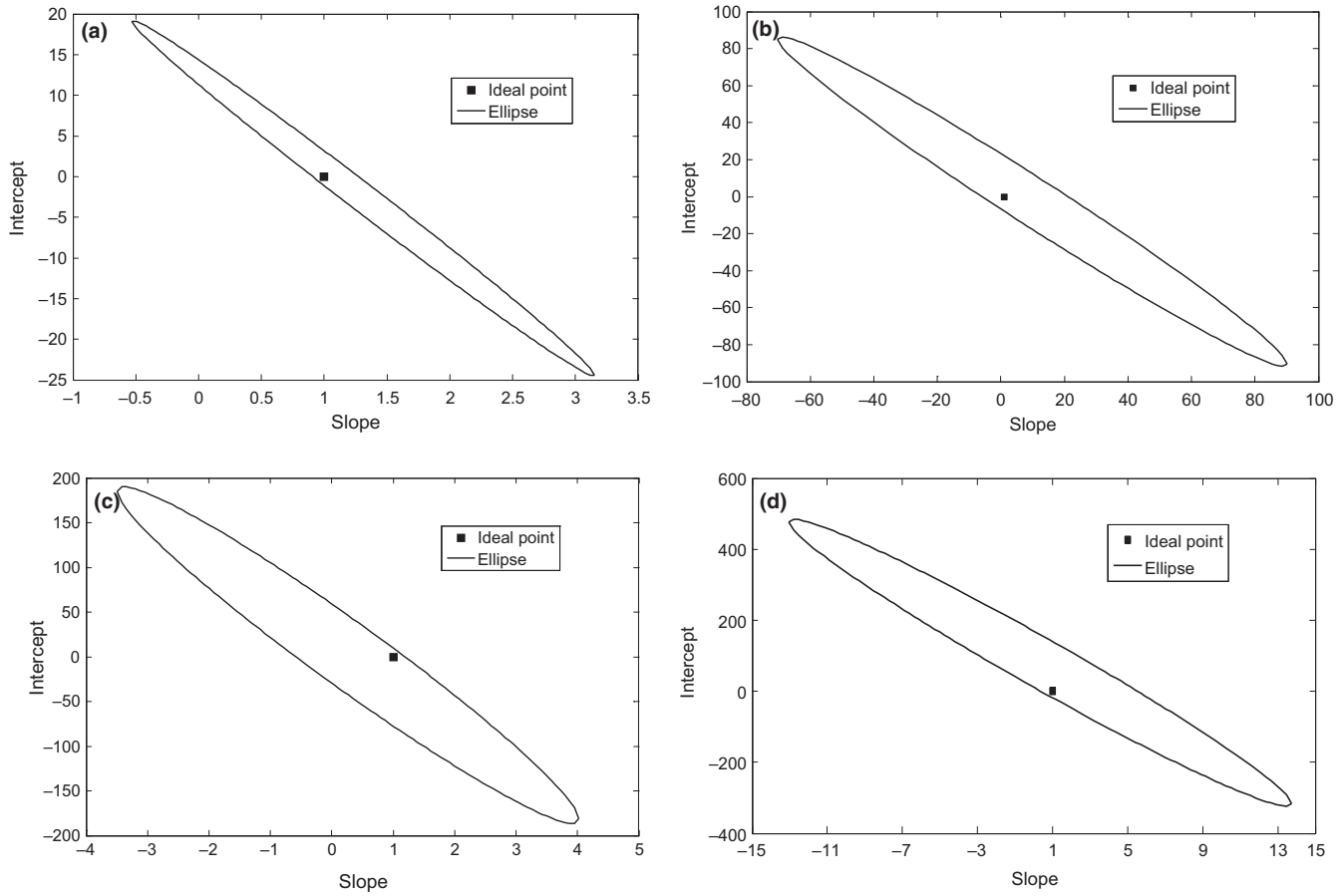


**FIGURE 1** Elliptical joint confidence region (EJCR) from correlation between in vitro and in vivo methods using weighted least-squares. Correlation between iron bioaccessibility and total iron retention (a), correlation between zinc bioaccessibility and total zinc retention (b), correlation between phosphorus bioaccessibility and total phosphorus retention (c) and correlation between in vitro protein digestibility and apparent protein digestibility (d)

correlated satisfactorily. In agreement, there were not significant differences between IPD and APD for each diet (Table 4).

## 4 | DISCUSSION

### 4.1 | Mineral bioavailability and apparent protein digestibility of diets

Chemical composition of a feed only provides information about the nutrient content in a diet, but not its nutritional value. If the nutrients are not well digested and absorbed in the gastrointestinal tract of the target species, the diet will have lower nutritional value (Abimorad et al., 2008).

Mineral bioavailability of $D_1$, $D_2$ and $D_3$ diets was measured using total body mineral retention parameter in juvenile *P. mesopotamicus* model. Iron, zinc and phosphorus retention was significantly higher for $D_3$ than $D_2$ and $D_1$ diets. These differences can be attributed to phytase activity, which was remained after extrusion process. Yan and Reigh (2002) studied the effects of fungal phytase at levels of 0, 500, 1,000, 2,000, 4,000 and 8,000 PU/kg diet on utilization of dietary protein and minerals by fingerling (12 g) channel catfish *Ictalurus punctatus* fed an all-plant-protein diet composed of soybean meal, corn and wheat middling's. They found phosphorus concentrations was significantly higher in bone of fish fed with >500 PU/kg than in bone of fish fed with 0 PU/kg. Moreover, the amount of zinc in bone of fish fed with diets containing 4,000 PU/kg was significantly higher than that in fish fed with 0 or 500 PU/kg. Phytase activity of $D_3$ was approximately 4,000 PU/kg, indicating the concentration of enzyme was enough to exert an effect on zinc and phosphorus retention in *P. mesopotamicus*. Note that under the physiological conditions existing in the gut, phytate chelates positively charged ions such as $Ca^{2+}$, $Mg^{2+}$, $Zn^{2+}$ and $Fe^{2+}$, thus reducing their bioavailability in fish. Moreover, most the phosphorus present in vegetable protein sources is bound to phytate and due to its low digestibility (Morales et al., 2014). The higher zinc and iron bioavailability of $D_3$ also was observed in white muscle zinc content and plasma iron concentration, which is a biomarker of iron store.

In contrast with this, no effect was observed for calcium retention in juvenile *P. mesopotamicus* model. Calcium absorption and metabolism are regulated by hormones related to vitamin D, and various feed additives may complement the efficacy of phytase in fish feed, like vitamin D analogues (cholecalciferol and ergocalciferol) (Cao et al., 2007).

Apparent protein digestibility of $D_3$ was higher than that obtained for the other diets. Phytase effect was also observed for other fish species such as carps (Bai, Qiao, Wei, Guo, & Qi, 2004) and rainbow trout (Foster, Higgs, Donsanjh, Rowshandeli, & Parr, 2000). Cheng and Hardy (2003) found phytase supplementation in expelled soybeans increased APD in rainbow trout (*Oncorhynchus mykiss*) respect to control diet (raw soybeans). Debnath, Pal, and Sahu (2005) reported APD of diets was significantly improved in *Pangasius pangasius* by enzyme supplementation up to 500 PU/kg, whereas the control group showed a low protein digestibility, confirming the established properties of phytate to form phytate–protein complexes resistant to proteolytic digestion. The increase in APD by phytase addition can be due to high pepsin activity. In this regard, Morales et al. (2014) reported the activity of gastric protease measured in gilthead sea bream fed on the phytase diet was around 60% higher than that of fish receiving the control diet. As it is known, It is noteworthy that the reduction in the hydrolysis of dietary protein due to the presence of phytate can be attributed not only to the formation of low digestible binary complexes with substrate proteins, but also in an indirect way to its binding to digestive proteases (Morales, Saenz de Rodrigañez, Márquez, Díaz, & Moyano, 2013).

## 4.2 | In vitro mineral bioaccessibility and protein digestibility of diets

As mentioned before, bioaccessibility of $D_1$, $D_2$ and $D_3$ diets was estimated using an in vitro method (Wolfgor et al., 2002), which measures mineral dialysability under controlled pH conditions after a digestion-simulating physiological process. In this regard, pepsin digestion pH was adjusted to stomach pH of *P. mesopotamicus* and PIPES buffer molarity was adjusted in order to obtain a final dialysate pH according to pH of intestinal content observed in in vivo model. Therefore, the in vitro bioaccessibility method used in our study reproduced the gastrointestinal pH of *P. mesopotamicus*, which is an important task since depending on iron's oxidation state and the presence of complexing agents, pH can cause iron precipitation and prevent in vitro dialysability or its in vivo absorption. Also, zinc solubility (which depend on pH) in the intestinal lumen is a major factor determining the uptake by enterocytes (Cousins, 1997).

Zinc and phosphorus in vitro bioaccessibility of $D_3$ was higher than $D_2$ and $D_1$ diets as observed in in vivo model. The exogenous factor identified as the most important absorption inhibitor of zinc is phytic acid (Fairweather-Tait, 1992). Phosphate groups of hexaphosphate and pentaphosphate inositol can form strong complexes with this mineral, lowering its solubility (Drago, 2016). Thus, phytase added to diet play an important role improving zinc absorption. Moreover, phytase can hydrolyse phytate increasing phosphorus availability of vegetable meal (Cao et al., 2007; Yan & Reigh, 2002). Phytase activity usually shows two wave crests: the highest activity around pH 5.0–5.5 and the second highest around pH 2.5 (Cao et al., 2007). Ji (1999) reported poor efficacy of phytase activity in agastric fish with digestive systems pH of 6.8–7.3. However, in fish with lower digestive pH, an increase in phytase activity was observed (Cao et al., 2007; Morales, Moyano, & Marquez, 2011). Thus, the thermo-resistant fungal phytase added to $D_3$ formulation was active at simulated gastric pH of the in vitro method used to predict bioaccessibility and the time of pepsin digestion was enough to reduce phytic acid in diet. In this regard, Yan and Reigh (2002) studied the effects of phytase on dephosphorylation of phytic acid in the gastrointestinal tract of *Ictalurus punctatus*. The levels of total phytate from the diet decreased from 32% to 94% in stomach contents of fish fed 1,000–8,000 PU/kg, respectively, within 2 hr after feeding. Note that $D_3$ had a residual phytase activity of 4,000 PU/kg. Finally, in vitro method was able to detect differences in bioaccessibility from diets with different formulations, as was the phytase addition to $D_3$.

In vitro protein digestibility values were similar to those found in vegetable extruded product such as extruded maize products added with a red seaweed *Porphyra columbina* (Cian et al., 2014), extruded whole grain red sorghum (Llopart et al., 2014) and extruded whole rice grain (Albarracín et al., 2015). In this regard, IPD of $D_3$ was higher than that obtained for other diets and approximately 11% higher than $D_1$. Knuckles, Kuzmicky, and Betschart (1985) evaluated the effects of sodium phytate and partially hydrolysed sodium phytate on in vitro pepsin digestion of casein and bovine serum albumin using a dialysis method. They found the inhibitory effect of phytate differed with the substrate and increased with dose level. At the highest phytate level, the digestion of casein and bovine serum albumin was reduced by 14% and 7% respectively. This result was associated to phytase activity which increased protein digestibility through breakdown of phytin–protein complexes (Cao et al., 2007; Gilani, Cockell, & Sepehr, 2005; Selle, Ravindran, Caldwell, & Bryden, 2000).

## 4.3 | Correlation between in vitro and in vivo methods

All methods based in the use of specific fish enzymes that have been published and propose to use them instead of mammal enzymes, used Pearson's correlation coefficient or ordinary least-squares (OLS) to compare in vivo and in vitro method (Dimes, Garcia-Carreno, & Haard, 1994; Dimes & Haard, 1994; Dimes et al., 1994). In these works, it is concluded that the methods with fish enzymes are better than with mammalian enzymes doing OLS. However, applying this test to the regression parameters derived from the least-squares method assumes that the results in the *x* axis (often the reference method) are error-free, or that the errors assigned to the reference method are negligible with respect to those given by the new method (*y*-axis). This is not true since the precision of both methods must often be taken into account. These precisions can be considered using the different existing approaches for calculating regression coefficients and related statistical parameters that consider errors in both axes (Olivieri & Goicoechea, 2007; Riu & Rius, 1997). Franco, Mantovani, Goicoechea, and Olivieri (2002) reported the dangers of not using a correct regression method for the comparison of analytical methods. In addition, the fact that in some cases the weighted least-squares (WLS) and bivariate

least-squares (BLS) methods can produce similar results, but very different from those provided by OLS (ordinary linear regression), is discussed by these authors. Thus, in this work, the correlations between in vivo and in vitro methods were performed by WLS, considering the elliptical joint confidence region (EJCR).

For iron, zinc and phosphorus both methods (in vitro mineral bioaccessibility and TBMR in *P. mesopotamicus*) were comparable and correlated satisfactorily. In the case of iron and zinc, the values obtained for each diet were not significantly different, indicating the in vitro method estimates accurately the TBMR in both, direction and magnitude. However, the values of phosphorus were significantly higher than those obtained for each diet with the in vitro method. Therefore, although the in vitro method reproduced the trend of the results, it was not accurate. For zinc and iron, a direct relationship between bioaccessibility and white muscle zinc content ($r^2$ = .9748) and plasma iron concentration ($r^2$ = .9734), respectively, was observed, indicating that the in vitro method correlate with these biomarkers, which resulted good indicators of zinc and iron nutritional status. IPD of diets was comparable and correlated satisfactorily with APD. Moreover, the values obtained for each diet were not significantly different, indicating that the in vitro method estimates accurately APD of feed for *P. mesopotamicus*. Finally, in vitro calcium bioaccessibility proved to be an inadequate method for estimating absolute calcium retention in *P. mesopotamicus*.

## 5 | CONCLUSION

This study has documented for the first time the use of in vitro mineral bioaccessibility and protein digestibility methods to estimate in vivo mineral retention and apparent protein digestibility of different fish feed. We correlated the results obtained from these in vitro methods and those achieved with traditional in vivo methods using a juvenile *P. mesopotamicus* model by weighted least-squares. However, this method could be checked in relation to other fish species and other growing stages. For iron, zinc and protein, the in vivo and in vitro methods were comparable and correlated satisfactorily. In vitro methods were able to reproduce accurately the results obtained from traditional in vivo assays using juvenile *P. mesopotamicus* model. Finally, these in vitro methods could be very useful as screening in aquaculture, to evaluate potential iron and zinc bioavailability and protein digestibility from the diets in a shorter time and cost than the traditional in vivo assays, allowing reformulation taking into account the amount of nutrient potentially available and adjusting them to the requirements.

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## CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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