Utilisation of fish or crab silage protein for cobia (*Rachycentron canadum*) – effects on digestion, amino acid distribution, growth, fillet composition and storage quality

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Dissertation for the degree philosophiae doctor (PhD)

at the University of Bergen

January 2009

The present work was funded by The Norwegian Agency for Development Cooperation through the project: ‘Improving training research capacity at University of Fisheries’ (SRV 2701). The experiments were carried out at the Institute of Aquaculture Research and the Institute of Biotechnology and Environment – Nha Trang University (new name of University of Fisheries), Vietnam from May 2006 to June 2007. The study was continued at the Department of Biology, University of Bergen under the supervision of Professor Ragnar Nortvedt.

Acknowledgements

First of all, I wish to express my thanks to Professor Ragnar Nortvedt, my supervisor, for his guidance, support, criticism and friendship from the beginning to the end of this study.

I would like to thank the leaders of the project SRV2701 and Nha Trang University Associate Professor Quach Dinh Lien, Dr. Vu Van Xung, Associate Professor Hoang Tung, Dr. Nguyen Dinh Mao, Associate Professor Lai Van Hung and Dr. Trang Si Trung for supporting my study. I am grateful to Associate Professor Ngo Dang Nghia at the Institute of Biotechnology and Environment and Mr. Trinh Van Lien at the Institute of Aquaculture Research, Nha Trang University for providing me with excellent working conditions and laboratory facilities. I want to thank component II, Aquaculture Faculty, Nha Trang University, Nha Trang Oceanography Institute and the Advanced Laboratory, Can Tho University in Vietnam for assistance with facilities. I want to thank all my colleagues at the Aquaculture Faculty, the Institute of Biotechnology and Environment and the Institute of Aquaculture Research, Nha Trang University for advice and collaboration in the execution of the present study.

Thanks to Adjunct Professor Gro-Ingunn Hemre and Dr. Maris Espe at the National Institute of Nutrition and Seafood Research (NIFES) for valuable guidance and criticism of the experimental design. Thanks also to Professor Helge Reinertsen (NTNU Trondheim) and Dr. Bjørn Eirik Olsen (NFC Tromsø), project advisers for their support, encouragement and friendship. Special thanks to my friend, Miss. Nguyen Thu Thao for invaluable help and technical advice. I would like to thank Mrs. Anne Ruth and Professor Rune Nilsen for their care, support and encouragement during my study.

I am very grateful to the teachers and staff in the Department of Biology, University of Bergen, Norway for their teaching, support and encouragement. Thanks to Mrs. Berit Øglænd for making my life comfortable here.

Finally, special thanks to my son, Phan Ngoc Anh, my dad, Mach Hoi and my husband, Terje Ellertsen for their love and support.

Bergen, January, 2009

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Abstract

The present study was carried out to test the ability to utilise fish silage to feed cobia *(Rachycentron canadum)* in aquaculture in Vietnam. All four experiments of the present study were carried out over 13 months from June 2006 to June 2007 at Nha Trang University in Vietnam. The experiments involved making acid-silages and testing the effects of silage-based diets on digestion, amino acid absorption, growth and the nutritional quality of the cobia fillets and their shelf life.

The local by-catch fish, lizardfish (*Saurida undosquamis*) and blue crab (*Portunus pelagicus*), were used to prepare 12 uncooked or cooked silages in study I. Different amounts of formic acid (85%) were mixed with the minced fish and crab and the silages were stored at ambient temperature (30 ± 2 ^{o}C) for 60 days. The antioxidant effect of adding ethoxyquin was also investigated. At the end of the experiment, the nutritional quality of all silages was stable. However, cooked silages showed a tendency for spoilage, particularly cooked crab silages. The antioxidant effect was only observed in fish-silage groups in which the TBARS values were significantly higher in the silage without addition of ethoxyquin than in the silage with it. Generally, the composition of raw materials was reflected in the composition of the silages. There were significant differences in composition between different raw material-based silages and between uncooked and cooked silages, particularly levels of ash, crude protein (CP) and non-protein nitrogen (NPN) at the end of the trial (for ash: 3.7 – 4.7% in fish vs. 10.7 – 11.5% in crab; for CP: 16.3 – 21.3% in fish vs. 8.6 – 9.0% in crab; and for NPN: 76.5 – 81.1% in uncooked fish silage vs. 20.0 – 21.3% in cooked fish silage, and 80.1 – 88.9% in uncooked crab silage vs. 54.7 – 57.3% in cooked crab silage). It was concluded that uncooked materials are more suitable for prolonged storage than cooked materials and it is probably not necessary to add antioxidants to silages made from materials with a low lipid content.

All three growth experiments were conducted at the Institute of Aquaculture Research in Cam Ranh, Nha Trang University, Vietnam. Studies II (cobia size about 24 g) and III (size about 100 g) were conducted for 6 and 3 weeks,

respectively, in indoor tanks supplied with circulated seawater with biological filtration and constant aeration; experiment IV (size about 500 g) was carried out in cages in a pond for 3 months.

Lizardfish or blue crab was used to prepare diets for study II, while only lizardfish was used for study III and IV. Yttrium oxide (Y_2O_3) was added (100 mg kg^{-1} diet) to the diets for the study of apparent digestibility coefficients (ADC) in study II. Test diets included either raw or uncooked fish or crab silage as part of the protein source.

In study II, weight gain (WG) and specific daily growth rate (SGR) were significantly higher in cobia fed the raw-based diets and fish-silage-based diet (FSD) than in fish fed crab-related silage-based diets (CSD and MSD) (185 – 286 vs. 34 – 90% and 2.49 – 3.21 vs. 0.68 – 1.53%/day). Feed conversion ratios (FCR) were significantly higher in the groups fed CSD and MSD diets than the groups fed the other diets (2.06 – 6.49 vs. 0.97 – 1.16), resulting in significantly lower protein productive values in the groups fed CSD and MSD than in the other groups (0.06 – 0.16 vs. 0.31 – 0.37). The FCR results were confirmed by significantly lower ADC values in fish fed CSD and MSD than in fish fed the other diets. The difference in the growth of fish fed raw or silage diets was probably related to the distribution of amino acids in the plasma and liver.

Study III showed significantly higher concentrations of most free essential amino acids at 6 and 12 h, but significantly lower levels at 48 h post-feeding in fish fed silage-based diets than in fish fed raw-based diet. At 48 h post-feeding, the total level of plasma free amino acids (FAA) was significantly higher in fish fed raw-based diet than in fish fed silage-based diets (4999 vs. 3390 – 4339 nmol AA ml^{-1} plasma). Similarly, at 48 h post-feeding FAA concentrations in the liver were significantly higher in fish fed 0%- or 13%-silage-based diets than in fish fed 26%- or 39%-silage-based diets. Faster amino acid absorption in the diets containing higher levels of fish silage led to a significantly higher concentration of plasma FAA earlier, which could result in an imbalance of amino acid concentrations for protein synthesis later on and eventually affect growth rate.

In study IV, no significant differences in nutritional composition were observed between the fillets of cobia fed raw- or silage-based diets for 3 months. Cobia fillets contained of a balance of amino acids (EAA/NEAA=1). The three groups of fatty acids; saturated fatty acid (SFA), monounsaturated acid (MUFA) and polyunsaturated fatty acid (PUFA) in cobia fillet were divided into quite similar proportions from 30 to 33% of TFA. PUFA accounted for 30% of total fatty acids with high levels of n-3 PUFA (69% of total PUFA). n-3 PUFA consisted mainly of docosahexaenoic acid (DHA) (46 – 49% of total PUFA) and eicosapentaenoic acid (EPA) (11 – 12% of total PUFA). The quality index method (QIM) was used to estimate the shelf life of whole gutted cobia stored in ice. QI scores and quantitative descriptive analysis (QDA) from both groups were correlated throughout storage (r^2 = 0.83 – 0.86). However, the total scores for QIM and QDA were low compared to the maximum scores given in the schemes (14 vs. 24 and 5 vs. 10, respectively) after 15 days storage in ice. In addition, the development of lipid oxidation and the growth of bacteria in cobia fillets were also below the acceptable limits after 15 days storage in ice. Thus, the final overall shelf life of the cobia was not determined in the present study, but it is at least 15 days.

In order to make efficient use of local by-catch fish and by-products from the processing industry for aquaculture in general and for cobia in particular, more studies on the preparation of silage and proportions used are needed, since nutritional demand varies between life stages and from species to species. Moreover, further studies on shelf life and the processing quality of cobia are also needed.

List of publications

This thesis is based on the following papers:

I. Mach, T.N.D. & Nortvedt, R. (2009). Chemical and nutritional quality of silage made from raw or cooked fish and crab. *Journal of The Science Food and Agriculture* (submitted)

II. Mach, T.N.D., Nguyen, D.M. & Nortvedt, R. (2009). Effects on digestibility and growth of juvenile cobia (*Rachycentron canadum*) fed fish or crab silage protein. *Aquaculture Nutrition* (accepted)

III. Mach, T.N.D. & Nortvedt, R. (2009). Free amino acids distribution in plasma and liver of juvenile cobia (Rachycentron canadum) fed increased levels of lizard fish silage. *Fish Physiology and Biochemistry* (submitted)

IV. Mach, T.N.D. & Nortvedt, R. (2009). Fillet composition and initial estimation of shelf life of cobia (*Rachycentron canadum*) fed raw fish and fish silage moist diets. *Aquaculture* (submitted)

List of abbreviations

ADC	Apparent digestibility coefficients
CCS	Cooked crab silage
CCSE	Cooked crab silage with ethoxyquin
CD	Raw crab diet
CFS	Cooked fish silage
CFSE	Cooked fish silage with ethoxyquin
CSD	Crab silage diet
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
EAA	Essential amino acid
FAA	Free amino acid
FCR	Feed conversion ratios
FD	Raw fish diet
FSD	Fish silage diet
HSI	Hepatosomatic index
MCC	Mixed cooked fish/ cooked crab
MCFRC	Mixed cooked fish/ raw crab
MD	Mixed raw fish/raw crab diet
MRFCC	Mixed raw fish/ cooked crab
MRR	Mixed raw fish/ raw crab
MSD	Mixed fish/crab silage diet
MUFA	Monounsaturated fatty acid
NEAA	Nonessential amino acid
NPN	Non-protein nitrogen
PCA	Principal component analyses
PPV	Protein productive value
PUFA	Polyunsaturated fatty acid
QDA	Quantitative descriptive analysis
QIM	Quality index method
RCS	Raw crab silage
RCSE	Raw crab silage with ethoxyquin
RFS	Raw fish silage
RFSE	Raw fish silage with ethoxyquin
SFA	Saturated fatty acid
SGR	Specific growth rate
TBARS	Thiobarbutiric acid reactive substances
TCA	Trichloroacetic acid
TFA	Total fatty acid
VSI	Viscerosomatic index
WG	Weight gain

1. INTRODUCTION

Cobia, *Rachycentron canadum* Linnaeus, 1766, are a migratory pelagic species that is found worldwide in tropical, subtropical and warm-temperate waters. It is the only species in the family Rachycentridae and its closest relatives are the remoras or suckerfish (Shaffer and Nakamura, 1989). Cobia are normally found in groups of 3 – 100 fish in shallow water along the shoreline when they are hunting for food during migration. As carnivores, cobia eat crustaceans, cephalopods and small fish such as mullet, eels, snappers and pinfish (Franks *et al.,* 1996; Arendt *et al.,* 2001). Their favourite food is crabs, so they are called “crabeaters”. Crustaceans account for 50–100% of prey in the stomach contents of juvenile cobia (Darracott, 1977; Smith, 1995; Franks *et al.,* 1996). Widely distributed, they are also called many other names such as black kingfish, ling and lemon fish. Cobia have an elongated body and grow up to 68 kg in weight and 2m in length (Wheeler, 1975; Kaiser and Holt, 2005). Cobia have a moderately long life span of at least 10 years (Richards, 1967; Franks *et al.,* 1999) and can live up to 15 years in the wild (Kaiser and Holt, 2005).

Cobia are highly prized by recreational fishermen but not by industrial fisheries because of their solitary existence. However, in marine aquaculture cobia are considered as a remarkable candidate species due to their fast growth, good fillet quality and high commercial prices. Cobia can reach the weight of 5 – 6 kg within one year and 8 – 10 kg in 16 months. With optimal feed and temperature they can grow from 30 g (55- to 75-day-old fingerlings) to 6 – 10 kg within 280 – 390 days (Su *et al.,* 2000). Moreover, cobia larvae can be produced by mass production, fry can be nursed by intensive or super-intensive rearing in tanks, and juveniles and adults can be adapted to aquafeeds such as wild-catch fish and moist or dry pellets (Liao *et al.,* 2004; Faulk and Holt, 2005; Faulk *et al., 2007;* Webb *et al.,* 2007; Benetti *et al.*, 2008a & b; Daniel *et al., 2008*). In a super-intensive recirculation system, the stocking density for fish sized from 10 to 100 g in nurseries was 594 fish/m^3 (Liao *et al*., 2004). Asian production of cobia has therefore increased rapidly, from 13 tons in 1996 to 2 400 tons in 2002 and 20 461 tons in 2004, with Chinese production comprising about 81% of the total in 2004 (Lungren *et al.,* 2006).

In Vietnam, marine aquaculture has developed since 1988 with concentration on grouper and lobster cage farms (Edwards *et al.,* 2004). Recently, cobia have attracted marine aquaculture farmers. Cobia culture in Vietnam began in 1999 with the first successful hatching at Cat Ba research station outside Hai Phong in the north. Now fingerlings are also supplied by some hatcheries in the centre of Vietnam. Cobia culture takes place along the Vietnamese coast from the north to the south. Both small- and large-scale farms exist. Small-scale, family-owned farms are traditional wooden raft cages, while large-scale, private or joint-venture companies use Norwegian-style circular plastic cages. Wild-caught small-sized fish are mostly used as feed in small farms whereas dry pellets are used in large farms.

Known as voracious feeders, cobia have been the focus of nutritional research, particularly studies on protein and lipid requirements (Chou *et al.,* 2001; Faulk and Holt, 2003; Turner and Rooker, 2005; Wang *et al.,* 2005; Craig *et al.,* 2006; Sun *et al*., 2006a & b; Zhou *et al.,* 2006 & 2007; Lunger *et al.,* 2007b; Niu *et al*., 2008a & b) or replacement of fish meal protein by other protein resources (Chou *et al.,* 2004; Zhou *et al.,* 2004 & 2005; Lunger *et al.,* 2006 & 2007a; Romarheim *et al.,* 2007). In laboratory studies on juvenile cobia, replacement of up to 40% of fish meal by soybean meal in diets had no effect on the growth rate **(**Chou *et al.,* 2004; Zhou *et al.,* 2005; Lunger *et al.,* 2007a; Romarheim *et al.,* 2007**).** However, another important protein source, fish silage, has not been studied using cobia.

Fish silage is made by adding organic or inorganic acid or a mix of both to offal, by-products or by-catch fish for storage purposes and to increase the value of the products. This is called acid-silage to differentiate it from fermented silage where fish is usually mixed with molasses or lactic acid bacteria or commercial enzymes or bacterial proteases (Tatterson and Windsor, 1974; Tatterson, 1976; James *et al.,* 1977; Tatterson *et al.,* 1979; Strøm *et al.,* 1979; Austreng and Asgard, 1986; Stone and Hardy, 1986; Levin *et al., 1989;* Rebeca *et al., 1991; Viana et al*., 1993; Shahidi *et al., 1995;* Zahar, 2002; Vazquez *et al.,* 2008). Potassium sorbate and ethoxyquin are also added to prevent mould, yeast growth and oxidation development, respectively. Fish silage contains a balance of essential amino acids and has a high nutrient value; therefore it is useful as a protein supplement in aquaculture and livestock production (Strøm *et al.,* 1979; Rungruangsak and Utne,

1981; Raa and Gildberg, 1982; Tatterson, 1982; Hardy *et al.,* 1983 & 1984; Jackson *et al.,* 1984a & b; Haard *et al., 1985; Anglesea and Jackson, 1985;* Lie *et al.,* 1988; Stone *et al.,* 1989; Haaland *et al.,* 1990; Dumas *et al., 1991;* Parrish *et al.,* 1991; Espe *et al.,* 1992 & 1999; Heras *et al.,* 1994, Fagbenro, 1994; Fagbenro and Jauncey, 1994, 1995b & 1998; Perez, 1995; Evers and Carroll, 1996; Viana *et al.,* 1996; Tocher *et al*., 1997; Viana *et al., 1999;* Goddard and Al-Yahyai, 2001; Goddard *et al.,* 2001; Raghunath and Gopakumar, 2002; Vidotti *et al*., 2002 & 2003; Goddard and Perret, 2005; Hevrøy *et al.,* 2005; Borghesi *et al., 2008*). Tropical countries have great potential to produce fish silage from capture, by-products from commercial and industrial processes, particularly by-catch fish (Durairaj *et al., 1976;* Gildberg and Raa, 1977; Yeoh and Merican, 1978; Kompiang *et al.,* 1979 & 1980; Potter *et al.*, 1979; Hood and Zall, 1979; Kompiang, 1981; Strøm and Eggum, 1981; Watkins *et al., 1982;* Tidemann *et al.,* 1984; Hall *et al*., 1985; Meyers, 1986; Meyers and Benjamin, 1987; Bertullo, 1992; Ames and Ward, 1995; Evers and Carroll, 1996; Fagbenro and Bello-Olusoji, 1997; Machin, 1999; Liaset *et al.,* 2000; Jialin and Lied, 2001; Plascencia-Jatomea *et al., 2002;* Rustad, 2003; Arruda *et al*., 2007; Geron *et al., 2007*). In Vietnam, total marine fishing was estimated at 2.6 million tons, of which by-catch accounted for 0.93 million tons in 2001 primarily from trawling in the inshore fisheries (RIMF, 2001).

It is well known that dietary amino acids affects levels of FAA in fish tissues, particularly EAA levels (Pion, 1976; Cowey *et al*., 1977; Nose *et al.,* 1978; Kaushik and Luquet, 1980; Dabrowski and Dabrowska, 1981; Walton and Cowey, 1982; Dabrowska, 1984; Ogata *et al.,* 1985; Cowey and Walton, 1989; Yokoyama and Nakazoe, 1991; Kaushik *et al*.,1994; Ogata and Murai, 1994; Yokoyama *et al*., 1994; Schuhmacher *et al*., 1997, Yamamoto *et al.,* 1998 & 2005). Dietary amino acid patterns were related to tissue FAA patterns, particularly a high relationship between dietary and plasma EAA (Nose, 1973; Kaushik and Luquet, 1977; Schlisio and Nicolai, 1978; Plakas et al., 1980; Dabrowski, 1982; Murai *et al.,* 1984a, b & 1987*;* Thebault, 1985; Fauconneau, 1985; Wilson *et al.,* 1985*;* Ogata, 1986; Walton and Wilson, 1986; Lyndon *et al*., 1993; Kaushik *et al*., 1994; Yamamoto *et al., 2000*). In order to find out relationships between different dietary silages and tissue FAA concentration, FAA concentration in plasma and livers were also examined in the present study.

Sensory evaluation is considered to be a rapid, cost-efficient and accurate method for the assessment of quality, shelf life and storage conditions of food (Nielsen, 1997; Martinsdottir, 2002). The first sensory method was developed by Torry Research Station (Shewan *et al*., 1953). A newer method, the Quality Index Method (QIM), is based upon a scheme developed by Tasmanian Food Research (Bremner, 1985). QIM has been developed for many species in European and Nordic countries (Larsen *et al*., 1992; Huss, 1995), including: red fish *Sebastes marinus* (Martinsdottir and Arnason, 1992), Atlantic mackerel *Scomber scombrus*, horse mackerel *Trachurus trachurus* and European sardine *Sardina pilchardus* (Andrade *et al.,* 1997; Gokoglu, *et al.,* 2004; Macagnanoa *et al., 2005; Erkan and Ozden, 2008*), brill *Rhombus leavis*, dab *Limanda limanda*, haddock *Melanogrammus aeglefinus*, pollock *Pollachius virens*, sole *Solea vulgaris* and turbot *Scophtalmus maximus* (Luten, 2000), hake *Merlucciuc merluccius* (Ruiz-Capillas and Moral, 2001), sharpsnout seabream *Diplodus puntazzo* (Hernandez *et al.,* 2001), gilthead seabream *Sparus aurata* (Kyrana *et al., 1997;* Huidobro *et al.,* 2000, 2001a & b; Tejada and Huidobro, 2002; Lougovois *et al.,* 2003), Atlantic salmon *Salmo salar* (Carbonell *et al.,* 2003; Sveinsdottir *et al.,* 2003), herring *Clupea harengus* (Nielsen and Hyldig, 2004), common octopus *Octopus vulagris* (Barbosa and Vaz-Pires, 2004), flounder *Paralichthys patagonicus* (Massa *et al.,* 2005), European cuttlefish *Sepia officinalis* and shortfin squid *Illex coindetii* (Vaz-Pires and Seixas, 2006; Sykes *et al., 2009*), hybrid striped bass *Morone saxatilis x Morone chrysops* (Nielsen and Green, 2007), Atlantic halibut *Hippoglossus hippoglossus* (Nortvedt and Tuene, 1998; Guillerm-Regost *et al., 2006),* sea bass *Dicentrarchus labrax* (Parisi *et al.,* 2002; Knowles *et al*., 2007), and Atlantic cod *Gadus morhua* (Bechmann *et al.,* 1998; Pastoriza *et al.,* 2002*;* Bonilla, 2004; Kent *et al., 2004;* Bonilla *et al.,* 2007). The most commonly used attributes for fish are the appearance of the eyes, skin and gills together with odour and texture. The development of QIM for a particular seafood or fish species involves the selection of appropriate and best fitting attributes in order to observe a linear increase in the QI during iced-storage time. The maximum storage time and thus the limit for rejection of fish can be determined by the sensory evaluation of cooked samples using Quantitative Descriptive Analysis (QDA) (Stone and Sidel, 1993; Sveinsdottir *et al.,*

2002 & 2003; Bonilla *et al.,* 2007; Nielsen and Green*,* 2007). The results from QDA should be used as a reference when developing QIM for fresh fish.

Fish silage has been popular as an animal feed in some Scandinavian countries (Hansen and Alsted, 1986; Arason *et al.,* 1990; Espe *et al.,* 1992; Liaset *et al.,* 2000); however, it is little used in Vietnam. This study was carried out to promote another way to utilise the low value fish in aquaculture, in order to make efficient use of local protein sources from by-products or by-catch fish, particularly in small-scale farms where aquafeeds can be produced in an artisanal manner from fish silage at low cost and with minimal equipment (Asgard and Austreng, 1981; Batista, 1986; Ives, 1991; Machin, 1999; Archer *et al.,* 2001; Bechtel, 2006). Moreover, this study also determined the effects of silage on fillet quality and storage of cobia by comparing the nutritional composition of fillets and shelf life using sensory evaluation.

2. AIMS OF THE STUDY

The aims of the study were to estimate the quality of acid silages made from the local by-catch fish and crab to test the use of silages as an ingredient in moist diets for cobia, and to investigate the effect of the silages on growth, feed efficiency, fillet quality and shelf life. Four experiments were carried out to achieve the following objectives.

- To determine the chemical and nutritional quality of silages made from raw or cooked by-catch fish and crab that are highly available species in the local market.

- To investigate the effects of different dietary silages on digestibility and growth of juvenile cobia.

- To study amino acid absorption in juvenile cobia by determining free amino acid distribution in the plasma and liver at different levels of dietary silage.

- To evaluate the effect of dietary silage on fillet composition and to investigate whether dietary silage has an effect on the shelf life of cobia stored in ice.

3. SUMMARY OF MATERIALS AND METHODS

3.1. Silage production

Lizardfish *(Saurida undosquamis*) and blue crab *(Portunus pelagicus*), both highly available in the local area, were selected for the present study. Silages were prepared from uncooked and cooked minces (Paper I). Potassium sorbate (2.2 g kg^{-1}) and ethoxyquin (250 mg kg^{-1}) were added as an anti-fungicide and anti-oxidant, respectively. The experiment was organised as a full factorial design with 2^3 factors: two species, cooked or raw with or without ethoxyquin, plus four centre points. Therefore there were twelve silages with three replicates of each. Twenty-five g kg^{-1} formic acid (85%) was used for fish silages, while 88 g kg^{-1} was used for crab silages. More formic acid was needed to balance the pH due to the high mineral content in the crab shell. The mixing ratios were 2.2 g kg^{-1} potassium sorbate, 125 mg kg^{-1} ethoxyquin and 46 g kg^{-1} of formic acid for all mixed fish/crab silages, allowing evaluation of a reduced ethoxyquin level in the mixed centre points. The mixtures were stored in 5-litre plastic buckets with lids at room temperature (30 ± 2 ^{o}C) for 60 days.

3.2. Experimental fish

Juvenile cobia (2000 fish about 10–12 cm each) from the Hoang Ky Hatchery were initially reared in two cages (3 x 3 x 2 m) in a pond at the Institute of Aquaculture Research in Cam Ranh, Nha Trang University, Vietnam and then were orderly used in studies II, III and IV. The fish were acclimatised to the experimental diets before selection for each experiment. Fish of similar size were selected for the three experiments (about 24 g for study II, 100 g for study III and 500 g for study IV). Studies II and III were conducted in indoor tanks supplied with circulated seawater with biological filtration and constant aeration for 6 weeks and 3 weeks, respectively; experiment IV was carried out in a pond for 3 months. All growth trials were carried out with three replicates of each treatment.

3.3. Diets and feeding

Based on the results of study I, uncooked silages were chosen for preparation of diets for the subsequent studies. Uncooked fish silage was used as an ingredient

for preparing moist diets in studies II, III and IV; uncooked crab silage was only used for study II. Formulate moist diets initially used raw fish and fish silage as ingredients. Sodium alginate + $CaCl_2$, wheat gluten + steaming or wheat gluten + microwave radiation were used as three alternative binding strategies to test stability. The leakage of nitrogen from the diets to water was used to evaluate stability. The results showed that the diets made from raw fish were more stable than those made from silage and higher stability was observed in steamed diets compared to the other diets.

Silages were prepared one month before use in the experiments. For experiments III and IV, after 2 weeks silages were solar-dried to reach a moisture content of approximately 45%. Diets for each experiment were formulated with intended isonitrogenous and isoenergetic composition. In study II, cobia was fed steamed moist diets consisting of raw or silaged lizardfish or blue crab: raw fish diet (FD), fish silage diet (FSD), raw crab diet (CD), crab silage diet (CSD), and mixed raw fish/crab diet (1:1, MD), mixed fish/crab silage diet (1:1, MSD) (Table 1, Paper II). Yttrium oxide (Y_2O_3) was added (100 mg kg^{-1} diet) to the diets to study the apparent digestibility coefficients (ADC). Before selection for study III and IV, fish were directly fed fresh fish. The bigger fish used in study III (100 g) and IV (500 g) did not accept steamed moist diet. Fresh moist diets are similar to fresh fish; these were therefore used instead of steamed moist diets (Table 1, Paper III and IV). Sodium alginate (from 7 – 30 g kg^{-1} diet) was used as a binder in 10% $CaCl_2$ solution. Four levels of concentrated fish silage (0, 130, 260 and 390 g kg^{-1} diet) were added to the diets in study III, while only two levels (0 and 200 g kg^{-1} diet) were used in study IV. In all experiments fish were hand-fed to apparent satiation twice daily (08:00 and 16:00) during a 30-min period, and the amount of diet consumed was recorded daily.

3.4. Sample collection

After collection, all samples for chemical analyses were stored at –80 ^{o}C for subsequent analyses, while samples for microbial counts and sensory assessment (QIM) were analysed immediately. Before sampling the fish were starved for 24 h (Papers II and IV) and anesthetised with MS 222 (Papers II and III) or killed by a strong blow to the head and cutting the gills (Paper IV). The faeces for apparent

digestibility coefficient (ADC) determination were collected as described by Austreng (1978) (Paper II). Arterial blood and liver for free amino acid analyses were sampled at 0, 6, 12, 24 and 48 h after the final meal (Paper III). Plasma was separated by immediately centrifuging at 1000 x *g* for 10 min and then freezing, and liver was frozen immediately (–80 ^{o}C).

3.5. Analytical methods

Chemical analyses

Moisture was determined by oven-drying at 105 ^{o}C for 48 h. Ash content was determined by ignition at 550 ^{o}C in a muffle furnace for 24 h. The pH in the silages was directly measured with a digital pH meter, while the pH in the diets was determined according to Fagbenro and Jauncey (1995a). Total nitrogen (N) was determined by a combustion method (CHNS-O analyzer Thermo Finnigan, USA) and crude protein was estimated as Nx6.25. Protein autolysis was estimated as soluble non-protein nitrogen (NPN) in 20% trichloroacetic acid (TCA) (Backhoff, 1976). Amino acids were determined by the EZ:faast method (Finnigan LCQ Advantage Max, USA). Tryptophan was not determined in this study due to the high costs of this specific analysis. Crude lipid was determined gravimetrically after extraction with ethylacetate (Losnegard *et al.*, 1979). Fatty acid composition in the cobia fillet and diets (Paper IV) was analysed as described by Lie *et al.* (1986), in which lipids were extracted from the samples with chloroform/methanol (2:1, v/v). Lipid oxidation (Paper I and IV) was estimated by the TBARS (thiobarbutiric acid reactive substances) method (Pikul *et al*., 1989). Y_2O_3 content was determined by ICP (Perkin Elmer, USA) (Paper II).

Free amino acids in plasma and liver were determined in study III. FAA in the liver and the diets were extracted by adding 1:2 (v/v) phosphate buffer (pH 7), spinning for 1 min and centrifuging at 2500 x *g* for 30 min (Hevrøy *et al.,* 2005). The supernatants were collected and the same analytical procedure was followed as for the plasma. The plasma and FAA extracted from livers and diets were continuously deproteinised by addition of 5% (w/v) sulphosalisylic acid (1:1, v/v) and then the samples were kept on ice for 30 min and centrifuged at 5000 x *g* for 15 min,

according to Espe and Njaa (1991). The supernatants were taken for FAA analyses by the EZ:faast kit (Finnigan LCQ Advantage Max).

Microbial analyses (Paper I and IV)

Bacteria, yeast and mould were determined immediately after sampling by the "Aerobic Plate Count at 30 ^{o}C: Surface plate method" (Health Protection Agency, 2004).

Shelf life study (Paper IV)

Ten employees from the Quality of Seafood Department, Seafood Processing Technology Faculty, Nha Trang University, participated in the development and evaluation of the QIM and QDA schemes for cobia (EEC, 1976; Howgate *et al.*, 1992; Jonsdottir, 1992; Larsen *et al.,* 1992; Huss, 1995). The schemes were applied to estimate the freshness and quality of the gutted cobia at 3, 5, 7, 9, 11, 13 and 15 iced-storage days. The fish were randomly and individually coded with a number unrelated to storage time and assessed within the QIM and QDA schemes (Table 2 and 3, Paper IV). After QIM assessment, the meat of the gutted fish was cooked in a steam oven at 100 ^{o}C for 7 min and consumed to determine its acceptability after storage time (QDA).

3.6. Formulas for calculation of parameters

The following formulations were used to calculate parameters in the growth experiments.

$$Survival\ (\%) = 100\,x\,\frac{final\ \ amount\ \ of\ \ fish}{initial\ \ amount\ \ of\ \ fish}$$

$$Viscerosomatic\ \ index\ (VSI\%) = 100\,x\,\frac{viscera\ \ \ weight\,(g)}{whole\ \ body\ \ weight\,(g)}$$

$$Hepatosomatic\ \ index\ (HSI\%) = 100\,x\,\frac{liver\ \ \ weight\,(g)}{whole\ \ body\ \ weight\,(g)}$$

$$Weight\ \ gain\ (WG\%) = 100\,x\,\frac{final\ \ body\ \ weight - initial\ \ body\ \ weight}{initial\ \ body\ \ weight}$$

$$Specific\ \ growth\ \ rate\,(SGR\%/day) = 100\,x\,\frac{\ln final\ \ weight - \ln initial\ \ weight}{days}$$

$$Protein\ \ productive\ \ value\,(PPV) = \frac{protein\ \ growth\,(g)}{protein\ \ \ intake\,(g)}$$

$$Food\ \ conversion\ \ ratio\,(FCR) = \frac{dry\ \ weight\,of\ \ \ feed\ \ \ intake\,(g)}{wet\ \ \ weight\ gain\,of\ \ \ fish\,(g)}$$

Apparent digestibility coefficients (ADC) were determined by the following equations according to Austreng (1978) and Guillaume *et al.* (2001).

For dry matter:

$$ADC\ (\%) = 100 - (100\,x\,\frac{\%\ indicator\ \ in\ \ diets}{\%\ indicator\ \ in\ \ faeces})$$

For crude protein, crude lipid and gross energy:

$$ADC\ (\%) = 100 - (100\,x\,\frac{\%\ indicator\ \ in\ \ diets}{\%\ indicator\ \ in\ \ faeces}\,x\,\frac{\%\ nutrient\ \ in\ \ faeces}{\%\ nutrient\ \ in\,diets})$$

3.7. Statistical analysis

The Statistica™ (version 7.0) software programme was used for analysis of variance (ANOVA). Significant differences ($p<0.05$) between means were tested by Duncan's multiple range test, according to Duncan (1955). Multivariate correlations between objects and variables were revealed by principal component analyses (PCA) using The Sirius™ (version 7.0) software programme, according to Kvalheim and Karstang (1987).

4. GENERAL DISCUSSION

4.1. Chemical composition and quality of silages (Paper I)

At the end of the experiment, all fish silages stored well with low and stable pH values (3.7 – 4.3) during storage; however, crab silages and mixed fish/crab silages showed signs of spoilage towards the end with pH values exceeding recommendation (>4.5, Espe and Lied, 1999) (Figure 2, Paper I). The pH values of the crab and mixed fish/crab silages fluctuated during storage (4.5 – 6.2). Moreover, maggots were observed in the cooked crab silages on the last few days of the experiment. Foaming observed in the crab silages during addition of formic acid led to the conclusion that no further pH adjustment was possible in the crab silages. Addition of more formic acid would also make it potentially more toxic as a feed ingredient. The increase in pH during the first five days was probably due to a reaction between the fish bones and crab shell and formic acid (Torrissen *et al.,* 1981; Espe *et al.,* 1989; Ariyani and Buckle, 1991; Espe and Lied 1999; Vizcarra-Magana *et al.,* 1999). The high mineral content in the crab shell probably necessitated the high level of formic acid added since the proportion of formic acid participated in a neutralising reaction with calcium salt in crustacean shells (Torrissen *et al.,* 1981; Ariyani and Buckle, 1991). According to Haaland and Njaa (1989) and Vizcarra-Magana *et al.* (1999), deamination reactions probably caused slight changes in pH during storage.

Formic acid is added to silage to prevent bacterial growth; therefore bacterial growth was an important parameter in evaluation of storage efficiency. Generally, more bacteria were found in the crab silages than in the fish silages (Table 2, Paper I). In the cooked silages, bacterial counts were significantly lower at day 0, but significantly higher at day 60, compared to the uncooked silages. 99.4% of bacteria in fish and 99.1% of bacteria in crab were killed after cooking; whiles in uncooked materials, after 15 days added formic acid 98.2% of bacteria in fish silages and 97.9% of bacteria in crab silages were killed; and 99.4% of bacteria in fish silage and 98.1% of bacteria in crab silages were killed after 30 days, compared to the total bacteria in their materials before adding formic acid (Table 2,

Paper I). A similar result was reported by Hoq and Bhuiyan (1995). After 30 days, the bacterial counts in all the silages were increased, with further increases after 45 days, with the exception of uncooked silages from fish and crab. It is known that formic acid does not prevent mould from growing during prolonged storage (Poulter *et al.,* 1979; Hoq and Bhuiyan, 1995). Mould spores were found at day 15, 30 and 45 but not at day 60. The cause of disappearance of mould at day 60 is unknown. Bacterial counts were lower in the fish silages than in the crab or mixed fish/crab silages during storage, which may be due to the lower pH in the fish silages. Moreover, in all relative-cooked silages, bacteria counts increased quickly after 45 days of storage, except in the cooked fish silage where the pH of the silage remained stable and under 4.5. This suggests that the cooked materials were not suitable for silage production.

Silage protein was converted into non-protein nitrogen (NPN) by endogenous enzymes during storage. All silages liquefied after four days of storage except for the cooked fish and mixed cooked fish/crab silages. Significant differences in NPN between cooked and uncooked materials and between silage groups were found during storage (Figure 3, Paper I). At the end of the trial, NPN levels were higher in uncooked silages than in cooked silages (fish: 79% vs. 25%; crab: 85% vs. 56%; mixed fish/crab: 70% vs. 51%); this was consistent with results reported by Espe *et al.* (1989, 1990 & 1992), Espe and Haaland (1992), and Wang and Einer (2001). Endogenous enzymes in the raw materials were inhibited after cooking, and therefore NPN values were lower in cooked silages than in uncooked silages (Backhoff, 1976; Wood *et al.,* 1985; Espe *et al.,* 1992; Fagbenro and Jauncey, 1993; Espe and Lied, 1999; Wang and Einer, 2001).

Lipid oxidation is an important parameter in evaluation of the quality of silages made from fatty fish. In the present study, crude lipid in crab was low (<0.4%), and therefore the role of antioxidants was only observed in fish silages (Figure 4, Paper I). TBARS values in both uncooked and cooked fish silages containing ethoxyquin were low and stable during storage, which was consistent with results from Jackson *et al.* (1984a), Hall *et al*. (1986) and Vizcarra-Magana *et al.* (1999). Antioxidants are able to inhibit the initial lipid oxidation (Jackson *et al.,* 1984a; Vizcarra-Magana *et al.,* 1999), whereas malondialdehyde, the initial lipid-oxidation, was present in the

raw materials. Diminishing TBARS values during storage were probably related to a reduction in malondialdehyde, which partly reacted with protein and amino acids in the silage and partly formed other secondary oxidation products (Buttkus and Bose, 1972).

Macronutrient (dry matter, ash, crude lipid and crude protein) and amino acid composition were determined to compare nutritional values between silages. Significant differences in macronutrients were found between fish and crab silages and between uncooked or cooked materials (Table 1, Paper I). During storage, the composition of macronutrients was quite stable, although the levels of dry matter were slightly increased due to evaporation of water or consumption of water in the process of hydrolysis of the protein and probably also of triglycerides (Jackson *et al.,* 1984a; Espe *et al.,* 1989). Similar results were reported by Raa and Gildberg (1982), Jackson *et al.* (1984a), Espe *et al.* (1990 and 1992), and Espe and Lied (1999). Higher levels of lipid and protein were observed in the fish silages, while higher levels of ash were found in the crab silages. In the same groups the levels of macronutrients were significantly higher in the cooked silages than in the uncooked silages (Table 1, Paper I). The levels in the mixed fish/crab silages were variable. Furthermore, no significant differences in lipid levels were found between the silages with or without ethoxyquin, which was in accordance with results reported by Jackson *et al.* (1984a). There were significant differences in amino acid profiles (mg amino acid g^{-1} protein) between fish and crab silages and between uncooked and cooked materials (Figure 1 and Table 3, Paper I). The profiles of mixed fish/crab silages were at an intermediate level, compared to the two other main groups (Figure 1, Paper I). Similar to protein, the amino acid composition of the silages was quite stable and mostly constant up to 60 days of storage. The same results were reported by Espe *et al.* (1989) and Espe and Lied (1999). Levels of most amino acids were higher in cooked materials and silages than in uncooked materials and silages in both fish and crab, which is consistent with Mackie (1973) and Wood *et al.* (1985). Levels of six amino acids: arginine, threonine and histidine, serine, proline and aspartic acid were significantly higher in the crab groups than in the fish groups, while only three amino acids (lysine, 4-hydroxyproline and glutamic acid) had significantly lower levels in the crab groups than in the fish groups. These differences might contribute to the different growth rates in the growth trials.

In conclusion, there were significant differences in nutritional values and storage ability between fish and crab silages and between uncooked and cooked materials. The high levels of pH in the crab and mixed fish/crab silages were probably involved in the high microbial counts at the end of the experiment, particularly for cooked crab silages. The levels of macronutrients and amino acids in most silages were quite stable until the end of the experiment, but the increasing bacterial counts which were observed in all the silages would limit storage for a prolonged time at the ambient temperature.

4.2. Diets

The diets in each experiment had almost equal nutritional composition, but significant differences in pH and NPN levels were observed among the raw- and silage-based diets. Particularly in study II, pH values were lower in CSD (5.95) and MSD (6.55) than in FSD (7.84) which was probably due to the addition of more formic acid to the crab silage than to the fish silage (Table 1, Paper II). In all experiments, NPN levels were higher in the silage diets than in the raw diets. Moreover, the amino acid composition was similar in all diets (Table 2 in Paper II and III, and Table 4 in Paper IV). However, in study II, hydroxyproline levels were lower in CD (4.5 mg g^{-1} protein) and CSD (4.6 mg g^{-1} protein) than in the other diets (7.5 – 10.5 mg g^{-1} protein). In study III, when silage levels added to the diets increased from 0% to 39%, the pH decreased from 7.74 to 6.43, while NPN increased from 164.2 to 400.3 g kg^{-1} total N (Table 1, Paper III). Similarly, an increasing tendency was observed when the amount of total FAA and the ratios of free EAA/NEAA increased from diet A to diet D (Table 2, Paper III). The fatty acid composition in the diets was determined in study IV. Monounsaturated fatty acids (MUFA) predominated at over 43% of the total fatty acids, while polyunsaturated fatty acids (PUFA) accounted for only 24% of the total fatty acids (TFA) with high levels of n-6 PUFA (67 – 70% of total PUFA, Table 5, and Paper IV). No differences in appetite were observed between fish fed raw-based diets and fish-silage diets in individual trials, except in fish fed CSD and MSD in study II. High levels of formic acid in the diets probably reduced the appetite of the fish. Generally, steamed moist diets were more stable than fresh moist diets; however fish showed more appetite for fresh moist diets than steamed moist diets. Since

cobia are carnivores, fresh moist diets are close to their natural food. In practical aquaculture, fresh moist diets are simpler to produce, but more difficult to control environment and disease infection from nature to farms.

4.3. Mortality

Mortality was very high in fish fed diets prepared from crab silage (62%) or mixed fish/crab silage (30%) in study II, while it was low in fish fed raw- or silage-fish-based diets (≤ 10 % for study II, < 2% for study III, and zero for study IV). Levels of crab silage added in CSD were twice as high as in MSD and mortality in the CSD group was twice as high as in the MSD group. Fish fed CSD and MSD jumped and swam continuously before they died, which indicated poor welfare. Dissection of the dead fish fed CSD and MSD showed liver damage. This may be due to the relatively high formic acid level in the diets.

4.4. Digestion, feed utilisation and growth (Paper II)

Weight gain and feed efficiency were significantly higher in fish fed raw-based diets and FSD than in fish fed CSD and MSD (WG: 185 – 286% vs. 34 – 90%, PPV: 0.31 – 0.37 vs. 0.06 – 0.16 and FCR: 0.97 – 1.16 vs. 2.06 – 6.49, Table 3, Paper II). Low feed efficiency in fish fed CSD and MSD was confirmed by ADC values which showed significantly lower values compared to fish fed the other diets (Figure 3, Paper II). Fish fed crab-related silage diets containing high levels of formic acid showed clear damage to the liver of dead fish and significantly lower levels of HSI, which may be involved with the stress response, reduced appetite, digestive efficiency and consequently the depression in weight gain. A previous study by Rungruangsak and Utne (1981) on rainbow trout showed that growth and proteolytic activity in the digestive tract were lower in fish fed a diet containing formic acid-treated silage than in fish fed diets containing hydrochloric acid-treated or sulphuric acid-treated silage. Similarly, a major carp fed on a formic-acid-silage-based diet achieved significantly lower growth than fish fed sulphuric-acid-silage-based diets (Ali *et al*., 1994).

The FCR of fish fed raw-based diets and FSD were consistent with studies by Chou *et al.* (2001 and 2004). In their studies, the FCR of juvenile cobia (30 – 35 g) fed

dietary casein or soybean protein ranged from 1.09 to 1.84. Other studies on juvenile cobia (8 – 26 g) fed up to a 50% fishmeal-based diet by Zhou *et al.* (2005), Lunger *et al.* (2006) and Romarheim *et al.* (2007) showed similar values of FCR from 0.9 to 1.9.

Moreover, in the present study ADC values were generally higher in lipid (74 – 96%) than in protein (61 – 75%). Although the ADC values were significantly higher in fish fed FD and MD than in fish fed the other diets, they were still lower compared to previous results reported for rainbow trout (Austreng, 1978; Refstie *et al*., 1997), red drum *Sciaenops ocellatus* (McGoogan and Reigh, 1996), salmon (Storebakken *et al.,* 1998) and cobia (Zhou *et al.,* 2004), with the exception of the ADC of crude lipid.

The faster growth rate in fish fed raw-mixed fish/crab diet than in fish fed either raw-fish or raw-crab diets (286% vs. 223 – 236%) was consistent with a study by Hammond in Shaffer and Nakamura (1989**).** In their trial, the cobia grew better when crab was added to their feed. It was therefore concluded that raw mixed fish/crab that is available in the local market is a potential ingredient in the cobia diet.

No significant differences in FCR and ADC values between fish fed FSD and FD were noted, but the growth rate and PPV were significantly lower in fish fed FSD than in fish fed FD. Fish silage has not been studied as feed ingredient in diets for cobia before, but the present results were consistent with previous studies on rainbow trout (12 – 16 g) (Hardy *et al*., 1983; Stone *et al*., 1989) and common carp (15 – 20 g) (Wood *et al*., 1985) fed diets containing 40–50% of fish silage. However, different results were reported in rainbow trout (95 – 135 g) (Rungruangsak and Utne, 1981) and Atlantic salmon (320 – 1400 g) (Jackson *et al.,* 1984b; Lie *et al.,* 1988; Espe *et al.,* 1992), in which fish fed 40 – 50% silage-based diets showed no differences in weight gain compared to fishmeal- or raw-fish-based diets. The lack of effect on the growth rate of Atlantic salmon fed silage-based diets was also reported by Jackson *et al.* (1984b), Lie *et al.* (1988), Parrish *et al.* (1991) and Lall (1991). In contrast, salmon fed a 25%-silage-based diet grew faster than fish fed a commercial dry pellet (Crampton *et al.,* 1982). Similarly, major carp (2.8 g) fed 26.4 – 53.1% silage-based diets showed higher weight gain than

those fed a fishmeal-based diet (Ali *et al*., 1994). Moreover, a higher growth response was observed in major carp fed a 40%-silage-based diet (Ali *et al.,* 1994) and Atlantic salmon (2 kg) fed a 10%-silage-based diet (Espe *et al.,* 1999) compared to both lower and higher levels of silage in the diets. Similarly, eel fed 10 – 20% silage protein diets grew faster than those fed fishmeal-based or commercial diets (Goncalves *et al*., 1989). The different results in the previous studies probably relate to the different fish sizes used in the experiments. Larger fish or adult fish may have utilised silage-based diets better than juvenile fish which is consistent with the study by Heras *et al*. (1994) in which Atlantic salmon fed a 22.0 – 24.6% silage-based diet had a poorer growth response than fish fed raw-based diets when small (190 g), but showed no significant differences between dietary treatments at a larger size (490 g).

Protein from fish-silage based diets was partially autolysed (from 14% of NPN in FD to 36% in FSD), which may explain the less efficient utilisation of protein in silage-based dietary groups. However, studies on salmon (Crampton *et al.,* 1982; Parrish *et al.,* 1991; Lall, 1991; Heras *et al*., 1994; Espe *et al.,* 1999), carp (Ali *et al*., 1994) and eel (Goncalves *et al.,* 1989) showed that fish fed partially autolysed protein utilised the protein with the same or higher efficiency than fish fed non-autolysed protein. According to Geiger (1947) fish efficiently utilise protein for growth when optimum concentrations of all amino acids are available in their tissues. In order to determine how much autolysed protein should be used in cobia diets to achieve optimum protein efficiency, more studies are needed.

4.5. Plasma and liver free amino acid response (Paper III)

Free amino acids in plasma

Five amino acids (arginine, methionine, valine, lysine and leucine) dominated approximately 82 – 87% of total plasma free EAA; glutamine and glycine accounted for 34 – 46% of total plasma free NEAA (Figures 1 and 2, Paper III). The maximum concentrations of FAA in plasma were variably observed post-feeding (6 – 48 h), and EAA almost peaked earlier than NEAA. At 6 h post-feeding five EAA reached the maximum levels, while six NEAA presented the minimum concentration.

Significant differences in FAA levels in plasma were found between dietary treatments. Concentrations of most EAA were significantly higher in fish fed diet D than in fish fed the other diets at 6, 12 and 24 h post feeding but significantly lower or similar levels were observed at 48 h (Figure 1, Paper III). Significantly different concentrations were observed in six EAA between fish fed diets A and B at 6, 12 and 48 h, and between fish fed diets B and C at 0, 6 and 12 h, whereas significantly different concentrations were found between fish fed diets A and C in all EAA (except valine) at most sampling times (Figure 1, Paper III). Similarly, concentrations of most NEAA were significantly higher at 6, 12 and 24 h but significantly lower levels were observed at 48 h post-feeding in fish fed diet D than in fish fed the other diets (Figure 2, Paper III). Most EAA peaked at 6 h post-feeding, therefore EAA/NEAA ratios for total plasma FAA were significantly higher (1.08 – 1.47) at 6 h in all groups, compared to the ratios after 24 h (0.59 – 0.72) (Figure 1, Paper III). Significantly lower ratios were observed in fish fed diet A at 6 h (1.08) and 12 h (0.70), while significantly higher ratios were noted in fish fed diet D at 6 h (1.47) and diet C at 12 h (0.96) post-feeding.

Higher concentrations of most plasma EAA in cobia fed diets C and D compared to fish fed the diets A and B were observed at 6 and 12 h post-feeding, suggesting that the higher the level of NPN in the diet, the more rapid the absorption of amino acids. At 48 h the levels of most plasma FAA were lower in fish fed silage diets than in fish fed the raw fish diet. Similar results were also observed in rainbow trout, carp, tilapia and yellowtail (Plakas *et al.,* 1980; Plakas and Katayama, 1981; Yamada *et al.,* 1981 & 1982; Walton and Wilson, 1986; Murai *et al.,* 1987; Stone and Hardy, 1988; Cowey and Walton, 1988; Schuhmacher *et al.,* 1995 & 1997; Aoki *et al.,* 2001). Rainbow trout fed fish silage-based diets or free amino acid diets showed faster and higher concentrations of plasma FAA compared to fish fed intact protein diets (Stone and Hardy, 1988; Yamada *et al.,* 1981; Walton and Wilson, 1986; Murai *et al.,* 1987; Cowey and Walton, 1988; Schuhmacher *et al.,* 1995 & 1997). The timing of the plasma FAA peak varies considerably from species to species and probably depends on many factors such as diet and experimental conditions. Plasma free EAA in carp and tilapia fed casein diets peaked at 4 h (Plakas *et al.,* 1980; Murai *et al.,* 1982; Yamada *et al*., 1982), whereas in rainbow trout fed casein diets the peak was within 12 – 36 h; however, in those fed an

amino acid diet the peak was between 4 – 12 h (Yamada *et al.,* 1981; Walton *et al*., 1986; Murai *et al.,* 1987; Schuhmacher *et al*., 1995). The maximum concentration of plasma free EAA was reported at 6 h after feeding in salmonids fed diets containing hydrolysed protein, casein or fishmeal (Nose, 1973; Walton *et al*., 1986; Espe *et al.,* 1993; Torrissen *et al.,* 1994; Sunde *et al.,* 2003). The earlier plasma free EAA peaked, the faster they declined which may lead to an imbalance in amino acid concentrations for subsequent protein synthesis (Geiger, 1947).

Liver free amino acids

Liver FAA contained more NEAA than EAA at all sampling times, except cystine and tyrosine (Figures 3 and 4, Paper III). Post-feeding concentrations of most FAA decreased and reached minimum levels at 6 h. After that the levels increased and peaked at 12 or 24 h and then declined during the next 24 h. There were significant differences in concentrations of liver FAA between dietary treatments, but a tendency for FAA distribution among the groups was not clear. Fish fed diet B showed a significantly lower concentration of most FAA at 12 h post-feeding, but significantly higher levels at 24 and 48 h compared to the other groups. Concentrations of most liver FAA were significantly lower in fish fed diets C and D than in fish fed diets A and B at 48 h. Significantly different ratios of EAA/NEAA in liver FAA were observed from 12 h post-feeding between dietary groups. EAA/NEAA ratios in liver FAA were significantly lower at 12 h (diets A and B) and at 48 h (diets C and D), but significantly higher at 24 h (diets A and B). NEAA dominated in the liver FAA, thus EAA/NEAA ratios were lower in the liver than in plasma (0.37 – 0.55 vs. 0.58 – 1.47) throughout the absorptive period, which is consistent with results reported by Walton and Wilson (1986) and Hevrøy *et al*. (2005). The significantly lower levels of liver FAA in fish fed diets C or D, compared to fish fed diets A or B at 48 h post-feeding, were probably related to the faster protein digestibility of the diets when higher levels of fish silage were added. High levels of lysine, leucine, valine, glutamine, alanine, and glycine were observed throughout the absorptive phase from dietary amino acids, dietary FAA, liver FAA to plasma FAA, which was consistent with results reported for salmonids (Walton and Wilson, 1986; Hevrøy *et al*., 2005). On the other hand, very high levels of aspartic acid and glutamic acid were found in dietary amino acids, dietary FAA and liver

FAA, but very low levels were found in the plasma FAA, which was in accordance with the result reported by Hevrøy *et al*. (2005). Very low levels of these two amino acids in the plasma after feeding were also reported in studies by Nose (1973), Wilson and Poe (1974), and Espe and Lied (1994). The results revealed transformation reactions of the amino acids from hepatopancreas to plasma. Very low levels of tryptophan were found in liver FAA and plasma FAA in the present study, which were consistent with a previous study on rainbow trout (Walton *et al*., 1986). Low levels of tryptophan in plasma FAA were also reported in salmonids (Nose, 1973; Ogata and Rai, 1985; Murai *et al*., 1987; Espe and Lied*,* 1994; Hevrøy *et al.,* 2005), carp (Plakas *et al*., 1980; Ogata and Arai, 1985), channel catfish (Ogata and Arai, 1985), and Atlantic cod (Lyndon *et al*., 1993). With a lower proportion compared to the other FAA, tryptophan is considered an important component of amino acids in diets. Tryptophan has an effect on growth rate and protein synthesis in mammals (Lin *et al.*, 1987; Cortamira *et al.*, 1991). Dietary tryptophan affected weight gains and feed efficiency ratios in studies on juvenile milkfish *Chanos chanos* (Coloso *et al.*, 1992) and juvenile Asian sea bass *Lates calcarifer* (Cosolo *et al*., 2004). In the present study, the level of tryptophan in plasma was higher in fish fed diet A than in fish fed the silage-based diets at 48 h post-feeding, which is likely to be related to the growth rate of fish. It is well known that growth is probably regulated by a series of controls, in which a balanced supply of FAA to the plasma and muscle tissue plays an important role. In the present study, levels of most plasma FAA were significantly lower in fish fed a silage-based diet than in fish fed intact-protein diets at 48 h post-feeding; this probably affected growth and feed conversion efficiency. In order to use silage as an ingredient for cobia in aquaculture, more studies are needed to find the optimum EAA balance that will fulfill both metabolism and growth demands under different environmental conditions and at different life stages.

4.6. Fillet quality response (Paper IV)

After three months feeding on moist diets with or without added fish silage, there were no significant differences ($p>0.05$) in the nutritional quality of the fillets between the two dietary groups (Tables 6, 7 and 8, Table IV). However, there was a tendency for a slightly higher proportion of fillet yield (49.9% vs. 47.7%) and proportion of fat in the fillets (3.6% vs. 3.2%) in fish fed a raw diet than in fish fed a silage diet. Cobia fillet consisted of approximately 3.4% of crude lipid and 20.0% of crude protein (Table 6, Paper IV). In general, the lipid content of fish fillets varies considerably (0.2 – 25% ww), since fish store lipid as energy depots, whereas protein content is quite stable (16 – 21%) (Stansby, 1962; Love, 1970). Typically, lean fish like cod (< 1% lipid in muscle) store lipid only in the liver, while fatty fish like salmonids and herring (up to 22% lipid in muscle) store lipid in fat cells distributed throughout the body (Murray and Burt, 1969). The lipid content in cobia fillet in the present study was quite similar to that in tuna *Thunus* spp. (4%) (Murray and Burt, 1969) and sea bass (5 – 6%) (Testi *et al*., 2006; Yanar *et al*., 2007; Yildiz *et al*., 2008). A balance of amino acids was found in cobia fillet with EAA/NEAA ratios of approximately 1. Lysine and leucine predominated at over 42% of EAA. The amino acid profile of the cobia fillets was fairly similar to that of rainbow trout (EAA/NEAA ratios about 1.1) (Unusan, 2007). The three groups of fatty acids in the cobia fillet accounted for similar proportions (Table 8, Paper IV). PUFA accounted for approximately 30% of TFA. n-3 PUFA was the major PUFA in cobia fillets (69% of total PUFA), although n-6 PUFA predominated in their diets (67 – 70% of total PUFA). PUFA in cobia fillet consisted mainly of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). MUFA shared approximately 31% of TFA with a high level of C18:1n-9 (64 – 65% of total MUFA). Finally, saturated fatty acids (SFA) accounted for 32% of TFA with a high proportion of C16:0 (61 – 62% of total SFA). DHA and EPA contents in cobia meat were reported to be 328 – 507 mg 100 g^{-1} and 280 – 485 mg 100 g^{-1}, respectively (source:www.seafarm.com.tw, 15/5/2008); the levels were 470 – 500 mg 100 g^{-1} for DHA and 105 – 129 mg 100 g^{-1} for EPA in the present study. Atlantic salmon fed diets with and without added fish silage showed no significant differences in fatty acid composition in their fillets (Lie *et al.,*

1988; Heras *et al*., 1994), which was consistent with the present result. In addition, PUFA levels accounted for 24.5 – 29.1% of total TFA with n-3 PUFA predominating (68 – 81% of total PUFA) in the salmon fillets. In comparison, n-3/n-6 ratios were lower in cobia fillets than in cod fillets (2.3 vs. 7.7 – 15.2) (Ackman and Burgher, 1964; Jangaard *et al.,* 1967; Addison *et al.,* 1968; Lie *et al*., 1986), but were similar to ratios in sea bass fillets (1.3 – 3.8) or sea bream fillets (1.8 – 2.9) (Testi *et al*., 2006; Yanar *et al*., 2007; Yildiz *et al*., 2008). In conclusion, cobia fillets have a good nutritional level of amino acids and fatty acids compared to other species in aquaculture.

4.7. Shelf life (Paper IV)

The suitability of the cobia for storage was determined based on sensory evaluation of the gutted fish, and lipid oxidation and microbial counts in the fillets. No significant differences in the parameters were observed between the two cobia groups throughout the trial (Figures 1, 2 and 5, Paper IV).

Sensory evaluation of gutted cobia

Scores for the attributes for gill (colour and mucus), and eyes (shape) increased more sharply, compared to those of skin (colour, mucus and odour) and texture during storage (Figure 1, Paper IV). The correlations (r^2) between the sum of all attributes' (QI) scores and the storage time show that the attributes gradually and naturally decayed during storage. In the present study, the r^2 value (0.83 – 0.84) was higher than that (r^2 = 0.74) reported by Martinsdottir *et al*. (2001), but lower than in the studies by Sveinsdottir *et al.* (2003), Nielsen and Hyldig (2004), Nielsen and Green (2007) and Bonilla *et al*. (2007) (r^2 = 0.85 – 0.99). Furthermore, the attributes also displayed in principal component analysis (PCA) (Figure 2, Paper IV). At the end of the storage trial, the scores for the attributes varied considerably, reaching 33 – 63% of the maximum scores of each attribute given in the scheme (Figure 1 and Table 2, Paper IV). Similarly, the high correlation of QDA (r^2 = 0.86) indicated that the quality of the fillets gradually deteriorated with time (Figure 3, Paper IV). At the end of the trial, the scores for the attributes varied significantly and reached to 40 – 70% of the maximum scores of each attribute given in the QDA scheme (Figure 3 and Table 3, Paper IV). The scores for QIM and QDA in the

present study were low and fluctuated, possibly due to confusion about attributes, individual differences in the use of the scale, or in precision (Næs *et al*., 1994). In Figure 4 (Paper IV), the QI scores given by the panellists differ from individual to individual. The variation in QI scores given by individual panellists was lowest at days 3 and 15, but highest at day 7 of storage in ice, which was probably due to clearer quality attributes at the beginning and at the end of the storage trial.

Lipid oxidation of cobia fillets

Seafood, and in particular fatty fish, which have high levels of unsaturated fatty acids, is sensitive to oxidation during storage, especially in iced storage. Therefore, lipid oxidation is considered to be one of the most important factors responsible for deterioration in the quality of fish during storage. It is well known that the initiation of lipid oxidation probably involves nonenzymatic and enzymatic reactions and that unsaturated fatty acids are more susceptible to oxidation than SFA due to the lower activation energy in the initiation of free radical formation for triplet oxygen auto-oxidation (Holman and Elmer, 1947; Lea, 1952*).* The development of lipid oxidation depends on several factors such as storage period, temperature, the presence of inhibitors or catalysts, the availability of oxygen and the amount of unsaturated fatty acids (Maclean and Castell, 1964; Castell *et al*., 1966; Castell and Bishop, 1969; Aubourg and Medina, 1999; Erickson, 2002). The fatty acid composition in the fillets of the two cobia groups was similar, which might explain the absence of significant differences in development of rancidity between them. TBARS values of both fillet groups rapidly increased from day 5 to day 10 (7 – 17 nmol g^{-1} fillet), but followed a slight decline from day 10 to day 15 (16 nmol g^{-1} fillet). The reduction in TBARS values at day 15 was probably due to a deficiency of substrate e.g. free fatty acids. According to Nunes *et al.* (1992), the acceptable limit of lipid oxidation for fish stored in ice is 70 – 110 nmol TBARS g^{-1} flesh (equivalent to 5 – 8 mg of malondialdehyde kg^{-1} flesh). The TBARS values in the present study were low compared to the recommended limit.

Microbial counts in cobia fillets

According to The International Commission on Microbiological Specifications for Food (ICMSF), the acceptable limit for total aerobic plate count (APC) of iced-

storage product is 10^7 cfu g^{-1} wet weight (ICMSF, 1978). No significant increase in microbial growth was observed in either cobia group during storage. The aerobic plate counts increased slowly from day 5 to day 10 ($0.25x10^4$ – $1.68x10^4$ cfu g^{-1}) and increased sharply from day 10 to day 15 (from $1.68X10^4$ to $9.55x10^4$ – $14.47x10^4$ cfu $g^{-1)}$. However, the values still satisfied the ICMSF recommendation.

Based on the above results from QIM and QDA for the gutted cobia, and for lipid oxidation and microbial counts of the fillet, the quality of the cobia was probably acceptable after 15 days of storage in ice.

5. GENERAL CONCLUSION

5.1. Silage production

- At the ambient temperature (30 ± 2 ^{o}C), the quality of all silages, except for cooked crab and mixed cooked silages was acceptable after up to 60 days of storage. Uncooked materials seemed to be more suitable for making silage than cooked materials.

- Crab had a lower level of crude protein than fish (8.5% vs. 16.2%), but a higher level of ash (9.6% vs. 3.6%). The composition of silages was almost the same as their respective raw materials; therefore there were significant differences between different raw material-based silages and between uncooked and cooked treatments.

- The high level of ash in crab required the addition of high levels of formic acid in the crab-related silages.

- No significant differences in composition of silages were found between treatments with or without antioxidant during storage, except for TBARS values in fish silage. It is probably not necessary to add antioxidants to silages made from material with a low lipid content.

5.2. Diets

- High levels of formic acid in crab-related silage probably reduced the appetite of the fish; fish fed raw-based or fish-silage-based diets had similar appetites.

- Cooked moist diets were more stable than fresh moist diets; however cobia had more appetite for fresh moist diets than cooked moist diets. Since cobia are carnivores, fresh moist diets are more similar to their original food. In general, fresh moist diets are simpler to produce, but more difficult to control in the aquaculture environment and may transfer disease infection from nature to farms.

5.3. Mortality

- Mortality was 0 – 10% in fish fed raw- or silage-based diets in all experiments, whereas mortality was very high in fish fed crab-related silage-based diets. Mortality was twice as high in fish fed CSD than in fish fed MSD (62 vs. 30%), and the level of crab silage in CSD was double that in MSD. Fish fed CSD and MSD jumped and swam continuously before they died and damage was observed in their livers. The high formic acid level in the crab silage was possibly related to high mortality.

5.4. Digestion, feed utilisation and growth

- The ADC values, feed efficiency and weight gain were significantly lower in fish fed crab-related silage-based diets than in fish fed fish-silage-based diets or raw-based diets. Therefore acid-crab silage should not be used in aquafeed for cobia.

- A significantly different growth rate was observed in fish fed raw fish-based or fish-silage-based diets in study II ($p<0.05$), even though no significant differences in ADC and feed FCR were found between the two groups ($p>0.05$). It can be concluded that fish silage can be used as a protein source for cobia diets, but that the proportion added to a diet needs more study.

5.5. Plasma and liver free amino acid response

- The significantly lower levels of most FAA in livers at 48 h post-feeding in fish fed 26%- or 39%-fish-silage-based diets than in fish fed 0%- or 13%-fish-silage-based diets were probably related to faster protein digestibility in the diets containing higher levels of fish silage.

- There was a significantly higher concentration of most free EAA in plasma at 6 and 12 h, but lower levels at 48 h post-feeding in fish fed fish-silage-based

diets than in fish fed raw-fish-based diets. This may result in an imbalance of amino acid concentrations for subsequent protein synthesis and eventually might affect the growth rate.

5.6. Fillet quality response

- No significant differences in nutritional composition were found between fillets of cobia fed diets with or without fish silage for three months.
- Cobia fillets had a high nutritional composition compared to other aquaculture species. Cobia fillets contained a balance of amino acids (EAA/NEAA = 1) and fatty acid groups, of which PUFA accounted for 30% of total TFA with high levels of n-3 PUFA (n-3/n-6 = 2.3).

5.7. Shelf life

- No significant differences in shelf life were reported between cobia fed diets with or without fish silage.

The scores for most attributes were low compared to the maximum values given in the QIM and QDA schemes, and the TBARS values and microbial counts were below acceptable limits in the cobia fillets at the end of storage. Therefore, the quality of the cobia was probably acceptable after 15 days of storage in ice.

6. FUTURE PERSPECTIVES

- Instead of using high levels of formic acid for crab or mixed fish/crab silage, other acids or molasses or lactic acid bacteria could be used to produce fermented silage.

- In order to use fish silage as an ingredient for cobia feed, the proportion added and the ratios of partially autolysed protein in the diets should be further tested to find the optimum EAA balance that will fulfill both metabolism and growth demands under different environmental conditions and at different life stages.

- The low scores for most shelf life attributes compared to the maximum values given in the QIM and QDA schemes by the end of the trial, were probably due to the short period of storage. Consequently, the shelf life of the cobia was not determined exactly in the present study, but it is at least 15 days. Further studies are needed in order to estimate the shelf life of cobia more accurately.

More studies are also needed to document the fillet yield and processing quality of cobia in relation to texture, fillet gaping, drip loss and colour, and how these characteristics are influenced by other feeding regimes, pre-slaughter stress and starvation.

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