A study of the discrete and interactive effects of different polysaccharides on the digestibility of diets fed to barramundi (*Lates calcirifer)*.

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Keywords : fibre, anti-nutritional, grain, lignin, cellulose, pectin, starch

Accepted for publication in: *Aquaculture*

Abstract

This study examined the single, paired and combined inclusion effect of a range of different polysaccharides types on the dry matter, protein and energy digestibility of diets fed to barramundi (*Lates calcarifer)*. The different polysaccharides included pregelatinised starch, cellulose, lignin and pectin. There were significant differences among the digestibility parameters of the diets with the different inclusion levels of each of the different polysaccharide types. Using a MANOVA analysis effects were noted for polysaccharide type, inclusion level and interaction terms on the digestibilities of dry matter, protein and energy. Cellulose addition resulted in a reduction in both dry matter and energy that was largely commensurate with its inclusion level, but its effect on protein digestibility was marginal. Starch had the least effect on any of the digestibility parameters of all the polysaccharide types examined. Pectin had the largest effect on dry matter, while lignin had the greatest impact on diet protein and energy digestion. In the diets with paired combinations of polysaccharides, lignin and pectin were responsible for negatively synergistic interactions in all digestibility parameters. These results show that different polysaccharide classes can have distinctly different effects on diet digestibility parameters. The results also show that some classes of polysaccharide have greater interactive effects than others.

1. Introduction

Aquaculture production is becoming increasingly reliant on the replacement of fishmeal with a combination of plant meals in feeds (Gatlin, et al., 2007). Already the use of terrestrial plant-derived raw materials in fish feeds is common place (Aslaksen et al., 2007; Gatlin et al., 2007; Hardy, 2010). Plant materials have recognised benefits including a reduction in formulation costs and improved functional characteristics (Krogdahl et al., 2010; Glencross et al., 2010). Less understood are the anti-nutritional effects caused by the introduction of non-starch polysaccharides (NSP) that are present in the carbohydrate fraction of many of these plant materials. The NSP content of many plant materials is recognised as a key factor which has been shown to affect the nutritional variability among these materials being fed to fish (Glencross et al., 2008, 2012a; 2012b).

Fish, like most monogastrics are generally capable of some level of starch digestion (Bergot and Breque, 1983; Amirkolaie et al., 2006; Enes et al., 2008; Moreira et al., 2008; Glencross et al., 2012a). However, it is the presence of the plant cell wall derived NSP's which has caused many complications due largely to their indigestible nature (Kraugerud et al., 2007; Hansen and Storebakken, 2007; Glencross, 2009). The non-nutritive value of most NSP's means they act largely as a bulking agent, which generally results in a reduction in feed digestibility with increased content in the feed. Although the non-nutritive value of many NSP's is well understood, what is less understood is the anti-nutritional and or interactive effects of the more complex NSP's which lead to a reduction in digestibility which exceeds their level of inclusion (Glencross et al., 2008; 2012b). An example of this is seen with the presence of lignin in diets of rainbow trout either via inclusion of some plant proteins or as a specific additive (Glencross et al., 2008; 2012b). With the common practice of blending different plant proteins and carbohydrates, it is therefore becoming increasingly important to consider not only the discrete effects but also the interactive of NSP's.

There have been various reports on the discrete effects of different NSP classes in fish diets (Glencross et al., 2003; Leenhouwers et al., 2004; 2007; Amirkolaie et al., 2005; Hansen and Storebakken, 2007; Glencross, 2009, Glencross et al., 2012b). Studies with rainbow trout have shown cellulose (insoluble NSP) inclusion in diets cause a reduction in dry matter and energy digestibility, but only a nominal effect on protein digestibility of the diet. (Hansen and Storebakken, 2007; Glencross, 2009, Glencross et al., 2012b). In a study by Glencross (2009) assessing insoluble and soluble NSP classes in lupins in rainbow trout diets, the classes were reported to cause different effects on diet digestibility. However, a further study detected only marginal differences in across soluble NSP classes of pectin and mannan (Glencross et al 2012b). The inclusion of lignosulphonate had the largest negative effect on diet digestibility,

particularly on protein digestibility (Glencross et al., 2012b). It was suggested that the cause of the larger variability in the earlier study may be due to an interaction effect and that the testing of blended NSP's should be considered. Therefore this study aims to examine the effects of the discrete and blended inclusion levels of different classes of NSP (cellulose, lignin and pectin) and starch on the digestible value of diets fed to barramundi, *Lates calcarifer*.

2. Materials and Methods

2.1 Diet preparation

The experiment design was based on a diet formulation strategy that used a diet-substitution approach, although the assessment of the digestible value of those ingredients was not the intent of this experiment (Glencross et al., 2007). To achieve this, a basal diet was formulated and prepared to include approximately 600 g/kg DM protein, 185 g/kg DM fat and an inert marker (yttrium oxide at 1 g/kg) (Table 1). Each polysaccharide ingredient or blend was added at to the test diets at 200 g/kg inclusion to a reciprocal-sample of the basal mash (see Table 1). The diets were made by the addition of water (about 25% of mash dry weight) to the mash whilst mixing to form a dough which was subsequently screw pressed using a pasta maker through a 4 mm diameter die. The moist pellets produced were then oven dried at 60°C for around 12 h before being allowed to cool to ambient temperature in the oven. The basal diet was prepared in a similar manner, but without the addition of any test ingredient. The source and composition of all ingredients is presented in Table 2.

2.3 Fish handling and faecal collection

Hatchery-reared barramundi (*Lates calcarifer*) were kept in a series of experimental tanks (300 L) supplied with aeration and heated flow-through seawater (salinity =35 PSU) at a flow rate of about 3 L/min maintaining dissolved oxygen at 6.2 ± 0.2 mg/L and temperature at 28.6 ± 0.2ºC (mean ± S.D.). Each of the tanks were stocked with 10 fish of 397.7 ± 68.9 g (mean ± S.D.; n = 40 from a representative sample of the population). Treatments were randomly assigned amongst 24 tanks, with each treatment having two replicates. The experiment was repeated and blocked by time to achieve a total of four replicates per treatment. The same batch of fish was used for both blocks, but with a randomisation of the fish between each block. The fish were allowed to acclimatise to their allocated dietary treatment for at least seven days before faecal collection commenced (Blyth et al., 2012).

The barramundi were manually fed once daily to apparent satiety, as determined over three separate feeding events between 0800 and 0900 each day. Faeces were collected the same afternoon (1600 – 1700) from each fish within each tank using stripping techniques based on those reported by Glencross (2011). Fish were anesthetised using AQUI-S (0.002 mL/L) in a separate smaller aerated tank. Once loss of equilibrium was observed, close attention was paid to the relaxation of the ventral abdominal muscles of the fish to ensure the fish were removed from the water before they defecated in the anaesthetic tank. The faeces were then expelled from the distal intestine using gentle abdominal pressure while the ventral abdominal muscles were relaxed. Hands were rinsed between handling each fish to ensure that the faeces were not

contaminated with urine or mucous. The faecal sample was placed in a small plastic vial and stored in a freezer at -20°C. Fish were not stripped on consecutive days in order to minimise stress on the animal and maximise feed intake prior to faecal collection. Faeces were collected over a six-day period, with each fish being stripped three times, once every second day. Faecal samples from different days were pooled within tank, and kept frozen at –20°C before being freeze-dried in preparation for analysis.

2.4 Chemical and digestibility analysis

Diet and faecal samples were analysed for dry matter, yttrium, nitrogen and gross energy content. Diets and ingredients were analysed for these same parameters and in addition for ash, total lipid, lignin, neutral-detergent fibre and acid-detergent fibre. Dry matter was calculated by gravimetric analysis following oven drying at 105ºC for 24 h. Total yttrium concentrations were determined after mixed acid digestion using inductively coupled plasma mass spectrophotometry (ICP-MS) based on the method described by (McQuaker et al., 1979). Protein levels were calculated from the determination of total nitrogen by CHNOS auto-analyser, based on N x 6.25. Gross ash content was determined gravimetrically following loss of mass after combustion of a sample in a muffle furnace at 550°C for 12 h. Total lipid content of the diets was determined gravimetrically following extraction of the lipids using chloroform:methanol (2:1). Gross energy was determined by ballistic bomb calorimetry. Dietary fibres were determined by digesting the defatted sample with multiple washes of acetone and ethanol. The resulting residue was corrected for undigested protein and ash according to the method of the Champ et al. (1998). Neutral-detergent fibre (NDF) samples were boiled with buffered NDF solution. The residue was collected on a coarse sintered glass crucible (Van Soest and Robertson, 1981). The acid-detergent fibre (ADF) was determined following a sample being reacted in 0.5M acid detergent solution and the residue was collected on a coarse sintered glass crucible after, the method of Van Soest and Goering (1970). Lignin was determined by reacting the ADF residue with cold 72% sulphuric acid. The sample was ashed and the residue measured gravimetrically (Van Soest and Robertson, 1981). Total carbohydrate content was determined based on dry matter – (protein+lipid+ash) content. Cellulose content was determined based on the ADF – Lignin. Hemicellulose content was determined based on NDF – ADF content.

Differences in the ratios of the parameters of dry matter, protein or gross energy to yttrium, in the feed and faeces in each treatment were calculated to determine the apparent digestibility (AD_{diet}) for each of the nutritional parameters examined in each diet based on the following formula (Maynard and Loosli, 1979):

$$AD_{diet} = \left(1 - \left(\frac{Y_{diet} \times Parameter_{faeces}}{Y_{faeces} \times Parameter_{diet}} \right) \right) \times 100$$

where Y_{diet} and Y_{faeces} represent the yttrium content of the diet and faeces respectively, and $Parameter_{diet}$ and $Parameter_{faeces}$ represent the nutritional parameter of concern (organic matter, protein or energy) content of the diet and faeces respectively.

2.5 Statistical analysis

All values are means unless otherwise specified. Effects of each of the discrete treatments on on the digestibility of dry matter, protein and gross energy in each of the diets were initially examined by a one-wayANOVA (Table 3). Levels of significance were determined using a Least Significant Difference (LSD) test. Limits for all critical ranges were set at $P < 0.05$. Further analysis of the effects of each NSP type were examined using Multiple regression to identify magnitude, vectors and significance of effects related to inclusion elvel of each NSP (Table 3). Additionally a stepwise forward regression was also employed to determine the additive effects of the different NSP classes which influenced each digestibility parameter (Table 5). These tests were undertaken using the Statistica™ v6.0 software (Statsoft®, Tulsa, OA, USA). Regression analysis was undertaken using the data analysis tools of Microsoft excel.

3. Results

3.1 Ingredient composition

There were substantial differences in the chemical characteristics of the four different polysaccharide ingredients used in this study. The measurement of ADF, NDF and lignin allowed the determination of lignin, cellulose and hemicellulose contents of each sample (Table 1). Lignin content was highest in the lignosulphate (99 g/kg DM) and lowest in the pectin (0 g/kg DM). Cellulose content was highest in the purified cellulose ingredient (827 g/kg DM) and lowest in the starch (1 g/kg DM) and pectin (0 g/kg DM). Similarly, hemicellulose was also highest in the purified cellulose ingredient (129 g/kg DM) and lowest in the starch (2 g/kg DM) and pectin (0 g/kg DM). Protein content in all test ingredients was low (<30 g/kg DM) as was also the total lipid content (<62 g/kg DM).

3.2 Diet digestibilities

There were distinct differences between the diets in terms of their dry matter and energy digestibility (Table 3), with differences among protein digestibilities of the diets generally being somewhat less that the other two parameters. A two-way ANOVA analysis (MANOVA) identified that there were significant effects of the different polysaccharide types (P=0.001) and also the inclusion levels (P=0.001) of each of the polysaccharide types on the digestibility of dry matter and energy of each diet (Table 3). A significant (P=0.001) interaction term was also defined between the type of polysaccharide and the level of its inclusion (Table 3). Regression analysis of each polysaccharide type and inclusion level clearly shows the effect of each polysaccharide type on the digestibilities of dry matter, protein and energy (Table 1 and 4).

The addition of cellulose caused a reduction in dry matter digestibility that was almost directly commensurate with the level of its addition (Table 1 and 4). Lignin caused a slightly larger reduction on dry matter digestibility (Table 1 and 4). Pectin caused a reduction in dry matter digestibility almost double that of its inclusion level (Table 1 and 4). Starch inclusion showed a nominal effect on dry matter digestibility.

The addition of the cellulose + starch blend was observed to make a reduction in dry matter digestibility that was close to commensurate with the level of cellulose addition (Table 1 and 4). The cellulose + pectin + starch + lignin blend was observed to make a reduction in dry matter digestibility that was near commensurate with the combined level of addition. The starch + lignin and cellulose + pectin + starch + lignin blends also had a similar such effect on dry matter digestibility. The pectin + lignin blend caused a reduction to dry matter digestibility almost double that of its inclusion level (Table 1 and 4). The cellulose + lignin blend had a

similar such effect on dry matter digestibility. The pectin + starch blend was also observed to cause a reduction in the dry matter digestibility which exceeded the level of NSP addition, but not to the same degree as observed with the pectin + lignin or cellulose + lignin blend.

The addition of starch had almost no impact on protein digestibility (Table 1 and 4). The cellulose and cellulose blends containing starch or pectin had a similar effect on protein digestibility. The pectin + starch blend caused only a minor reduction in protein digestibility. Howver, the addition of pectin, cellulose + lignin and the cellulose + pectin + starch + lignin blend caused a reduction in protein digestibility which exceeded the pectin + starch blend, but was lower than total inclusion level. The lignin and starch + lignin blend were observed to cause a reduction protein matter digestibility that was close to commensurate with the level addition. The pectin + lignin blend was observed to make a reduction in protein matter digestibility which exceeds the level of addition.

The cellulose blends containing starch or pectin caused a reduction in energy digestibility (Table 1 and 4), slightly less than the level of addition. The addition of cellulose or starch was observed to make a reduction in energy matter digestibility that was close to commensurate with the level of addition. Pectin, had a similar such effect on energy digestibility. The starch + lignin, starch + pectin and cellulose + pectin + starch + lignin blends caused a reduction in energy digestibility which exceeded total addition. The pectin + lignin and cellulose + lignin blend had a reduction effect on dry matter digestibility almost double the inclusion level. Lignin caused a reduction in protein digestibility greater than the pectin + starch blend, but lower than the pectin + lignin blend.

Step wise regression indicated that pectin was the dominant fibre affecting dry matter digestibility, followed by lignin and then cellulose (Table 5). The presence of pectin, lignin and cellulose in the model explained 36.9% of the variation in diet dry matter digestibility. The addition of starch did not add to the model for diet dry matter or protein digestibility. Lignin was the dominant fibre class affecting diet protein digestibility, followed by pectin and then cellulose (Table 5) accounting for 80.3% of the variation in digestibility. Lignin was also the dominant fibre class affecting diet energy digestibility, followed by pectin, cellulose and then starch (Table 5) accounting for 31.2% of the variation in digestibility.

4. Discussion

It has been demonstrated that different polysaccharide types cause varying effects on nutrient and energy digestibilities when included in fish diets (Glencross, 2009; Glencross et al., 2012b). It is now understood that the presence of these complex carbohydrates has a significant bearing on the variability in many of the nutritional responses by fish to diets (Aslaksen et al., 2007). While the discrete effects of different polysaccharides have been studied, little information is available on the potential interactive effects among the different polysaccharides classes. As raw materials contain different polysaccharide types and are typically added in a combination rather than added in isolation to aquaculture feeds, it is important to consider the implications of the addition of several different types of these polysaccharides to the nutritional value of feeds. It has been suggested that it is the interactive effect of these different polysaccharides that contributes to the variable effects seen in the digestibility values of many raw materials (Glencross et al., 2008; 2012b).

4.1 Polysaccharide class discrete effects

Each of the dietary non-starch-polysaccharide classes had a clear effect on the digestibility of dry matter content of diets fed to barramundi. This is a clear contrast to the effect seen with the pregelatinsed wheat starch, which had little effect on dry matter digestibilities. This difference clearly shows that although this species of fish can digest pregelatinised starch that it has almost no ability to digest any of the non-starch-polysaccharide (NSP) classes tested in this study.

With the majority NSP classes the decline in dry matter digestibility is directly related to the level of inclusion on of each NSP sample. This effect was seen directly with cellulose and to a lesser degree with lignin. These observations are similar to those observed by Glencross et al. (2012b), who also reported a significant decline in organic matter digestibility of diets with different levels of cellulose inclusion when fed to rainbow trout. Similar such effects were also reported by Hansen and Storebakken (2007), who also reported a significant decline in organic matter digestibility of diets with high cellulose inclusion when fed to rainbow trout. Glencross (2009) also noted the same effects with cellulose on dry matter and energy digestibilities and also the insoluble NSP content of lupin kernel meals. These observations combined with the present ones support that in most cases the NSP are simply acting as non-nutritive filler and have limited greater interactive effect on diet dry matter digestibility.

However, in the present study, the single or blended addition of pectin or lignin resulted in reductions in digestibility at levels which exceed their inclusion level and are indicative of an

interactive anti-nutritional factor. Pectin is a soluble fibre, commonly used as a gelling agent in products for human consumption. It has been suggested that NSP's in soluble form are most detrimental to fish (Reftsie, et al., 1999), due to their ability to disrupt digestive function by increasing the viscosity of intestinal contents (Francis et al., 2001). When pectin is blended with any of the fibre types it is possible this interaction is occurring and leading to reduced diet digestibility. A similar interaction was observed with the cellulose + lignin blend diet.

A multivariate approach by Glencross et al (2008) assessed over seventy lupin meals with rainbow trout. It was shown in that study that lignin content negatively affected nitrogen digestibility and that in combination lupin protein level and lignin content was the strongest predictor of protein digestibility. This result was supported by a subsequent study with rainbow trout in which lignin inclusion (as lignosulphate) in particular was observed to have a significant effect on diet protein digestibilities (Glencross et al., 2012b). That study assessed three inclusion levels 25, 50 and 100g/kg and interestingly, the effect on protein digestibility ceased to be linear after the 50 g/kg inclusion level. It was suggested that this could be due to a saturation effect of the lignosulphate on whatever loci it is disrupting in the digestion process. These findings are consistent with observations from other studies on the lignin fibre class, with lignosulphanate also found to have significant effects on diet digestibility even at very low inclusion levels (Glencross., 2009; Glencross et al., 2008; 2012b).

The different dietary NSP types had clear effects on the digestibility of the energy content of diets fed to barramundi. The response of diet energy digestibility to inclusion of the different NSP's, eithersingly or blended, is consistent with the fact that these fish are obtaining nominal energetic value from the presence of NSP's in the diet. This is also consistent with recent findings that show a limited energetic value from the inclusion of NSP's in diets for trout (Glencross et al., 2012b). Interestingly, when lignin (100g/kg) was pair combined with any of the fibre classes it resulted in an exacerbated reduction in diet energy digestibility indicative of a negatively synergistic interaction. However, this was not observed in the diet which contained a 50g/kg inclusion of each of the fibre classes, suggesting that providing the inclusion of lignin is $\leq$ 50g/kg it is unlikely to significantly disrupt energy digestibility in barramundi.

4.2 Conclusions

This study demonstrates that there are both discrete and interactive effects of the different polysaccharides on the nutrient and energy digestibilities of diets fed to barramundi. While the direct manipulative approach of the present study provides some clear indications on which NSP from different feed grains might affect their own digestibility, it would be useful to follow up this work with an assessment of a broad suite of feed grains and cross reference these

observations using a multivariate analysis approach, similar to that done by Glencross et al (2008) with lupin meals, but instead the proposed study should examine a cross section of the different feed grains available. Being able to corroborate the results from the present study with that from a multivariate analysis of actual feed grains will help consolidate the hypothesis that it is the NSP complexity in these raw materials that is a key cause of nutritional value variability. Secondly further research to identify why both pectin and lignin have the effects that the do would be useful to understand the mode of action of these NSP on the digestion of diets by fish.

Acknowledgements

The provision of fish by BettaBarra is gratefully acknowledged.. Special thanks to Stuart Arnold for constructive comment and editorial on an early draft.

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Tables and Figures

Table 1. Formulations, composition and digestibility coefficients of the experiment diets (all values except ADC's are g/kg).

Ingredient	Basal	A	B	C	D	E	F	G	H	I	J	K	Pooled SEM
Fishmeal	764	611.2	611.2	611.2	611.2	611.2	611.2	611.2	611.2	611.2	611.2	611.2	
Fish oil	100	80	80	80	80	80	80	80	80	80	80	80	
Wheat gluten	130	104	104	104	104	104	104	104	104	104	104	104	
Cellulose	-	200	-	-	-	100	100	100	-	-	-	50	
Pectin	-	-	200	-	-	100	-	-	100	100	-	50	
Pregelled starch	-	-	-	200	-	-	100	-	100	-	100	50	
Lignin	-	-	-	-	200	-	-	100	-	100	100	50	
Vitamin premix	5	4	4	4	4	4	4	4	4	4	4	4	
Yttrium oxide	1	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	
Dry matter	937	944	920	944	932	937	937	941	929	919	934	923	
Protein	637	499	488	513	518	501	515	519	501	515	474	519	
Lipid	197	189	157	177	174	171	150	171	167	151	163	161	
Ash	155	124	130	121	157	129	125	141	129	128	143	135	
Total Carbohydrates	11	188	226	190	151	200	209	170	204	206	220	185	
- Starch	49	10	15	268	0	28	138	67	224	87	300	156	
- ADF	45	181	34	51	79	106	135	125	58	74	77	99	
- NDF	33	177	26	39	63	96	128	115	45	63	68	92	
- Lignin	6	11	8	3	29	5	0	16	1	9	11	9	
- Cellulose	39	170	26	47	50	100	135	109	57	65	66	90	
- Hemicellulose	12	4	8	12	15	10	7	10	12	11	9	6	
Gross Energy	22.07	20.56	20.95	20.78	22.18	20.38	21.18	20.65	20.60	20.83	21.74	20.59	
Dry matter ADC	0.742	0.591	0.456	0.744	0.550	0.592	0.655	0.458	0.508	0.443	0.598	0.591	0.021
Protein ADC	0.908	0.941	0.810	0.916	0.692	0.875	0.932	0.790	0.848	0.650	0.710	0.767	0.015
Energy ADC	0.814	0.651	0.628	0.640	0.562	0.686	0.676	0.487	0.608	0.456	0.456	0.620	0.021

* Vitamin and mineral premix includes (IU/kg or g/kg of premix): Vitamin A, 2.5MIU; Vitamin D3, 0.25 MIU; Vitamin E, 16.7 g; Vitamin K,3, 1.7 g; Vitamin B1, 2.5 g; Vitamin B2, 4.2 g; Vitamin B3, 25 g; Vitamin B5, 8.3; Vitamin B6, 2.0 g; Vitamin B9, 0.8; Vitamin B12, 0.005 g; Biotin, 0.17 g; Vitamin C, 75 g; Choline, 166.7 g; Inositol, 58.3 g; Ethoxyquin, 20.8 g; Copper, 2.5 g; Ferrous iron, 10.0 g; Magnesium, 16.6 g; Manganese, 15.0 g; Zinc, 25.0 g. **estimated from the Carbohydrate content of each test ingredient and its inclusion level in each diet.

Table 2. Measured nutrient composition of the experimental ingredients (all values are g/kg DM unless otherwise indicated)

	Fishmeal	Gluten	Cellulose	Starch	Lignin	Pectin
Dry Matter	954	912	930	891	891	887
Protein	682	801	7	7	7	30
Lipid	101	80	9	16	6	6
Ash	183	9	1	3	669	53
Total Carbohydrates*	35	110	983	975	318	911
- Starch	28	302	0	997	31	23
- ADF	72	91	928	0	0	7
- NDF	44	91	925	0	0	5
- Lignin	6	187	290	0	613	0
- Cellulose	66	0	637	0	0	7
- Hemicellulose	28	0	2	0	0	3
Gross Energy	18.3	22.8	17.2	16.8	21.0	14.9

[a] Pregelatinised wheat starch: Manildra, Auburn, NSW, Australia. [b] Cellulose and Pectin: Sigma Chemical Company, St Louis, MO, USA. [b] Calcium ligninosuphate: Dustex, Canningvale, WA, Australia.*calculated based on dry matter – (protein+ash+fat)

Table 3. Multiple regression analyses of ingredient digestible values

	Parameter	Vector	Std.err.	Coefficients	Std.err	T(43)	p-value
ADC-DM	Intercept			0.753	0.061	12.364	0.000
	Cellulose	-0.347	0.166	-0.008	0.004	-2.097	0.042
	Pectin	-0.658	0.166	-0.015	0.004	-3.972	0.000
	Starch	-0.087	0.166	-0.002	0.004	-0.528	0.600
	Lignin	-0.555	0.166	-0.013	0.004	-3.351	0.002
ADC-PRO	Intercept			0.914	0.023	39.263	0.000
	Cellulose	0.094	0.092	0.001	0.001	1.019	0.314
	Pectin	-0.413	0.092	0.007	0.001	-4.480	0.000
	Starch	-0.070	0.092	0.001	0.001	-0.757	0.453
	Lignin	-0.894	0.092	0.014	0.001	-9.711	0.000
ADC-E	Intercept			0.838	0.062	13.433	0.000
	Cellulose	-0.417	0.173	-0.009	0.004	-2.405	0.021
	Pectin	-0.523	0.173	-0.012	0.004	-3.106	0.004
	Starch	-0.386	0.173	-0.009	0.004	-2.226	0.031
	Lignin	-0.750	0.173	-0.017	0.004	-4.327	0.000

Table 4. Step wise regression analyses of ingredient digestible values

Parameter	Variable	Step	Multiple-R	Multiple-R^2	R^2 Change	F – to enter	P-value
ADC-DM	Pectin	1	0.392	0.154	0.154	8.346	0.006
	Lignin	2	0.546	0.298	0.145	9.290	0.004
	Cellulose	3	0.607	0.369	0.071	4.921	0.032
ADC-PRO	Lignin	1	0.790	0.624	0.624	76.393	0.000
	Pectin	2	0.888	0.789	0.165	35.154	0.000
	Cellulose	3	0.896	0.803	0.014	3.245	0.078
ADC-E	Lignin	1	0.394	0.155			
	Pectin	2	0.451	0.203	0.048	2.722	0.106
	Cellulose	3	0.483	0.233	0.030	1.713	0.197
	Starch	4	0.559	0.312	0.079	4.957	0.031