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Application of *in vitro* digestibility methods in aquaculture: constraints and perspectives

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Abstract

In vitro assays simulating the digestion process have been extensively used in the evaluation of the nutritional quality of foods and feeds for humans and terrestrial animals. Such techniques have been comparatively less used in nutrition of aquacultured species, although in recent years the interest in developing alternatives to the conventional *in vivo* digestibility assays has increased. Using 65 published papers as a reference, this review summarizes the different approaches and configurations used to date in the simulation of the digestion of food by aquatic animals, as well as the main limitations detected in the different methods and the potential advantages derived from their application. Some recommendations are suggested to develop suitable *in vitro* assays oriented either to the nutritional evaluation of feed ingredients or to get a more detailed knowledge on the interactions among digestive enzymes and feed components.

Key words: *in vitro*, aquaculture, nutrition, feeds, pH-stat.

Introduction

Digestion is a highly complex process providing animals with the energy and nutrients required for their maintenance and growth. For this reason, many researchers have been interested in the total or partial modelling of the digestion process using a variety of different *in vitro* methodologies. The *in vitro* approach has been used by nutritionists, who have tried to reproduce physiological condition of the digestive tract, with a variable degree of accuracy, the compartments and working conditions existing in the digestive tract of the target species to have an insight on how feeds or food are processed or digested. For this purpose, different types of bioreactors and operating conditions have been developed to reach the two main objectives of these experiments; (i) to predict the expected biological performance after the digestion in terms of the potential bioavailability of a given nutrient or chemical substance (predictive models) or (ii) to obtain a better understanding of the different processes, interactions and factors affecting the hydrolysis of protein, lipid and carbohydrate in feeds (during the digestion process (explanatory models).

As pointed out by Coles *et al.* (2005), limitations and drawbacks of *in vitro* methods do not discount them from being a valuable research tool. A key point is to determine whether the main concern of such methods is to develop a routine laboratory tool for evaluating feedstuffs and foods, with good repeatability, rapid turnaround of results and relative accuracy or to get a deeper insight into the biological processes taking place *in vivo*.

In vitro digestion methods and approaches used with terrestrial animals

The development and use of *in vitro* chemical reactor techniques to simulate the digestion of foods and feeds began over 60 years ago and have included studies with ruminants (Kitessa *et al.* 1999; Mabjeesh *et al.* 2000), monogastric animals (Boisen & Eggum 1991; Swaisgood & Catignani 1991; Savoie 1994; Boisen 2000) or humans (Woolnough *et al.* 2008; Hur *et al.* 2011; Butts *et al.* 2012; Guerra *et al.* 2012). The more extended digestive model used in ruminant nutrition was initially developed by Tilley and Terry (1963) and simulated the degradation of organic matter and complex carbohydrates in the rumen contents by incubating feed samples in the presence of ruminal fluid. With several modifications (Michalet-Doreau & Ould-Bah 1992), this technique is still routinely used, together with other systems such as the gas-fermentation technique

(Getachew *et al.* 1998; Wilfart *et al*., 2007). It is now widely accepted that these assays provide a reliable estimation of the nutritive values of forages and compound feeds in different species of livestock (cows, sheeps and goats).

In a similar manner, a large number of *in vitro* assays have been used to simulate digestion in terrestrial monogastrics; the majority of these oriented to the nutritional evaluation of dietary proteins. The simplest methods are based on the use of simple batch reactors in which protein substrates are incubated in the presence of enzymes like pepsin (Parsons 1991), trypsin (Saunders *et al.* 1973), papain (Buchanan 1969), pronase (Taverner & Farrell 1981) or renin (Bhatty 1982) and after a given reaction time, the final digestive products or nondegraded substrates are quantified. A different method employed to assess the efficiency of digestive function on proteins is based on the pH changes produced by the release of protons as a result of enzyme hydrolysis; these are the pH-drop (Hsu *et al.* 1977) and pH-stat assays (Pedersen & Eggum 1983). The latter has been widely used in the evaluation of nutritional quality in processed foods because it is relatively simple and rapid and its reproducibility has proved to be high in interlaboratory assays (McDonough *et al.* 1990). Nevertheless, buffering capacity of some components present in feeds seems to be an obstacle in the pH-drop assay and both methods have been criticized for their simplicity, considering the complex processes taking place in the different parts of the digestive tract, as well as the interactions between hydrolysis of protein and other feed components. Despite the drawbacks, these methodologies are widely used today to model the hydrolysis of lipids (Martin *et al.* 2010; McClements & Li 2010; Li *et al.* 2011). Consequently, the use of assays simulating every phase of the digestion using suitable pH, enzyme concentrations and reaction times is suggested to achieve a suitable modelling of the complex physicochemical transformations present in the gut (Guerra *et al.* 2012). Furuya *et al.* (1979) developed a method oriented to simulate both the gastric and the intestinal phases, which included the use of pepsin and intestinal fluid obtained from fistulated pigs. A similar method, based on the use of pepsin and pancreatin, was developed by Büchmann (1979) to predict faecal digestibility of protein in rats. Some other models have an even more complex configuration to simulate the transformation of the digesta in the pig colon using microbial cellulases or a mixture of carbohydrases (Van Der Meer & Perez 1992; Boisen 2000).

Nevertheless, all the described methods are still relatively simple because all of them are based on the incubation of feed samples with enzymes in closed vessels adapted to this purpose. To simulate the complex process of hydrolysis and further intestinal absorption of nutrients, Gauthier *et al.* (1982) developed a dialysis cell system, based on a two-step hydrolysis using pepsin and pancreatin, taking place within a dialysis tube with a molecular weight cut-off 1000 Da, this allowing separation of products as they were formed. This method has been used to evaluate kinetics of enzymatic hydrolysis of macromolecules like protein (Galibois *et al.* 1989) or starch (Drake *et al.* 1991). In a similar manner, Cave (1988) developed a continuous filtration technique using 500 Da MWCO membranes or end filtration using 10 000 Da MWCO membranes to separate digested and undigested materials, applied to predict nitrogen digestibility in poultry feeds. Porter *et al.* (1984) developed a system based on the immobilization of enzymes on glass particles on which the circulating substrate was passed. The so-called immobilized digestive enzyme assay (IDEA) avoids autohydrolysis of enzymes and contamination of final products and the same enzyme can be used several times. Nevertheless, the assays are very complex to develop and require more time to conduct the assay.

Some researchers have gone one step further developing very complex gastrointestinal models. Havenaar and Minekus (1996) constructed a computer-controlled dynamic model simulating several physiological features of stomach and intestine (pH changes, peristaltic movements, transit rates, biliary and pancreatic secretions). Other similar models have been developed to simulate stomach functionality (Kong & Singh 2010; Wickham *et al.* 2012) or the microbial environment at the large intestine(Macfarlane & Macfarlane 2007; Payne *et al.* 2012). As these latter models were developed to reproduce with detail the human digestive system, they are expensive and difficult to conduct, being mostly used in pharmacological tests and less frequently to assess nutritional quality of feeds.

In spite of the great number of methodologies and experiments performed to date, no standard *in vitro* digestibility assay has been developed. Even for those assays which have been used for years, like the Rumen Simulation Technique (Czerkawski & Breckenridge 1977), the attempts to develop standard protocols are quite recent (Martínez *et al.* 2011). Gastrointestinal models used in human nutrition are in a similar situation and still require standardization of the protocols and assay conditions a task recently developed by some researchers (Hollebeeck *et al.* 2013).

In vitro digestion models and approaches used with aquatic animals

General aspects

The simulation of digestion in aquatic animals is based to a large extent upon the methodologies already developed for terrestrial animals and humans. To date, around 64 studies have been published dealing with *in vitro* digestibility relative to aquatic animals, including 45 with fish, 15 with crustaceans and 4 with molluscs (these studies are summarized in Tables 1–3). A review of the different papers reveals that

Table 1 *In vitro* digestibility assays performed on different molluscs. IVIVC means that an *in vitro*/*in vivo* correlation was evaluated in the study

Hydrolysis	pH	Enzyme source	E:S ratio	T °C	Time (min)	Ingredients evaluated	Measured parameters	Species	Reference	IVIVC
Simple closed reactors										
Alkaline	7.0	Enzymes from crystalline style/bovine pancreatin + fungal cellulase	– 1.5 U/mg subst. 0.165 U/mg subst.	20	60	C_{14} marked microcapsules	Released C_{14}	*Crassostrea gigas*	Langdon 1989;	N
Alkaline	7.2	Extracts from the whole organism	–	20	1440	4 species of phytoplankton	Peptides + reducing sugars + fatty acids	*Hyriopsis bialatus*	Areekijseree *et al.* 2006	N
Alkaline	8.2	Extracts from digestive gland and intestine	–	30	1440	10 species of phytoplankton	Released amino acids + reducing sugars	*Hyriopsis bialatus*	Supannapong *et al.* 2008	N
pH-stat										
Alkaline	8.0	Porcine trypsin + bovine chymotrypsin porcine peptidase	160 U trp +3.2 U quimotrp +2.9 U pept/mg protein	37	35	34 ingredients	Degree of hydrolysis	*Haliotis midae*	Shipton & Britz (2002)	Y

in the case of crustaceans, they have focused on the simulation of the digestive hydrolysis of protein-rich feed ingredients in penaeid shrimp (Ezquerra *et al.* 1997; Lazo *et al.* 1998; Lemos *et al.* 2000, 2004, 2009; Córdova-Murueta & García-Carreño 2002) and lobster *Panulirus argus* (Perera *et al.* 2010). In the case of fish, 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*). Moreover, some studies have focused on the development of *in vitro* techniques for the evaluation of larval feeds (Alarcón *et al.* 1999; Tonheim *et al.* 2007; Martínez-Montaño & Lazo 2012). As with crustaceans, the majority of studies with fish to date have been concentrated on the nutritional quality of protein-rich feed ingredient sources and in particular fishmeal. The focus of these studies on protein hydrolysis is in stark contrast to the majority of studies with terrestrial animals, which have focused on the development of methods to assess carbohydrate hydrolysis. This may be related to the different role that each of these major nutrients has in the metabolism and hence in nutritional requirements of each group of organisms. Also, few studies have been conducted on the *in vitro* digestive evaluation of lipids (Koven *et al.* 1997) or carbohydrates (Cousin *et al.* 1996; Omondi & Stark 1996; Simon 2009).

Configurations

The different configurations used in the simulation of digestion in aquatic animals can be divided into the following categories.

Simple digesters (one step)

1 Closed vessel (without product removal)
- **i** With measurement of products or undigested substrate (simple reactors)
- **ii** With measurement of pH changes (pH-drop; pH-stat)

2 Semi-permeable vessel (with products removal)
- **i** Membrane reactors

Compound digesters (two steps)

1 Two closed vessels
- **i** simple reactor + pH-stat
- **ii** pH-stat acid/pH-stat alkaline

2 Closed vessel + membrane reactor

3 Other configurations (i.e. pH-stat + everted intestine)

A wide range of simple digesters based on the use of a reaction vessel on which substrates and enzymes are mixed under controlled conditions being final products of the reaction measured after a given time have been applied to simulate digestion in molluscs (Langdon 1989; Supannapong *et al.* 2008), crustaceans (Lan & Pan 1993; Divakaran *et al.* 2004; Simon 2009) and fish (Eid

Table 2 *In vitro* digestibility assays performed on different crustaceans. IVIVC means that an *in vitro/in vivo* correlation was evaluated in the study

Hydrolysis	pH	Enzyme source	E:S ratio	T °C	Time (min)	Ingredients evaluated	Measured parameters	Species	Reference	IVIVC
Simple closed reactors										
Alkaline + acid	7.5 4.0	Extract from digestive gland	0.03 mg active protein/mg substrate protein	30	240 120	6 ingredients 2 feeds	Tyrosin	*Penaeus monodon*	Lan & Pan (1993)	N
Alkaline	7.0	Extracts from digestive gland	–	40	60	8 carbohydrate sources	Reducing sugars	*Pennaeus vannamei*	Cousin *et al.* (1996)	Y
Alkaline	–	Extracts from digestive gland	–	30	1440	12 carbohydrate sources	Reducing sugars	*Penaeus indicus*	Omondi & Stark (1996)	N
Alkaline	8.5	Extracts from digestive gland	–	37	60	6 feeds	Amino acids	*Litopenaeus vannamei*	Divakaran *et al.* (2004)	N
Alkaline	7.5	Extracts from digestive gland	–	30	180	3 feeds	Amino acids	*Artemesia longinaris*	Fernández Gimenez *et al.* (2009)	Y
Alkaline		Extracts from gut fluid	0.0073 U total protease/mg protein	30	1440	5 feeds + 7 ingredients	Undigested protein	*Litopenaeus vannamei*	Fox & Lawrence (2009)	Y
Acid	5.0	Foregut and digestive gland extracts	–	37 20	60 360	13 carbohydrate sources	Glucose	*Jasus edwardsii*	Simon 2009;	N
pH-shift										
Alkaline	8.0	Trypsin/Trypsin + chymotrypsin + peptidase/Trypsin + chymotrypsin + peptidase + pronase	–	37	10 10 20	8 ingredients	Relative digestibility to casein	*Penaeus vannamei*	Lazo *et al.* (1998)	N
pH-stat										
Alkaline	8.0	Extract from digestive gland/Porcine trypsin + bovine chymotrypsin + porcine peptidase + pronase	0.21 U total alkaline protease/mg protein 0.38 U total alkaline protease/mg protein	25	60	7 animal and plant meals	Degree of hydrolysis	*Litopenaeus vannamei*	Ezquerra *et al.* (1997)	Y
Alkaline	8.0	Extract from digestive gland/Porcine trypsin + bovine chymotrypsin + porcine peptidase + pronase	–	25	60	6 plant meals	Degree of hydrolysis	*Penaeus vannamei Penaeus californiensis*	García-Carreño *et al.* (1997)	N
Alkaline	8.0	Extract from the digestive gland	–	23, 25, 27	60	6 meals	Degree of hydrolysis	*Litopenaeus vannamei*	Ezquerra *et al.* (1998)	Y
Alkaline	8.0	Extract from digestive gland/Porcine trypsin + bovine chymotrypsin + porcine peptidase + pronase	2.5 U total protease/ 80 mg protein/	28	60	10 feeds + fish, krill and squid hydrolysates	Degree of hydrolysis	*Litopenaeus vannamei*	Córdova-Murueta & García-Carreño (2002)	Y
Alkaline	8.0	Extract from digestive gland	4 U total alkaline protease/80 mg protein	25	35	6 commercial feeds	Degree of hydrolysis	*Litopenaeus vannamei*	Lemos & Nunes (2008)	N
Alkaline	8.0	Extract from digestive gland	4 U total alkaline protease/80 mg protein	25	60	26 ingredients	Degree of hydrolysis	*Litopenaeus vannamei*	Lemos *et al.* (2009)	Y

Table 2 (continued)

Hydrolysis	pH	Enzyme source	E:S ratio	T °C	Time (min)	Ingredients evaluated	Measured parameters	Species	Reference	IVIVC
Alkaline	8.0	Extract from digestive gland	–	37	60	3 safflower meals	Degree of hydrolysis	*Litopenaeus vannamei*	Galicia-González *et al.* (2010)	Y
Alkaline	8.0	Extract from digestive gland	–	28	5	3 feeds + 7 ingredients	Degree of hydrolysis	*Litopenaeus vannamei*	Maldonado *et al.* (2012)	Y
Membrane reactor										
Alkaline	7.5	Extract from digestive gland	25 U/mg protein	26	360	5 fishmeals	Amino acids	*Panulirus argus*	Perera *et al.* (2010)	N

& Matty 1989; Bassompierre *et al.* 1998; Weerasinghe *et al.* 2001). Digesters used to evaluate the course of protein hydrolysis by changes in the pH have been used extensively, accounting to nearly half of total reported nutritional studies in aquatic organisms. These methods are based on the same principle: during proteolysis, protons are released from the cleaved peptide bonds at alkaline pH, resulting in a decrease in pH. Assuming a correlation between the initial rate of peptide release and protein digestibility, the latter can be predicted by (i) recording the decrease in pH (pH-drop method) or (ii) keeping the pH constant with continuous addition of NaOH and measuring NaOH consumption (pH-stat method). This latter method, developed by Pedersen and Eggum (1983), has been particularly used in the *in vitro* simulation of digestion by crustacean species. Simple digesters reproducing one step of enzyme hydrolysis (mainly the intestinal stage) by mixing enzymes or enzyme extracts with the desired substrates and measuring end products or undigested substrate have been also used to a great extent (over a third of all published studies). More complex configurations, that is, two-step systems provided with semi-permeable membranes or the technique of everted intestine have been used less frequently.

Membrane reactors have also been used by some researchers (Moyano & Savoie 2001; Hamdan *et al.* 2009; Morales & Moyano 2010; Márquez *et al.* 2012) and are based on the initial design of Savoie and Gauthier (1986). The device is formed by an inner reaction chamber separated from an outer chamber by a dialysis membrane of variable MWCO (1000–3500 Da). The amino acids, reducing sugars or minerals released during hydrolysis pass across the membrane and are continuously removed from the outer chamber by the continuous flow of buffer controlled by a peristaltic pump. Both reaction chambers are placed in a thermal water bath that maintains the system at the desired temperature. This system provides an estimation of the total amount of nutrients and other molecules potentially absorbed by the intestine after enzyme hydrolysis. Nevertheless, a better estimation of nutrient absorption *in vitro* can be provided by the everted intestine technique (Bamford *et al.* 1972). This uses segments from different parts of the intestine maintained in oxygenated Ringer solution to measure the movement of molecules across the cellular membrane barrier under different conditions. In combination with pH-stat, it has been used to assess differences in the absorption of amino acids after hydrolysis of different proteins in different fish species (Rosas *et al.* 2008; Martínez-Montaño *et al.* 2010). Nevertheless, such a configuration can be considered a combination of *in vitro* and *in vivo* techniques and tends to be quite

Table 3 *In vitro* digestibility assays performed on different fish. IVIVC means that an *in vitro/in vivo* correlation was evaluated in the study

Hydrolysis	pH	Enzyme source	E:S ratio	T °C	Time (min)	Ingredients evaluated	Measured parameters	Species	Reference	IVIVC
Simple closed reactors										
Alkaline	6.2	Intestinal extracts	–	25	1800	Algae, Bacteria	Amino acids	*Hypophthalmichthys molitrix*	Bitterlich (1985)	N
Alkaline	9.5	Intestinal extracts	29.8 BAEE Units/ mg protein	37	1440	9 ingredients	Residual nitrogen	*Cyprinus carpio*	Eid and Matty (1989)	N
Acid	5.1	Intestinal extracts	–	25	60	Laminarine	Glucose + disacarides	*Chondrostoma nasus Oreochromis* sp.	Sturmbauer (1991)	N
Acid + alkaline	–	Extracts from different gut parts	–	30	60, 120, 180, 1020	3 marked lipids (C_{14})	Lipid hydrolysis	*Scophtalmus maximus*	Koven *et al.* (1997)	Y
Acid	3.0	Porcine pepsin	10 U/mg protein	45	960	8 ingredients 15 feeds	–	*Oncorrhynchus mykiss*	Gomes *et al.* (1998)	Y
Alkaline	7.8	Pyloric caeca extracts	0.05 U SAPNA/ mg protein	15	1440	3 fishmeals	Amino acids	*Oncorrhynchus mykiss*	Bassompierre *et al.* (1998)	N
Alkaline		Trypsin + chymotrypsin + protease; Intestinal extracts from salmon and tuna	192 U BAEE U + 102 U BTEE + 5.7 U protease/ mg protein	25	720	8 feeds	Amino acids	*Thunnus maccoyi*	Carter *et al.* (1999)	N
Alkaline	8.0	Cod trypsin + bovine quimotrypsin + porcine LAP + amylase + wheat germen lipase + bile salts/ Intestinal extracts	–	30	240	Zooplancton	Residual nitrogen	*Clarias gariepinus* (larvae)	García-Ortega *et al.* (2000)	N
Acid + alkaline	3.0 9.0	Stomach + pyloric caeca extracts/Porcine pepsin/ Porcine trypsin + bovine chymotrypsin + porcine peptidase	–	37	1080 60	12 ingredients	Soluble phosphorus	*Oncorrhynchus mykiss*	Weerasinghe *et al.* (2001)	N
Alkaline	8.2	Intestinal extracts	–	15	1440	27 fishmeals	Amino acids	*Salmo salar*	Rungruangsak-Torrissen *et al.* (2002)	Y
Alkaline	8.0	Trypsin + chymotrypsin + bacterial protease	–	22	720	13 meals	Amino acids	Fish larvae	Tonheim *et al.* (2007)	N
Alkaline	8.0	Trypsin + chymotrypsin + bacterial protease	–	22	720	4 feeds	Soluble protein Amino acids	Fish larvae	Nordgreen *et al.* (2009)	N
Alkaline	9.0	Intestinal extract	2.5 U/mg protein	25	90	8 meals	Protein fractions by SDS-PAGE	*Solea senegalensis*	Sáenz de Rodrigánez *et al.* (2011b)	N

Table 3 (continued)

Hydrolysis	pH	Enzyme source	E:S ratio	T °C	Time (min)	Ingredients evaluated	Measured parameters	Species	Reference	IVIVC
Acid + alkaline	3.0 8.5	Whole larval extracts	–	37	30	4 feeds	Tyrosin	*Atractosteus tropicus (larvae)*	Aguilera *et al.* (2012)	N
Alkaline	8.2	Extracts from whole body or digestive tracts	–	30	1440	5 natural feeds + 8 commercial feeds +18 ingredients	Amino acids + reducing sugars	*Beta splendens*	Thongprajukaew *et al.* (2013)	N
pH-shift/pH-drop										
Alkaline	–	–	–	37	10 720	6 ingredients	–	*Symphysodon aequifasciata*	Chong *et al.* (2002)	Y
Alkaline	7.8	Porcine trypsin	3.600 BAEE U/mg protein	15	10	4 experimental feeds	Relative digestibility to casein	*Oncorrhynchus mykiss*	Fenerci and Erdal (2005)	Y
Alkaline	8.0	Extracts from the whole digestive tract	–	–	10	9 ingredients	Relative digestibility to casein	*Anabas testudineus*	Ali *et al.* (2009)	N
Alkaline	8.0	Extracts from the whole digestive tract	–	–	10	4 ingredients	Relative digestibility to casein	*Orechromis nilotica*	Sultana *et al.* (2010)	N
pH-stat										
Acid or alkaline	8.0	Porcine pepsin/Porcine trypsin + bovine chymotrypsin + porcine peptidase	–	–	–	10 diets including different fishmeals	Undigested residue/ Degree of hydrolysis	*Salmo salar*	Anderson *et al.* (1993)	Y
Alkaline	8.0	Extract from pyloric caeca/ Porcine trypsin + bovine chymotrypsin + porcine peptidase + pronase	BAEE U/mg protein: Trp 3.500 Chymotrp 25 Peptidase 17 Pronase 5.3	37	60–80	6 poultry meals	Degree of hydrolysis	*Oncorhynchus mykiss*	Dong *et al.* (1993)	Y
Alkaline	7.7	Intestinal digesta	2.0 TAME U/mg	15	690	Protein concentrate	Protein fractions	*Oncorrhynchus mykiss*	Grabner (1985)	N
Alkaline	8.0	Purified extract from pyloric caeca	–	37	120	Fishmeals	Degree of hydrolysis	*Oncorrhynchus mykiss*	Dimes and Haard (1994)	Y
		Purified extract from pyloric caeca					Degree of hydrolysis	*Oncorrhynchus mykiss*	Dimes *et al.* (1994a,b)	Y
Alkaline		Purified extract from pyloric caeca				Casein	Degree of hydrolysis	*Oncorrhynchus kisutch*	Haard *et al.* (1996)	N
Alkaline						16 fishmeals	Degree of hydrolysis	*Salmo salar*	Anderson *et al.* (1997)	N
Alkaline	8.0	Whole larval extracts	0.8 U total protease/mg protein	25	90	Larval feeds	Degree of hydrolysis	*Sparus aurata*	Alarcón *et al.* (1999)	N

Table 3 (continued)

Hydrolysis	pH	Enzyme source	E:S ratio	T °C	Time (min)	Ingredients evaluated	Measured parameters	Species	Reference	IVIVC
Alkaline	8.0	Purified extracts from pyloric caeca	9 BAPNA mU + 53 SAPNA mU/ mg protein	25	600	17 ingredients	Degree of hydrolysis	*Gadus morhua*	Tibbetts *et al.* (2011)	Y
Alkaline	8.0	Larval extracts	3.75 U total alkaline protease/ mg protein	37	90	9 ingredients	Degree of hydrolysis	*Paralichthys californicus* (larvae)	Martínez-Montaño and Lazo (2012)	N
Acid + alkaline	2.0 9.0	Stomach and intestinal extracts/ commercial enzymes	5 U total protease/mg protein	37	90	3 feeds	Degree of hydrolysis	*Totoaba macdonaldi*	Minjarez-Osorio *et al.* (2012)	N
Membrane reactors										
Alkaline	7.8	Extract from pyloric caeca	0.05 SAPNA U/mg protein	15	1.440	3 Fishmeals	Amino acids released	*Oncorrhynchus mykiss*	Bassompierre *et al.* (1997a)	N
Alkaline	7.5	Extract from digestive gland	25 U/mg protein	26	360	5 Protein meals	Amino acids released	*Panulirus argus*	Perera *et al.* (2010)	N
Alkaline	8.5	Extract from pyloric caeca	1.68 protease/mg protein	25	360 min	8 feeds	Amino acids released	*Sparus aurata*	Sáenz de Rodrigánez *et al.* (2011a)	N
Simple reactor + pH-stat										
Acid + alkaline in trout; alkaline in carp	3.8 7.7 6.7	Intestinal and stomach digesta	5.6 U acid protease + 2.0 alkaline (TAME) U/mg protein	15 23	900 + 690 300	Leguminous meal	Protein fractions	*Oncorrhynchus mykiss* *Cyprinus carpio*	Grabner and Hofer (1985)	N
Acid + alkaline	3.8 + 7.8	Extracts from stomach and pyloric caeca	0.024 SAPNA U/mg	–	1.440 + 1.500	3 fishmeals	Soluble amino groups	*Oncorrhynchus mykiss*	Bassompierre *et al.* (1997b)	N
Alkaline acid + alkaline	8.0 2.0 + 8.0	Extracts from pyloric caeca and duodenum – Porcine pepsin + extracts from pyloric caeca and duodenum	2.5 U total protease/mg protein 10 U acid protease +2.5 U alkaline protease/mg protein	37 37	90 60 + 90	9 ingredients	Degree of hydrolysis	*Sparus aurata*	Alarcón *et al.* (2002)	N
Acid + alkaline	2.0 + 7.8	Porcine pepsin + Intestinal extracts	–	37	30 + 60	Sardine hydrolysate	Degree of hydrolysis	*Thunnus orientalis*	Martínez-Montaño *et al.* (2010)	N
Simple reactor + membrane reactor										
Acid + alkaline	2.0 + 7.7	Stomach and intestinal extracts/ porcine pepsin + porcine pancreatin	12.5 U acid protease + 2.5 U alkaline protease/mg protein	25 37	30 + 360	Fish and soybean meals	Degree of hydrolysis vs Amino acids released	*Sparus aurata*	Moyano and Savoie (2001)	N

Table 3 (continued)

Hydrolysis	pH	Enzyme source	E:S ratio	T °C	Time (min)	Ingredients evaluated	Measured parameters	Species	Reference	IVVC
Acid + alkaline	2.0 + 8.0	Stomach and pyloric caeca extracts	200 U total protease/mg protein	22 37	120	Commercial feed	Amino acids and reducing sugars released	*Argyrosomus regius*, *Dicentrarchus labrax* *Oreochromis niloticus*	Hamdan *et al.* (2009)	N
Acid + alkaline	2.0 + 9.0	Stomach and pyloric caeca extracts	31 U acid protease + 19 U alkaline protease/mg protein	37	60 + 180	Fish and soybean meals	Amino acids and phosphorus	*Oncorhynchus mykiss*	Morales and Moyano (2010)	N
Acid + alkaline	4.0 + 8.5	Stomach and pyloric caeca extracts	50 U acid protease + 27 U alkaline protease/mg protein	25	120 + 240	4 feeds	Amino acids released	*Salmo salar*	Morken *et al.* (2012)	Y
Acid + alkaline	4.0 + 8.5	Stomach and pyloric caeca extracts	31 U acid protease + 19 U alkaline protease/mg protein	37	60 + 180	8 feeds	Amino acids released	*Oncorhynchus mykiss*	Márquez *et al.* (2013)	Y

complex because the intestinal portion must be maintained alive using specialized equipment.

Operating conditions

Equally important as the use of a suitable configuration of the model is to adapt the main operating conditions to those existing in the digestive tract of the selected target species. Within this context, there are several main factors to consider; class, source and amount of enzymes, pH, temperature and reaction time. Most assays aimed to simulate *in vitro* digestion of aquatic animals have used enzyme extracts obtained from different parts of the digestive system (digestive gland in the case of crustaceans, stomach pyloric caeca and proximal intestine in the case of fish). In some cases, the whole organism or their own digesta are used as a source of enzymes (Grabner & Hofer 1985; Alarcón *et al.* 1999; Sultana *et al.* 2010). A significant number of studies also use commercially available enzymes from mammals or microbial origin (Anderson *et al.* 1993; Lazo *et al.* 1998; García-Ortega *et al.* 2000; Shipton & Britz 2002; Tonheim *et al.* 2007; Nordgreen *et al.* 2009), and several studies have compared the results obtained using both types of enzyme sources (Dong *et al.* 1993; Dimes *et al.* 1994a; Ezquerra *et al.* 1997; Carter *et al.* 1999; Moyano & Savoie 2001; Córdova-Murueta & García-Carreño 2002; Minjarez-Osorio *et al.* 2012).

The amount of enzyme activity reportedly used by researchers in their assays has been highly variable (see Tables 1–3), and in most cases, the ratio of enzyme to substrate (E:S) used is not clearly indicated nor justified on a physiological basis. In many cases, the composition of the enzyme mixture is merely adapted from other studies in spite of these have been performed on a different species. The pH selected for the assays is usually 2.0 or 3.0 in studies including an acid phase of the digestion and 7.5–9.0 when simulating the alkaline phase. The reactors are generally maintained at mild temperatures ranging from 15 to 25°C, although in many cases a body temperature for mammals is used (37°C). Higher temperatures of 40 or 45°C are scarcely employed (Cousin *et al.* 1996; Gomes *et al.* 1998). The length of the assays is also highly variable, ranging from 5 to 15 min (Bassompierre *et al.* 1998; Lazo *et al.* 1998; Maldonado *et al.* 2012) to 12 and even 24 h (Eid & Matty 1989; Bassompierre *et al.* 1997a; Rungruangsak-Torrissen 2007; Supannapong *et al.* 2008; Fox & Lawrence 2009; Thongprajukaew *et al.* 2013), although in most cases lasts <3–4 h.

Parameters measured

The parameters used to measure the action of the enzymes within the different models are very diverse. In the case of

pH-stat assays, the evaluated parameter is always the degree of hydrolysis (DH,%; Rutherfurd 2010), while in other types of reactors, the extension of the enzyme action is measured using different methodologies to quantify the amount of amino acids released; the o-phthaldialdehyde method (Hamdan *et al.* 2009; Perera *et al.* 2010; Sáenz de Rodrigánez *et al.* 2011b; Morales *et al.* 2013), the TNBS method (Divakaran *et al.* 2004; Rungruangsak-Torrissen 2007; Supannapong *et al.* 2008; Marmon & Undeland 2013) or the HPLC (Bitterlich 1985; Berge *et al.* 2004; Martínez-Montaño *et al.* 2011; Marquez *et al.* 2013). Other parameters, such as the amount of released tyrosin (Lan & Pan 1993), the relative digestibility to casein (Lazo *et al.* 1998; Fenerci & Erdal 2005; Sultana *et al.* 2010) or the amount of undigested protein (García-Ortega *et al.* 2000; Fox & Lawrence 2009), have been used. Grabner (1985) developed a methodology in which the hydrolysis of protein was assessed by a detailed evaluation of the protein fractions released, and this approach obtained good estimations of the *in vivo* process, but it was expensive and time-consuming. Other studies assess the effect of enzymes on specific substrate proteins using electrophoresis SDS-PAGE (Alarcón *et al.* 2002; Perera *et al.* 2010; Sáenz de Rodrigánez *et al.* 2011b; González-Zamorano *et al.* 2013). In studies aimed to evaluate carbohydrate hydrolysis, the parameter used is the production of reducing sugars (Cousin *et al.* 1996; Omondi & Stark 1996; Supannapong *et al.* 2008; Hamdan *et al.* 2009), while only one study estimating *in vitro* hydrolysis of lipids evaluated the release of 14C-labelled lipid substrates (Koven *et al.* 1997).

In vitro digestion models in aquatic animals; critical issues

In spite of the significant amount of information provided to date, some of the configurations and operating conditions used in many of the described studies seem not to accomplish properly with the requisites needed for an accurate simulation of the *in vivo* digestion process. For example, in many cases, only the intestinal stage of the digestion but not the gastric phase is simulated; such an approach may be suitable in the case of decapod crustaceans, but incorrect for many fish species with a functional stomach. It has been demonstrated that stomach digestion of protein has a remarkable effect on further processing due to the effect of both acid pH, with affects protein solubility (de Jonge *et al.* 2009) and pepsin (Alarcón *et al.* 2002). Also, stomach digestion has also been shown to inactivate some trypsin inhibitors present in protein-rich plant ingredients (Krogdahl & Holm 1981; Alarcón *et al.* 2001). Finally, total bioavailability of minerals (P, Fe) is conditioned to a great extent by their solubilization at acid pH (Morales & Moyano 2010). Taking this into account, it follows that both the quantitative and qualitative results of protein hydrolysis obtained when using configurations not including a simulation of stomach digestion may be quite different from those existing in the live fish. In this sense, gastrointestinal models simulating the two steps of hydrolysis should be most accurate (Moyano & Savoie 2001; Morales & Moyano 2010).

Many studies are based in the use of the pH-stat methodology; this system has several important advantages (easy to operate, short reaction time, repeatability), but also some potential limitations. Perhaps the more important is the need to maintain an alkaline pH (>8.0) to ensure that all the protons do not remain attached to the amino groups of the released amino acids, thus limiting the possibility of simulation only to the alkaline phase of the digestion (Márquez & Vázquez 1999; Rutherfurd 2010). In addition, it operates in a small closed reactor on which final products are quickly accumulated, can interfere with the hydrolysis and cannot be separated from the initial substrate for analysis. On the other hand, the measurement may be influenced by the buffering capacity of the protein source and in the case of using semi purified enzyme extracts and complex substrates, the release of protons as a result of lipids hydrolysis by lipases may interfere with the determination of protein hydrolysis itself.

Several studies are carried out using commercial enzymes purified from microorganisms or from mammalian; nevertheless, different authors have demonstrated that fish digestive enzymes, and particularly, proteases have different features than those in other vertebrates, mainly their affinity for substrates, reaction speed, thermal optimum or sensitivity to inhibitors (Díaz-López *et al.* 1998; Alarcón *et al.* 1999; Kumar *et al.* 2007). While assays performed with bacterial or mammal enzymes may be useful to show differences between different substrates in their susceptibility to hydrolysis under similar conditions, results probably will be quite different from those obtained in live animals. Hence, assays performed using digestive enzyme extracts obtained from a given species should give a more accurate picture on the potential bioavailability of nutrients in such species. These aspects have been well addressed by several authors (Dimes *et al.* 1994b; Haard *et al.* 1996).

On the other hand, in most cases, there is a lack of explanation for the rationale to determine the amount of enzymes used in the assays, and it seems that they were selected only to produce a clearly measurable effect. The absence of a standardized relationship between the enzyme/substrate ratio used in the assays and that presumably existing in the digestive tracts of the different species may produce results not directly related to the *in vivo* process, while this has been an important point with *in vitro* digestibility assays conducted with terrestrial animals and humans (Smeets-Peeters *et al.* 1998; Ulleberg *et al.* 2011). In the

case of aquatic animals and with some exceptions (Grabner & Hofer 1985; Morales & Moyano 2010; Morken *et al.* 2012), this aspect has not been well explained or developed. Again, this suggests that most of the results obtained in the assays may be far from the true biological response and that the objective of most studies has been to develop simple and useful approaches oriented to a comparative evaluation of ingredients, but not to necessarily assess their bioavailability on a biological basis. Considering this, the pH of the *in vitro* assays should also be based on *in vivo* measurements. Although pH values ranging from 7.5 to 9.0 may not reflect the conditions of the digestive tract of most species, a pH of 2.0 used in assays including an acid phase may be quite far from the real conditions, because the pH existing in the fish stomach usually is much higher, and this clearly affects pepsin functionality and hence net results (Márquez *et al.* 2012).

The temperature used in the reactions clearly affects not only total time required for the assays but also the possible correlation to *in vivo* results (Ezquerra *et al.* 1998). For example, incubation temperatures reportedly used in the assays have varied from 15 to 37°C; the first value has a physiological basis as it is similar to the body temperature of many aquatic organisms, but it may result in very slow reactions. High temperatures are not justified from a physiological basis, but they allow getting results on a shorter time. Taking this into account, the reaction times used in the assays seems to be a compromise between the need to obtain significant and reliable results and their feasibility at lab scale and show a high variability; ranging from the 10 min used in the experiments based in the use of pH-drop up to 24 h in some assays performed with simple digesters. Clearly, it would be inappropriate to reproduce the digestion times existing in live animals, because they are influenced by several factors (age, food composition, temperature, etc.) and show a great variability. Nevertheless, too short reaction times reveal the limitations associated with the models based in closed and small reactors, while long reaction times may not be adequate mainly due to problems associated with bacterial contamination, saturation by reaction products and autohydrolysis of enzymes.

In relation to the types of substrates evaluated in the assays, it is worthwhile to mention that ingredient particle size is not specified in most of the published papers, but it is an important aspect with a high influence on the final results. In addition, with some exceptions (i.e. Morken *et al.* 2012; Márquez *et al.* 2013), most studies were carried out on single ingredients that have not been subjected to the processing conditions used in feed preparation (steam pelleting or extrusion). Thermal treatment leads to physicochemical and chemical changes that affect protein quality (Camire 1998). Heat-induced changes in the protein structure may render the protein less susceptible to hydrolysis by digestive proteases (Phillips 1989). It may have an important effect on the result of the *in vitro* assay and hence in the comparative ranking obtained when ingredients with a different sensitivity to thermal processing are evaluated.

Problems with the comparison of *in vitro/in vivo* digestibility values

Digestion could be defined in a simple manner as the transformation of food/feeds into more simple components that can be absorbed by the intestine of animals. Digestibility would be an indicator of the efficiency of such process related either to the whole food/feed or to a specific nutrient, being usually expressed under the form of percentages. Other related terms to be considered in this context are bioaccessibility and bioavailability. Bioaccessibility can be defined as the fraction of a nutrient released from the food matrix that becomes available for further hydrolysis and absorption (Hedrén *et al.* 2002). Bioavailability is a concept associated with the efficiency of intestinal absorption and metabolic use of an ingested nutrient (Gregory *et al.* 2005) defined as the fraction of that nutrient becoming available for physiological functions or storage. Although the total amount of a nutrient present in a feed may be potentially bioaccessible, in practice, it may never be absorbed during digestion (Faulks & Southon 2005). The bioavailability of nutrients is usually measured in blood plasma or as net incorporation of the nutrients to animal biomass (Pack *et al.* 1995; Lall & Anderson 2005). Almost all researchers who use *in vitro* models to simulate the digestion stages in the digestive tract of animals invariably indicate that one of their main objectives is to measure the *digestibility* of nutrients and/or ingredients. Nevertheless, this is not correct when considering the above indicated definition of digestibility and what is really measured when using bioreactors, which is mostly the amount of hydrolysed products released by enzymes from different substrates under controlled conditions. For this reason, the correlation between *in vitro* and *in vivo* estimations of digestibility may be not easy. Only one-third of the papers evaluated in this study tried to establish correlations between results obtained both *in vivo* and *in vitro*. In this sense, it must be taken into account that the concordance between results will increase with the ability of the *in vitro* assay to simulate stages of the digestion resulting in products similar to those produced by the *in vivo* digestion. As the digesta is transformed as progresses within the digestive tract, from the stomach (chyme) up to the distal intestine (faeces), a better correlation can be achieved using models able to simulate the different phases of the process (Fig. 1). Hence, it is presumed that assays based in the use of simple closed digestors simulating single acid or alkaline hydrolysis will

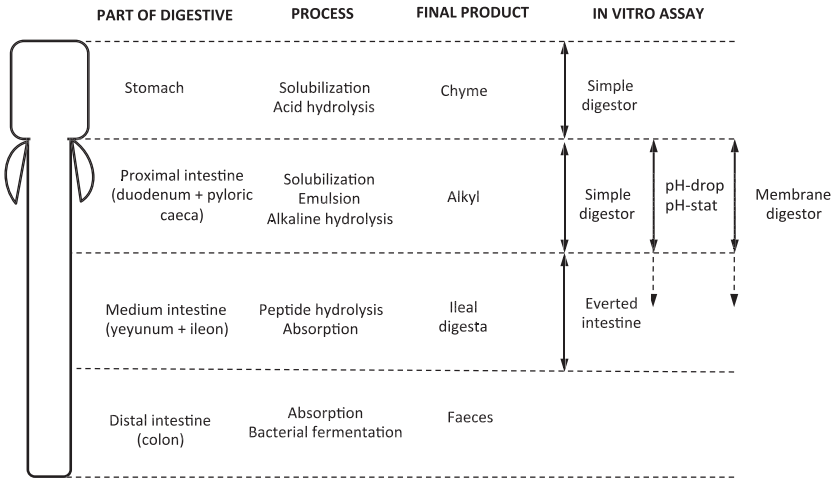

Figure 1 Correlation between processes taking place within the digestive tract and *in vitro* assays used to simulate them.

produce results quite different of those present *in vivo*. As detailed previously, although in most fish stomach digestion is not as important as in mammals, acid digestion may quantitative and qualitatively affect the net result of whole digestion process and this should explain why results obtained in many *in vitro* assays show a poor correlation to values measured *in vivo* (Nengas *et al.* 1995; Gomes *et al.* 1998). Hence, these models simulating more advanced stages of the digestion (production of alkyl) may offer a better fitting, particularly when the subject species are carnivorous fish with a well-developed stomach digestion.

On the other hand, the composition of the ingredients affects to a great extent the possibility of getting a good correlation. Without some exceptions (i.e. collagen), ingredients of animal origin are generally more easily digested and they suffer small transformations after hydrolysis and absorption; hence, the composition of the ileal digesta is quite similar to that of faeces. In contrast, ingredients of plant origin present compounds like carbohydrate polymers that are difficult to hydrolyse by most aquatic species, being also fermentation substrates in the colon. In this case, the composition of the faecal mass may be quite different of the ileal digesta and the possibility of *in vitro/in vivo* correlation becomes difficult. These differences are clearly evidenced in studies on which correlations are established separately for animal or plant ingredients (Tibbetts *et al.* 2011). On the other hand, robust and reliable correlations require a large number of data, but with some exceptions (Gomes *et al.* 1998; Shipton & Britz 2002; Lemos *et al.* 2009; Tibbetts *et al.* 2011), the number of ingredients evaluated is <10. Although several authors have found good correlations between values of *in vitro* and *vivo* bioavailability (Chong *et al.* 2002; Shipton & Britz 2002; Lemos *et al.* 2009; Márquez *et al.* 2013), in most cases, such correlation do not exist or do not follow a simple, linear model (Dimes *et al.* 1994b; Fenerci & Erdal 2005). A possible explanation could be that hydrolysis and absorption of products in the digestive tract are very complex processes difficult to reproduce properly *in vitro*. As indicated before, the physical (mixing, disaggregation, solubilization) or chemical (effect of pH and ions, hydrolysis by specific enzymes) transformations of a given food component can be simulated in a bioreactor, but not the effect of antinutritional factors, dietary fibre or the activity and effect of intestinal microbiota. This latter point is very important; the estimation of digestibility is usually carried out by a mass balance between the amount of ingested nutrient and the fraction recovered in faeces, but the composition of faecal matter may be influenced to a great extent by internal secretions, microbial biomass or products of their metabolism. Hence, the differences between the amounts of amino acid ingested and those present in faeces is affected to a great extent by the degradation and synthesis produced by resident intestinal microbiota (Fuller & Tomé 2005). Depending on the amino acid and the type of ingredient, values of *in vivo* digestibility calculated from faecal analysis can either over or subestimate the true ileal digestibility (Moughan & Smith 1985; Moughan 2003). A more precise form of determining digestibility would require sampling of digesta prior to entering the large intestine (ileal digestibility). This process requires cannulation, a technique commonly used in studies with some terrestrial animals (Boisen & Moughan 1996) but up to date not used in aquaculture nutrition (Fig. 2). Taking this into account, it can be concluded that although *in vitro* assays are able to reproduce to a certain extent some of the main processes taking place in the gut (i.e. currently only protein hydrolysis), they do not offer a reliable measure of digestibility. They seem to be more useful in the evaluation of the initially mentioned other parameters: *bioaccessibility* or *bioavailability* of nutrients. Furthermore,

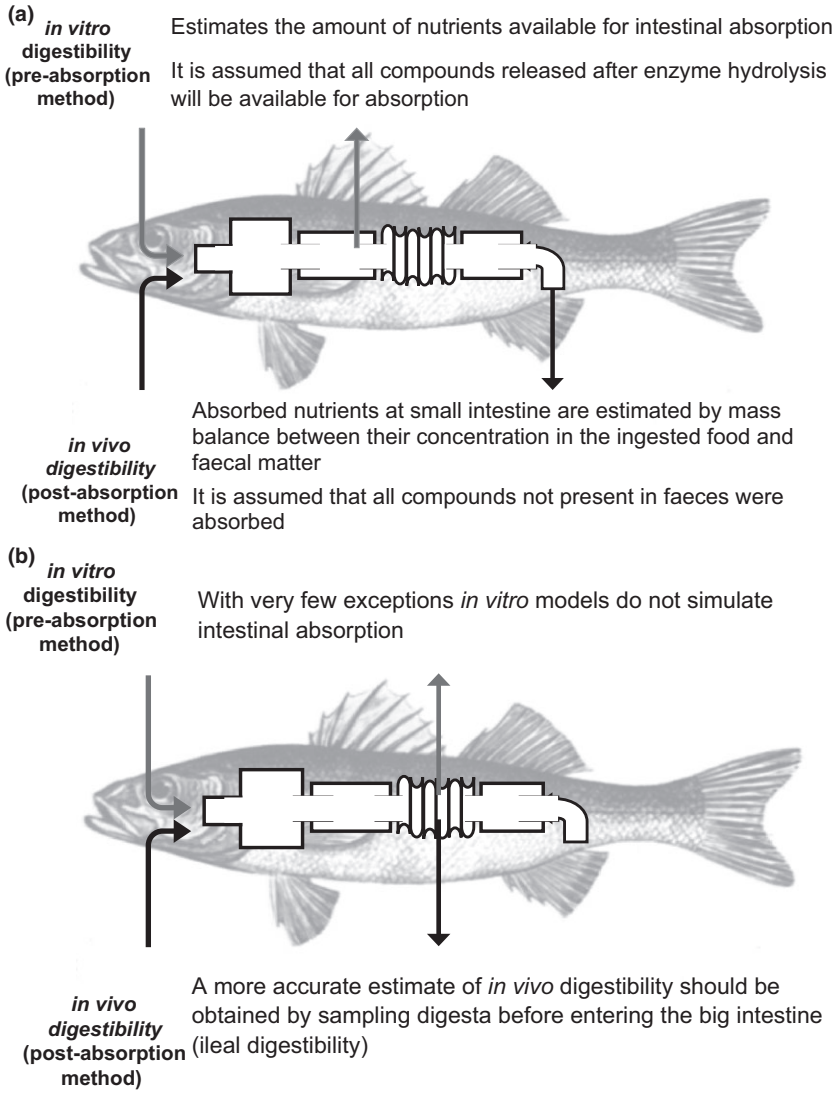

Figure 2 Comparison between *in vitro* and *in vivo* digestibility assays; (a) Data obtained in most experiments try to correlate quite different parameters, (b) Type of data that could be ideally correlated.

the assessment of *in vivo* digestibility could be considered a post-absorption way to estimate bioavailability, while *in vitro* assays would provide a pre-absorption estimation of this parameter (Table 4).

Conclusions and perspectives

One important challenge for the global feed industry today is to find a rapid and reliable method that can be used to predict accurately the nutritional value of raw ingredients, by-products and processed feeds for a given species in order to reduce to minimize the laborious and expensive *in vivo* assays. To date, *in vitro* methods simulating the digestive function in aquatic animals have been mostly used to rank feed ingredients and to a certain extent, to predict the biological performance of a given feed formulation at lab scale but are currently not routinely used by the aquafeed industry. Practical application of *in vitro* digestibility assays would require the use of a simple reliable method, because the more complex a method, the greater the possibility that errors are introduced into it through multiple steps in the methodology and through operator error and interlaboratory differences. Methods based on the use of pH-stat may accomplish this objective, although the operating conditions should be based and adapted to a greater extent to the physiological parameters existing in the selected species. In addition, for any *in vitro* method to be considered robust and repeatable, it needs to prove that it can produce the same results, within reasonable limits of statistical variation, at multiple laboratories. With some exception (Cruz Suárez 2008), there is still a lack of inter-laboratory investigation to test or standardize the *in vitro* digestibility methods used in aquaculture. If these points were properly addressed, the aquafeed

Table 4 Comparison between *in vivo* and *in vitro* methods for the estimation of nutrient bioavailability

	Apparent digestibility	Controlled hydrolysis
Typology	*In vivo*; post-absorptive	*In vitro*; pre-absorptive
Basis	It is assumed that the fraction of nonabsorbed nutrient will appear in faeces	It is assumed that the fraction of nutrient released by enzymes will be available for absorption
Constraints	The amount and composition of the nutrient may be modified by intestinal microbiota Requires special facilities Long experiments Offers limited information on the effect of factors on bioavailability of nutrients	Hydrolysis may not reproduce that taking place in the digestive Not all nutrients released will be absorbed Requires the use of enzymes obtained from the live organism Much lower hydrolysis rates than in the live organism
Advantages	The digestion process is real	Do not require expensive facilities Short experiments Offers a lot of information on the effect of factors on the bioavailability of nutrients
Main orientation	Mostly zoothecnical; offers a quantitative estimation of bioavailability	Mostly physiological; offers a qualitative estimation of bioavailability

industry could benefit from a proper technique that may reduce to a great extent the number of *in vivo* assays required to evaluate ingredients and feeds, this resulting in savings in cost and time.

Other recent approaches also emphasize this same objective; this is the case of assays based in near-infrared spectroscopy (NIRS). This technique has been used extensively to measure the crude compositional characteristics of feed grains (Alomar *et al.* 2003; Decruyenaere *et al.* 2009) and also to estimate faecal digestibility of forages in wild and domestic ruminants (Decruyenaere *et al.* 2009; Boval *et al.* 2004), rabbits (Núñez-Sánchez *et al.* 2012) and pigs (Van Barneveld *et al.* 1998; McCann *et al.* 2006). In recent years, progress has been made towards measuring digestibility of nutrients in shrimp or fish species (Maneerot *et al.* 2006; Glencross *et al.* 2008). Its potential application by the aquafeed industry is still limited by the complexity of developing suitable calibration models reflecting the particular characteristics of each different fish species and the variability in the composition of feedstuffs. The use of *in vitro* techniques may facilitate to obtain the calibration parameters required by the application of NIRS in the estimation of digestibility for those aquatic organisms on which *in vivo* determination of apparent digestibility may be difficult to perform because of their size or sensitivity to stress. In this sense, *in vitro* assays should be in-between the *in vivo* evaluation and other rapid and alternative *ex vivo* techniques used to estimate digestibility of feeds.

In addition, although bioavailability of nutrients measured using *in vitro* assays must be correlated with parameters measured *in vivo*, common measures of digestibility may not be the most suitable parameter to consider. In any case, the availability of accurate *in vivo* data is crucial for critical evaluation of any potential *in vitro* method. It is worthwhile to point out that each type of measurement can be valuable for a different reason. While animal nutritionists are more concerned with protein digestibility either true or apparent, digestive physiologists are more interested in the digestion process itself, as well as in the composition and amounts of products released to the intestinal lumen and potentially absorbed. (Savoie *et al.* 2005). It seems that results obtained with *in vitro* assays that evaluate differences in the release rate of amino acids after hydrolysis (Márquez *et al.* 2012) or their intestinal absorption (Martínez-Montaño *et al.* 2010; Martínez-Montaño & Lazo 2012) avoiding the interference with intestinal microbiota can correlate better to the potential bioavailability of protein than to its *in vivo* digestibility measured under the conventional conditions. It follows that both approaches can be complementary and that *in vitro* bioavailability assays can correlate better with other indicators of biological efficiency like the Conversion Index or the Specific Growth Rate, as suggested by results obtained by Dimes *et al.* (1994a,b) or Rungruangsak-Torrissen *et al.* (2002). These more complex *in vitro* simulation of the digestion process may be highly valuable from a more scientific point of view to get a better understanding of the interactions between digestive enzymes and different nutrients, water, ions and other chemical compounds that may be present or delivered with the feeds (antinutritional factors, antibiotics, pregiotics and probiotics). This would probably require more sophisticated configurations, including sequential acid and alkaline hydrolysis and separation of reaction products, as well as different analytical techniques.

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