FEED QUALITY FOR IMPROVED SURVIVAL AND ROBUSTNESS OF CLEANER FISH

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Feed quality for improved survival and robustness of cleaner fish

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ABSTRACT

The aim of study was to improve the quality of moist feed for Ballan wrasse with use of different binders and inclusion level of the binders, as well as optimizing the immersion bath to improve gelling of alginate. Quality of moist feed was evaluated as water stability and texture. Experimental diets were formulated mainly based on a commercial brood stock feed mixed with minced shrimps and a binder. Gelatinized starch was tested at three inclusion levels 10%, 15%, 20%. Gelatin was tested at 2.5%, 5% and 7.5% inclusion level. Sodium alginate was tested at 1.5% and 3% level in the diet. The gelling of alginate was tested with use of 2% and 4% $CaCO_3$ and with immersion of pellets in two solutions, either $CaCl_2$ 5% or a mix of $CaCl_2$ 3% + formic acid 3%. The alginate pellets were also produced with or without $CaCO_3$ added in the dough, followed by immersion in a solution of $CaCl_2$ 5% or mix of $CaCl_2$ 3% + formic acid 3%. A combination of all three binders was also tested in different immersion solutions.

The results showed that water stability was not affected by binder type or inclusion level. Among the pellets with sodium alginate, alginate level and $CaCO_3$ level did not improve water stability. Improved water stability was found for pellets immersed in $CaCl_2$ 5% compared to a mix of $CaCl_2$ 3% + formic acid 3%.

The pellet texture was affected by binder type and inclusion level. Gelatinized potato starch gave highest texture, followed by alginate and gelatin. Texture was significant improved with increasing binder level. Texture on moist feed produced with alginate was also improved with use of $CaCO_3$ in the dough. Recommended inclusion level is 2%. Immersing alginate pellets in a mix of $CaCl_2$ 3% + formic acid 3% improved texture compared to $CaCl_2$ 5% .The overall conclusion from the experiment was that water stability was unaffected or even impaired by immersing pellets in formic acid or $CaCl_2$. Texture of the pellet was easier to manipulate by the inclusion level of binder and immersion solution.

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1. INTRODUCTION

1.1. Salmon farming and sea lice problem

Over the past 40 years, the aquaculture industry in Norway experienced an incredible development. The production has grown from a modest 531 tons in 1971 to approximately 1 million tons in 2010, with a first hand value worth 34.7 billion NOK (Norwegian Ministry of Fisheries and Coastal Affairs, 2012). Seafood exports accounted for about 6 percent of total Norwegian exports with the value estimated to NOK 54 billion (app. USD 9 billion) in 2010 (The Norwegian Ministry of Fisheries and Coastal Affairs, 2012). The growth in the production began at the early 1980s when large-scale salmon production made a break through. Today, salmon makes up 90 percent of the total sale of Norwegian fish farming, followed by rainbow trout, the second dominating species (The Norwegian Ministry of Fisheries and Coastal Affairs, 2012).

However, the rapid development of salmon farming has resulted in increased problems with sea lice infestation on farmed salmon. The species causing the problems are *Lepeophteirus salmonis* and *Caligus elongates* (Ecofish, 2013; Pike and Wadsworth, 1999; Todd et al., 2000). The sea lice is an ecto parasite inflicting damage to their hosts thought their feeding activity on the host's body. The fish infected with sea lice have lower growth rate, loss of scales, and in worst case get open skin lesions making the fish more prone to secondary infection. Consequently the fish may get a lower market value (Costello, 2006; Pike and Wadsworth, 1999). It is also a concern that increased sea lice infestation on farmed fish is a threat to wild salmonids. Norwegian farmers were the first to experience problems associated with sea as early as 1960s. The Scottish farmers succumbed in mid 1970s. It is now widely regarded not only as the parasite causing greatest problem in salmonid farming, but also as one of the major threats to future growth of salmonid farming (Ecofish, 2013; Pike, 1989).

The economic cost of sea lice has over the years increased for the salmon farmers. The most significant cost for controlling sea lice includes production loss, treatment cost, reduced fish growth, reduced food conversion efficiency and the money invested for research on methods to combat sea lice (Costello, 2009). According to the latter author, the estimated cost of sea lice control in 2006 was more than 305 million Euro across the world. However, Norway was the country spending most money to control sea lice with more than 131 million Euros.

1.2. Combating sea lice

Controlling the sea lice on farmed salmonids is depending on the use of chemicals. According to Horsberg (2010), formaldehyde treatment was the first chemical applied to combat sea lice, but was soon replaced by the organophosphates metrifonate, dichlorvos and azamethiphos. Later an antiseptic agent, hydrogen peroxide, was introduced due to resistance development against organophosphates. Chitin synthesis inhibitors (diflubenzuron and teflubenzuron), pyrethroids (cypermethrin and deltamethrin) and the avermectin emamectin benzoate were subsequently introduced as control agents (Horsberg, 2010). In several countries, resistance problems have occurred. Resistance against organophosphates were evident already in the early 1990s, and from mid 2000, resistance against emamectin has been evident in almost every salmon farming regions. Resistance against pyrethroids is spreading, and even for hydrogen peroxide, resistance problems have occurred. No resistance problems have yet been reported for the chitin synthesis inhibitors (Horsberg, 2010). In addition to the resistance, chemicals in the treatment of sea lice infestations are normally subsequently released to the aquatic environment and may have impact on other aquatic organisms and their habitat (ICES WGEIM, 2004; Johnson et al., 2004). Therefore, the demand for new strategies for sea lice control based on non-chemical control options rather than chemical treatments. At present the industry in collaboration with Norwegian Universities and research institutes have several ongoing projects to find alternative strategies to prevent sea lice infestations on salmonids (Kyst, 2013).

One of the non-chemical methods to combat sea lice is use of cleaner fish such as the Ballan wrasse (*Labridae*). The Ballan wrasse eats lice off the salmon and has suggested being one important weapon in the battle fighting sea lice. Ballan wrasse placed in the cage together with salmon juveniles will symbiotically grow up alongside each other. The introduction of Ballan wrasse has shown to be successful in controlling sea lice infestation on farmed Atlantic salmon. The cleaner fish were even shown to be more efficient in controlling sea lice than conventional chemical treatment method resulting in less disease outbreak and reduced stress of salmon (Deady et al., 1995; Figueiredo et al., 2005; Treasurer, 1994).

One of the main obstacles of using Ballan wrasse within the salmon industry is to have enough of them. Until now, the use of Ballan wrasse has been based on wild catch. In the wild, Ballan wrasse of the size needed are in short supply (Ecofish, 2013; Treasurer, 2012). The use of cleaner fish, wrasse, has been adopted widely in Norway and Scotland as part of an integrated pest management control programme. Although wrasses were stocked with salmon in the 1990s, there has been renewed and more sustained use of wrasse in the last 4 years. Large numbers of wrasse collected in the wild have been stocked, with estimates of numbers up to 10 million per annually in Norway (Skiftesvik et al., 2013) .

The stocking density of Ballan wrasse to salmon in salmon cages is 2-4 percent (Ottesen et al., 2013). Based on this stocking density, the annual estimated demand is 7-15 million individuals per year. Providing this species in adequate numbers requires on land hatcheries. A base population is usually established from collection of wild Ballan wrasse, and offspring from these are used to establish breeding populations and fish to be put in the sea. Today there are intensive research going on to solve the biological bottlenecks in the production of wrasse in tanks, and there are many research questions that need to be resolved to meet the future demand for cleaner fish. One major question is related to feed and nutrition of the fish. There is an overall lack of nutritional knowledge, what to feed the fish and how during different life stages. One major gap of knowledge is how to feed the Ballan wrasse in the base population. Often moist diets are used; however, these diets are labour demanding and often result in poor water quality. Poor water quality is leading to increased mortality. It is therefore an urgent need to improve feed quality in order to improve the hatching success.

One of the success criterions in salmonid farming has been the use of high quaity extruded dry pellets. However, fish caught from the wild does not readily accept to eat (extruded) dry pellets. Thus, feeding of Ballan wrasse broodstock (fish taken from the wild) is usually carried out with moist feed. Common practice today is to grind extruded cod pellets, mix it with a binder and produce it into a moist pellet. Shrimp can also be added as a palatability enhancer for Ballan wrasse. Use of moist feed often results in poor quality of the water. It is therefore a need to develop new protocols for improved feed technology to Ballan Wrasse.

1.3. Binders to improve quality of moist feed

Binders are useful to improve the quality of moist feed and to reduce leaking of nutrients to the water. Overall, binders can be categorized as digestible binders and indigestible binders. Digestible binders are favored because they supply nutrients. Examples of digestible binders are proteins and gelatinized starch. Such binders are commonly used in modern extruded fish feed (Sorensen, 2011; Sørensen et al., 2010; Yogendra, 2011). In moist feed, however, indigestible binders such as guar gum and alginate, were used to promote a pellet with high water stability and good technical quality (Storebakken, 1985). Indigestible binders are less attractive because they only promote good pellet structure, but they supply no nutrients. Alginate, and in particular guar gum, have shown to have a negative effect on digestibility of protein and fat (Storebakken, 1985). Based on these early investigations, guar gum is not recommended in moist feed and the inclusion level of alginate should be minimized to the level needed for good technical quality.

Alginate is an anionic copolymer composed of homopolymeric regions of 1,4-linked β-D-mannuronic (M blocks) and α-L-guluronic acid (G blocks), Alginate is extracted from seaweed a dilute alkaline solution (Paolucci et al., 2010). Gelling of alginate takes place when the divalent Ca^{2+} replace Na^{+} , creating interchain ionic binding between the molecular units (M –or G block) of alginate. This exchange take place at low pH (Donati and Paoletti, 2009; Draget et al., 2009; Paolucci et al., 2010; Rezende et al., 2004) and is explaining why pellets were soaked in formic acid.

1.4. Aim of study

The hypothesis of the present research is that quality of moist feed can be improved by use of alternative digestive and indigestive binders in combination with alternative gelling technology. The overall aim of the present study was to investigate use of different binders and gelling technology to improve the water stability of moist feed for Ballan wrasse.

2. MATERIALS AND METHODS

2.1. Production of experimental diets

The experimental diets were produced at the Norwegian University of Life Sciences (UMB), Department of Animal and Aquacultural Sciences, Aas, Norway. Twenty six moist diets (30% water content) were produced in order to optimize the binders and technology, aiming at improved water stability and pellet quality. The diet, types and levels of binders, level of $CaCO_3$, $CaCl_2$, and bath solution were set up as following (Table 1). Control diet was made following formulation from Sundalsora.

Three different binders were used. Gelatinized starch (Lygel F60, Lykeby Culinar AB, Sweden) at three levels, 10%, 15% and 20% (Diets 1, 2, 3). Gelatin (Rousselot® 100 FG8, Rousselot Angouleme SAS, Angouleme, France) was also tested at three levels 2.5%, 5% and 7.5% (Diets 4, 5, 6). Alginate (Scogin MV Alginate is a sodium alginate produced by FMC Biopolymer; alginate had to be dissolved in hot water before it was mixed into the dough) was tested at two levels 1.5% and 3% with or without supplementation of $CaCO_3$. Gelling of pellets made with alginate was tested in immersion bath with formic acid 5%, solution of $CaCl_2$ (5%) or in solution of mix of $CaCl_2$ 3% and Formic acid 3% (Diet 7-17b).

Diets with combination of three binders also was made and tested with use of different immersion bath. The immersion baths were $CaCl_2$ 5% (19a) or a mix of $CaCl_2$ 3% + Formic acid 3% (19b). No immersion was also tested (19c).

The dry ingredients were mixed thoroughly by use of a mixer. Water (30%) was added to the dry ingredients, and the mixer was run for another 10 minutes. The dough was shaped using the mincer with die size 5 mm. Pellets were manually cut by scissors to length 10 to 15 mm. Water stability and sinking velocity was ensured in a pre-study by immersing pellets in salt water (3.3%). Pellets made with alginate were immersed in bath solution (Formic acid 5%, $CaCl_2$ 5% or mix of Formic acid 3%+ $CaCl_2$ 3%) for 15 minutes. The immersed pellets were taken out from the solution and left to rest on a tray for one hour before the feed samples were put in plastic bags and stored in 4^oC for further analysis.

Formic acid was added to the dry mix in diets 12, 14, 16, 18. However, those diets were dissolved during the immersion of acid or in salt water therefore were thus not produced.

Diets 11a, 13a, 15a, 17a were made by immersing diets 11, 13, 15, 17 in solution of $CaCl_2$ 5%.

Diet 11b, 13b, 15b, 17b were made by immersing diets 11, 13, 15, 17 in combination of $CaCl_2$ 3% % + Formic acid 3%.

Diet 19a, 19b, 19c were produced by immersing diet 19 in solution $CaCl_2$ 5%, mix of $CaCl_2$ 3% and no bathing, respectively.

Table 1 Diets, binder types, binder level, Agents added to dry mix and immersion solution

No	Binder	Binder level (%)	Agents added to dry mix	Immersion solution
1	Gelatinized starch	10	No	No immersion
2	Gelatinized starch	15	No	No immersion
3	Gelatinized starch	20	No	No immersion
4	Gelatin	2.5	No	No immersion
5	Gelatin	5.0	No	No immersion
6	Gelatin	7.5	No	No immersion
7	Sodium alginate	1.5	2% $CaCO_3$	Formic acid 5%
8	Sodium alginate	3.0	2% $CaCO_3$	Formic acid 5%
9	Sodium alginate	1.5	4% $CaCO_3$	Formic acid 5%
10	Sodium alginate	3.0	4% $CaCO_3$	Formic acid 5%
11a	Sodium alginate	1.5	No	$CaCl_2$ 5%
11b	Sodium alginate	1.5	No	$CaCl_2$ 3% + Formic acid 3%
12	Sodium alginate	1.5	1% Formic acid	$CaCl_2$ 5%
13a	Sodium alginate	3.0	No	$CaCl_2$ 5%
13b	Sodium alginate	3.0	No	$CaCl_2$ 3% + Formic acid 3%
14	Sodium alginate	3.0	1% Formic acid	$CaCl_2$ 5%
15a	Sodium alginate	1.5	2% $CaCO_3$	$CaCl_2$ 5%
15b	Sodium alginate	1.5	2% $CaCO_3$	$CaCl_2$ 3% + Formic acid 3%
16	Sodium alginate	1.5	2% $CaCO_3$	$CaCl_2$ 5%
17a	Sodium alginate	3.0	2% $CaCO_3$	$CaCl_2$ 5%
17b	Sodium alginate	3.0	2% $CaCO_3$	$CaCl_2$ 3% + Formic acid 3%
18	Sodium alginate	3.0	2% $CaCO_3$ + 1% Formic acid	$CaCl_2$ 5%
19a	Gelatinized starch + Gelatin + Sodium alginate	10 + 2.5 + 0.5	1% $CaCO_3$	$CaCl_2$ 5%
19b	Gelatinized starch + Gelatin + Sodium alginate	10 + 2.5 + 0.5	1% $CaCO_3$	$CaCl_2$ 3% + Formic acid 3%
19c	Gelatinized starch + Gelatin + Sodium alginate	10 + 2.5 + 0.5	1% $CaCO_3$	No

Table 2 Composition of diets

Composition (%)	**Diet**																			**Control diet**
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	
Skretting	39.2	34.2	29.2	54.8	52.3	49.8	45.7	44.2	43.7	42.2	47.7	46.7	46.2	45.2	45.7	44.7	44.2	43.2	43.3	54.6
Shrimp	20.8	20.8	20.8	20.8	20.8	20.8	20.8	20.8	20.8	20.8	20.8	20.8	20.8	20.8	20.8	20.8	20.8	20.8	20.8	20.8
Water	30	30	30	21.9	21.9	21.9	30	30	30	30	30	30	30	30	30	30	30	30	21.9	21.9
Gelatinized starch	10	15	20	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	10	*
Gelatin	*	*	*	2.5	5.0	7.5	*	*	*	*	*	*	*	*	*	*	*	*	2.5	*
Sodium alginate	*	*	*	*	*	*	1.5	3	1.5	3	1.5	1.5	3	3	1.5	1.5	3	3	1.0	*
$CaCO_3$	*	*	*	*	*	*	2	2	4	4	0.0	0	0	0	2	2	2	2	0.5	*
Formic acid added to dry mix	*	*	*	*	*	*	*	*	*	*	0	1	0	1	0	1	0	1		*
Sum	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	

** is denoted for not inclusion*

Control diet was made following formulation from Sundalsora.

2.2. Testing of physical pellet quality

2.2.1. Water stability test

The water stability test was carried out at IHA lab, UMB University, Norway. The procedure carried out according to (Baeverfjord et al., 2006) method with the shaking water bath (Julabo labortecknik GmbH, Seelbath, Germany) (software version: SW22 n2.6). A sample of about 10 gram pellets were placed into wire net 3mm hole size baskets, 8 cm in diameter (Weighted the basket to know it’s weight before pellets be placed). Pellets with baskets were weighted then placed into a 600 ml glass beaker filled with 300 ml distilled water. Six beakers were placed into shaking water bath each testing cycle. Parameters set for one cycle were temperature 25^{o}C, 110 rpm shaking frequency for 180 minutes. Each diet was replicated three times (some samples were four times). After shaking process finished, the baskets with feed sample was gently taken out of water bath and weighted again. The feed was then placed into a heating cabinet drying at 104^{o}C for at least 18h. The baskets with sample were weighted again immediately when the samples were taken out from the heating cabinet. Dry matter after incubation was calculated as the net weight of sample after drying (the weight of basket with sample after drying substrate the weight of basket) divide the net weight of sample before drying (the weight of basket with sample before drying substrate the weight of basket) in percent.

Dry matter of sample was determined by following procedures:

- Weight of the cup
- A sample of about 10 gram grinded pellets placed into a porcelain cup. Weight of the cup + sample
- Net weight of sample is the (Weight the cup + sample) subtracts the weight of cup
- Placed the cup + Sample in drying chamber for at least 18h at 104oC
- Weight the cup + sample after drying immediately
- Net weight after drying is the Weight the cup + sample after drying substrate the weight of cup
- Drying mater is the percent of net weight of sample after drying by the net weight of sample before drying in percent

Stability value was calculated as the dry matter after incubation by dry mater of sample

2.2.2. Texture test

The texture of the pellets was carried out at NOFIMA, As, Norway. An average of thirsty pellets from each diet was chosen for the texture test. The length of pellets was measured manually by using electronically digital caliper. The hardness strength at rupture and diameter of pellets were measured by Texture Analyser (TA-XT2®, Model 1000R; SMS Stable Micro Systems, Blackdown Rural Industeries, Surrey, UK). The force data was recorded at 25%, 50%, 60%, 70% of the pellet diameter. Because texture at 70% of the diameter showed the greatest variation, these values were used for the statistical analysis and presented in the result part. The procedure was carried out as described by (Aas et al., 2011).

2.2.3. Statistical analysis

Data collected were analyzed by using the SAS software 9.2 (SAS Institute Inc., Cary, NC, USA) to investigate if there are significant different among diets ($P < 0.05$). The data was analyzed by use of analysis of variance (ANOVA), proc glm procedure. The data was analyzed by use of one way ANOVA or two ways ANOVA. Results were presented as mean value ± standard error.

Based on data collected, seven models were tested to investigate significant effect of binders, $CaCO_3$ or immersion solution on pellet quality. The models tested were:

- Comparing mean value of physical quality for all diets (ONE WAY- ANOVA).
- Comparing effect of binder type, level of binder on physical quality. Diets 1, 2, 3, 4, 5, 6 and Control were used and analyzed by use of TWO WAY ANOVA.
- Effect of alginate level and CaCO3 level were tested using diets 7, 8, 9, 10 and Control and analyzed by use of TWO WAY ANOVA.
- Effect of alginate level in CaCl2 bath or in mix of CaCl2 3% + Formic acid 3% bath were tested with use of Diets 11a, 11b, 13a, 13b and Control diet.
- Effect of alginate level with or without CaCO3 added to the dough soaked in CaCl2 with or without formic acid. Diets 11a, 11b, 13a, 13b, 15a, 15b, 17a, 17b and Control diet were used for this analysis.
- Comparing effect of all binder combination with or without in CaCl2 bath solution or Formic acid. Diets 19a, 19b, 19c were used for the test.

3. RESULTS

3.1. Water stability

A comparison of water stability of all diets was made by one way ANOVA. Significant differences in water stability were observed (Figure 1). Diet 19c (mix of binders, no bathing) gave the highest stability value while diet 19b (mix of binders, bathed in $CaCl_2$ 3% and formic acid 3%) had lowest value. No significant difference was noted among the others diets.

3.1.1. Effect of type and level of binders on water stability

In order to test the effect of binder type and level of binder on water stability, the data was sorted. Diets 1, 2, 3, 4, 5, 6, 11a, 13a were used to test the effect of binder type, binder level and interaction between binder type* binder level on water stability. The results showed that there was a significant effect of binder type. Gelatinized starch gave the highest water stability, though the numerical values showed only modest differences (Table 3). Level of binder (Table 4) or the interaction binder type and level did not give any significant differences in water stability (Tables 5).

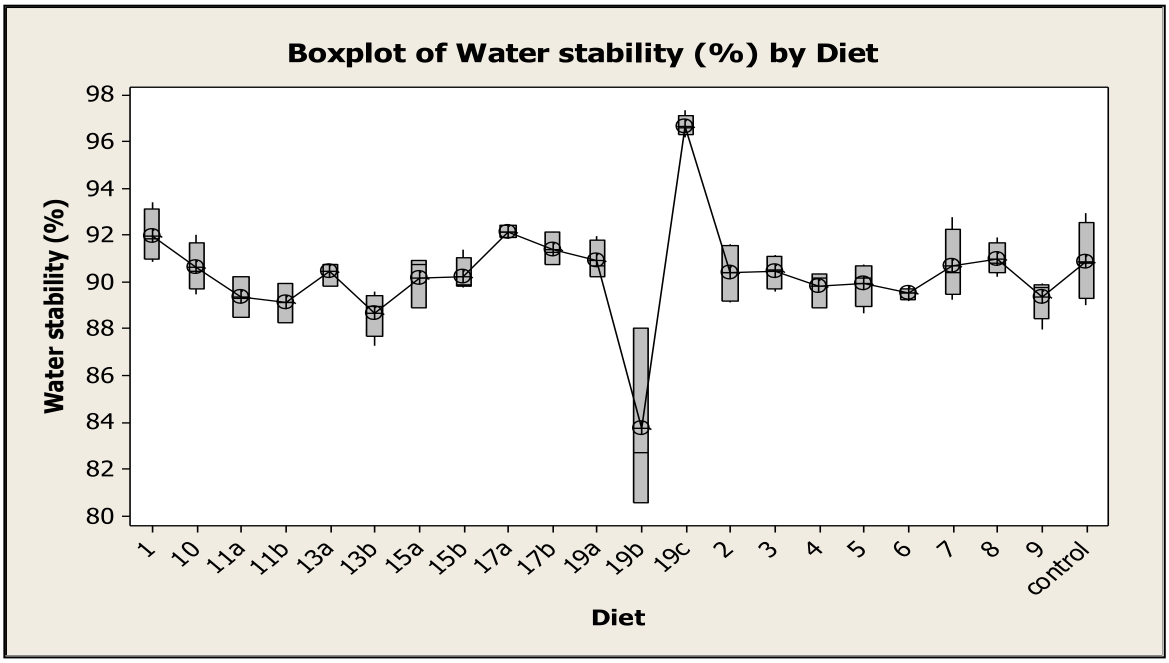

Fig.1. Water stability of different diets

Table 3 Effect of binder on water stability

Binders	**Stability mean**
Control	90.87± 0.82
Starch	90.95± 0.36
Gelatin	89.75 ± 0.20
Alginate	89.72 ± 0.20

Table 4 Effect of binder level on water stability

Binders	**Level**	**Stability**
Control	0	90.87± 0.51
Starch	10 %	92.0 ± 0.51
Starch	15%	90.37 ± 0.51
Starch	20%	90.47 ± 0.51
Gelatin	2.5%	89.83± 0.59
Gelatin	5%	89.95 ± 0.51
Gelatin	7.5%	89.50 ± 0.51
Alginate	1.5%	89.33± 0.59
Alginate	3%	90.30 ± 0.72

Table 5 Effect of interaction of binder type * binder level on water stability

Binder* level	**Stability mean**
0	90.87± 0.82
1	91.07 ± 0.56
2	90.16 ± 037
3	89.98 ± 0.26

**Definition:*

- 0 *is binder level 0- Control diet*
- 1 *is the lowest level of binder, i.e. average of diets with starch, gelatin, and alginate added at inclusion level 10%, 2.5%, 1.5%, respectively*
- 2 *is the intermediate level of binder, i.e. average of diets with starch, gelatin, alginate added at inclusion level 10%, 2.5%, 1.5%, respectively*
- 3 *is the highest level of binder, i.e. the average of diets with starch, gelatin and alginate added at 20%, 7.5% and 3%, respectively.*

3.1.2. Effect of alginate level and $CaCO_3$ level on water stability

The data was sorted according on diets 7, 8, 9, 10 and Control to compare the effect of alginate level and $CaCO_3$ level on water stability. The two way ANOVA analyses showed that water stability was not affected by alginate level (Table 6), $CaCO_3$ level (Table 7) or the interaction between amount of alginate and $CaCO_3$ (Table 8).

Table 6 Effect of alginate level on water stability

Alginate level	Stability mean
Control diet	90.87 ± 0.82
1.5%	90.04± 0.48
3%	90.79 ± 0.30

Table 7 Effect of $CaCO_3$ level on water stability

$CaCO_3$ level	Stability mean
Control diet	90.87 ± 0.82
2%	90.82 ± 0.39
4%	90.00 ± 0.40

Table 8 Effect of interaction between alginate level and CaCO3 level on water stability

Alginate level	$CaCO_3$ level	Stability mean
Control diet	Control diet	90.87 ± 0.61
1.5%	2%	90.70 ± 0.61
1.5%	4%	89.37 ± 0.61
3%	2%	90.95 ± 0.61
3%	4%	90.62 ± 0.61

3.1.3. Effect of alginate level and bath solution $CaCl_2$ 5% or mix of $CaCl_2$ 3% + Formic acid 3% on water stability

Table 9 Effect of alginate level on water stability

Alginate level	Stability mean
Control diet	90.87^{a} ± 0.82
1.5%	89.23^{b} ± 0.31
3%	89.46^{ab} ± 0.46

Table 10 Effect of bath solution on water stability

Bath solution	Stability mean
Control diet	90.87^{a} ± 0.82
$CaCl_2$ 5%	89.90^{ab} ± 0.37
Mix solution	88.88^{b} ± 0.33

$^{a, b}$ *different letters are denoting differences among means*

"Mix" *is denoting for combination of $CaCl_2$ 3% + formic acid 3%*

Table 11 Effect of interaction between alginate level * bath solution on water stability

Alginate level	Bath solution	Stability mean
Control diet	0	90.87 ± 0.55
1.5%	$CaCl_2$ 5%	89.33 ± 0.63
1.5%	Mix	89.13 ± 0.63
3%	$CaCl_2$ 5%	90.46 ± 0.63
3%	Mix	88.70 ± 0.55

"Mix" *is denoting for combination of CaCl2 3% + formic acid 3%*

Diets 11a, 11b, 13a, 13b, and Control diet were used to analyze the effect of alginate level and gelling of pellets in different bath solutions ($CaCl_2$ 5% or mix of $CaCl_2$ 3% and Formic acid 3%). The results showed that level of alginate and bath solution tended to affect water stability (Tables 9, 10), while the interaction alginate level* bath solution did not affect water stability ($P>0.05$) (Tables 11).

3.1.4. Effect of alginate level with or without $CaCO_3$ added to the dough soaked in $CaCl_2$ with or without formic acid on water stability.

Table 12 Effect of alginate level on water stability

Alginate level	Stability mean
Control diet	$90.87^{a} \pm 0.82$
1.5%	$89.76^{b} \pm 0.26$
3%	$90.53^{ab} \pm 0.43$

Table 13 Effect of bath solution on water stability

Bath solution	Stability mean
Control diet	$90.87^{a} \pm 0.82$
$CaCl_2$ 5 %	$90.54^{ab} \pm 0.36$
Mix	$89.81^{a} \pm 0.34$

$^{a, b}$ *different letters are denoting differences among means*

*"*Mix*" is denoting for combination of $CaCl_2$ 3% + formic acid 3%*

Table 14 Effect of $CaCO_3$ level on water stability

$CaCO_3$ level	Stability mean
2%	$90.94^{a} \pm 0.30$
0%	$89.71^{b} \pm 0.32$

$^{a, b}$ *different letters are denoting significant differences among means*

Table 15 Effect of interaction of alginate level* bath solution* $CaCO_3$ level

Alginate level	Bath solution	$CaCO_3$ level	Stability mean
Control diet	Control diet	0	90.87 ± 0.49
1.5%	$CaCl_2$ 5%	0	89.33 ± 0.57
1.5%	$CaCl_2$ 5%	2%	90.20 ± 0.57
1.5%	Mix	0	89.13 ± 0.57
1.5%	Mix	2%	90.22 ± 0.49
3%	$CaCl_2$ 5%	0	90.46 ± 0.56
3%	$CaCl_2$ 5%	2%	92.16 ± 0.56
3%	Mix	0	88.70 ± 0.49
3%	Mix	2%	91.40 ± 0.56

*"*Mix*" is denoting for combination of $CaCl_2$ 3% + formic acid 3%*

Diets 11a, 11b, 13a, 13b, 15a, 15b, 17a, 17b were used to investigate the effect of alginate level with or without $CaCO_3$ added to the dough soaked in $CaCl_2$ with or without formic acid. The results showed that water stability was tended to affect by alginate level (Table 12), bath solution (Table 13) and $CaCO_3$ inclusion level (Table 14). However, the three way interaction between those factors was not significant (Table 15).

3.1.5. Effect of combination of all binders on water stability

Diets 19a, 19b, 19c were formulated with all binders, including gelatinized starch, gelatin and alginate. The results showed that diet 19c (no bathing) had the highest stability, while diet 19b (bath in mix solution) had lowest stability, compared to other diets (Table 16).

Table 16 Effect of combination of all binders on water stability

Diet	Stability mean
Control diet	$90.87^{b} \pm 0.82$
19a	$90.90^{b} \pm 0.42$
19b	$83.77^{c} \pm 0.20$
19c	$96.67^{a} \pm 0.23$

$^{a,\,b,c}$ *different letters are denoting significant differences among means*

Table 17 Texture measured at 70% nedtr (N) for all diets

Diets	N	Mean of texture
3	29	$75.7^{a} \pm 3.02$
19c	30	$58.5^{b} \pm 1.77$
2	30	$45.9^{c} \pm 1.12$
13a	31	$40.4^{dd} \pm 0.09$
10	30	$39.5^{de} \pm 1.17$
17b	20	$38.4^{de} \pm 1.08$
8	30	$36.6^{e} \pm 1.08$
13b	25	$36.5^{e} \pm 1.26$
6	30	$26.5^{f} \pm 0.93$
17a	25	$25.6^{fg} \pm 0.64$
15b	20	$25.1^{fg} \pm 0.71$
5	34	$23.9^{fh} \pm 0.69$
9	30	$23.5^{gi} \pm 0.65$
7	30	$22.3^{gj} \pm 0.72$
11a	60	$20.7^{kj} \pm 0.38$
Control	30	$20.6^{kj} \pm 0.72$
1	30	$19.2^{ki} \pm 0.62$
19a	25	$18.9^{jk} \pm 0.59$
4	30	$18.5^{k} \pm 1.00$
15a	26	$13.8^{ll} \pm 0.40$
19b	30	$12.3^{l} \pm 0.60$

$^{a,\,b,c\ldots l}$ *different letters are denoting significant differences among means*

3.2. Texture

The load at different diameter of the pellet was recorded. The greatest load and variation among the diets was observed when load was recorded at 70% of the diameter. These data was therefore used to analyze the treatment effects. When texture of all data was analyzed by one way ANOVA, significant differences were observed among diets (Table 17). Textured varied between 12.3 N (diet 19b) to 75.7 N (diet 3).

3.2.1. Effect of type and level of binders on texture

The Two way- ANOVA analysis showed that texture was significantly affected by binders (Table 18), level of inclusion (Table 19) as well as binders* levels (Table 20). Gelatinized starch gave the highest texture. The lowest texture was observed for gelatin and control diet, but no significant difference was observed between gelatin and control diet (Table 18).

Table 18 Effect of binder type on texture

Binder type	Texture mean
Starch	$47.8^{a} \pm 2.67$
Alginate	$31.3^{b} \pm 1.38$
Gelatin	$23.7^{c} \pm 0.61$
Control	$20.6^{c} \pm 0.72$

[a, b,c] different letters are denoting significant differences among means

Table 19 Effect of level binder on texture

Binder level	Texture mean
0	$20.6^{c} \pm 0.27$
1	$19.6^{c} \pm 0.43$
2	$37.3^{b} \pm 1.10$
3	$52.4^{a} \pm 3.58$

[a, b,c] different letters are denoting significant differences among means

Table 20 Effect of interaction between binder type and binder level on texture

Binder type	Binder level	Texture mean
0	0	20.6 ± 1.25
1	1	19.2 ± 1.25
1	2	47.4 ± 1.25
1	3	76.9 ± 1.25
2	1	19.03 ± 1.25
2	2	24.2 ± 1.17
2	3	27.7 ± 1.25
3	1	20.5 ± 1.25
3	2	42.1 ± 1.23

**Definition:*

Binder

- *1- starch*
- *2- Gelatin*
- *3- Alginate*

Level

- *0 is binder level 0- Control diet*
- *1 is the lowest level of binder, i.e. average of diets with starch, gelatin, and alginate added at inclusion level 10%, 2.5%, 1.5%, respectively*
- *2 is the intermediate level of binder, i.e. average of diets with starch, gelatin, alginate added at inclusion level 10%, 2.5%, 1.5%, respectively*
- *3 is the highest level of binder, i.e. the average of diets with starch, gelatin and alginate added at 20%, 7.5% and 3%, respectively*

The highest texture was also found at the highest level of starch 20%. Control diet had the lowest texture, but was not significantly different from the lowest inclusion level of binder (Table 19).

For the interaction between binder type and level, starch at the highest inclusion level (20%) gave the significantly highest texture compared to all other combinations (Table 20).

3.2.2. Effect of alginate and $CaCO_3$ level on texture

The results showed that texture was significantly affected by alginate level. The significantly highest texture was observed for pellets with 3% alginate. No differences were observed between 1.5% alginate and the control diet (Table 21).

Table 21 Effect of alginate level on texture

Alginate level	Texture mean
Control diet	20.60^b ± 0.72
1.5%	22.42^b ± 0.48
3%	37.99^a± 0.81

$^{a, b}$ different letters are denoting differences among means

For the $CaCO_3$ level, the lowest texture was observed for the control diet with no $CaCO_3$ added. Diets with $CaCO_3$ added to the dough gave significantly higher texture compared to the control, but there were no significant difference between 2% and 4% $CaCO_3$ added to the dough (Table 22).

Table 22 Effect of $CaCO_3$ level on texture

$CaCO_3$ level	Texture mean
Control diet	$20.6^b \pm 0.72$
2%	$29.4^a \pm 1.13$
4%	$30.9^a \pm 1.29$

$^{a, b}$ different letters are denoting differences among means

The interaction between alginate level and $CaCO_3$ level did not have a significant effect on texture (Table 23).

Table 23 Effect of interaction between alginate level and CaCO3 level on texture

Alginate level	$CaCO_3$ level	Texture mean
Control	0	20.6 ± 0.89
1.5%	2%	22.3 ± 0.89
1.5%	4%	22.5 ± 0.89
3%	2%	36.6 ± 0.89
3%	4%	39.4 ± 0.89

3.2.3. Effect of alginate level and bath solution ($CaCl_2$ 5% or mix of $CaCl_2$ 3% + Formic acid 3%) on texture

Texture was significantly affected by all variables in the model (alginate level and bath solution as well as the interaction.

The highest alginate level gave significant highest texture compared to lower level of alginate and Control diet ($P< 0.0001$) (Table 24).

Table 24 Effect of alginate level on texture

Alginate level	Texture mean
Control diet	$20.60^b \pm 0.72$
1.5%	$20.71^b \pm 0.38$
3%	$38.61^a \pm 0.79$

$^{a, b}$ different letters are denoting differences among means

The bath solution $CaCl_2$ 5% gave significantly highest texture compared to the mix of $CaCl_2$ 3% and Formic acid 3% (Table 25). The Control diet had the significantly lowest texture ($P=0.033$).

For the combination of alginate level and bath solution ($CaCl_2$ 5% or mix of $CaCl_2$ 3% and formic acid 3%), the texture was significantly highest for the highest level of alginate, while bath solution contributed less on texture (P=0.011) (Table 26).

Table 25 Effect of bath solution on texture

Solution	**Texture mean**
Control diet	20.60^{c} ± 0.72
$CaCl_2$ 5%	30.63^{a} ± 1.38
Mix	27.94^{b} ± 1.24

$^{a, b,c}$ *different letters are denoting differences among means*

"Mix" is denoting for combination of $CaCl_2$ 3% + formic acid 3%

Table 26 Effect of interaction between alginate level * bath solution

Alginate level	**Solution**	**Texture mean**
Control	0	20.60 ± 0.79
1.5%	$CaCl_2$ 5%	20.58± 0.79
1.5%	Mix	20.84 ±0.79
3%	$CaCl_2$ 5%	40.36 ±0.78
3%	Mix	36.45 ± 0.87

"Mix" *is denoting for combination of $CaCl_2$ 3% + formic acid 3%*

3.2.4. Effect of alginate level with or without $CaCO_3$ added to the dough soaked in $CaCl_2$ with or without formic acid on texture

The results are significant affected by alginate level (P< 0.0001), $CaCO_3$ (P<0.0001), bath solution (P<0.0001), and 3-way interaction (P<0.0001).

The highest texture was observed for the highest level of alginate while no significant differences were noted between the control diet and the diet with lowest content of alginate (Table 27).

No addition of $CaCO_3$ into dry mix gave a significantly higher texture compared to supplementation of 2% $CaCO_3$ into the dry mix (Table 28).

The bath treatment showed that the highest texture was obtained for pellets treated in the mix of $CaCO_3$ 3% and formic acid 3%, followed by $CaCl_2$ 5%. The lowest was observed for the control diet (Table 29).

Table 27 Effect of alginate level on texture

Alginate level	**Texture mean**
Control diet	20.60^{b} ± 0.72
1.5%	19.84^{b} ±0.46
3%	35.35^{a} ± 0.75

$^{a, b}$ *different letters are denoting differences among means*

Table 28 Effect of $CaCO_3$ level on texture

$CaCO_3$ level	**Texture mean**
Control diet	27.56^a ±0.81
2%	24.92^b ±0.98

$^{a, b}$ different letters are denoting differences among means

Table 29 Effect of bath solution on texture

Bath solution	**Texture mean**
Control diet	20.60^c ± 0.72
$CaCl_2$ 5%	25.61^b ± 1.00
Mix	29.53^a ± 0.90

$^{a, b}$ different letters are denoting differences among mean

"Mix" *is denoting for combination of $CaCl_2$ 3% + formic acid 3%*

Significant differences were also noted for the three way interaction alginate level* bath solution * $CaCO_3$ (Table 30) .Overall this three way interaction showed that level of alginate mainly dictated the texture. Also it showed that the mix of bath solution ($CaCl_2$ 3% + formic acid 3%) gave higher texture than use of 5% solution of $CaCl_2$ with or without $CaCO_3$ added to the dough.

Table 30 Effect of interaction between alginate level* Bath solution * $CaCO_3$ level on texture

Alginate level	**Bath solution**	**$CaCO_3$ level**	**Stability mean**
Control diet	Control diet	0	20.60 ± 0.73
1.5%	$CaCl_2$ 5%	0	20.58 ± 0.73
1.5%	Mix	0	20.84 ± 0.73
1.5%	$CaCl_2$ 5%	2%	13.77 ± 0.79
1.5%	Mix	2%	25.10 ± 0.89
3%	$CaCl_2$ 5%	0	40.36 ± 0.72
3%	Mix	0	36.45 ± 0.80
3%	$CaCl_2$ 5%	2%	25.65 ± 0.80
3%	Mix	2%	38.34± 0.89

"Mix" *is denoting for combination of $CaCl_2$ 3% + formic acid 3%*

3.2.5. Effect of combination of all binders on texture

Diets 19a, 19b, 19c were formulated with all binders, including gelatinized starch, gelatin and alginate. The results showed that diet 19c (no bath treatment) had the highest texture and diet 19b (bath in mix solution) had lowest texture compared to others (Table 31).

Table 31 Effect of combination of all binders on texture

Diet	**Texture mean**
Control diet	20.60^b± 0.72
19a	18.98^b ± 0.59
19b	12.27^c± 0.60
19c	58.49^a ± 1.77

$^{a, b,c}$ *different letters are denoting differences among means*

4. DISCUSSION

The aim of the study was to improve the quality of moist feed for Ballan wrasse. Different types of binders were evaluated at different inclusion level. Another aim was to improve the gelling technology by use of different bath solution $CaCl_2$ 5% or combination of $CaCl_2$ 3% + formic acid 3% with or without $CaCO_3$ added to the dough. The experimental diets were formulated based on a commercial brood stock feed mixed with minced shrimps and a binder. Formic acid used for lowering pH is important for gelling of alginate (Draget et al., 1996; Velings and Mestdagh, 1995). The gelling process also depends on Ca^{2+} ions replacing Na^+ to make the final water stable structure in the pellets. Two sources of Ca^{2+} ions that easily dissolve were therefore tested. Testing of different binders and immersion agents gave as expected different water stability and texture of the pellets when all diets were compared.

Overall the water stability was in the range 84% (diet 19b) to 97% (diet 19c) (Fig. 1) when the two most extreme values were included in the dataset. Taking out these two values, water stability for other 21 diets were in the range from 87 to 92%. The low water stability of diet 19b (combination of all three binders, immersion into $CaCl_2$ 3% + formic acid 3%) may be explained by pellets dissolving in the immersion solution. Apparently, no gelling took place during immersion, and the pellets dissolved because gelatinized starch and gelatin solubilized. This theory is strengthened by the high water stability of diet 19c. Diet 19c also contained all three binders and was made without immersion of pellets in bath.

The texture was affected both by binder types and level of inclusion. The gelatinized starch resulted in the highest texture followed by alginate and gelatin. The results indicate that gelatinized starch result in a stiffer gel compared to alginate and gelatin. In general carbohydrates are characterized as good binders because the macromolecular structure consists of several polar functional groups. The high polarity allows absorption of significant amounts of water and they are crucial for the formation of hydrogels making three dimensional gels (Paolucci et al., 2010). Both the gelatinized starch and the sodium-alginate are carbohydrate binders. The higher texture of pellets produced from gelatinized starch compared to alginate in the present study may be explained by the carbohydrate source. Gelatinized potato starch also gave the highest texture in extruded feed (Sorensen, 2011; Sørensen et al., 2010; Yogendra, 2011). In the study of Sorensen et al, 2009 gelatinized potato starch was tested against wheat, wheat starch, potato starch, wheat + wheat starch. Gelatinized potato starch gave the highest hardness of extruded feed in comparison with others. These results indicate that the effect of starch as a binder vary with the ingredient source and pre-processing. Gelatin is a protein binder. The mode of action of a protein binder differs from carbohydrates. Gelation of the protein is important to activate the binding forces. Gelation usually takes place with heat and water as the driving forces to unfold the native protein structure. During cooling, protein strands will aggregate forming a certain order of protein and particles in a matrix. Strength of the matrix will depend on protein structure, amino acid composition, presence of salt, heat treatment, pH, water holding capacity (Maximo, 2010). The lowest texture in the present experiment was obtained with gelatin as a binder.

The significant improved texture with increasing inclusion level of binders level is in line with several other studies (Igbinosun, 1988; Rodríguez-Miranda et al., 2012; Rosas et al., 2008; Rosas, 2008). For the present experiment, texture was almost doubled for each level of starch and alginate, suggesting that the highest inclusion level should be used. For gelatin, there was only modest improvement of using 7.5% inclusion compared to 5% suggesting that there is little room for improvement by using the highest level of inclusion. It was reasonable to expect that increasing inclusion level of binder will result in improved texture. However, in order to reduce the cost of the diet, it is important to determine an optimal inclusion level of expensive binder (Ali, 1988; Timothy, 2005). Nutritional value of the binders also needs to be taken into consideration when inclusion level is established. There are several publications showing a negative effect on feed utilization and nutrient digestibility when levels of indigestible binders are increased (Argüello-Guevara and Molina-Poveda, 2012; Igbinosun, 1988; Paolucci et al., 2010; Rosas, 2008; Storebakken, 1985). Nutritional value and feed utilization of the experimental diets were not evaluated in the present experiment.

The interaction between binder type and level showed that the three binders gave the same texture at lowest inclusion level. Doubling the inclusion level of gelatinized starch and alginate gave a fourfold and twofold improvement of texture, respectively. Gelatin did not have the same power, and only resulted in approximately 20% higher in texture.

4.1. Effect of type and level of binders on water stability and texture

Surprisingly, water stability was not affected by binder or by the inclusion level (Table 3). Water stability showed low variation among the diets and ranged between 90-91% both for binders and inclusion level. With such low variation among treatment means, no significant effect of binder or inclusion level could be expected. Overall, water stability of the moist feed in the present experiment was nearly in the same range as water stability for commercial extruded feed (Yafei, 2012). Yafei (2012) reported that water stability in the range 92-94%. Extruded feed is expected to have higher water stability than moist feed, indicating that the water stability of the moist pellets tested in the present experiment was generally high.

4.2. Effect of alginate level and $CaCO_3$ level on water stability and texture

According to the RUBIN technology, calcium ions are important for gelling of pellets in order to get a water stable texture of the moist feed. Different levels of $CaCO_3$ (as calcium donor) were therefore studied in combination with two levels of alginate. Surprisingly the water stability showed no effect of alginate level (Table 6), or $CaCO_3$ level (Table 7) or their interaction (Table 8). The present results are in contrast to earlier findings. (Argüello-Guevara and Molina-Poveda, 2012) showed that pellets with 5% sodium alginate had better water stability than 3% inclusion. Improved water stability was also reported by other researchers testing alginate at inclusion level 0-4% (Igbinosun, 1988; Rodríguez-Miranda et al., 2012).

The improved texture with higher alginate inclusion is in line with (Rodríguez-Miranda et al., 2012). The latter authors showed that alginate at inclusion level 0, 0.5%, 1.5%, and 2% gave improved texture values in the range from 1.98 to 3.31 N.

Adding $CaCO_3$ to the dough (prior to immersion) gave higher pellet texture compared to the control diet (Table 22). However, no differences were observed between the two inclusion levels (2% and 4%). The significant improvement in texture compared to the control diet was most likely not explained by the $CaCO_3$. The significant interaction between alginate level and $CaCO_3$ (Table 23) showed that alginate level and not $CaCO_3$

contributed to the improvement of texture. These results suggest that 2% inclusion of $CaCO_3$ supply a surplus of cations for gelling and are sufficient.

4.3. Effect of alginate level and bath solution $CaCl_2$ 5% or Mix of $CaCl_2$ 3% + formic acid 3% on water stability and texture

The results showed that alginate level and bath solution tended to improved water stability. Improved water stability with increasing alginate level is in line with other studies (Argüello-Guevara and Molina-Poveda, 2012; Igbinosun, 1988; Rodríguez-Miranda et al., 2012). Use of $CaCl_2$ (5%) as the only immersion solution gave higher water stability compared to the combination of $CaCl_2$ (3%) + formic acid (3%). These results suggest that the presence of formic acid had no effect on water stability. This result was unexpected because according to (Draget et al., 1996), gelling of alginate need to take place at low pH. The present results however, suggest that water stability in the pellets depended on concentration of $CaCl_2$. The same trends were observed for the texture results. Texture was improved by alginate level and bath solution while the combination of alginate level* bath solution contributed less. These findings were expected and in line with other researchers (Argüello-Guevara and Molina-Poveda, 2012; Draget et al., 1996; Igbinosun, 1988; Rodríguez-Miranda et al., 2012).

4.4. Effect of alginate level with or without $CaCO_3$ added to the dough soaked in $CaCl_2$ with or without formic acid on water stability and texture

Adding $CaCO_3$ to the dough improved water stability slightly suggesting that gelling of alginate was more efficient with the Ca^{2+} ions already present in the mash. Unexpectedly, no differences were observed between immersion pellets in $CaCl_2$ (5%) or a combination of $CaCl_2$ (3%) + formic acid (3%). As earlier discussed the findings suggest that $CaCl_2$ is more important for binding than formic acid.

Surprisingly the texture results showed that diets added $CaCO_3$ into the dry mix gave a lower texture compared to control diets with no addition of $CaCO_3$. Overall the texture of these pellets was low because only the lowest (2%) inclusion level of $CaCO_3$ was tested. Also, the control diet was not soaked while the experimental diets were soaked. As earlier discussed, immersion gave a significant reduction in texture and water stability. Because immersion showed an overall reduction of texture and water stability, it is hard to explain why highest texture was observed for pellets soaked in a combination of $CaCl_2$ (3%) + formic acid (3%) or $CaCl_2$, compared to the control diet.

5. CONCLUSION

The present study showed that the water stability were in the range 84-97%, but was not affected by binders or inclusion level. Texture of the pellets was in the range 12.3-75.9 N and was affected by binder and inclusion level. Gelatinized starch gave the highest texture followed by alginate and gelatin. At lowest inclusion level all binders gave the same texture, while a doubling of binder content increased texture by a fourfold for gelatinized starch, twofold for alginate and only 20% for gelatin. The results also showed that inclusion level of $CaCO_3$ added to the dough had no effect on water stability or texture. An inclusion rate of 2% of $CaCO_3$ was sufficient to provide a water stable pellet. Gelling of pellets with sodium alginate in the dough was improved when 5% $CaCl_2$ was used instead of 3% $CaCl_2$, or a combination of $CaCl_2$ (3%) + formic acid (3%). In conclusion, quality of moist feed for Ballan wrasse can be improved by optimizing inclusion level of different binders and $CaCO_3$ added to the dry mix. Immersion of pellet in acid or $CaCl_2$ had a negative effect on water stability while texture was improved.

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