Chickens on weed

The potential of seaweed for broiler nutrition

Lotte Stokvis

Propositions

1. Intact seaweed is currently a challenging ingredient for providing digestible macro-nutrients in broiler diets.
   (this thesis)

2. “Past performance is no guarantee for future results” also applies to the use of enzymes to improve nutrient digestibility of seaweed co-products.
   (this thesis)

3. Consumer demand for increased animal welfare impacts human wellbeing through an increased environmental and economic burden.

4. The interactions between researchers during a physical conference mitigate the environmental impact of traveling to the venue.

5. Studying towards a PhD degree confirms the ‘butterfly effect’ as it is extremely sensitive to apparently unimportant events.

6. During hard times it is important to keep working hard and to stop working.

Propositions belonging to the thesis, entitled

‘Chickens on weed – The potential of seaweed for broiler nutrition’

Lotte Stokvis

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Thesis

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Chapter 1

General introduction

The importance of this research

By 2050, the world population is expected to have grown to 9.7 billion people (United Nations, 2017). Furthermore, the improving living standards and urbanisation are driving factors in the global increase in meat, egg and milk consumption (Boland *et al.*, 2013). Poultry production is a key factor in providing the growing world population with sufficient animal protein. The production of poultry meat is very efficient in terms of unit of feed provided versus unit of edible animal protein produced, compared to the production of for example beef (Brameld and Parr, 2016). In order to achieve this efficient poultry production, highly digestible diets, often based on cereals (corn, wheat) and soybeans, need to be fed (Figure 1.1). This often results in a competition for resources also used for human consumption (the food-feed competition), and more recently for biofuel production. Hence, this intensifies the search for novel dietary ingredients that neither compete for resources with other sectors, nor for arable land- and freshwater use.

Seaweed is such a novel dietary ingredient of interest. Neither arable land, nor fresh water is needed for production, and additionally, there is currently no significant competition as a food source. Furthermore, high yields in terms of biomass per unit of surface area can be achieved (Buschmann *et al.*, 2017). Therefore, I present in this thesis a study of the nutritional value of seaweed for livestock, and explore the potential of seaweed as an ingredient in broiler diets.

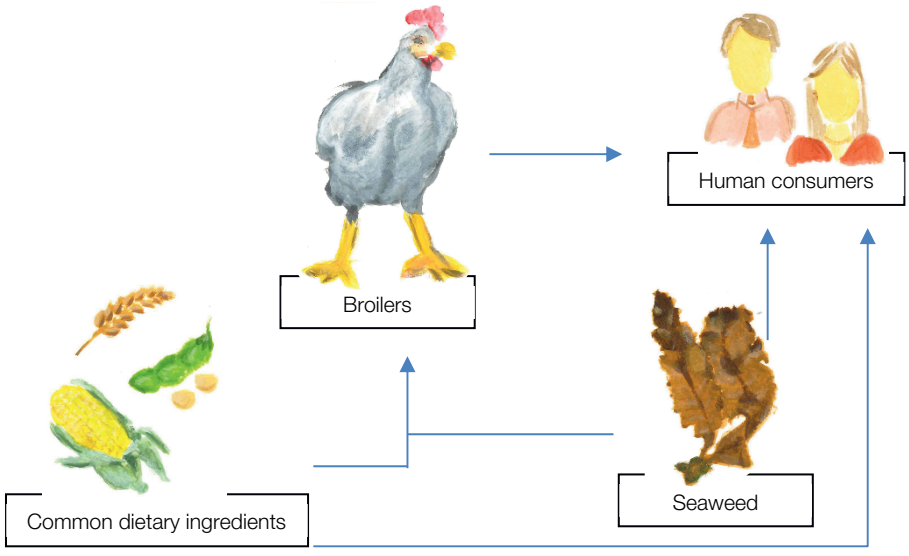

Figure 1.1. Schematic overview of the use of resources for broiler meat production.

Seaweed

A wide variety of seaweed species exists. Based on their pigmentation, the approximately 10,000 known seaweed, or macro-algae species are classified into brown (*Phaeophyceae*), red (*Rhodophyceae*) and green (*Chlorophyceae*) seaweed species (Figure 1.2). The chemical composition of the wide range of seaweed species shows a large variation within and between these seaweed classes, as well as within species (a.o. Angell *et al.*, 2016a; Biancarosa *et al.*, 2017). Such variation depends, amongst others, on time of harvest, geographical location, depth in the water column and environmental factors (Schiener *et al.*, 2015; Boderskov *et al.*, 2016; Sharma *et al.*, 2018). All seaweed species spoil relatively quickly after harvest, and hence need to be consumed or processed quickly (e.g. Paull and Chen, 2008).

Figure 1.2. Examples of brown (*Phaeophyceae*, 1-3), green (*Chlorophyceae*, 4-5) and red (*Rhodophyceae*, 6-7) seaweed species. 1: *Saccharina spp.,* 2: *Laminaria digitata*, farmed on a line system; 3: *Ascophyllum nodossum*; 4 & 5: *Ulva spp.*; 6: *Palmaria spp.*; 7: *Gracilaria spp.*. Pictures by Job Schipper.

Nutritional composition of seaweed

Protein ~ In brown seaweed, the protein content is usually below 150 g/kg dry matter (DM), and it is lower compared to red (below 322 g/kg DM) and green (below 287 g/kg DM) species (Angell *et al.*, 2016b; Øverland *et al.*, 2019). The latter two can have protein levels comparable to, for example, that of soybean meal. Protein content and amino acid (AA) composition are also subject to large variations between and within seaweed species and classes.

An estimation of the protein content in land-based plants is generally based on the assumption that 16% of the protein is N, using a nitrogen-to-protein conversion factor of 6.25 (Rutherfurd and Moughan, 2018). Despite the variation existing in the true conversion factor of different land-based plants, the factor is often implemented in calculations of protein in animal diets. In seaweed, however, a higher quantity of the nitrogen is present as non-protein nitrogen (NPN), like ammonia, nitrate and nitrite, compared to land-based plants (Makkar *et al.*, 2016; Øverland *et al.*, 2019). This NPN cannot be utilized by monogastrics and poultry to any significant extent. Due to the higher quantity of NPN, using a nitrogen-to-protein conversion factor of 6.25 would overestimate the protein content of seaweed. Hence, a lower generic seaweed nitrogen-to-protein conversion factor of 5.0 is proposed to more correctly estimate the protein content (Angell *et al.*, 2016b). A better and more direct, but more elaborate and expensive method for determining the protein content of seaweed is by adding up the analysed contents of individual amino acids, from which then a true nitrogen-to-protein conversion factor can be calculated (Mariotti *et al.*, 2008).

Carbohydrates ~ Seaweed carbohydrate composition differs largely from that of terrestrial (land-based) plants. The main structural and storage carbohydrates for brown, red and green seaweed are listed in Table 1.1. In most major land-based crops, starch is the main storage carbohydrate. Starch consists of glucose, and more specifically amylose and amylopectin. Laminarin is also a polysaccharide of glucose. The storage polysaccharides of seaweed and land-based plants differ in the way they are linked and do or do not form a branched structure, which has an effect on solubility and degradability (Hizukuri *et al.*, 1981; Bertoft *et al.*, 2008; Bertoft, 2017). The main structural carbohydrates of seaweed consist of other components than lignin, making seaweed more flexible compared to land-based plants. The latter use amongst others lignin, although lignin-like carbohydrates are observed in some seaweed species (Martone *et al.*, 2009). Generally, the dietary fibre level of seaweed is higher compared to that of land-based plants (Holdt and Kraan, 2011).

Table 1.1. Main pigments and carbohydrates of brown, red and green seaweed species compared to those in land-based plants.

Component	Brown seaweed *Phaeophyceae*	Red seaweed *Rhodophyceae*	Green seaweed *Chlorophyceae*	Land-based plants
Main pigments	Fucoxanthin, Chlorophyll a, β-carotene[3,4,5]	Phycobilliprotein, chlorophyll a[4,5]	Chlorophyll a and b, carotene, xanthophyll[4,5]	Chlorophyll a and b, carotene, xanthophyll, anthocyanin[1,2]
Structural carbohydrates	Alginate, fucoidan[3,4,5]	Carrageenan[5]	Xylan, ulvan[5]	Cellulose, hemicellulose, pectin[2]
Storage carbohydrates	Laminarin[3,4,5]	Floridean starch (amylopectin)[3,5]	Floridean starch (amylopectin)[5]	Starch (amylose + amylopectin)[2]

[1]Alkema and Seager, 1982.
[2]Hartmann and Trumbore, 2016.
[3]Makkar *et al.* 2016.
[4]Verma *et al.*, 2017.
[5]Øverland *et al.*, 2019.

Lipids ~ The lipid content is usually below 40 g/kg DM in seaweed (Mišurcová, 2012), and consequently the fat content of seaweed does not contribute much to the energy value of a diet (Biancarosa *et al.*, 2017). However, the proportion of polyunsaturated fatty acids is relatively high (Ginneken *et al.*, 2011), and the content of eicosapentaenoic acid (EPA) can be far above 50% of the total fatty acid content (Santos *et al.*, 2017). This is contrary to most land-based crops. These crops only produce alpha-linolenic acid (ALA), although dietary ALA can be converted to EPA, be it at an inefficient rate of less than 15%. This increases the nutritional value of seaweed fat, thereby significantly contributing to the polyunsaturated fatty acid content of a poultry diet.

Minerals and heavy metals ~ The ash content of seaweed is high, ranging from 200-350 g/kg DM (Evans and Critchley, 2014). It can provide many nutritionally important minerals and trace elements like iodine, calcium, potassium, iron, magnesium, manganese and cobalt (Torres *et al.*, 2019). Next to potentially favourable minerals and trace elements, seaweed can also accumulate heavy metals. Particularly arsenic, lead, cadmium and mercury can be present in high levels (Holdt and Kraan, 2011). Despite the high levels of for example arsenic, these are mostly present in organic form, being of lower toxicity compared to inorganic arsenic (Holdt and Kraan, 2011; Biancarosa *et al.*, 2017).

Seaweed in livestock diets

The use of seaweed in livestock diets has been of increasing interest in the last decade (Buschmann *et al.*, 2017; Øverland *et al.*, 2019). At present, seaweed is not used in livestock diets at a large scale. Only a limited number of studies have been published that included seaweed for its nutritional value. Mostly, the inclusion levels of seaweed in animal diets in the literature are below 5% on an as is basis (Gahan *et al.*, 2009; McDonnell *et al.*, 2010; Abudabos *et al.*, 2013). Other studies focussed on effects other than those resulting purely from the nutritional value (bioactive effects) of seaweed.

Furthermore, knowledge about the nutritional value for livestock by assessment of the digestibility of macronutrients is scarce.

In general, a large drawback of seaweed as dietary ingredient is the high mineral content (e.g. salts; Rubio *et al.*, 2017). It may lead to overconsumption of water and consequently to a high water content of excreta (diarrhoea; Guiry and Blunden, 1991; Koreleski *et al.*, 2010) and thus wet litter conditions and potentially resulting in footpad lesions in poultry (Shepherd and Fairchild, 2010). Furthermore, the high ash content decreases the nutrient and energy density of the diet.

Based on its chemical composition, seaweed might be a better dietary ingredient for ruminants than for monogastrics and poultry (Makkar *et al.*, 2016). The microbiota in the rumen of ruminants can also utilize the NPN and high levels of structural carbohydrates present in seaweed, contrary to monogastrics and poultry, although the digestibility is still low (Cabrita *et al.*, 2017).

The diet of Orkney sheep in the North Ronaldsay Islands mainly consists of seaweed: mostly brown species (*Laminaria digitata* and *Laminaria hyperborea*) and some red and green species (Hansen *et al.*, 2003). Their microbiota have adjusted towards a composition that aids the digestion of the seaweed (Greenwood *et al.*, 1983; Orpin *et al.*, 1985), and these sheep were able to fulfil their nutritional needs throughout the year with eating almost exclusively seaweed (Hansen *et al.*, 2003). When the green seaweed *Ulva lactuca* was fed to male lambs at a 20% inclusion level (Arieli *et al.*, 1993), the digestibility of protein was observed to be low (40%) and that of energy moderate (60%). When 20% *Chaetomorpha linum* and *Ruppia maritima* were fed to growing lambs, performance was not negatively affected, except for a slight increase in feed conversion ratio of the lambs fed the *C. linum* diet compared to lambs fed a control diet without seaweed (Ktita *et al.*, 2010). A more recent study in sheep included 25% *Ulva laetevirens* or *Gracilaria vermiculophylla*, finding low fibre digestibilities (Cabrita *et al.*, 2017). Complementary information on the digestibility of other seaweed species to accurately evaluate the nutritional value of seaweed, and in most other ruminant species, is hitherto lacking.

For monogastrics and poultry, the high protein levels in some red and green seaweed species may provide a good protein source (Angell *et al.*, 2016a). The generally high structural carbohydrate levels are not well digestible in these species (Makkar *et al.*, 2016). This potentially reduces the availability of the cell contents, reducing the nutritional value of such seaweeds for these species (a.o. Ventura *et al.*, 1994).

When 10% *Ascophyllum nodossum* (brown seaweed) meal was added to a pig diet, weight loss was observed compared to a control diet without seaweed (Jones *et al.*, 1979). This was likely due to the low protein content of the seaweed meal. A residue (dried residue remaining after alginate extraction) from this seaweed was also deemed unsuitable as a protein and energy source for pigs (Whittemore and Percival, 1975). The few studies reported in literature studying seaweed for its nutritional value thus conclude that the included seaweeds are less suitable for pig nutrition at these levels. More recently seaweed inclusion in pig diets has been studied, albeit with low inclusion

levels of 0.05 to 1% and predominantly studying effects on health and meat quality. For example, the 1% inclusion of brown seaweed *Ascophyllum nodossum* in the diet of newly weaned piglets only caused a numerical increase in body weight at the end of the experimental period (11 days post weaning; Dierick *et al.*, 2009).

When broilers were fed 2-6% of the brown seaweed *Sargassum dentifebium* in their finisher diet (El-deek *et al.*, 2011), birds showed a decreased performance compared to broilers fed a control diet without seaweed, based on final body weight (-3.3%) and feed intake (+5.0%), as well as feed conversion ratio (+11%). After inclusion of 0, 10, 20 and 30% *Ulva laetevirens* (green seaweed), a decreased feed intake (-1.5% to -5.1%), body weight gain (-11.8% to -24.5%) were observed in chicks and cockerels fed diets with increasing seaweed inclusion levels (Ventura *et al.*, 1994). These authors concluded that this seaweed was not to be advised for inclusion in poultry diets in concentrations of 10% or higher.

Generally, the sporadic studies reporting the effects of the inclusion of seaweed in livestock diets for its nutritional value hinder a clear conclusion. To be able to include seaweed in livestock diets for its nutritional value, more in-depth knowledge is needed on the nutritional composition and digestibility of multiple seaweed species.

Methods to evaluate the nutritional value of seaweed for livestock

For a proper and detailed evaluation of the nutritional value of novel dietary ingredients like seaweed, different techniques are available. The *in vitro* and *in vivo* techniques commonly used to estimate nutritional values of dietary ingredients will be discussed in the following sections. The definition of nutritional value used throughout this thesis is the value that is present based on the macronutrient composition.

In vitro *methods*

First, *in vitro* digestibility models can be used, to estimate the nutritional value of novel dietary ingredients for livestock. Multiple *in vitro* digestibility models can be applied, depending on the available seaweed material and the animal species and research question of interest.

First of all, the chemical composition of the ingredient or diet of interest can be analysed, to report the nutritional composition. This reflects the potential that the ingredient has as dietary ingredient, and can explain whether this ingredient might be more suited for specific animal species, related to for example different fermentation capacities of animal species.

In further *in vitro* analyses, the digestibility and fermentation of ingredients can be simulated. For ruminants, a gas production test can be performed (e.g. Cone *et al.*, 1997). This mimics the digestion and fermentation in the gastro-intestinal tract of ruminants, and is performed with gastro-intestinal juices of the animal species of focus.

For instance, the *in vitro* simulated digestibility of multiple seaweed species was compared between seaweed fed and non-seaweed fed Orkney sheep, using rumen fluid of those animals in an *in vitro* simulated model (Greenwood *et al.*, 1983). For monogastrics, other models can be applied, like the one developed by Boisen and Fernandez (1997). Depending on the question at hand, this model takes into account separate steps for mimicking breakdown of nutrients in the stomach (gastric step), digestion and potential absorption in the small intestine and fermentation capacity in the large intestine.

Multiple factors can be adjusted to most closely reflect the processes occurring in the animal of interest. For example, dietary ingredients can be ground to reflect feed particle size after chewing and the temperature can be adjusted to reflect the body temperature of the animal species of focus. Additionally, shaking or stirring can be applied during the analyses to reflect peristalsis and the enzyme cocktails used during the process of breaking down the nutrients can be adjusted to reflect more closely the enzyme mixtures and buffering capacity as observed in the animal species of focus. These *in vitro* analyses and models estimate the nutritional value of ingredients or diets for livestock, and are an important step in the process of evaluation of novel dietary ingredients.

The combined results of the nutritional composition and the *in vitro* digestibility model can highlight differences in the nutritional value between dietary ingredients or diets. One should keep in mind though, that these *in vitro* simulated models always reflect only part of the complex digestive processes that occur in animals. Important interactions between for example nutrients and gastro-intestinal juices, or interactions of ingredients with the intestinal lining and microbiome throughout the gastro-intestinal tract are not simulated in the *in vitro* digestibility models. This is the reason that *in vitro* simulated digestibility models do not always closely reflect the results obtained if exactly the same diet or dietary ingredients are fed to animals, even when conditions are very well controlled. Because of this, it is to date still important to also study the effects of novel dietary ingredients within the animal of focus.

In vivo *methods*

The *in vivo* evaluation of the nutritional value of dietary ingredients can be performed in ruminants, monogastrics and poultry. In this thesis the focus will be directed towards the *in vivo* evaluation of seaweed and seaweed products for broiler chickens. Towards this aim, different experimental setups can be implemented. In all cases, animals will be fed the ingredient of interest. This can either be done by 1) formulating a diet that fulfils the needs of the animal (basal diet), and dilute this basal diet with an amount of the dietary ingredient of interest. Or 2) formulating a control diet and diet including the ingredient of interest, while correcting for differences in protein, amino acids, or metabolizable energy. In both cases, the digestibility is calculated based on the difference in the amount of specific nutrients in the diet and excreta. This can be done by the collection of all excreta of the animal over a known period of time. Another

method is the marker method, including indigestible components that follow the chyme while not interacting with nutrients or the digestive tract of the animal (De Vries and Gerrits, 2018). They are added to the diet and by using the concentration of the nutrients in the diets and intestinal content or faeces, in relation to the concentration of the marker, the apparent nutrient digestibility can be calculated. In this case, all differences in nutrient digestibility between birds fed the basal or control diet and the diet containing the ingredient of interest are ascribed to this nutrient of interest. This method of calculation is known as the difference method (Kong and Adeola, 2014).

Knowledge gaps

In recent decades, the focus for animal diets was mostly directed towards increasing efficiency, whereas currently the focus also entails more sustainable and future proof human and animal diets and dietary ingredients. This includes using resources for animal feed that are not human-edible, are more locally sourced, and do not compete for arable land or fresh water (e.g. Zanten *et al.*, 2018). Although seaweed might play a valuable part in this shift towards more sustainable and future proof concepts, however, the gaps in knowledge about intake and digestibility of seaweed, and the animals' response hereto, is so far hindering the inclusion of seaweed in animal diets. If seaweed is to be included in animal diets at inclusion levels that contribute to the nutritional value of the diet, the digestibility of intact seaweed, or products thereof, needs to be studied in more detail. Furthermore, the effects of these seaweeds other than those attributed to its nutritional value need to be investigated, such as health-related effects, to be able to conclude whether seaweed is suitable as dietary ingredient in livestock diets.

Research objectives and outline

Based on the gaps in knowledge, the objective of this thesis was to investigate the potential value of seaweed for animal nutrition, with particular reference to broilers. To this end, the following chapters sequentially investigate the chemical composition, *in vitro* and *in vivo* digestibility and health-related effects of selected seaweed products.

In Chapter 2, the nutritional value and the variation therein of intact seaweeds obtained from coastal waters in Northwestern Europe as a source of macronutrients are presented for the application in animal feed. Therefore, the chemical composition of included seaweeds was analysed, and the *in vitro* digestibility was evaluated by use of multiple *in vitro* digestibility models. The seaweed species included in this chapter were brown: *Saccharina latissima*, *Laminaria digitata* and *Ascophyllum nodossum*, red: *Palmaria palmata* and *Chondrus crispus*, and green species: *Ulva lactuca*. Chapter 3 describes the effect of washing, ensiling and extraction processes on the nutritional value and digestibility of seaweed products for broilers. The nutritional value of included seaweed products was evaluated based on nutrient content, an *in vitro* digestibility

model and an *in vivo* experiment in broilers. The seaweed species included in this chapter were brown: *Saccharina latissima* and *Laminaria digitata*, and green species: *Ulva lactuca*. Chapter 4 discusses the effects of a proteolytic enzyme pre-treatment of green seaweed species *Ulva laetevirens* and red seaweed species *Solieria chordalis* co-products, resulting from a biorefinery process, before inclusion in a broiler diet. The effects were studied based on performance, *in vivo* digestibility of diets and seaweed co-products, and health-related parameters. The latter included analyses of intestinal content pH, histo-morphological parameters of the small intestine and plasma cytokine levels. The experiment described in Chapter 5 investigated a proteolytic enzyme pre-treatment to improve digestibility of *Ulva laetevirens* seaweed co-products when included in standard corn-soybean-based and European protein-based diets. Additionally, the effects on health and performance were further investigated. Finally, Chapter 6 presents a synthesis, an integrative discussion of the research and results from this thesis.

Chapter 2

Evaluation of seaweeds from marine waters in Northwestern Europe for application in animal nutrition

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Abstract

The increasing demand for animal protein by the growing world population intensifies the exploration of novel feed sources. This study evaluated the nutritional value of six intact seaweed species (*i.e.* the brown species *Saccharina latissima, Laminaria digitata,* and *Ascophyllum nodossum,* the red species *Palmaria palmata,* and *Chondrus crispus* and green species *Ulva lactuca*), collected from the coast of Ireland, Scotland and France as an ingredient for animal feed. The nutrient composition, *in vitro* digestibility, and *in vitro* gas production simulating rumen fermentation, were determined. The nutrient contents (g/kg dry matter), both between and within species, were highly variable, ranging from 45–248 for crude protein, 351–691 for non-starch polysaccharides, and 173–445 for ash. Overall, the brown seaweeds had the highest non-starch polysaccharides content, whereas samples of the red and green seaweeds had an amino acid content up to 265 g/kg dry matter. All samples had a substantial non-protein nitrogen fraction, varying from 0.12–0.29 of nitrogen. The fibre fractions of brown seaweeds showed different properties than land-based plants, as illustrated by a lower analysed neutral detergent fibre than acid detergent fibre content. The ileal organic matter and nitrogen digestibility, as well as the total tract organic matter digestibility (mean digestibility coefficients: 0.81, 0.89 and 0.88, respectively) were lower for all seaweeds compared to soybean meal (digestibility coefficients: 0.84, 0.98 and 0.97, respectively). *S. latissima, L digitata, P. palmata* and *U. lactuca* had a higher maximum gas production than alfalfa, but lower than sugar beet pulp. Based on the protein content and amino acid-pattern, intact *P. palmata* and *U. lactuca* would be a valuable protein source for farm animals, with the high non-starch polysaccharides and non-protein nitrogen contents and a low *in vitro* digestibility potentially limiting their use as a feed ingredient for monogastric species. The fermentability of *L. digitata, S. latissima* and *P. palmata* indicate that these intact seaweeds may have a higher nutritional value in ruminants. The high ash content in all seaweed species hampers the use of intact seaweed for both ruminants and monogastrics. Extraction of protein and other favourable components, while reducing the ash content, seems an important step towards seaweed inclusion in animal diets. Further identification and characterisation of seaweed polysaccharides is required to understand and improve the digestibility of seaweed fractions.

Keywords: seaweed, animal feed, chemical composition, *in vitro* digestibility, gas production

Introduction

The expected population growth to 9.7 billion in 2050 (United Nations, 2017) coupled with increased living standards and urbanisation are important drivers for the expected global increase in meat, egg and milk consumption in the next decades (Boland *et al.*, 2013). Concomitant with this increase is the increase in demand for feed ingredients and as such the development of novel sources of biomass for feed ingredient production. The extension of marine biomass production by wild harvest and large scale cultivation of seaweed could significantly contribute to biomass production for feed as well as food. Advantages of seaweed production are the use of salt instead of fresh water, sea instead of arable land-based production, and the high productivity in terms of biomass produced per unit of surface area (Buschmann *et al.*, 2014; 2017).

The chemical composition of multiple species of the three classes (brown, red and green seaweeds, based on their pigmentation) of seaweed shows large variation between, and also within species, depending on e.g. season of harvest, geographical characteristics and environmental factors (Holdt and Kraan, 2011; Schiener *et al.*, 2015; Boderskov *et al.*, 2016; Sharma *et al.*, 2018). At present, seaweed is not used in large scale commercial animal feed production to any significant extent. Detailed knowledge of the nutritive value of seaweed for feed is, hitherto lacking with only a few studies reporting *in vitro*, *in situ* or *in vivo* digestibility of seaweed (e.g. Greenwood *et al.*, 1983; Molina-Alcaide *et al.*, 2017). This knowledge is essential for the successful application of seaweeds as an ingredient in the diet of various production as well as companion animals. In addition, the number of published performance studies using intact seaweed as a source of macronutrients in production animals is limited. Most of the studies used low inclusion levels of 0.3 up to 4 g of seaweed per kg of diet on an as-is weight basis (Gahan *et al.*, 2009; McDonnell *et al.*, 2010; Abudabos *et al.*, 2013) or focussing on the effects of bioactive compounds of seaweed rather than its nutritional value (e.g. Novoa-Garrido *et al.*, 2014).

The aim of this study was to evaluate the nutritive value and the variation in nutritive value of intact seaweeds obtained from coastal waters in Northwestern Europe as a source of macro nutrients, for application in animal feeds, by analyses of the chemical composition and use of *in vitro* digestibility models.

Material and methods

Seaweeds

In total 13 different samples of six seaweed species classified as either *Phaeophyta* (brown), *Rhodophyta* (red) or *Chlorophyta* (green) were evaluated. The study includes the brown species *Laminaria digitata*, *Saccharina latissima*, and *Ascophyllum nodosum*, red species *Palmaria palmata* and *Chondrus crispus* and green species *Ulva*

lactuca. The seaweed species were collected, mainly from wild populations, along the coast of Ireland, Scotland and France in 2013 with Table 2.1 providing an overview of the seaweed species and their origin. All six Scottish samples were harvested in August at the coast of the island Bute (West coast of Scotland). After drying on the coast by wind, air and sun, they were milled in a blender into course particles (± 10 mm^2). These seaweeds were harvested and processed by Justseaweed (Rothesay, United Kingdom). The Irish samples of *A. nodossum*, harvested in summer, and *C. crispus*, harvested in spring, and *U. lactuca*, harvested in May, were processed by Ocean Harvest Technology (Ocean Harvest Technology Ltd, Milltown, Ireland). After harvesting near the coast of Galway, Ireland, the seaweeds were dried by wind, air and sun, and milled with a hammer mill over a 300 and 100 µm screen, respectively. The French samples of *S. latissima*, *P. palmata* and *U. lactuca* were harvested in September at the coast of Plouarzel (nearby Brest, France). After drying on the coast by wind, air and sun, they were milled with a Subaru mill in rough parts (± 2 cm^2). These seaweeds were harvested and processed by Teranga Sea Weeds (Brest, France). The Irish *L. digitata* was provided by North Seaweed (North Seaweed BV, Kapelle, the Netherlands) and originated from the coast of Ireland. All samples were stored at room temperature until the start of the experiment.

Table 2.1. Overview of the brown, red and green seaweed species harvested along the coastal regions of Northwestern Europe used in the experiment.

Seaweed classification	Species	Harvesting location	Harvesting date
Brown Algae	*Laminaria digitata*	Bute (S)	08-2013
	Laminaria digitata	Ireland (I)	2013
	Saccharina latissima	Bute (S)	08-2013
	Saccharina latissima	Plouarzel, Bretagne (F)	09-2013
	Ascophyllum nodosum	Bute (S)	08-2013
	Ascophyllum nodosum	Galway (I)	Summer 2013
Red Algae	*Palmaria palmata*	Bute (S)	08-2013
	Palmaria palmata	Plouarzel, Bretagne (F)	09-2013
	Chondrus crispus	Bute (S)	08-2013
	Chondrus crispus	Galway (I)	Spring 2013
Green Algae	*Ulva lactuca*	Bute (S)	08-2013
	Ulva lactuca	Plouarzel, Bretagne (F)	09-2013
	Ulva lactuca	Galway (I)	05-2013

S=Scotland, F=France, I=Ireland.

Chemical composition

Prior to analyses, all seaweeds were ground using a laboratory mill (Peppink 200 AN, Olst, the Netherlands) equipped with a 1 mm sieve. The samples were analysed using official methods to determine moisture (dry matter: DM, ISO 6496, 1999), nitrogen (N, ISO 5983, 2005), ether extract (crude fat; ISO 6492, 1999), ash (ISO 5984, 2002), the HCl-insoluble ash was determined as ash-HCl, crude fibre (ISO 6865, 2000), starch (ISO 15914, 2004), total sugar (European Commission, 2009), tryptophan (ISO 13904,

2005) and other amino acids (AAs, ISO 13903, 2005). Acid detergent fibre (ADF) and acid detergent lignin (ADL) were analysed according to ISO 13906, 2008. Neutral detergent fibre (NDF) was analysed according to ISO 16472, 2006. Organic matter (OM) was calculated as 1000–ash. Crude protein (CP) was calculated as N×5.00 (Angell *et al.*, 2016b). For this purpose, the tyrosine and proline content were calculated based on their ratio to phenylalanine for each seaweed species (ratio calculated from data by Biancarosa *et al.* (2017)). Non-protein N (NPN) was calculated, as well as the N to protein (N:P) conversion factors K_P and K_A. K_P is the ratio between the sum of the anhydrous AAs (g/kg DM) and N (g/kg DM), and K_A is the ratio between the sum of anhydrous AAs (g/kg DM) and amino acid N (AA-N; g/kg DM). True protein was calculated as the sum of anhydrous AAs (g/kg DM). The fraction of non-starch polysaccharides (NSP) was calculated as OM minus CP, crude fat, starch and total sugar. The commonly used feed ingredients soybean meal (SBM), alfalfa and sugar beet pulp were used as reference ingredients.

In vitro *digestibility of DM, OM and N*

In vitro incubations were performed according to a modified two- and three-step method for pigs according to the procedures as described by Boisen and Fernandez (1997) to analyse the *in vitro* ileal and total tract digestibility. Reduced substrate concentrations were used, as described by Bikker *et al.* (2016), because of the high viscosity of seaweed samples during incubation. The reference ingredient soybean meal (SBM) was included in the *in vitro* digestibility studies. A two-step *in vitro* incubation was performed to simulate the digestive process in the stomach and small intestine to estimate the ileal digestibility of the substrates. The two-step *in vitro* incubations were conducted in fourfold, of which 2 replicates were used to determine N digestibility and 2 replicates to determine OM digestibility. The three-step *in vitro* incubation was performed including an 18h incubation with Viscozyme (Viscozyme® L, Sigma Aldrich, Germany) to simulate the digestive process in the stomach, small intestine and large intestine of a pig to estimate total tract digestibility. The three-step *in vitro* incubations were conducted in duplicate to determine OM digestibility.

Rumen fermentation

Potential fermentation in the rumen of the seaweed samples was measured by the gas production technique during incubation in rumen fluid as described by Cone *et al.* (1996). As insufficient seaweed was available to acclimatise the cows to the substrate, rumen fluid was obtained from dairy cows kept on a ration of maize and grass silage. Each sample was incubated in triplicate and a blank run (rumen fluid without sample) in duplicate. The cumulative gas production over time was described using a two-phase model based on asymptotic maximum gas production (ml/g OM), time at which half of this gas production was reached and a parameter determining the shape of the curve for different substrates (Groot *et al.*, 1996). The first phase describes the fermentation of the soluble fraction, whereas the insoluble fraction is fermented in the

second phase. The duration of each of the two phases was not set at the length generally used for land-based plants, but was determined by the model to find the best possible fit. Maximum gas production was corrected for protein content with an increase of 2.5 ml per percent true protein in the OM of the analysed products as calculated based on total AA content, according to Cone and Van Gelder (1999). Data from Hulshof *et al.* (2016) and Cone *et al.* (2008) were used for comparison to SBM, sugar beet pulp and alfalfa meal as reference ingredients. These products were analysed in the same lab, using the same techniques as used for the seaweed samples in the current experiment.

Statistical analyses

The *in vitro* digestibility data were analysed as an unbalanced 3×6×3 factorial design, with the factors seaweed classification (brown, red and green), species within classification (brown species: *L. digitata*, *S. latissima* and *A. nodossum*, red species: *P. palmata* and *C. crispus* and green species: *U. lactuca*), and origin (Scotland, Ireland and France). Because of some variation in season of harvest among different locations (see Table 1), the effect of origin may include an influence of season of harvest. These effects cannot be separated. The residual maximum likelihood procedure (Genstat, 19th edition) was used to test the effects of classification, species, and origin on *in vitro* digestibility parameters and curve fitting parameters of the gas production test. Data are presented as means with standard error of difference per parameter. All statements of significance are based on testing at $P<0.05$. A regression analysis was performed with a generalized linear model. First, an all-subset regression was conducted for *in vitro* ileal OM digestibility, ileal N digestibility, total tract OM digestibility and corrected maximum gas production as dependent variables. Possible explanatory variables included in the model were ash, CP, crude fat, sugar, starch, NSP, NDF, ADF and ADL (all expressed in g/kg DM). All possible combinations of a model with a maximum of three explanatory variables were compared to avoid over-parametrisation in the dataset with 13 seaweed samples. Non-significant variables were excluded and the significant model with the highest explained variation was selected. In addition, regression analysis was conducted with corrected maximum gas production as dependent variable and *in vitro* OM and N digestibility as independent variable to determine whether the results from the *in vitro* digestibility test had a predictive value for the gas production.

Results

Chemical composition

Ash content of the seaweeds ranged from 173 in Irish *U. lactuca* to 445 g/kg DM in Irish *C. crispus* (Table 2.2). Variation in ash content occurred between and within species from different locations. Likewise, CP content varied substantially, with the highest content in *U. lactuca* from Ireland (248 g/kg DM) and France (168 g/kg DM),

and the lowest content in Scottish *A. nodosum* (45 g/kg DM). Crude fat content among seaweeds varied between 7 g/kg DM (Irish *C. crispus*) and 38 g/kg DM (Scottish *A. nodossum*). In most brown seaweed species, sugar and starch content was very low, whereas they were higher in most of the red and green species. Most species contained high NSP levels (>500 g/kg DM), with the highest value in Scottish *C. crispus* (691 g/kg DM), and the lowest NSP level in *C. crispus* of Irish origin (351 g/kg DM). Within the NSP fraction, substantial variation in the different types of fibre (NDF, ADF and ADL) was observed, both within and between species, depending on origin. In most species, the HCL-ash content ranged between 2 and 20 g/kg DM, but in *C. crispus* 152 g/kg DM HCL-ash was determined.

The AA profiles of the seaweed samples, together with the N content and the N:P conversion factors are presented in Table 2.3. The AA-N content was lower in seaweeds than in SBM (70.7–87.9 vs. 94.0 g/100 g N). Consequently, the AA content (in g AA-N/100 g N) of most essential AA, apart from methionine and threonine, was lower than in SBM. The NPN ranged from 12.1–29.4% of N compared to 6.0% of N for SBM. The conversion factor K_P ranged between 4.16 and 5.01 for the seaweed samples, and K_A ranged from 5.55–5.89.

In vitro *simulated ileal digestibility*

Organic matter digestibility ~ The simulated ileal OM digestibility coefficient of the seaweeds ranged from 0.44 (Irish *A. nodossum*) to 0.81 (French *S. latissima*; Table 2.4). The OM digestibility coefficient of SBM was 0.84. Both classification and species within classification affected the OM digestibility, but their effects depended on the origin as indicated by the interactions, with P=0.008 and 0.005, respectively. The OM digestibility coefficient of Irish *A. nodossum* (0.44) was lower compared to the Scottish variety (0.62). In contrast, the OM digestibility coefficient of Irish *U. lactuca* was higher (0.67) than Scottish *U. lactuca* (0.54) and French *U. lactuca* (0.51). For the other species, origin did not have a significant effect on OM digestibility coefficients. The multiple regression analyses indicated that 79.5% of the observed variation was explained by the selected significant model (P<0.001) including the variables (in g/kg DM) ash (P=0.012), NDF (P<0.001) and ADF (P<0.001; Table 2.5). All three terms included in the model were negatively correlated with the ileal OM digestibility, with coefficients -0.0006, -0.0012 and -0.0011 for ash, NDF and ADF, respectively.

Table 2.2. Analysed nutrient content of selected seaweed species, harvested along the coastal regions of Northwestern Europe.

		g/kg	g/kg DM										
Seaweed classification	Species (origin)	DM	Ash	OM [a]	CP [b]	Cfat	Cfibre	Sugar	Starch	NSP [c]	NDF	ADF	ADL	HCl-ash
Brown	*L. digitata* (S)	891	275	725	92	16	73	2	2	618	120	200	36	7
	L. digitata (I)	923	367	633	82	11	69	1	1	545	91	164	28	7
	S. latissima (S)	874	243	757	74	10	71	6	1	668	122	185	23	11
	S. latissima (F)	902	273	727	117	12	62	1	1	603	96	171	7	6
	A. nodossum (S)	883	214	786	45	38	54	30	1	679	162	331	180	3
	A. nodossum (I)	910	411	589	57	10	88	1	1	530	152	298	48	11
Red	*P. palmata* (S)	939	209	791	141	12	35	35	17	594	312	50	6	7
	P. palmata (F)	949	228	772	134	13	28	48	22	555	347	42	5	4
	C. crispus (S)	883	176	824	98	17	31	6	26	691	392	40	9	2
	C. crispus (I)	899	445	555	125	7	45	3	90	351	190	53	14	152
Green	*U. lactuca* (S)	842	243	757	70	23	76	24	75	567	385	141	70	20
	U. lactuca (F)	880	260	740	168	35	76	12	73	457	329	143	69	8
	U. lactuca (I)	883	173	827	248	21	57	7	42	530	259	135	69	11
	Soybean meal [d]	900	73	927	531	28	ND	134	10	224	90.6	51.4	UD [f]	ND
	Sugar beet pulp [e]	917	79	921	88	14	ND	234	4	581	356	182	8	ND
	Alfalfa [e]	927	125	875	174	27	ND	54	19	601	419	318	77	ND

DM=dry matter, OM=organic matter, CP=crude protein, Cfat=crude fat, Cfibre=crude fibre, NSP=non-starch polysaccharides, NDF=neutral detergent fibre, ADF=acid detergent fibre, ADL=acid detergent lignin, S=harvested in Scotland, F=harvested in France, I=harvested in Ireland, UD=under detection limit, ND=not determined. Each value in the table is based on one analysis in duplicate.

[a]Calculated as 1000–ash.

[b]Calculated as nitrogen×5.00.

[c]Calculated as 1000–ash–crude protein–crude fat–starch–sugars.

[d]Values based on analysis conducted by Hulshof *et al.* (2016).

[e]Values based on analysis conducted by Cone *et al.* (2008).

[f]Detected ADL content was 0, which is under the limit for accurate detection of ADL.

Table 2.3. Individual AA content (g/100 g N), true protein expressed as anhydrous AAs (g/kg DM) and N content (g/kg DM), non-protein nitrogen (% of N) and N to protein conversion factors of selected seaweed species compared to soybean meal (reference ingredient).

	Seaweed classification													
	Brown						Red				Green			
Component	*L. digitata*		*S. latissima*		*A. nodosum*		*P. palmata*		*C. crispus*		*U. lactuca*			SBM [a]
g AA-N/100 g N	S	I	S	F	S	I	S	F	S	I	S	F	I	
Lysine	4.9	5.0	5.6	4.2	4.0	4.2	6.3	6.7	7.3	4.7	4.4	5.7	4.7	7.5
Methionine	0.9	0.8	1.2	0.8	1.2	1.2	1.1	1.1	0.9	0.8	1.0	1.1	1.1	0.8
Cysteine	1.5	1.9	1.6	1.1	1.0	1.0	1.7	2.2	1.8	1.0	1.4	0.9	0.7	1.0
Threonine	3.2	3.1	3.4	2.7	3.1	3.1	3.3	3.4	3.2	3.2	4.1	3.4	3.1	2.9
Tryptophan	0.8	0.8	1.0	0.7	1.1	1.0	0.9	0.9	0.9	0.7	0.9	1.4	1.0	1.1
Leucine	3.7	3.5	4.6	3.2	3.9	4.4	4.2	4.4	3.5	4.0	4.3	4.7	4.3	5.2
Isoleucine	2.0	2.0	2.4	1.8	2.1	2.5	2.4	2.5	2.0	2.8	2.5	2.4	2.2	3.1
Histidine	3.0	3.5	2.9	2.3	2.7	3.1	3.1	3.3	3.0	2.5	2.5	3.4	1.6	4.7
Phenylalanine	2.0	1.9	2.4	1.7	2.2	2.4	2.1	2.2	2.1	2.3	2.6	2.9	2.6	2.8
Arginine	7.4	6.9	8.5	5.9	6.8	8.2	10.0	11.1	11.5	11.1	8.8	9.9	12.9	14.7
Asparagine + Aspartic acid	9.0	9.7	10.5	7.8	8.9	8.9	9.8	10.8	8.9	8.9	11.6	12.8	11.8	11.3
Serine	3.3	3.3	3.7	2.8	3.0	3.2	4.0	4.2	4.1	3.6	4.6	4.1	3.7	4.1
Glutamine + Glutamic acid	10.9	11.5	12.9	22.4	13.0	9.5	10.5	11.3	7.6	7.7	9.7	8.5	9.6	16.2
Glycine	5.4	5.4	6.1	4.6	5.0	5.7	6.9	6.9	7.8	6.2	7.1	6.8	6.0	5.0
Alanine	17.1	14.0	9.2	12.2	5.6	5.1	7.3	7.5	4.9	5.6	9.0	7.4	6.8	4.4
Valine	3.4	3.2	3.9	3.3	3.3	3.6	4.2	4.4	3.4	3.6	4.7	4.1	3.8	3.7
Proline [b]	3.0	2.9	3.0	2.1	2.6	2.9	3.4	3.5	2.9	3.2	3.2	3.5	3.1	3.7
Tyrosine [b]	1.1	1.1	1.3	0.9	1.1	1.2	1.3	1.4	1.2	1.3	1.5	1.6	1.5	1.7

Table 2.3 (continued). Individual AA content (g/100 g N), true protein expressed as anhydrous AAs (g/kg DM) and N content (g/kg DM), non-protein nitrogen (% of N) and N to protein conversion factors of selected seaweed species compared to soybean meal (reference ingredient).

	Seaweed classification													
	Brown						Red				Green			
Component	*L. digitata*		*S. latissima*		*A. nodosum*		*P. palmata*		*C. crispus*		*U. lactuca*			SBM [a]
g AA-N/100 g N	S	I	S	F	S	I	S	F	S	I	S	F	I	
AA-N (g/100 g N)	82.7	80.2	84.1	80.8	70.7	71.2	82.5	87.9	76.9	73.2	83.9	84.5	80.4	94.0
AA (g/kg DM)	102.0	87.9	84.5	128.3	43.8	55.3	155.5	156.9	98.1	121.8	79.9	190.9	265.0	523.6
Anhydrous AA (g/kg DM) [c]	86.5	74.8	72.2	109.6	37.5	47.4	132.8	134.1	83.8	104.1	68.1	163.3	226.9	451.2
AA-N (g/kg DM) [d]	15.2	13.1	12.4	18.9	6.4	8.1	23.3	23.5	15.1	18.3	11.7	28.3	39.9	79.9
N (g/kg DM)	18.3	16.3	14.8	23.4	9.0	11.4	28.2	26.8	19.6	25.0	13.9	33.5	49.7	85.0
Non-protein N (% of N)	17.3	19.8	15.9	19.2	29.4	28.8	17.5	12.1	23.1	26.8	16.1	15.5	19.7	6.0
N:protein factor, K_P[e]	4.72	4.58	4.89	4.69	4.16	4.16	4.71	5.01	4.27	4.17	4.89	4.87	4.57	5.31
N:protein factor, K_A[f]	5.71	5.70	5.81	5.81	5.89	5.84	5.71	5.70	5.55	5.70	5.83	5.77	5.68	5.65

S=harvested in Scotland, F=harvested in France, I=harvested in Ireland, AA-N=amino acid nitrogen, DM=dry matter.

[a]Soybean meal values based on analysis conducted by Hulshof *et al.* (2016).

[b]Values calculated based on their ratio to phenylalanine from Biancarosa *et al.* (2017).

[c]Anhydrous AA (g/kg DM): weight as found when AAs are bound in protein.

[d]Based on N content of each individual amino acid.

[e]Ratio between sum of anhydrous AAs (g/kg DM) and N (g/kg DM) as described by Mariotti *et al.* (2008).

[f]Ratio between sum of anhydrous AAs (g/kg DM) and AA-N (g/kg DM) as described by Mariotti *et al.* (2008).

Nitrogen digestibility ~ The *in vitro* N digestibility coefficients of the seaweeds ranged from 0.25 (Scottish *A. nodossum*) to 0.89 (French *S. latissima*), while that of SBM was 0.98 (Table 4). Both classification, species within classification, and origin affected the N digestibility, but their effects were interdependent as indicated by the interactions (all with P<0.001). The N digestibility coefficients of French *S. latissima* and *U. lactuca* (0.89 and 0.74) were higher compared to their Scottish varieties (0.71 and 0.69). The N digestibility coefficients of all seaweeds of Irish origin were higher than that of their respective Scottish varieties (*A. nodossum* (0.60 vs. 0.25), *C. crispus* (0.82 vs. 0.72), *L. digitata* (0.82 vs. 0.72) and *U. lactuca* (0.80 vs. 0.69)). Overall, *A. nodosum* had a lower N digestibility coefficient than the other species.

Multiple regression analyses indicated that 88.4% of the observed variation was explained by the selected significant model (P<0.001) including the variables (in g/kg DM) CP (P=0.004) and ADL (P<0.001; Table 2.5). Crude protein was positively correlated (coefficient=0.0012), whereas ADL was negatively correlated (coefficient=-0.0027) with the ileal N digestibility.

Table 2.4. *In vitro* digestibility coefficients of OM and N of selected seaweed species, determined with a modified Boisen method (Boisen and Fernandez, 1997) to simulate ileal and total tract digestibility.

			Ileal digestibility		Total tract digestibility
Seaweed classification	Species	Origin	OM	N	OM
Brown	*L. digitata*	S	0.707^{bcd}	0.715^{cd}	0.807^{d}
		I	0.742^{abc}	0.820^{b}	0.869^{ab}
	S. latissima	S	0.755^{abc}	0.717^{cd}	0.843^{bcd}
		F	0.812^{a}	0.886^{a}	0.883^{a}
	A. nodossum	S	0.621^{de}	0.249^{f}	0.646^{f}
		I	0.441^{g}	0.597^{e}	0.739^{e}
Red	*P. palmata*	S	0.662^{cd}	0.826^{b}	0.860^{abc}
		F	0.625^{de}	0.821^{b}	0.842^{bcd}
	C. crispus	S	0.707^{bcd}	0.715^{cd}	0.764^{e}
		I	0.764^{ab}	0.824^{b}	0.807^{d}
Green	*U. lactuca*	S	0.540^{ef}	0.688^{d}	0.736^{e}
		F	0.514^{fg}	0.737^{c}	0.755^{e}
		I	0.672^{bcd}	0.799^{b}	0.828^{cd}
	Soybean meal		0.842	0.979	0.970
	Standard error of differences		0.0447	0.0145	0.0179
	P-value				
	Classification		<0.001	<0.001	0.002
	Species		<0.001	<0.001	<0.001
	Origin		0.458	<0.001	<0.001
	Classification × Origin		0.008	<0.001	<0.001
	Species × Origin		0.005	<0.001	0.256

OM=organic matter, N=nitrogen, S=harvested in Scotland, F=harvested in France, I=harvested in Ireland. Each digestibility coefficient value is based on 2 replicate measurements.

[a-g]Means within a column without a common superscript differ significantly (P<0.05).

Table 2.5. Multiple regression analyses performed for *in vitro* ileal OM and N digestibility, total tract OM digestibility (see table 4) and the corrected maximum gas production (see table 6).

	Model			P-value[a]						
	P-value	Adjusted R^2	SE	Ash	CP	Cfat	Sugar	NDF	ADF	ADL
Ileal OM digestibility	0.137	11.3	0.102			0.137				
	0.003	61.4	0.067					0.002	0.003	
	<0.001[c]	79.5	0.049	0.012				<0.001	<0.001	
Ileal N digestibility	<0.001	74.3	0.082							<0.001
	<0.001[c]	88.4	0.055		0.004					<0.001
	<0.001	88.9	0.054	0.256	0.003					<0.001
Total tract OM digestibility	<0.001	61.7	0.042							0.001
	0.001	68.1	0.038		0.015	0.001				
	<0.001[c]	82.1	0.029		0.014			0.011		<0.001
Corrected maximum gas production [b]	0.076	19.1	0.850							0.076
	0.051	33.8	0.769				0.093			0.034
	0.036[c]	46.1	0.694				0.024	0.103		0.014

CP=crude protein, Cfat=crude fat, NDF=neutral detergent fibre, ADF=acid detergent fibre, ADL=acid detergent lignin, OM=organic matter, N=nitrogen.

[a]The variables starch and non-starch polysaccharides were also included in the multiple regression analyses, but not selected in any model and thus not shown here.

[b]In each of the generated models for the gas production test one term did not significantly correlate to the variation in the gas production test. The model with three terms was selected, as the best and only significant model.

[c]Indicate the selected models, based on significance of the model and terms included as explanatory variables.

In vitro *simulated total tract digestibility*

Organic matter digestibility ~ Total tract OM digestibility coefficients ranged from 0.65–0.88 for the seaweed samples, and was 0.98 for SBM (Table 2.4). The factors classification, species within classification and origin effected the OM digestibility, but were interdependent as indicated by their interactions ($P<0.001$). Mean total tract OM digestibility coefficients were highest for *L. digitata* (0.84), *S. latissima* (0.86) and *P. palmata* (0.85) and lowest for *A. nodossum* (0.69). The OM digestibility coefficients of seaweeds of Irish origin was higher than that of their respective Scottish varieties. The total tract OM digestibility coefficient of brown seaweeds from France were higher compared to their Scottish varieties (0.88 vs. 0.77).

Multiple regression analyses indicated that 82.1% of the observed variation was explained by the selected model ($P<0.001$) including the variables (in g/kg DM) CP ($P=0.014$), NDF ($P=0.011$) and ADL ($P<0.001$; Table 2.5). Only CP was positively correlated with total tract OM digestibility, with correlation coefficients of 0.0005, -0.0002 and -0.0011 for CP, NDF and ADL, respectively.

In vitro *gas production*

The results of the *in vitro* gas production test are shown in Table 2.6 and Figure 2.1. The protein corrected maximum gas production of all seaweeds was below that of the

Table 2.6. Curve fitting parameters of the two-phase model for selected seaweed species in the gas production (GP) test.

Seaweed classification	Species	Origin	Asymptotic GP (mL/g OM)		Maximum GP (mL/g OM)	Corrected maximum GP (mL/g OM)[a]	T_{half} (h)		Shape	
			phase 1	phase 2			phase 1	phase 2	phase 1	phase 2
Brown	*L. digitata*	S	111.3^{b}	186.6^{a}	297.9^{abc}	327.9	1.7	16.5	2.14	1.91
		I	135.3^{b}	66.5^{bc}	201.8^{bcde}	231.4	2.8	27.1	1.74	7.15
	S. latissima	S	145.9^{b}	161.7^{ab}	307.6^{ab}	331.4	2.2	20.6	1.97	1.87
		F	84.0^{b}	191.4^{a}	275.5^{abc}	313.3	1.9	13.8	2.61	1.83
	A. nodossum	S	66.0^{b}	33.7^{c}	99.7^{de}	111.8	6.0	15.4	2.09	31.48
		I	53.8^{b}	64.9^{bc}	118.7^{de}	138.6	3.5	20.7	1.27	5.02
Red	*P. palmata*	S	282.1^{a}	52.0^{bc}	337.3^{a}	379.3	3.7	28.3	2.03	2.16
		F	306.5^{a}	15.1^{c}	321.7^{ab}	365.1	4.8	36.2	1.89	11.11
	C. crispus	S	49.1^{b}	40.8^{c}	89.9^{e}	115.4	2.1	22.4	1.58	3.86
		I	65.0^{b}	48.5^{bc}	113.4^{de}	160.2	4.0	25.4	1.37	25.78
Green	*U. lactuca*	S	133.7^{b}	78.5^{abc}	212.2^{bcd}	234.7	5.7	12.0	1.22	3.53
		F	103.8^{b}	82.7^{abc}	186.5^{cde}	241.6	5.5	27.7	1.28	3.28
		I	93.1^{b}	32.8^{c}	125.8^{de}	194.4	6.0	26.6	1.20	4.97
Sugar beet pulp			194.0	207.8	401.8	425.7		5.0	2.65	3.90
Alfalfa			202.5	0.0	198.9	248.6	7.2	0.0	1.09	0.00
Standard error of differences			45.64	53.89	54.97		4.17	9.65	0.777	16.89
P-value										
	Classification		<0.001	0.004	0.247		0.413	0.117	0.165	0.780
	Species		<0.001	0.009	<0.001		0.743	0.568	0.573	0.460
	Origin		0.817	0.422	0.356		0.993	0.238	0.623	0.966
	Classification × Origin		0.600	0.824	0.708		0.987	0.544	0.894	0.623
	Species × Origin		0.575	0.046	0.137		0.542	0.695	0.701	0.182

OM=organic matter, GP=gas production, Thalf=half timeS=harvested in Scotland, F=harvested in France, I=harvested in Ireland.
Each digestibility value in the table is based on 3 replicate measurements.
Sugar beet pulp and alfalfa were included as reference ingredients.
[a]Maximum gas production corrected with an increase of 2.5 mL per percent TP in OM of the seaweed and reference ingredients, according to Cone and van Gelder (1999).
[a-g]Means within a column without a common superscript differ significantly (P<0.05).

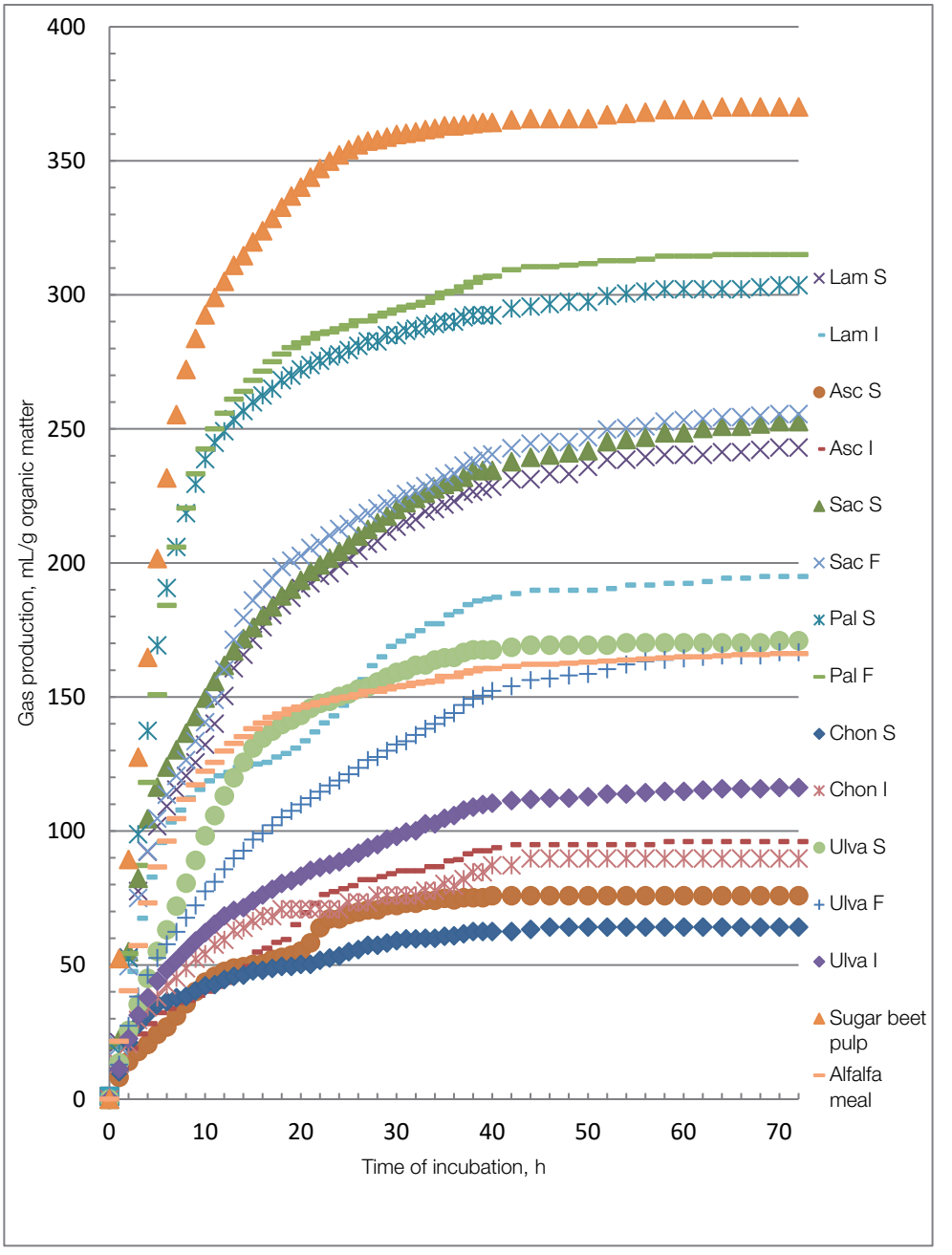

Figure 2.1. Cumulated gas production (mL/g OM) over a 72h incubation period of the selected seaweeds (Table 1), and sugar beet pulp and alfalfa meal as reference ingredients.

S=Harvested in Scotland, F=Harvested in France, I=Harvested in Ireland.
Lam=*Laminaria digitata*, Asc=*Ascophyllum nodosum*, Sac=*Saccharina latissima*, Pal=*Palmaria palmata*, Chon=*Chondrus crispus*, Ulva=*Ulva lactuca*.

reference ingredient sugar beet pulp (425.7 ml/g OM), but the gas production of *P. palmata*, *S. latissima* and *L. digitata* was above that of alfalfa meal (248.6 ml/g OM). The uncorrected maximum gas production of *P. palmata, S. latissima, L. digitata* and *U. lactuca* was significantly higher than that of the other seaweeds. Phase 1 asymptotic gas production was affected by classification (P<0.001) and species (P<0.001), with *P. palmata* showing a significantly higher gas production than all other seaweeds. In the second phase, asymptotic gas production was also affected by classification (P=0.004) and species (P=0.009), but these effects were interdependent because of the interaction between species and origin. The gas production was only affected by species (P<0.001). No differences between seaweeds were found for rate of gas production, or switching characteristics (shape) between phase 1 and 2.

The multiple regression analyses showed that there was no significant model only including terms that significantly correlated with the corrected maximum gas production. The selected significant model (P=0.036) indicated that 46.1% of the observed variation was explained including the variables (in g/kg DM) sugar (P=0.024), NDF (P=0.103) and ADF (P=0.014; Table 2.5). Only sugar was positively correlated with the corrected maximum gas production, with coefficients 0.0434, -0.0039 and -0.0131 for sugar, NDF and ADL, respectively.

The multiple regression analyses relating the *in vitro* OM and N digestibility analyses to the corrected maximum gas production showed that total tract OM digestibility was positively correlated (correlation coefficient: 9.63) with the gas production test (P=0.010), explaining 42.4% of the variation.

Discussion

The aim of this study was to evaluate the nutritive value of intact seaweeds, common in marine waters around Northwestern Europe, for application in animal feed as source of macro nutrients.

Chemical composition

The chemical composition of seaweeds as observed in this study showed a varying protein content, with an interesting AA pattern, together with relatively high levels of NPN. High NSP contents were observed and sugar, starch and fat were only present at low levels, together with a very high ash content in all seaweeds. The obtained results, including the large variation in chemical composition within and among seaweed species, are largely in line with results for brown, red and green species as described in literature (Øverland *et al.*, 2019; Makkar *et al.*, 2016 and Biancarossa *et al.*, 2017).

The CP content ranged between 45 and 248 g/kg DM, with the relatively high values for the red and green compared to the brown species, which generally agrees to values in the literature (e.g. Øverland *et al.*, 2019). Nonetheless, the higher levels within the

range of CP as reported in literature for brown, red and green algae (up to 168, 376 and 352 g/kg DM respectively) were not observed here. This could be due to the inclusion of seaweeds from other regions in those studies, with seaweeds grown under other environmental conditions like water temperature, availability of nutrients, and another stage of maturity at harvest. The CP values as reported by Øverland *et al.* (2019) were based on a N:P conversion factor of 5.00. Thus the observed differences cannot be explained by the use of different conversion factors.

For seaweed, a conversion factor of 5.00 is proposed (Angell *et al.*, 2016b), based on the higher NPN in seaweed compared to standard land-based feed stuffs, for which a factor of 6.25 is commonly used. We observed an average NPN content of 200 g/kg N compared to 60 g/kg N in SBM. Two conversion factors were calculated based on the AA content, one calculating the ratio of anhydrous AA (g/kg DM) to AA-N (g/kg DM; K_A), and the other also taking into account the NPN by calculating the ratio of anhydrous AA (g/kg DM) to N (g/kg DM; K_P) both based on Mosse (1990). Because of the potentially high NPN content of seaweed, the conversion factor K_P is most applicable when evaluating intact seaweed as protein source for animal diets (Lourenço *et al.*, 2002; Templeton and Laurens, 2015; Biancarosa *et al.*, 2017; Angell *et al.*, 2016b).

A proposed conversion factor of 5.00 provided a more accurate calculation of true protein than a factor of 6.25. Nonetheless, the variation in actual conversion factors as calculated from our data, ranged from 4.16–5.01, showing that one factor for seaweed in general, as well as for seaweed classification, does not accurately reflect the true protein content. Because the K_P factors of samples within species are similar, we suggest using a conversion factor per species, which could be based on literature values and data mining, using already available data on true conversion factors.

The observed AA content, AA-N and N of the brown, red and green seaweed species showed a pattern, with the highest AA-N content in *P. palmata*, and *U. Lactuca* and the lowest content in *A. nodosum*. However, due to the large variation, even within species, provision of average contents has little value. The NPN can be utilized by rumen microbiota, but not by monogastric animals. This indicates that intact seaweed is a better protein source for ruminants, although the nitrogen content of most studied seaweed species was relatively low for using intact seaweed as protein supplement in ruminant diets.

The mean contents of the AAs methionine, cysteine, threonine, and valine (g AA-N/100 g N) were 20–50% higher than in SBM, whereas the contents of some other essential AAs, *i.e.* lysine, leucine, isoleucine, histidine, and arginine were 10–30% lower. The AA pattern and levels of individual AAs, as well as the variation observed, correspond with data reported in the literature (Øverland *et al.*, 2019; Biancarosa *et al.*, 2017), although most of the AA levels observed in our study are slightly higher than the values reported for the same seaweed species harvested around the coastal areas of Norway. Thus, with adequate supplementation with other protein sources or individual AA, the AA pattern of a seaweed containing feed could meet the requirements of both

monogastric and ruminant species. Nonetheless, the variation in AA profile within and between species was quite substantial. Therefore, it is recommended to evaluate the protein and AA content of each batch of seaweed before inclusion in animal diets, and to build a database to obtain more quantitative insight in the variation in AA content and pattern among and within seaweed species.

The NSP content was high in all seaweed samples, in accordance with data in the literature (Øverland *et al.*, 2019). The polysaccharides in the NSP, NDF and ADF fractions are not enzymatically digestible, and thus of low nutritive value for poultry, whereas they may be potentially fermentable in the large intestine of pigs (Nielsen *et al.*, 2014; Metzler-Zebeli *et al.*, 2010). For ruminants, the NSP content contributes as a substrate for rumen microbiota, thus potentially contributing to the short chain fatty acids and protein availability (Belanche *et al.*, 2012).

Seaweeds contain different polysaccharides compared to SBM or other land plants (Rupérez and Toledano, 2003; Jiménez-Escrig and Sánchez-Muniz, 2000; CVB, 2018), and it is not known in which analysed dietary fraction these polysaccharides are included. Although useful for evaluation of digestibility in animals, the analyses of NSP, NDF, ADF and ADL do not provide detailed information regarding specific polysaccharides and the actual nutritional value of the seaweeds. A striking example was observed in the brown seaweeds, in which the analysed ADF fraction was up to twofold greater than the NDF fraction. To our knowledge, this has not been reported elsewhere in literature, although the NDF and ADF of seaweeds are not often analysed. We speculate that the higher ADF than NDF fraction might be caused by the presence of specific polyphenolic compounds, which can precipitate in an acidic environment, but not in an neutral environment (Manev *et al.*, 2013; Soest van *et al.*, 1991). Further analyses are required for a better identification and quantification of the specific polysaccharides of seaweed, their inclusions in different analytical fractions and their contribution to the nutritional value.

The analyses of the carbohydrates after complete hydrolyses of the seaweeds (Data not shown) showed a species specific carbohydrate composition, in agreement with species specific polysaccharide fractions as reported by Øverland *et al.* (2019). Again, a large variation in composition was observed between and within species, for example with galactose mainly observed in red species, fucose in brown species and rhamnose in green species.

Regarding the energy content of seaweed, all seaweeds were very low in sugar, starch and fat content, which is in line with data in the literature (Holdt and Kraan, 2011; Øverland *et al.*, 2019). Because of the low fat content, the contribution to the nutritional value of seaweeds is low and fatty acid composition (data not shown) will not be further discussed within the scope of this paper. Nonetheless, the fatty acid content and composition might be relevant for the study of potential bioactive properties.

The high ash content in all seaweed species in this study hamper the inclusion of intact seaweed in animal diets, and indicates that the composition of the ash fraction

needs to be taken into account (data not shown). Especially poultry are susceptible to high mineral concentrations, as for example indicated by a maximum tolerable level of sodium chloride of 17, 30 and 45 g/kg DM for poultry, pigs and growing beef cattle (NRC, 2005). A too high mineral content in pigs and poultry feed may cause wet litter and diarrhoea, which may result in a decrease in performance (Guiry and Blunden, 1991; Koreleski *et al.*, 2010). Studies in sheep and goats showed that a high ash content of the diet due to inclusion of seaweed also resulted in a higher water intake and urine excretion in these animal species, but it had no negative effect on the digestibility of the diets (Marin *et al.*, 2009; Castro *et al.*, 2009). These results may suggest that the impact may be more detrimental in monogastric species. A potential method of reducing ash content of seaweed, is to rinse the intact seaweed with fresh water. This would reduce the ash content, and consequently increase the relative content of other nutrients, but also other soluble nutrients might be lost during washing (Magnusson *et al.*, 2016). Based on the high mineral content of all seaweed samples, these intact seaweeds are not suitable to be used in animal diets at high inclusion level.

This paper does not address the heavy metal content in the seaweeds. Nonetheless, these do need to be taken into account when formulating diets. Heavy metals can accumulate in large amounts in seaweed, which limits the use of intact seaweed in animal feed (Øverland *et al.*, 2019).

The large variation in chemical composition among and within seaweed species hampers seaweed inclusion in animal diets. In the literature, this variation is attributed to multiple environmental factors, like season of harvest (Holdt and Kraan, 2011; Schiener *et al.*, 2015; Øverland *et al.*, 2019; Sharma *et al.*, 2018), depth of the seaweed in the water column (Sharma *et al.*, 2018), and light and nutrient availability in the water (Boderskov *et al.*, 2016). Due to the characteristics of the seaweed samples in our study, the origin of the samples was to some extend confounded with the time of harvest. Hence, these two factors could not be fully separated. The variation also depends on the stage of the reproductive cycle, where results can be contradictory between species regarding changes in levels of *e.g.* main structural component during the reproductive phase (Skriptsova *et al.*, 2012). The variation in our results illustrate that dried seaweed meal is not a standardized feed material with a predictable chemical composition.

Within land-based feedstuffs, variation in nutrient levels also occurs (CVB, 2018), but to a lesser extent than in seaweed. Whereas each batch of land-based feedstuffs is preferably analysed for its nutrient composition before diet formulation, this practice will be even more required for effective inclusion of seaweeds in animal diets. Also within species grown at the same location, the variation is considerable (*e.g.* Marinho-Soriano *et al.*, 2006 and Schiener *et al.*, 2015). Cultivation of seaweed might be of interest, since it has been shown that there is a potential for the selection of seaweed, possibly leading to a higher yield of a more preferred, and possibly more constant or predictable, chemical composition (Li *et al.*, 2008; Westermeier *et al.*, 2010).

In vitro *digestibility*

The Boisen *in vitro* digestibility test has been developed to simulate the digestibility in the stomach, small intestine and large intestine of pigs in three distinct steps. Although developed for pigs, this method has been used for poultry without using the third step, since the contribution of large intestinal fermentation in birds is negligible (Losada *et al.*, 2010).

The *in vitro* simulated ileal OM digestibility of the seaweeds ranged from very low to reasonably good (OM digestibility coefficients: 0.44-0.81), The highest values were observed for *L. digitata*, *S. latissima*, and *C. crispus* but the mean digestibility was still about 10%-points lower than in SBM (0.84). The mean simulated ileal N digestibility coefficients of all seaweed species apart from *A. nodosum* was relatively high, generally above 0.75, but well below the N digestibility of SBM of 0.98. This could be explained by part of the N being bound to, or entrapped in, poorly digestible polysaccharides, *e.g.* cell wall structures (Lahaye and Robic, 2007). This would also explain the low OM digestibility (MacArtain *et al.*, 2007). In the multiple regression analyses performed on the *in vitro* digestibility, the fibre fractions NDF, ADF and ADL were included in the selected regression models and correlated negatively with the ileal and total tract digestibility. This emphasises the importance of further identification and characterisation of seaweed polysaccharides in different fibre fractions, to understand, predict and improve the digestibility of seaweed fractions in farm animals. Extraction of protein or other favourable components might be a good step forward towards seaweed inclusion in animal diets (Øverland *et al.*, 2019; Bjarnadóttir *et al.*, 2018; Bikker *et al.*, 2016). Indeed, Bikker *et al.* (2016) observed a 5% increase in an *in vitro* protein digestibility of *U. lactuca* extracted fraction compared to the intact seaweed.

We acknowledge that the *in vivo* validation of the observed *in vitro* results is lacking, since there are only few *in vivo* studies published with intact seaweed added at a substantial level, *e.g.* >50 g/kg DM or more, since most studies focussed on the effects of the bioactive compounds of seaweed rather than the nutritional value (*e.g.* Abudabos *et al.*, 2013; McDonnell *et al.*, 2010). Nonetheless, when taking into account the low protein content, the high NPN content, and low N digestibility relative to SBM, neither of the seaweeds investigated here would seem to be a high quality protein source for monogastric animals.

In vitro *rumen fermentation*

The gas production model for land-based feed materials generally includes predefined time periods for each phase (Van Gelder *et al.*, 2005). However, seaweed consists of very different polysaccharides and sugars compared to land-based plants, and the behaviour of the different fractions in the gas production test is not well known. Therefore, a basis to predefine the duration of the phases to set time periods was not available. The seaweeds *L. digitata*, *S. latissima*, and *P. palmata* had the highest mean corrected maximum gas production in the *in vitro* gas production

test. The values varied from approximately 65–90% of the corrected maximum gas production of sugar beet pulp, and were well above the result for alfalfa. This indicates their potential value in ruminant diets. The maximum gas production as recorded for seaweeds in this experiment was consistently about a factor 10 higher than in the study of Molina-Alcaide *et al.* (2017), who determined the asymptotic gas production of the species *L. digitata* (18 ml/g OM) and *P. palmata* (38 ml/g OM). Although a large variation in chemical composition and consequently in gas production may be expected between and within seaweed species in these two studies, we suspect the large differences between studies to be due to methodological differences. Nonetheless, the ratio between the maximum gas production of the two species is quite similar between the two studies.

The difference in the time at which half of the gas was produced between the seaweeds and sugar beet pulp, especially in the second phase, was likely caused by differences in the fermentability of specific polysaccharides in the sugar beet pulp *versus* those in the seaweeds. The behaviour of the specific NSP components of the seaweed during fermentation is important to understand the kinetics of the fermentation of seaweed in ruminant diets, but this has not been studied comprehensively. The lower gas production of the seaweeds *A. nodossum* and *U. lactuca* might be caused by a relatively high lignin content, which is poorly fermentable, although *C. crispus* does not contain a high lignin content and also had a notably low gas production. The negative impact of lignin was confirmed by the results of the multiple regression analyses of the *in vitro* data, with the highest negative impact of the ADL fraction. The ADL fraction was also shown to negatively correlate to digestibility of land-based plants using an *in vitro* rumen fermentation approach (Buxton and Russell, 1988). The negative correlation enhances the importance of further identification and characterisation of seaweed polysaccharides in different fibre fractions. Characterisation of the fermentability of individual NSP components is also of importance for the inclusion of seaweed in ruminant diets, where the intact seaweeds with high NSP content might be a nutritionally valuable feed stuff (Choct, 1997; Metzler-Zebeli *et al.*, 2010; Susmel and Stefanon, 1993).

Fermentability might be higher when using rumen fluid from animals that are adapted to a seaweed diet. Relative high OM digestibility coefficients of 0.84-0.97 (Tilley and Terry method) were observed for several seaweed species among which the brown *L. digitata* and *S. latissima* and the red species *P. palmata* using rumen fluid of seaweed fed Orkney sheep (Greenwood *et al.*, 1983). When sheep were fed seaweed diets, the microbial composition of the rumen changed towards more favourable microbial composition for seaweed fermentation, leading to an up to twofold higher digestibility of nutrients (Orpin *et al.*, 1985).

A regression analysis was performed relating the *in vitro* digestibility to the corrected maximum gas production, to determine the agreement between the *in vitro* models. Only the total tract OM digestibility correlated significantly with the gas production, explaining 43% of the variation. This correlation was expected since the *in vitro* total tract digestibility analyses included an enzyme step containing a range of

carbohydrates including arabinase, cellulase, beta-glucanase, hemicellulase and xylanase activity, mimicking the fermentation in the large intestine of pigs. This would come closest to the fermentation determined in the *in vitro* gas production test. The low percentage of variation in corrected maximum gas production explained by total tract OM digestibility (43%) indicates that the *in vitro* total tract OM digestibility is not a sufficient indicator for maximum gas production.

Conclusion

The results of this study demonstrate a large variation in nutrient composition, *in vitro* digestibility, and nutritional value, both between and within seaweed species. Therefore, use of mean nutrient contents has little value and the rational use in animal diets will require adequate analysis of each batch of product. Based on the protein content and AA pattern, some of the red and green seaweed species (*e.g. P. palmata* and *U. lactuca*) would be a valuable protein source for farm animals. However, several characteristics including a high NSP and NPN content and a low *in vitro* digestibility relative to SBM limit the use of intact seaweed in monogastric species, especially poultry. Since NPN can be used by rumen microbiota and some species (*e.g. L. digitata*, *S. latissima* and *P. palmata*) demonstrated a relatively high *in vitro* fermentability, intact seaweed may have a higher nutritional value for ruminants. Nonetheless, the high ash content in all seaweed species, potentially including heavy metals, hampers the use of intact seaweed for both ruminants and monogastrics. Washing seaweeds may reduce this high ash content, but also result in a loss of other soluble components. Extraction of protein and other favourable components seems an important step forward towards seaweed inclusion in animal diets. This would also allow to drastically reduce the high ash and mineral content in the intact seaweed. Further identification and characterisation of seaweed polysaccharides are required to understand, predict and improve the digestibility of seaweed fractions in farm animals, especially since the fibre fractions comprised up to 40% of DM in the selected seaweed samples. *In vivo* studies are required to validate the value of future seaweed products.

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Chapter 3

Evaluation of the nutritional value of seaweed products for broilers chickens' nutrition

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Abstract

This study investigates the effect of washing, ensiling and extraction processes on the nutritional value of seaweed products for broilers, based on nutrient content and *in vitro* and *in vivo* digestibility. The effects of ensiling, washing and extraction processes were evaluated using *Saccharina latissima, Laminaria digitata* and *Ulva lactuca*, with 2, 4, and 6 hour incubations in an *in vitro* simulated digestibility model, to obtain insight into the kinetics of digestibility. In an *in vivo* study, 160 Ross 308 male broilers were fed (day 14 to 22) a basal grower diet, or the basal grower diet with 100 g/kg of *S. latissima* silage or silage residue. Performance and ileal and total tract nutrient digestibility were determined. Washing and ensiling reduced the ash content, but also the *in vitro* organic matter digestibility (both $P<0.001$). Washing also reduced nitrogen digestibility ($P<0.001$). Extraction of seaweed decreased *in vitro* organic matter and nitrogen digestibility. Feeding seaweed diets to broilers resulted in a higher feed conversion ratio (1.62 versus 1.86 and 1.77 for broilers fed the basal, silage and silage residue diets respectively, $P<0.001$) without increase in final body weight. Feeding *S. latissima* silage residue compared to silage resulted in a slightly better broiler performance and a higher amino acid digestibility. In conclusion, washing, ensiling and extraction processes reduce the nutritional value of the seaweed products, and do not make seaweed suitable for inclusion in broiler diets. To create suitable seaweed products for inclusion in broiler diets, a further reduction in the ash content and increase in digestibility is needed.

Keywords: seaweed; feed ingredient; nutrition; *in vitro* digestibility; *in vivo* digestibility; broiler

Introduction

The increasing world population (United Nations, 2017) and increasing demand for animal protein (Boland *et al.*, 2013) have stimulated the exploration of novel feed sources, including seaweed for farm animals (Makkar *et al.*, 2016; Buschmann *et al.*, 2017). Advantages of seaweed production are the use of salt instead of fresh water, sea instead of arable land-based production, and the high productivity in terms of biomass produced per unit of surface area (Buschmann *et al.*, 2017). Nonetheless, the inclusion of a substantial percentage of seaweed in animal diets is hampered by the high content of ash and poorly digestible carbohydrates (Sharma *et al.*, 2018), the low digestibility (Bikker *et al.*, 2016, 2020), the limited shelf life (Paull and Chen, 2008; Stévant *et al.*, 2017) and the high cost of production (Van den Burg *et al.*, 2013). To address some of these disadvantages, seaweed can be washed to reduce the ash (minerals, sand) content on the outside of the leaves (Neveux *et al.*, 2014), and the product can be ensiled to extend the shelf life of fresh harvested seaweed (Herrmann *et al.*, 2015). A biorefinery approach, using seaweed fractions for different purposes, can increase the economic feasibility of the use of seaweed (Torres *et al.*, 2019). Co-products from the biorefinery of seaweed may be valuable and cost-effective ingredients in diets for farm animals, including broilers (Bikker *et al.*, 2016; Torres *et al.*, 2019). Although studies have been conducted with a low inclusion level of seaweed in broiler diets (Abudabos *et al.*, 2013; Choi *et al.*, 2014), little information is available on the effect of processing on the nutritional value of seaweed and seaweed products.

This study was conducted to investigate the effect of washing, ensiling and extraction processes on the nutritional value and digestibility of seaweed products for broilers. The nutritional value and digestibility were evaluated based on nutrient content, *in vitro* simulated digestibility and an *in vivo* study in broilers.

Material and methods

Within the framework of this study, *in vitro* and *in vivo* experiments were conducted. In the *in vitro* experiment, the effects of ensiling, washing and extraction processes on simulated nutrient digestibility of different species of seaweed (*Saccharina latissima* (*S. latissima*, sugar kelp), *Laminaria digitata* (*L. digitata,* oarweed) and *Ulva lactuca* (*U. lactuca,* sea lettuce)) were investigated. Subsequently, the nutrient digestibility of *S. latissima* silage and silage residue was determined in broilers. Because of the large variation in chemical composition between and within seaweed species (Biancarosa *et al.*, 2017; Bikker *et al.*, 2020), this study focussed on species common in marine waters in Northwestern Europe.

In vitro *experiment*

The effects of ensiling and washing of seaweed on nutritional value were studied using fresh and ensiled *S. latissima*. The effect of extraction of valuable sugars on nutritional value were studied using *S. latissima*, *L. digitata* and *U. lactuca*.

The unwashed, washed, fresh and ensiled *S. latissima* samples included in the *in vitro* analyses were provided by Hortimare (Heerhugowaard, the Netherlands) from the production location Kverhella, Norway (61.00°N, 4.70°E). For the production of silage, cubic meter containers were filled with fresh *S. latissima*, covered with plastic sheets with a bag filled with water on top as an air tight seal. A drain was installed at the bottom of the container to drain remaining seawater. The silage was stored at room temperature for 4 weeks. Washed fresh and ensiled *S. latissima* samples were produced by placing the unwashed material on plastic sheets and flushing it with a substantial amount of tap water without immersing the seaweed material. After removal of superficial water with paper towel, the *S. latissima* was oven dried (Thermo Heraeus OMH750, Thermo Fisher Scientific, Breda, The Netherlands) at 39°C for three days, until approximately 900 g/kg dry matter (DM) was reached.

A biorefinery approach with extraction of mannitol (*S. latissima* and *L. digitata*) or rhamnose (*U. lactuca*) was adopted as a method to improve the economic feasibility of the use of seaweeds. Unwashed *S. latissima* silage and fresh *L. digitata* (provided by Ocean Harvest, Galway, Ireland) were used to determine the nutritional value of the residue after aqueous extraction of mannitol by ECN (Petten, the Netherlands) as described by Van Hal and Huijgen (2014). In short, seaweed was treated with water of a salinity of less than 20 g/kg and a pH between 3.0 and 9.0, which solubilizes the mannitol in the water. The water with mannitol is then drained, leaving the residue product. Fresh *U. lactuca*, cultivated by WUR-IMARES in Yerseke, the Netherlands, was used for rhamnose extraction using acid hydrolysis with HCl, in a 20 L autoclave (Kiloclaaf, Büchi Labortechnik AG, Flawil, Switzerland) at 100°C for 60 min by ECN (Petten, the Netherlands). The residues of mannitol and rhamnose extraction were used in dried form. All seaweed samples were oven dried at 39°C until approximately 900 g/kg DM was reached. The analysed nutrient composition of the seaweed products used in the *in vitro* study are given in Table 3.1.

All samples were ground to pass a 1 mm sieve. The *in vitro* simulated digestibility analyses were performed according to an adjusted Boisen two-step method (Boisen and Fernandez, 1997) as described in the study of Bikker *et al.* (2016). Briefly, 1 g samples were incubated with 75 mL 0.1 M phosphate buffer solution (pH 6.0) and 0.2 M HCl solution until a pH of 2.0 was reached. One mL pepsin solution (25 g/L, 2000 International Federation of Pharmaceuticals U/g) was added, and samples were incubated at 39°C for 2 h under constant stirring. Thereafter, 30 mL 0.2 M phosphate buffer (pH 6.8) was added, plus NaOH until a pH of 6.8 was reached. One mL pancreatin (100 g/L) was added and the incubation was continued for 4 h under the same conditions. To obtain insight in the digestion kinetics of seaweed, the *in vitro* incubations were terminated after two, four or six hours, representing the gastric and

Table 3.1. Analysed nutrient content of seaweed products as used in the *in vitro* digestibility study.

	Analysed nutrient composition			
	g/kg	g/kg DM		
Seaweed product	DM	Ash	OM[1]	N
Saccharina latissima fresh unwashed	924	465	535	10.8
Saccharina latissima fresh washed	913	425	575	13.6
Saccharina latissima silage unwashed	922	374	626	20.5
Saccharina latissima silage washed	907	290	710	21.6
Saccharina latissima silage residue[2]	925	266	734	18.0
Laminaria digitata unwashed fresh	940	241	759	11.9
Laminaria digitata residue[3]	909	226	774	13.4
Ulva lactuca unwashed fresh	861	363	637	23.3
Ulva lactuca residue[4]	926	83	917	43.4
Reference ingredient				
Soybean meal[5]	900	73	927	85.0

DM, dry matter, OM, organic matter, N, nitrogen.
[1]Calculated as 1000–Ash.
[2]Residue after extraction of mannitol from *Saccharina latissima* silage unwashed material.
[3]Residue after extraction of mannitol from *Laminaria digitata* fresh unwashed material.
[4]Residue after extraction of rhamnose from *Ulva lactuca* fresh unwashed material.
[5]Values based on analyses conducted by Hulshof *et al.* (2016).

small intestinal digestion, respectively. The *in vitro* incubations were conducted in four-fold, of which two replicates were used to determine nitrogen (N) digestibility and two replicates to determine DM and organic matter (OM) digestibility.

In vivo *experiment*

The *S. latissima* silage and silage residue used in the *in vivo* experiment were produced by Hortimare (Heerhugowaard, the Netherlands) and originated from the production location Kverhella, Norway (61.00°N, 4.70°E). This seaweed species was selected because of its high availability in this area, suitability for cultivation, and as part of a project investigating the long term storage as *S. latissima* silage and subsequent biorefinery. The silage residue was produced by first crushing the silage, to improve the efficiency of the extraction process. Thereafter the silage was soaked in fresh water under alkaline conditions (pH>9, by adding Na_2CO_3) for 24h without stirring. The silage residue was the remaining product after draining the water for subsequent extraction of the soluble components from the drained water. The seaweed products were oven dried at 39°C for 3 days until a DM content of approximately 900 g/kg was reached. The chemical composition of the *S. latissima* products is included in Table 3.2, showing a somewhat higher ash, N and crude fibre content and lower non-starch polysaccharide content in *S. latissima* silage residue compared to the silage. In the silage residue product, the observed sodium content was twice as high compared to the silage product, whereas the chloride content was twice as low. In addition, small differences were observed in amino acid content expressed per 100 g N and a higher sum of amino acid nitrogen (AA-N) in the silage residue.

Table 3.2. Analysed nutrient composition and calculated energy content of *Saccharina latissima* silage and silage residue[1] products as used in an *in vivo* study in broilers.

	Seaweed products	
Nutrient content (g/kg dry matter)	*S. latissima* silage	*S. latissima* silage residue
Dry matter (g/kg)	896	897
Ash	242	263
Organic matter[2]	758	737
Nitrogen	23.8	26.2
Fat	20	20
Crude fibre	157	196
Sugar	<1	0
Starch	5	4
Non-starch polysaccharides[3]	583	550
Calcium	35.7	35.4
Phosphorous	1.4	1.7
Sodium	17.8	41.1
Chloride	46.1	26
Amino acids (g AA-N/100 g nitrogen)		
Lysine	4.8	5.9
Methionine	1.4	1.4
Cysteine	1.3	1.1
Threonine	3.3	3.5
Isoleucine	2.8	2.9
Arginine	8.5	9.5
Phenylalanine	2.7	2.6
Histidine	2.8	3.1
Leucine	4.9	5.1
Valine	4	4.2
Alanine	7.4	6.2
Asparagine + Aspartic acid	9.4	9.9
Glutamine + Glutamic acid	9.2	10.5
Glycine	7.1	6.6
Serine	3.6	3.8
Sum of AA-N	73	76.2
Calculated energy content (kJ/g dry matter)[4]	11.88	11.23

AA-N, nitrogen from amino acids.
[1]Residue after the aqueous extraction of Saccharina latissima silage.
[2]Calculated as 1000–ash.
[3]Calculated as organic matter–crude protein (as N×6.25)–fat–starch–sugar.
[4]Calculated as gross energy=22.6×crude protein (as N×6.25)+38.8×fat+17.5×starch+16.7×sugar+ 18.6×residue (as organic matter–crude protein (as N×6.25)–fat–starch–sugar) (Milgen *et al.*, 2018).

Animal housing and experimental design ~ The experiment was conducted at the experimental animal facility Carus of Wageningen University & Research (Wageningen, the Netherlands). All experimental procedures were approved by the Animal Care and Use Committee of Wageningen University & Research, the Netherlands (AVD401002015196). A total of 160 one-day-old male Ross 308 broiler chickens

(Hatchery Morren BV, Lunteren, the Netherlands) were housed in 4 pens with concrete floors and wood shavings (2 kg/m2) as bedding material during the pre-experimental period from 1 to 14 days of age. All birds received a commercial starter diet. All chickens were vaccinated against infectious bronchitis at arrival at the experimental facility, and against Newcastle disease at day 11. At day 14, the 160 chickens were distributed over 16 pens (0.87m×1.10m, slatted floors) with 10 birds per pen, while pen weight was kept within a 3% difference from mean pen weight. Three dietary treatments, a basal diet and a basal diet with either *S. latissima* silage or silage residue, were allocated to pens in a completely randomized design. From day 14–22, the basal diet was fed to 6 replicate pens and each seaweed diet was fed to 5 replicate pens. A standard temperature and lighting schedule was applied. During the entire experimental period, birds had ad libitum access to feed and water. At the end of the experiment (day 22) all chickens were euthanized by 0.5 mL T61 injection to the wing vein.

Experimental diets ~ All chickens received a standard broiler starter feed (Apparent metabolizable energy (AME) 12.3 MJ/kg, dig. Lys 11.9 g/kg) during the first 13 days and the experimental diets from day 14 to day 22. The experimental diets were formulated to meet the requirements for broilers in the grower phase (CVB, 2016). All experimental grower diets were supplemented with 5 g/kg titanium dioxide (TiO_2) as indigestible marker. The seaweed diets were supplemented with 100 g/kg dried *S. latissima* silage or *S. latissima* silage residue before mixing and pelleting at a diameter of 3.2 mm dye. The dietary ingredients and calculated nutrient content of the diets are given in Table 3.3.

Measurements ~ Feed intake was monitored on a weekly basis. All chickens were weighed at arrival at the experimental facility. Body weight (BW) was determined per pen after allocation to the treatments at day 14 and determined again at day 22. Excreta were collected from day 20 to day 22, after which all animals were euthanized and ileal contents were collected per pen.

Chemical analyses ~ All seaweed products were analysed using official methods described to determine moisture (DM; ISO 6496, 1999), ash (ISO 5984, 2002), and N (ISO 5983, 2005). The seaweed products and diets used in the *in vivo* study were also analysed for amino acids (AAs; ISO 13903, 2005), ether extract (fat; ISO 6492, 1999), crude fibre (ISO 6865, 2000), sugar (EC, 2009), starch (ISO 15914, 2004), Ca (ISO 6869, 2000), P (ISO 6491, 1998), Na (ISO 27085, 2009), and Cl (ISO 6495, 2015). The ileal digesta were analysed for DM, N, AAs, Ca and P as described above and for TiO_2 as marker for digestibility (Short *et al.*, 1996). The OM was calculated as 1000 minus ash. Non-starch polysaccharide content was calculated as OM minus crude protein, crude fat, starch and total sugars (CVB, 2016). Excreta were analysed for DM, ash, fat and crude fibre.

Table 3.3. Ingredients and calculated and analysed nutrient composition of the basal diet and diets containing 100 g/kg *Saccharina latissima* silage and silage residue, as fed to broilers to study the digestibility of the processed seaweed products.

	Diet		
Ingredient (g/kg)	Basal diet	Basal with *S. latissima* silage	Basal with *S. latissima* residue[1]
Maize starch	600.00	538.65	538.65
Soybean meal	120.00	107.73	107.73
Oat hulls	80.00	71.82	71.82
Casein	66.10	59.33	59.33
Dextrose	51.89	46.58	46.58
Soybean oil	23.33	20.94	20.94
Limestone	11.38	10.22	10.22
Mono-calcium phosphate	13.38	12.01	12.01
Magnesium oxide	1.00	0.89	0.89
L-Lysine HCl	1.38	1.24	1.24
DL-Methionine	3.13	2.81	2.81
L-Threonine	1.52	1.36	1.36
L-Arginine	3.36	3.02	3.02
L-Isoleucine	0.60	0.54	0.54
L-Valine	0.95	1.00	1.00
Salt	3.33	0.00	0.00
Sodium bicarbonate	1.74	0.37	0.00
Potassium carbonate	6.91	1.03	0.00
Diamol	0.00	10.58	11.98
Vitamin and mineral premix	5.00	5.00	5.00
Titanium dioxide	5.00	5.00	5.00
Saccharina silage	0.00	100.00	0.00
Saccharina silage residue	0.00	0.00	100.00
Calculated nutrient content (g)			
Apparent metabolizable energy (MJ)	12.80		
Retainable phosphorous	3.20		
Analysed nutrient composition (g/kg dry matter)			
Dry matter (g/kg)	878	880	868
Ash	56	75	73
Organic matter[2]	944	925	927
Nitrogen	25	24	24
Fat	32	30	25
Crude fibre	23	38	37
Sugar	66	57	59
Starch	620	555	283
Non-starch polysaccharides[3]	70	133	410
Calcium	8.4	11.0	11.2
Phosphorous	5.4	5.0	4.9
Sodium	2.2	2.2	4.1
Chloride	2.0	4.6	2.7

Table 3.3 (continued). Ingredients and calculated and analysed nutrient composition of the basal diet and diets containing 100 g/kg *Saccharina latissima* silage and silage residue, as fed to broilers to study the digestibility of the processed seaweed products.

	Diet		
	Basal diet	Basal with *S. latissima* silage	Basal with *S. latissima* residue[1]
Analysed nutrient composition (g/kg dry matter; continued)			
Amino acids (g AA-N/100 g nitrogen)			
Lysine	10.9	10.6	11.5
Methionine	6.2	6.0	6.0
Cysteine	1.1	1.3	1.4
Threonine	7.3	7.3	7.7
Isoleucine	7.4	7.3	7.8
Arginine	9.9	9.9	10.5
Phenylalanine	7.4	7.5	7.9
Histidine	3.8	3.6	3.9
Leucine	12.3	12.2	13.0
Valine	9.6	9.7	10.1
Alanine	5.4	5.9	6.2
Asparagine + Aspartic acid	13.0	13.1	14.1
Glutamine + Glutamic acid	28.0	26.5	28.5
Glycine	4.4	4.8	5.1
Serine	7.6	7.5	8.0
Sum of AA-N	134.3	133.2	141.7

AA-N, nitrogen from amino acids

[1]Residue after the aqueous extraction of *Saccharina latissima* silage

Calculations and statistical analyses ~ Performance parameters were calculated using feed intake and BW measurements over time. Apparent pre-caecal digestibility and apparent total tract digestibility of nutrients in the experimental diets were calculated, using Ti as indigestible marker according to the following equation:

$$D(X) = \left(1 - \frac{[Ti]diet \times [X]sample}{[Ti]sample \times [X]diet}\right)$$

where D(X) is the digestibility of nutrient x and $[Ti]_{diet}$, $[Ti]_{sample}$, $[X]_{diet}$ and $[X]_{sample}$ are the concentrations of Ti and nutrient X in the diet and digesta or faecal sample, respectively. The apparent pre-caecal digestibility and apparent total tract digestibility of nutrients in the seaweed co-products were calculated applying the difference method (Kong and Adeola, 2014), ascribing differences in digestibility between a basal diet and a basal diet including a component of investigation, assuming additivity.

All statistical analyses were performed using Genstat statistical software (VSN International, 2020). The effect of ensiling and washing on *in vitro* OM and N digestibility of *S. latissima*, in dependence of incubation time, was analysed with ANOVA using a 2 (no ensiling or ensiling) × 2 (no washing or washing) × 3 (2, 4 or 6h incubation time) factorial arrangement. The effect of species and mannitol or rhamnose extraction on the *in vitro* simulated OM and N digestibility of included seaweed products, in dependence of incubation time, was analysed with ANOVA using a 3 (*S. latissima*, *L. digitata* or *U. lactuca*) × 2 (mannitol or rhamnose extraction) × 3 (2, 4 or 6h incubation

time) factorial arrangement. The experimental unit for all *in vitro* analyses were separate runs in the simulated digestibility analyses. An ANOVA was used to determine differences in growth performance, and *in vivo* pre-caecal and total tract digestibility between *S. latissima* silage and silage residue. Pen was the experimental unit for BW, body weight gain, feed intake, feed conversion ratio (FCR) and the ileal and faecal digestibility. All data are presented as least square means. Differences were considered significant at $P<0.05$.

Results

In vitro *experiment*

Washed fresh and silage products from *S. latissima* had a lower ash content and a higher OM and N content than unwashed fresh and silage products (Table 3.1). Washed and unwashed silage products had a lower ash content and a higher OM and N content compared to washed and unwashed fresh *S. latissima*. The *S. latissima* and *L. digitata* residues were slightly different from the original products, with a lower ash and higher OM content. Additionally, *L. digitata* residue had a marginally higher N content compared to the unwashed fresh *L. digitata*. The *U. lactuca* residue on the other hand, had a substantially lower ash content, and consequently higher OM and N content compared to the unwashed fresh *U. lactuca*.

As summarized in Table 3.4, both ensiling and washing reduced the *in vitro* OM digestibility of *S. latissima* products ($P<0.001$ for both ensiling and washing). Washing, but not ensiling, reduced the *in vitro* N digestibility ($P<0.001$ and $P=0.057$ for washing and ensiling respectively). The *in vitro* OM and N digestibility of all *S. latissima* products increased significantly during incubation from 2 to 4h, with a small and largely insignificant increase during incubation from 4 to 6h.

As summarized in Table 3.5, extraction reduced the *in vitro* OM and N digestibility of all seaweed products ($P<0.001$). The biggest reduction in OM and N digestibility was observed in the *U. lactuca* residue (Interaction Species Extraction, $P<0.001$). For L. digitata the highest OM and N digestibility was observed, with the lowest values observed for *U. lactuca* (OM digestibility: 0.68, 0.59 and 0.46, Species $P<0.001$, and N digestibility 0.62, 0.51 and 0.45, Species $P<0.001$ for *L. digitata*, *S. latissima* and *U. lactuca* respectively). The *in vitro* OM and N digestibility of all seaweed products increased significantly during incubation from 2 to 4h, with a small and largely insignificant increase during incubation from 4 to 6h ($P<0.001$ for OM and N digestibility). A larger increase in OM and N digestibility was observed for *L. digitata* and *S. latissima* with an increasing incubation time compared to *U. lactuca* (Interaction Species×Incubation Time $P<0.001$ for OM and N digestibility).

Table 3.4. Effect of ensiling and washing on *in vitro* organic matter and nitrogen digestibility of *Saccharina latissima*, determined with a modified Boisen method (Boisen and Fernandez, 1997) to simulate digestibility in the stomach and small intestine of monogastric species (*in vitro* experiment).

		OM digestibility			N digestibility		
Ensiling	Washing treatment	2h	4h	6h	2h	4h	6h
Fresh	Unwashed	0.53^{cA}	0.73^{bA}	0.75^{aA}	0.49^{bA}	0.81^{aA}	0.79^{aA}
	Washed	0.41^{cC}	0.64^{bB}	0.66^{aB}	0.41^{bB}	0.75^{aB}	0.75^{aAB}
Silage	Unwashed	0.42^{cB}	0.64^{bC}	0.66^{aC}	0.51^{bA}	0.76^{aB}	0.80^{aA}
	Washed	0.29^{cD}	0.59^{bD}	0.59^{aD}	0.40^{bB}	0.70^{aC}	0.72^{aB}
Soybean meal (reference)		0.76	0.86	0.84	0.89	0.98	0.98
LSD		0.045			0.047		
		P-value		SED	P-value		SED
Ensiling		<0.001		0.008	0.057		0.009
Washing		<0.001		0.008	<0.001		0.009
Incubation time		<0.001		0.010	<0.001		0.011
Ensiling × Washing		0.325		0.012	0.146		0.012
Ensiling × Incubation time		0.176		0.015	0.090		0.015
Washing × Incubation time		0.051		0.015	0.227		0.015
Ensiling × Washing treatment × Time		0.595		0.021	0.628		0.022

OM=organic matter, N=nitrogen, LSD=least significant difference, SED=standard error of differences.
Each digestibility value is based on 2 replicate measurements.
[a-c]Means within a row without a common superscript differ significantly (P<0.05) in OM or N digestibility.
[A-D]Means within a column without a common superscript differ significantly (P<0.05) in OM or N digestibility.

Table 3.5. Effect of extraction processes of fresh and ensiled seaweed on *in vitro* organic matter and nitrogen digestibility of selected seaweed products, determined with a two-step method as described by Boisen and Fernandez (1997) to simulate digestibility in the stomach and small intestine (*in vitro* experiment).

Seaweed species	Extraction	OM digestibility			N digestibility		
		2 h	4 h	6 h	2 h	4 h	6 h
S. latissima (ensiled)	Starting material	0.42^{bA}	0.64^{aC}	0.66^{aB}	0.51^{cA}	0.76^{bB}	0.80^{aA}
	Residue	0.22^{bC}	0.57^{aD}	0.55^{aC}	0.28^{bD}	0.61^{aD}	0.58^{aD}
L. digitata (fresh)	Starting material	0.44^{bA}	0.79^{aA}	0.80^{aA}	0.44^{bB}	0.83^{aA}	0.83^{aA}
	Residue	0.33^{cB}	0.70^{aB}	0.65^{bB}	0.40^{cC}	0.80^{aA}	0.76^{bB}
U. lactuca (fresh)	Starting material	0.41^{bA}	0.64^{aC}	0.64^{aB}	0.41^{bBC}	0.71^{aC}	0.69^{aC}
	Residue	0.20^{bC}	0.39^{aE}	0.41^{aD}	0.16^{cE}	0.39^{bE}	0.43^{aE}
Soybean meal (reference)		0.76	0.86	0.84	0.89	0.98	0.98
LSD		0.051			0.036		
		P-value		SED	*P*-value		SED
Species		<0.001		0.010	<0.001		0.007
Extraction		<0.001		0.008	<0.001		0.006
Incubation time		<0.001		0.010	<0.001		0.007
Species × Extraction		<0.001		0.014	<0.001		0.010
Species × Incubation time		<0.001		0.017	<0.001		0.012
Extraction × Incubation time		0.149		0.014	0.617		0.010
Species × Extraction × Incubation time		0.015		0.025	0.002		0.017

OM=organic matter, N=nitrogen, LSD=least significant difference, SED=standard error of differences.
Each digestibility value is based on 2 replicate measurements.
[a-c]Means within a row without a common superscript differ significantly (P<0.05) in OM or N digestibility.
[A-E]Means within a column without a common superscript differ significantly (P<0.05) in OM or N digestibility.

In vivo *experiment*

Inclusion of *S. latissima* silage and silage residue in the broiler diets enhanced feed intake (Table 3.6; P<0.001) without an effect on body weight gain. Consequently, an impaired FCR was observed in birds fed the seaweed supplemented diets (P<0.001). Birds fed the *S. latissima* residue diet showed a lower FCR compared to birds fed the *S. latissima* silage diet (1.77 and 1.86 for the residue and silage diet fed birds respectively). Inclusion of *S. latissima* silage and silage residue in the diet resulted in a lower pre-caecal digestibility of N (P<0.001) and most AAs (P=0.022 to P<0.001) compared to the basal diet (Table 3.7). Inclusion of *S. latissima* silage and silage residue in the diet also resulted in a lower total tract digestibility of OM (P<0.001) and ash (P<0.001) compared to the basal diet. No differences were observed in the calculated N, Ca and P digestibility between the *S. latissima* silage and silage residue product (Table 3.8). The average pre-caecal N digestibility was 0.68. The pre-caecal digestibility of all AAs, apart from methionine, was lower for the *S. latissima* silage than for the *S. latissima* silage residue.

Table 3.6. Effect of inclusion of 100 g/kg *Saccharina latissima* silage and silage residue on growth performance of broilers in the period that the experimental diets were fed (day 14 – 22; *in vivo* experiment).

Performance parameters	Diet			SED	*P*-value
	Basal diet	Basal with *S. latissima* silage	Basal with *S. latissima* residue[1]		
Initial body weight day 14 (g/bird)	504.4	507.0	511.3	5.20	0.435
Final body weight day 22 (g/bird)	1044	1020	1061	14.7	0.054
Day 14 to 22					
Feed intake (g)	890[b]	954[a]	974[a]	14.6	<0.001
Body weight gain (g/bird)	540	513	550	13.5	0.052
Feed conversion ratio (g/g)	1.65[c]	1.86[a]	1.77[b]	0.024	<0.001

SED=standard error of differences.
Each value is based on 6 (basal diet) or 5 (seaweed diets) replicate pens with 10 birds each.
[1]Residue after the aqueous extraction of *Saccharina latissima* silage.
[a-c]Means within a row without a common superscript differ significantly (P<0.05).

Discussion

This study investigated the effect of washing, ensiling and extraction of valuable components from seaweed on the nutritional value of seaweed and seaweed residues for broilers.

As generally observed (e.g. Øverland *et al.*, 2019; Bikker *et al.*, 2020), the intact seaweed samples in this study had a high ash content, over 250 g/kg DM. Poultry are sensitive to high dietary mineral levels (National Research Council, 2005), which may

Table 3.7. Effect of inclusion of 100 g/kg *Saccharina latissima* silage and silage residue in broiler diets on the *in vivo* pre-caecal and total tract nutrient digestibility of the diets (*in vivo* experiment).

Apparent pre-caecal digestibility	Diet			SED	P-value
	Basal	Basal with *S. latissima* silage	Basal with *S. latissima* residue[1]		
Nitrogen	0.936[a]	0.917[b]	0.925[b]	0.003	<0.001
Calcium	0.787	0.761	0.765	0.029	0.623
Phosphorus	0.864	0.861	0.871	0.019	0.884
Amino acids					
Lysine	0.960[a]	0.947[b]	0.954[ab]	0.003	0.003
Methionine	0.976[a]	0.970[b]	0.971[b]	0.002	0.006
Cysteine	0.831[ab]	0.815[b]	0.836[a]	0.006	0.022
Threonine	0.927[a]	0.917[b]	0.929[a]	0.003	0.009
Isoleucine	0.946[a]	0.925[b]	0.939[a]	0.003	<0.001
Arginine	0.951[a]	0.943[b]	0.951[a]	0.002	0.002
Phenylalanine	0.962[a]	0.930[c]	0.945[b]	0.003	<0.001
Histidine	0.955[a]	0.933[c]	0.944[b]	0.003	<0.001
Leucine	0.954[a]	0.928[c]	0.942[b]	0.003	<0.001
Valine	0.946[a]	0.934[b]	0.944[a]	0.003	0.001
Alanine	0.872[a]	0.732[b]	0.768[b]	0.018	<0.001
Aspartic acid	0.928[a]	0.898[c]	0.918[b]	0.004	<0.001
Glutamic acid	0.956[a]	0.935[c]	0.948[b]	0.002	<0.001
Glycine	0.905[a]	0.881[b]	0.896[a]	0.005	<0.001
Serine	0.927[a]	0.911[b]	0.928[a]	0.003	<0.001
Apparent total tract digestibility					
Ash	0.413[a]	0.262[b]	0.253[b]	0.015	<0.001
Organic matter	0.821[a]	0.726[c]	0.751[b]	0.009	<0.001
Fat	0.888[a]	0.794[b]	0.907[a]	0.016	<0.001
Crude fibre	-0.029	0.072	0.013	0.062	0.293

SED=standard error of differences.
Each value is based on 6 (basal diet) or 5 (seaweed diets) replicate pens with 10 birds each.
[1]Residue after the aqueous extraction of *Saccharina latissima* silage.
[a-b]Means within a row without a common superscript differ significantly (P<0.05).

lead to water overconsumption and result in diarrhoea and a reduced performance (Guiry and Blunden, 1991; Koreleski *et al.*, 2010). The high ash content in seaweed hampers the inclusion of seaweed in poultry diets at nutritionally significant levels (e.g. >50 g/kg). Washing seaweed can reduce the ash content as observed by Neveux *et al.* (2014), who reduced the ash content of *Ulva ohnoi* by 43% and that of *Derbesia tenuissima* by 83% with washing 3 times for 1 min by immersion in tap water, stirring and draining the water at each wash. Milledge *et al.* (2018) found a reduction in ash content of 21 g/kg in *Sargassum muticum*, after washing freshly harvested seaweed once in running tap water for 30 s. The ash composition in the latter study showed that sodium chloride in the ash fraction was reduced from 515 g/kg ash to 425 g/kg ash due to washing. In this study, a relative reduction in ash content of 9% in fresh *S. latissima* and 22% in *S. latissima* silage was observed. This smaller reduction compared to the study of Neveux *et al.* (2014) might be explained by difference in seaweed

Table 3.8. Pre-caecal amino acid and mineral digestibility of *Saccharina latissima* silage and silage residue in broilers (*in vivo* experiment).

	Saccharina latissima			
Apparent pre-caecal digestibility	Silage	Residue[1]	SED	P-value
Nitrogen	0.66	0.69	0.062	0.587
Calcium	0.71	0.72	0.089	0.891
Phosphorus	0.71	-0.53	2.255	0.597
Amino acids				
Lysine	0.79	0.91	0.040	0.014
Methionine	0.90	0.90	0.030	0.863
Cysteine	0.74	0.85	0.022	0.001
Threonine	0.84	0.95	0.030	0.006
Isoleucine	0.72	0.90	0.030	<0.001
Arginine	0.88	0.96	0.016	0.001
Phenylalanine	0.67	0.85	0.022	<0.001
Histidine	0.57	0.86	0.046	<0.001
Leucine	0.67	0.87	0.035	<0.001
Valine	0.83	0.93	0.024	0.003
Alanine	0.10	0.35	0.098	0.033
Asparagine + aspartic acid	0.64	0.86	0.033	<0.001
Glutamine + glutamic acid	0.54	0.88	0.045	<0.001
Glycine	0.78	0.86	0.024	0.007
Serine	0.75	0.94	0.026	<0.001
Apparent total tract digestibility				
Ash	-0.04	-0.12	0.058	0.215
Organic matter	0.02	0.13	0.106	0.334
Fat	-2.04	0.77	0.403	<0.001
Crude fibre	0.20	0.07	0.173	0.474

SED=standard error of differences.
Each value is based on 5 replicate pens with 10 birds each.
[1]Residue after the aqueous extraction of *Saccharina latissima* silage.

species, but also by the more gentle washing treatment applied in the current study. Other studies addressed the effect of washing *Saccharina spp.* on specific minerals, applying different temperatures (16°C and 32°C) and durations (1, 2, 6 and 22h) of washing (Stévant *et al.*, 2018). From their study, the authors could not conclude whether the measured loss of dry weight due to tap water soaking treatments was due to nutrients being removed from the seaweed or water being taken up by the seaweed due to osmosis. Although some of their washing procedures did sufficiently reduce cadmium and iodine content in the *Saccharina spp.* for human consumption, they only did so to an extent that allows for ingestion of very small quantities of seaweed (3.3 g dry weight per day). Nonetheless, the reduction in ash content in this study was not adequate to substantially increase the seaweed inclusion in broiler diets.

Moreover, washing also drastically reduced the *in vitro* OM and N digestibility. The applied *in vitro* digestibility model is based on the solubility of the tested materials,

hence, the reduction in soluble nutrients in washed samples automatically resulted in a lower *in vitro* digestibility. Other studies report a loss of nutrients like soluble fibres during washing, due to the difference in osmotic pressure between salt and fresh water, although digestibility was not taken into account (Stévant *et al.*, 2018). It should be noted that not all soluble material is necessarily digestible (Choct *et al.*, 2010), hence the *in vitro* digestibility based on solubility might overestimate the digestibility of (intact) seaweed with a high content of soluble nutrients.

In this study, the impact of ensiling *S. latissima* on composition and nutritional value for broiler chickens was determined. Ensiling reduced the ash content, and increased the protein content by approximately a twofold, with a lesser increase in OM content. Furthermore, a substantial decrease in OM digestibility was observed, as well as a small decrease in N digestibility.

In the literature, a decrease in fibre and an increase in protein content was observed as a result of a seaweed fermentation process using *U. lactuca* (Felix and Brindo, 2014), which is in line with our observations for *S. latissima* silage. The latter authors hypothesised that the increase in protein content was caused by microorganisms utilizing fibres, consequently increasing microbial biomass. The decrease in ash content and extra protein available in ensiled seaweed improved the nutritional value of seaweed silage. Nevertheless, the strong decrease in OM digestibility indicates that the nutrients in the OM of seaweed silage cannot be utilized by broilers as well as the OM in fresh seaweed. The changes in digestibility might be largely explained by the process of ensiling. During ensiling, a part of the fluid from the silage was drained, and with that fluid also soluble nutrients will have drained. This might explain the decrease in OM digestibility, since the simulated digestibility model is based on solubility of the samples.

The nutritional value of seaweed residues was evaluated after extraction of components that can be used for food or chemical application to contribute to the economic value of seaweed production. Because of the high costs of production, harvest and processing of seaweed, such a biorefinery approach may be required for the economic feasibility of seaweed production (Van den Burg *et al.*, 2013; Torres *et al.*, 2019). After extraction, a higher ash and OM content, and specifically in *U. lactuca* a lower N content were observed, in combination with a lower OM and N digestibility compared to the original material. The reduction in ash content and increase in OM and N content due to extraction should, in theory, improve the nutritional value. However, the lower digestibility negatively affected the nutritional value of the seaweed residue products for broilers. Extraction of both *S. latissima* and *L. digitata* did not only result in removal of mannitol, but also of a substantial portion of other nutrients and minerals, presumably due to washing and cell disruption caused by osmosis (Van Hal and Huijgen, 2014). This loss of soluble nutrients might have caused the decrease in digestibility as observed for the residues of *S. latissima* and the *L. digitata*, and showed a similar effect on digestibility as washing *S. latissima*. In *U. lactuca* a large increase in N content was observed, although the digestibility was largely decreased. The extraction process for *U. lactuca* was performed in an acidic environment at 100°C. A large part of the minerals, and the polysaccharides rhamnose, glucuronic acid (both part of Ulvan, the

main polysaccharide in *Ulva spp.*), glucose and xylose were extracted (Groenendijk *et al.*, 2016), as also described in literature (Kidgell *et al.*, 2019). Potentially, the more severe acidic extraction at a higher temperature solubilized and removed more nutrients than the watery extraction did in *S. latissima* and *L. digitata*, explaining the differences in remaining soluble nutrients, thus in chemical composition, and with that digestibility. For example, Ulvan (main structural component of *Ulva spp.*) extraction was dependent on temperature and duration of the extraction procedure (Kidgell *et al.*, 2019). Other authors observed a relative increase of 20% and 5% in OM and N digestibility, respectively, of an *U. lactuca* product resulting from a biorefinery approach (Bikker *et al.*, 2016). These authors hypothesised that the enzymatic hydrolysis applied in their study degraded a large portion of the ileal indigestible carbohydrates, improving digestibility for monogastrics. This indicates that such an enzymatic hydrolysis, either as part of the extraction process or by itself, might be a good treatment to increase digestibility of seaweed products.

To quantify seaweed digestion in broilers, an *in vivo* experiment was carried out with *S. latissima* silage and silage residue added to broiler diets. In the residue product, sugars and other soluble nutrients were extracted with a watery alkaline extraction process by bruising and thereafter soaking and draining the seaweed silage. Nonetheless, the chemical composition did not show large differences between the silage and silage residue product. During the ensiling process, excess fluid was drained from the silage, already leading to a loss of soluble nutrients from the silage product. This explains at least part of the reason for the small differences in chemical composition between the silage and silage residue products. Furthermore, the sodium content was increased in the silage residue product. Sodium carbonate was added to establish alkaline extraction conditions, although the electrolyte balance was levelled between diets by adding potassium. Inclusion of either of the seaweed products significantly enhanced the feed intake of both treatment groups without beneficial effect on BW gain, consequently showing an increased FCR. Birds fed the silage residue diet performed better than birds fed the silage diet based on the lower FCR and higher final BW, which was likely caused by the low digestibility of the seaweed products. Literature on brown seaweed fed to poultry at nutritionally significant levels (>50 g/kg) is scarce. In one study, 20–60 g/kg fresh, boiled and autoclaved brown seaweed *Sargassum dentifebium* was added to finisher diets for broilers (El-deek *et al.*, 2011). These authors did not observe differences in chemical composition or metabolizable energy of the seaweed products due to boiling for 20 min or autoclaving at 121°C for 20 min. Additionally, no differences in the analysed metabolizable energy of the experimental diets, due to concentration or pre-treatment of the seaweed products added to the diets, were observed. Despite the lack of differences, birds fed the seaweed diets performed worse based on BW, BW gain, feed intake and FCR with increasing concentrations of seaweed products added and with increasing severity of treatment of the seaweed product. The authors did not provide a clear explanation of these results, although they did not measure digestibility of the seaweed products. Furthermore, the differences in behaviour of the chyme in the intestinal tract of the birds, due to the inclusion of the differently treated seaweed products, were not analysed.

The digestibility of crude fibre was close to zero in the current study, both in the basal diet and the seaweed diets. This might be related to the other dietary ingredients being highly soluble, which might enhance shorter retention times of the digesta in the GIT. The negative apparent total tract digestibility values of for example certain inorganic components of the silage and silage residue products, indicated an interaction effect of the seaweeds with the basal diet.

Some differences were observed between the silage and silage residue products used in the *in vitro* and *in vivo* study. Compared to the silage product, the residue product used in the *in vitro* studie showed a higher OM and a lower N content, as well as a lower OM and N digestibility. The opposite was observed for the products used in our *in vivo* study: compared to the silage product, in the residue product a lower OM and higher N content were observed, without differences in OM and N digestibility. The alkaline extraction process applied to the residue product used in the *in vivo* experiment, might have extracted different nutrients compared to non-alkaline conditions, leading to differences in chemical composition. Furthermore, large intra-species variations in chemical composition may explain part of the observed differences, since the products used in the *in vitro* and *in vivo* study originated from different batches. Intraspecies differences are ascribed to, amongst others, season of harvest, geographical characteristics and environmental factors (Schiener *et al.*, 2015; Boderskov *et al.*, 2016; Sharma *et al.*, 2018). Additionally, when comparing *in vitro* and *in vivo* digestibility, it should be taken into account that the *in vitro* digestibility values reflect the solubilization of nutrients. Since not all soluble material is digestible, the *in vitro* analyses might overestimate digestibility. Furthermore, the seaweed products within the diets fed to the broilers might have interacted with the diet. For example, the viscosity of the chyme in the intestinal tract of the broilers might be altered by viscous substances (polysaccharides) in the seaweed products, which may influence the digestibility of the diets as a whole (Holdt and Kraan, 2011; Burg *et al.*, 2012; Matthiesen *et al.*, 2021). This means that the digestibility of the seaweed products in the *in vivo* experiment, as calculated by difference in digestibility compared to the basal diet, does not only reflect the digestibility of the seaweed products but also their impact on the digestibility of the basal diet.

Conclusion

The results of this study demonstrate that washing reduces the ash content of seaweed products, but simultaneously may reduce the nutritional value of the seaweed. Ensiling, as well as an extraction process as part of a biorefinery approach also reduces the nutritional value of seaweed.

From this study we conclude that the process of washing or ensiling alone does not make seaweed suitable for inclusion in broiler diets. Additional steps to be taken in order to create suitable feed ingredients out of seaweed products include a further reduction of the ash content, and an increase in digestibility. The latter might be

achieved by different methods, like enzymatic hydrolysis, using suitable enzymes for seaweed species, related to the different chemical composition compared to land based plants. It is important to also gain more understanding of the behaviour of seaweed products in *in vitro* digestibility analyses as well as their behaviour in the gastro intestinal tract of broilers. This may allow to evaluate more precisely the nutritional value of the seaweed products. With this, a better understanding of the interaction between the seaweed products and the basal diets, including the consequences for the birds, their performance, and their health can be obtained.

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Chapter 4

A proteolytic enzyme treatment to improve *Ulva laetevirens* and *Solieria chordalis* seaweed co-product digestibility, performance and health in broilers

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† Dr. Van Krimpen sadly passed away during the conduct of this study.

Poultry Science, submitted

Abstract

To explore the potential use of seaweed co-products for broiler diets, this study investigates whether an enzyme treatment of seaweed co-products improves performance, *in vivo* digestibility and health in broilers. In total, 360 Ross 308 male broilers were fed one of five experimental diets: a basal diet, or a basal diet including the *U. laetevirens* or *S. chordalis* co-product, with or without proteolytic enzyme pre-treatment of the seaweed. The starter (d0-13) and grower (d14-21) diet contained 5 and 10% (w/w) seaweed product, respectively. Inclusion of seaweed in the broiler diets increased body weight gain (+14%), and feed intake (+12%) in the third week of the experiment. Birds fed the *U. laetevirens* compared to the *S. chordalis* diets had a higher body weight gain (+11%), and a lower feed conversion ratio (-7%). Seaweed inclusion reduced diet apparent faecal and ileal digestibility ($P<0.05$). Birds fed *U. laetevirens* vs. *S. chordalis* diets had a 10% reduced villus length. Enzymatic treatment reduced the digestibility of most nutrients, and increased crypt depth in birds fed the *U. laetevirens* diets, whereas the opposite was observed for the birds fed the *S. chordalis* diets (Seaweed×Enzyme $P=0.035$). Untreated versus treated seaweed in the diets led to lower (-60%) plasma Interleukin-13 levels. In conclusion, the proteolytic enzyme pre-treatment of the seaweed co-products did not improve performance or health-related parameters, and even reduced digestibility of the diets. Dietary inclusion of *U. laetevirens* co-products did improve performance based on growth and FCR. Inclusion of *U. laetevirens* in broiler diets had a slight negative effect on duodenal villus length, and a positive effect on crypt depth. The inflammation response was strongly reduced in birds fed the untreated *U. laetevirens* diet, making the *U. laetevirens* co-product of interest for future research.

Keywords: seaweed; broiler nutrition; novel feed source; digestibility; health

Introduction

To ensure a more sustainable broiler production, and to meet the increasing demand for animal derived foods, novel feed ingredients for broiler diets, especially protein sources, are receiving significant attention. Seaweed has several favourable characteristics as a protein source for animals, including a lack of required arable land or fresh water during production, as well as a current absence of significant competition as a food source. Additionally, seaweed can have a high protein content of up to 38% on dry matter (DM) basis and many species have a high concentration of important fatty acids (Biancarosa *et al.*, 2017; Øverland *et al.*, 2019).

Besides these positive characteristics, the current use of seaweeds as a feed ingredient also provides a number of challenges. For example, the nutritional composition of seaweed is highly variable, both within and between species, and generally the content of certain minerals is relatively high (Biancarosa *et al.*, 2017; Øverland *et al.*, 2019). The latter can be harmful when fresh seaweed is used as a feed ingredient, as for example an excessive sodium content may lead to the overconsumption of water and consequently induce diarrhoea (Koreleski *et al.*, 2010). Moreover, untreated seaweeds are relatively poorly digested by monogastric animals (Bikker *et al.*, 2020) and fresh seaweed has a limited shelf life after harvest (Paull and Chen, 2008; Stévant *et al.*, 2017). Currently inclusion of whole seaweed as a feed ingredient for simple stomached animals is not economically viable.

A biorefinery approach creating multiple fractions is suggested to overcome a number of the above-mentioned drawbacks (Bikker *et al.*, 2016; Torres *et al.*, 2019; Bikker *et al.*, 2020). For example, high value components or fractions can be extracted leaving a more cost-effective by/co-product for animal feed (Torres *et al.*, 2019). Due to the low DM content of seaweed, pressing is a possible way of fractionation which will result in a liquid fraction containing many soluble nutrients and cell contents, and a solid fraction, or co-product, containing mostly cell walls and insoluble complexes.

To be able to include seaweed co-products in animal feed, the co-products need to have an acceptable mineral (i.e. salt) and heavy metal content (Besada *et al.*, 2009), and a high nutrient digestibility for simple-stomached animals. The mineral content of seaweed or seaweed co-products can be reduced by washing with fresh water (Neveux *et al.*, 2014), while improvement in nutrient digestibility might be achieved through the use of one or a combination of enzymes. Regarding the latter, multiple feed grade enzymes are currently commercially available such as proteolytic and carbohydrytic enzymes, each targeting specific molecular bonds. Although studies have been conducted using seaweed (co-products) in animal feed (e.g. Abudabos *et al.*, 2013; Matshogo *et al.*, 2020), little information is available on the effects of treatments aimed to increase the digestibility of seaweed co-products, or the effect of such treatments on the nutritional value of the seaweed co-products for broiler chickens (Van Krimpen and Hendriks, 2019). Simultaneously, there is a lack of knowledge of the effects of seaweed co-products on bird health, and whether these seaweed co-products are suitable, or even favourable, for inclusion in broiler diets.

This study investigated whether a proteolytic enzyme treatment to seaweed co-products can improve performance and *in vivo* pre-caecal and total tract digestibility when included in a diet for broilers, and investigated effects on select health-related parameters (intestinal content pH, histology, plasma cytokine levels). The seaweed co-products tested were the remaining solid fractions after pressing and washing of the green seaweed *Ulva laetevirens* (*U. laetevirens*) and the red seaweed *Solieria chordalis* (*S. chordalis*).

Material and methods

The animal experiment was conducted at the facility of Wageningen University & Research in Lelystad, the Netherlands. All experimental procedures were approved by the Animal Care and Use Committee of Wageningen University & Research, the Netherlands (AVD401002015196).

Seaweed harvesting and processing

Both seaweed species were obtained from and processed by Olmix S.A. (Olmix Group, Bréhan, France) and harvested from a beach in France near Guisseny on September 30th, 2014 and Saint Hilaire de Riez on May 13th and 14th, 2019, respectively. Upon harvest and immediate washing with fresh water, *U. laetevirens* was frozen and *S. chordalis* was directly processed. *U. laetevirens* was defrosted, ground to 50-1000 nm particles (Inotec I175CDI-75D, Reutlingen, Germany) and pressed twice using a belt press (Flottweg BFRU 800, Vilsbiburg, Germany) at 6 bar with intermediate rehydration (DM=196 g/kg) with fresh water, while *S. chordalis* was only pressed once (DM=171 g/kg). The enzymatic treatment consisted of addition of 0.5% Alcalase (Soufflet Biotechnologies, ≥3000 U/g) and Neutrase (Novozymes; 0.8 AU-N/g) to the seaweed cake (co-product) on a dry weight basis at 50°C for 5 h under low agitation, followed by a 5 min enzyme inactivation step at 90°C. Both untreated and enzyme treated seaweed co-products were air-dried at 60°C for 48 h followed by 24 h at 50°C up to 90% dry matter (DM). Finally, all seaweed co-products were ground to pass a 1 mm sieve before inclusion in the experimental diets. The nutrient composition of the four seaweed co-products including their detailed mineral and amino acid (AA) composition is presented in Table 4.1.

Animals and housing

A total of 360 one-day-old male broilers with an average body weight (BW) of 44.4±0.73 g (Ross 308, Probroed and Sloot, Groenlo, the Netherlands) were randomly assigned to one of 30 pens with 12 birds per pen and pen weight being kept within a 3% difference from the average pen weight. Each pen (1.5 m×1.0 m) had a solid floor covered with wood shavings. All birds were vaccinated against infectious bronchitis at

Table 4.1. Analysed nutrient content of untreated (-) and enzymatically[1] treated (+) seaweed (*Ulva laetevirens* and *Solieria chordalis*) co-products.

Nutrient	*U. laetevirens*		*S. chordalis*	
Component	-	+	-	+
Gross nutrient content (g/kg dry matter)				
Dry matter (DM, g/kg)	943.6	942.3	942.3	919.5
Ash	151.8	151.3	328.7	321.0
Nitrogen (N)	49.1	49.4	38.2	35.7
Crude fat	16.5	19.6	5.6	5.8
Crude fibre	112.5	120.7	100.4	104.3
Sugar	4.1	5.0	16.7	13.7
Starch	19.5	21.1	20.1	8.3
Non starch polysaccharides[2]	562.7	556.1	437.7	472.6
Macro-minerals (g/kg DM)				
Calcium	11.8	12.0	15.1	15.7
Phosphorus	2.0	2.0	2.4	2.5
Potassium	18.5	17.9	85.3	84.1
Sodium	8.8	8.6	13.1	12.9
Chloride	11.1	8.8	40.4	39.3
Magnesium	17.3	17.0	4.7	4.8
Sulphur	37.9	37.1	56.6	54.1
Micro-minerals (mg/kg DM)				
Iron	846	847	2165	2186
Copper	<5	5.4	8.6	10.2
Manganese	30	30	167	173
Zinc	32	29	74	72
Arsenic	12.0	11.2	7.7	7.7
Cadmium	0.29	0.31	0.26	0.29
Cobalt	0.4	0.4	1.2	1.3
Mercury	<0.01	<0.01	0.07	0.08
Lead	1.4	1.3	5.0	5.5
Nickel	4.8	4.7	5.2	5.9
Selenium	2.6	2.5	11.9	12.6
Amino acids (AA, g AA-N/100 g N)				
Lysine	6.1	6.0	4.8	5.0
Methionine	1.1	1.1	0.9	0.8
Cysteine	1.3	1.2	2.2	2.2
Threonine	3.6	3.5	2.8	2.6
Tryptophan	1.2	1.2	1.1	0.9
Leucine	4.5	4.3	3.6	3.2
Isoleucine	2.7	2.6	2.4	2.2
Histidine	3.5	3.6	3.9	3.7
Phenylalanine	2.7	2.6	2.2	2.1
Arginine	10.4	10.2	15.8	15.5
Asparagine + aspartic acid	10.5	10.3	8.2	8.0
Serine	4.0	3.9	2.7	2.5
Glutamine + glutamic acid	8.4	8.2	7.3	6.9
Glycine	6.8	6.7	6.7	6.5
Alanine	8.2	8.0	4.9	4.7
Valine	4.3	4.2	3.7	3.4
Proline	3.3	3.3	3.6	3.7
Tyrosine	1.5	1.5	1.7	1.6
Taurine	0.0	0.0	0.4	0.5

Table 4.1 (continued). Analysed nutrient content of untreated (-) and enzymatically[1] treated (+) seaweed (*Ulva laetevirens* and *Solieria chordalis*) co-products.

Nutrient	*U. laetevirens*		*S. chordalis*	
Component	-	+	-	+
Total amino acids (g/kg DM)	276.6	273.2	193.6	174.2
Amino acid-nitrogen (g/kg DM)	41.2	40.7	30.1	27.2
Amino acid-nitrogen (g/100 g N)	83.9	82.5	78.8	76.2
Protein (g/kg DM)[3]	236.2	233.2	165.9	149.2
N:protein factor, K_p[4]	4.81	4.72	4.34	4.18
N:protein factor, K_a[5]	5.73	5.73	5.51	5.48

AA-N=amino acid nitrogen

[1]Alcalase (Soufflet Biotechnologies, ≥3000 U/g) and Neutrase (Novozymes; 0.8 AU-N/g).

[2]Calculated as NSP=1000–ash–(N-content×5.0)–crude fat–(starch+sugars).

[3]Sum of anhydrous amino acids.

[4]Sum of anhydrous amino acids (g/kg DM) to nitrogen (g/kg DM) as per Mariotti *et al.* (2008).

[5]Sum of anhydrous amino acids (g/kg DM) to amino acid-nitrogen (g/kg DM) as per Mariotti *et al.* (2008).

arrival, and against Newcastle disease at d 13. At d 16, the bedding material and solid floors were removed and replaced with slatted floors to enable collection of excreta. Each pen was assigned to one of five treatments in a completely randomized block design with six replicate pens per treatment. Ambient temperature was maintained at 32°C for the first three days and thereafter was gradually reduced to 22°C on d 21. A 23L:1D photoperiod was applied on the day of arrival, where after the dark period was increased by 1 h every day until a 16L:8D light schedule was achieved. Birds had ad libitum access to feed and water. At the end of the experiment at d 21, all birds were euthanized by an intravenous sodium pentobarbital injection in the wing vein.

Experimental diets

All starter (d 0-13) and grower (d 14-21) diets were formulated to meet or exceed the nutrient requirements for broilers (CVB, 2019). The grower diet was supplemented with 5 g/kg titanium (Ti) dioxide as an indigestible marker to allow determination of digestibility values. All diets were produced by Research Diet Services (Wijk bij Duurstede, the Netherlands) and fed as pellets (starter: 2.5 mm, grower: 3.2 mm). The seaweed containing diets consisted of the basal diet with either 5 (starter) or 10% (grower) seaweed co-product. The ingredients of the diets and analysed nutrient composition of the grower diets are presented in Tables 4.2 and 4.3, respectively.

Performance measurements

Feed intake (FI) and water intake were monitored weekly per pen. Average BW per pen was determined upon arrival at the experimental facility, and again at day 7, 14 and 21. The feed conversion ratio (FCR) over a period was calculated as: total pen FI over the period/(pen BW end of period–pen BW start of period+pen BW of dead or culled birds) with FI per bird corrected for mortality calculated as: FCR×BW gain.

Table 4.2. Composition of the basal and untreated (-) and enzymatically[1] treated (+) seaweed (*Ulva laetevirens* and *Solieria chordalis*) co-product containing starter (d 0 to 13) and grower (d 14 to 21) diets for broilers.

Ingredient (g/kg)	Starter diet					Grower diet				
	Basal	*U. laetevirens*		*S. chordalis*		Basal	*U. laetevirens*		*S. chordalis*	
		-	+	-	+		-	+	-	+
Maize starch	552.3	524.3	524.3	524.3	524.3	600.0	543.1	543.1	543.1	543.1
Soybean meal	200.0	189.8	189.8	189.8	189.8	120.0	108.7	108.7	108.7	108.7
Ulva laetevirens	-	50.0	50.0	-	-	-	100.0	100.0	-	-
Solieria chordalis	-	-	-	50.0	50.0	-	-	-	100.0	100.0
Oat hulls	80.0	75.9	75.9	75.9	75.9	80.0	72.4	72.4	72.4	72.4
Dextrose	50.0	47.4	47.4	47.4	47.4	55.4	50.2	50.2	50.2	50.2
Casein	36.3	34.4	34.4	34.4	34.4	61.5	55.7	55.7	55.7	55.7
Soya oil	19.4	18.4	18.4	18.4	18.4	23.8	21.6	21.6	21.6	21.6
Monocalcium phosphate	17.5	16.6	16.6	16.6	16.6	9.8	8.9	8.9	8.9	8.9
Chalk	8.4	8.0	8.0	8.0	8.0	12.6	11.4	11.4	11.4	11.4
Premix[2]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Titanium dioxide	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Potassium carbonate	3.8	2.0	2.0	2.0	2.0	6.9	2.8	2.8	2.8	2.8
Sodium bicarbonate	2.5	3.0	3.0	3.0	3.0	2.3	0.4	0.4	0.4	0.4
Magnesium oxide	0.5	-	-	-	-	1.0	-	-	-	-
Salt	2.7	-	-	-	-	2.8	-	-	-	-
DL-Methionine	4.1	3.9	3.9	3.9	3.9	3.7	3.4	3.4	3.4	3.4
L-Arginine	3.3	3.1	3.1	3.1	3.1	4.3	3.9	3.9	3.9	3.9
L-Lysine HCl	3.0	2.8	2.8	2.8	2.8	2.5	2.3	2.3	2.3	2.3
L-Threonine	2.1	2.0	2.0	2.0	2.0	-	-	-	-	-
L-Valine	1.8	1.7	1.7	1.7	1.7	1.4	1.3	1.3	1.3	1.3
L-Leucine	1.7	1.6	1.6	1.6	1.6	1.4	1.3	1.3	1.3	1.3
L-Isoleucine	0.6	0.6	0.6	0.6	0.6	0.6	0.5	0.5	0.5	0.5
Diamol	-	4.5	4.5	4.5	4.5	-	2.1	2.1	2.1	2.1
AME (MJ/kg)	12.30	11.80	11.80	11.80	11.80	12.85	11.90	11.90	11.90	11.90

AME=apparent metabolizable energy

[1]Alcalase (Soufflet Biotechnologies, ≥3000 U/g) and Neutrase (Novozymes; 0.8 AU-N/g).

[2]Provided per kg of diet: vitamin A, 10,000 IU; vitamin D_3, 2,500 IU; vitamin E, 50 mg; vitamin K_3, 1.5 mg; vitamin B_1, 2.0 mg; vitamin B_2, 7.5 mg; vitamin B_6, 3.5 mg, vitamin B_{12}, 20 µg; niacin, 35 mg; D-pantothenic acid, 12 mg; folic acid, 1.0 mg; biotin, 0.2 mg; Fe, 80 mg; Cu, 12 mg; Mn, 85 mg; Zn, 60 mg; I, 0.8 mg; Se, 0.15 mg.

Table 4.3. Analysed nutrient content of the basal and untreated (-) and enzymatically[1] treated (+) seaweed (*Ulva laetevirens* and *Solieria chordalis*) co-product containing grower diets as fed to the broilers.

Nutrient	Basal	*U. laetevirens*		*S. chordalis*	
Component		-	+	-	+
Gross nutrient content (g/kg dry matter (DM))					
Dry matter (g/kg)	888.9	888.9	890.2	889.7	889.7
Ash	54.3	59.6	59.1	73.8	73.3
Nitrogen	23.2	26.0	26.4	25.6	25.3
Crude fat	22.8	21.3	21.2	18.9	21.5
Crude fibre	35.8	43.9	42.6	42.4	42.4
Sugars	62.8	59.0	59.8	60.9	54.4
Starch	588.3	537.0	533.2	546.7	531.1
Non-starch polysaccharides[2]	155.7	193.4	194.7	171.8	193.0
Titanium	3.9	3.9	3.9	3.9	4.0
Amino acids (g AA-N/100 g nitrogen)					
Lysine	8.4	8.3	8.4	8.1	7.9
Methionine	2.5	2.4	2.3	2.2	2.2
Cysteine	0.7	0.8	0.8	0.9	0.9
Threonine	2.8	2.8	2.7	2.7	2.6
Tryptophan	1.1	1.1	1.1	1.0	0.9
Leucine	5.1	4.9	4.9	4.7	4.6
Isoleucine	3.2	3.1	3.1	3.0	2.9
Histidine	4.8	4.4	4.6	4.6	4.3
Phenylalanine	2.5	2.5	2.5	2.4	2.3
Arginine	15.6	14.5	14.7	15.3	15.2
Asparagine + aspartic acid	8.2	8.4	8.4	8.0	7.6
Serine	3.8	3.4	3.2	3.4	3.4
Glutamine + glutamic acid	15.8	14.3	14.3	14.2	14.0
Glycine	3.2	3.9	4.0	3.8	3.5
Alanine	3.5	4.3	4.3	3.6	3.4
Valine	4.6	4.6	4.6	4.4	4.3
Proline	5.7	5.2	5.2	5.2	5.1
Tyrosine	1.9	1.8	1.9	1.9	1.8
Taurine	0.0	0.0	0.0	0.1	0.1
Total amino acids (g/kg DM)	144.2	157.0	160.0	151.1	145.9
Amino acid-nitrogen (g AA-N/kg DM)	21.7	23.5	24.0	22.9	22.1
Amino acid-nitrogen (g/100 g N)	93.4	90.7	91.1	89.4	87.1
Protein (g/kg DM)[3]	124.6	135.4	137.7	130.4	126.0
Nitrogen:protein factor, K_p[4]	5.37	5.22	5.22	5.10	4.97
Nitrogen:protein factor, K_a[5]	5.75	5.75	5.73	5.71	5.71

[1]Alcalase (Soufflet Biotechnologies, ≥3000 U/g) and Neutrase (Novozymes; 0.8 AU-N/g).
[2]Calculated as NSP=1000–ash–(N-content×5.0)–crude fat–(starch+sugars).
[3]Sum anhydrous amino acids.
[4]Sum anhydrous amino acids (g/kg DM) to nitrogen (g/kg DM) as per Mariotti *et al.* (2008).
[5]Sum anhydrous amino acids (g/kg DM) to amino acid-nitrogen (g/kg DM) as per Mariotti *et al.* (2008).

Sample collection and chemical analyses

Excreta were collected qualitatively from d 19 to 21, after which all birds were euthanized and ileal contents were collected from the distal 20 cm of the ileum, anterior to the ileo-caecal valve. Excreta and ileal digesta were stored at -20°C until further

processing. Before chemical analyses, excreta and ileal digesta were freeze dried, and all samples were ground using a 1 mm screen. All seaweed co-products and diets were analysed for DM (ISO 6496, 1999), ash, (ISO 5984, 2002), nitrogen (N; ISO 5983, 2005), ether extract (crude fat; ISO 6492, 1999), fibre (ISO 6865, 2002), starch (ISO 15914, 2004), sugar (European Commission, 2009), tryptophan (ISO 13904, 2005) and other AAs (ISO 13903, 2005) as well as Ti (Short *et al.*, 1996). Furthermore, Ca, P, Na, K and Cl were analysed (ISO 27085, 2009; ISO 6495, 2015). In the seaweed co-products, Fe, Mn, Mg, Zn and Cu were analysed (ISO 27085, 2009) as well as As, Cd, Pb, Hg, Co, Se, Ni and S (DIN EN 15763, 2009). The ileal digesta samples were analysed for DM, ash, N, AAs and Ti, and the excreta samples were analysed for DM, ash, fat, fibre, starch, sugar and Ti. Organic matter (OM) was calculated as 1000–ash. Non-starch polysaccharide content (NSP) was calculated as 1000–ash–(N-content×5.0)–crude fat–(starch+sugars).

Health-related parameters

At d 21, from two birds per pen with a BW close to the average pen BW, additional samples were collected. Before euthanasia, a blood sample was collected (4 mL) from the left wing vein of those two birds for analysis of interleukin 13 (IL-13) and haptoglobin levels. Blood was centrifuged at 2500 rpm for 15 min, the plasma (>500 µL) was transferred to Micronic tubes, and stored at -20°C pending analyses. ELISA kits specific for chicken haptoglobin (AbClonal, Woburn, United States) and IL-13 (Elabscience, Houston, Texas, United States) were used to determine cytokine levels according to the manufacturers' instructions.

Of those same two birds, after euthanasia full and empty gizzard weight were determined for potential effects of the seaweed co-products on gizzard development. The gizzard was separated from the proventriculus and the duodenum, and the full gizzard weighed. Gizzard contents were removed by rinsing with demineralized water and gentle drying using a paper towel before the empty gizzard was weighed.

After euthanasia of the same two birds per pen, a 1 cm long piece of the duodenum was collected for histological analyses. The duodenum was separated from the gizzard and the jejunum. The pancreas was then removed from the duodenal loop. The 1 cm long piece of the proximal duodenum was dissected just before the loop. This tissue sample was gently rinsed in a physiological salt solution (0.9% NaCl) to remove any remaining digesta from the intestine before being stored in a phosphate buffered 10% formalin fixative at 4°C until further analyses. Before analyses, tissue samples were rinsed twice with tap water, and once with 70% alcohol, upon storage in 70% alcohol. The samples were cut in rings of ~3 mm length, placed in histology cassettes and embedded in paraffin using the Leica TP1020 tissue processor (Leica Microsystems B.V., Amsterdam, The Netherlands). The embedded tissue samples were cut in 5 µm thin sections, stretched, and placed on glass slides. Samples were stained using Mayer's haematoxylin and eosin standard staining protocols. A Lyca DM6b microscope and LASX software (Leica Microsystems B.V., Amsterdam, The Netherlands) were

used to measure villi length, crypt depth and tunica muscularis thickness. Villus length was defined as the distance from the tip of a villus to the villus-crypt junction. Crypt depth was defined as the distance from the villus-crypt junction to the circular muscle layer. The tunica muscularis thickness was defined as the distance between the start of the circular muscle layer to the serosa. The villi length to crypt depth ratio was calculated.

After euthanasia, the jejunum was separated from the duodenum and ileum of the same two birds per pen. Jejunal content was gently squeezed out and stored in a 5 mL Eppendorf tube. The jejunal content of both birds per pen was pooled to obtain sufficient material to measure pH using a Mettler Toledo Seven2Go™ pH Meter (Mettler-Toledo AG, Analytical CH-8603, Schwerzenbach, Switzerland).

Calculations and statistical analyses

Performance parameters were calculated using FI and BW measurements over time. Apparent pre-caecal digestibility and apparent total tract digestibility of nutrients in the experimental diets were calculated, using Ti as a marker according to the following equation:

$$DC(X) = \left(1 - \frac{[Ti]diet \times [X]sample}{[Ti]sample \times [X]diet}\right) \times 100$$

where DC(X) is the apparent digestibility coefficient of nutrient X in % and $[Ti]_{diet}$, $[Ti]_{sample}$, $[X]_{diet}$, and $[X]_{sample}$ are the concentrations of Ti and nutrient X in the diet and digesta or excreta sample in g/kg, respectively. The apparent pre-caecal digestibility and apparent total tract digestibility of nutrients in the seaweed co-products were calculated applying the difference method (Kong and Adeola, 2014) assuming additivity.

Data were analysed using SAS statistical software (version 9.4, SAS Institute Inc., Cary, NC). For all, except for histological data, a general linear model with contrast statements was used to 1) determine differences between birds fed the basal diet and those fed the seaweed containing diets and 2) determine the effects of seaweed co-product and enzymatic treatment in a 2×2 factorial design. For histological data, a similar approach was taken as described above for all parameters except for muscularis thickness. Because of the non-linear distribution of residuals, a generalized linear model was used for the latter. Model assumptions and goodness of fit were evaluated through normal distribution of residuals, and data were square root or log transformed when necessary. One pen (untreated *S. chordalis*) was excluded of the digestibility analysis based on an outlier test with studentized residuals >3 standard deviations from the sample mean. Data are presented as means unless stated otherwise with differences among means with a probability <0.05 considered significant.

Results

Nutritional composition

The *U. laetevirens* untreated and enzymatically treated co-product contained lower amounts of ash (15 vs. 32%) and more N (4.9 vs. 3.7%), AAs (27 vs. 18%) and NSP (56 vs. 46%) compared to the two *S. chordalis* co-products (Table 4.1). The mineral composition differed between seaweed species and was not majorly impacted by the enzymatic treatment. The *S. chordalis* co-products contained higher levels of almost all macro and micro minerals (including heavy metals), whereas the *U. laetevirens* co-products contain higher concentrations of magnesium and the heavy metals cadmium and arsenic. All analysed micro minerals in the seaweed co-products were well within the limitations based on the European regulations for ingredients in animal diets, except for iron (EG 1334/2003; European Commission, 2002). High iron levels of over 2100 mg/kg DM were observed in the treated and untreated *S. chordalis* co-products. The AA composition differed slightly between *U. laetevirens* and *S. chordalis* with the enzymatic treatment having no major effect on any of the AAs. Low levels of taurine were observed in *S. chordalis*, but not in *U. laetevirens*.

Solieria chordalis containing diets had 24 and 35% more ash compared to *U. laetevirens* and the basal diets, respectively (Table 4.3). All seaweed containing experimental grower diets had more fibre (18-22%), NSP (10-25%) and N (9-14%) and less starch (7-9%) compared to the basal diet.

Performance

Performance data are summarized in Table 4.4. In the first week of the experiment, FCR was higher for birds fed the seaweed diets compared to birds fed the basal diet (1.02 vs. 0.95; P=0.006). Furthermore, the FCR was higher for birds fed the *U. laetevirens* compared to the *S. chordalis* diets (0.99 vs. 1.05; P=0.014). In the second week, the same basal vs. seaweed diet effect was observed (P=0.008) as in week 1. Contrary to the first week, however, FCR was lower for birds fed *U. laetevirens* compared to the *S. chordalis* diets (1.18 vs. 1.23; P=0.001). Furthermore, an increased water intake was observed of birds fed the seaweed diets compared to those fed the basal diet (880 vs. 763 mL; P=0.003) and for birds fed the *S. chordalis* diets compared to those fed the *U. laetevirens* diets (943 vs. 817 mL; P<0.001). In the third week, BW gain was higher for birds fed the seaweed compared to those fed the basal diet (355 vs. 310 g; P=0.002), and higher for birds fed the *U. laetevirens* compared to the *S. chordalis* diets (374 vs. 336 g; P=0.007). Simultaneously, FI in the same week was higher for birds fed the seaweed diets compared to birds fed the basal diet (662 vs. 589 g; P=0.001), while no effect of seaweed species was observed. For birds fed *U. laetevirens* compared to *S. chordalis* diets, the FCR was lower (1.81 vs. 1.94; P<0.001). Water intake of birds fed the *S. chordalis* diets was higher than that of birds fed the *U. laetevirens* diets (1392 vs. 1171 mL; P=0.003).

Table 4.4. Effects of 5% (week 1 and 2) and 10% (week 3) inclusion of untreated (-) and enzymatically[1] treated (+) seaweed (*Ulva laetevirens* and *Solieria chordalis*) co-products in a broiler diet (basal) on performance parameters.

Period	Basal diet (B)	Seaweed diets (S)				SEM	P-values[3]			
Parameter[2]		*U. laetevirens*		*S. chordalis*			B vs. S	S	Enzyme (E)	S×E
		-	+	-	+					
Day 0-7										
Body weight gain (g)	135	126	133	144	126	2.8	0.719	0.389	0.398	0.060
Feed intake (g)	128	129	141	141	125	3.0	0.393	0.746	0.777	0.052
Feed conversion ratio (g/g)	0.95	1.03	1.06	0.98	0.99	0.011	0.006	0.014	0.322	0.698
Water intake (mL)	424	448	566	499	406	26.9	0.407	0.408	0.851	0.119
Water:feed (mL/g)	3.33	3.46	3.18	3.54	3.23	0.19	0.616	0.809	0.130	0.820
Day 7-14										
Body weight gain	326	321	324	322	303	3.6	0.354	0.193	0.300	0.154
Feed intake	378	379	380	396	370	4.6	0.761	0.738	0.261	0.208
Feed conversion ratio	1.16	1.18	1.17	1.23	1.22	0.007	0.008	0.001	0.590	0.831
Water intake	763	820	814	987	899	19.6	0.003	<0.001	0.152	0.215
Water:feed	2.02	2.16	2.14	2.49	2.43	0.04	<0.001	<0.001	0.341	0.623
Day 14-21										
Body weight gain	310	380	367	334	338	6.6	0.002	0.007	0.712	0.528
Feed intake	589	672	675	646	654	9.3	0.001	0.251	0.778	0.911
Feed conversion ratio	1.90	1.77	1.84	1.94	1.94	0.016	0.241	<0.001	0.116	0.135
Water intake	1193	1161	1180	1396	1387	32.0	0.213	0.003	0.946	0.837
Water:feed	2.03	1.73	1.75	2.16	2.11	0.04	0.130	<0.001	0.785	0.548
Day 0-21										
Body weight gain	771	827	825	801	767	9.7	0.144	0.071	0.421	0.485
Feed intake	1094	1180	1194	1184	1146	14.0	0.020	0.498	0.723	0.422
Feed conversion ratio	1.42	1.43	1.45	1.48	1.49	0.006	<0.001	<0.001	0.004	0.632
Water intake	2380	2430	2559	2882	2691	57.4	0.047	0.023	0.800	0.193
Water:feed	2.18	2.06	2.14	2.44	2.34	0.04	0.254	<0.001	0.918	0.086

[1]Alcalase (Soufflet Biotechnologies, ≥3000 U/g) and Neutrase (Novozymes; 0.8 AU-N/g).

[2]Each value is based on 6 replicate pens with 12 (Basal diet, *U. laetevirens*-, *U. laetevirens*+, *S. chordalis*+ diet) or 7 (*S. chordalis*- diet) birds each.

[3]Statistical contrasts: Basal vs. seaweed: Basal diet vs. (*U. laetevirens*-, *U. laetevirens*+, *S. chordalis*- and *S. chordalis*+ diets), Seaweed: (*U. laetevirens*- and *U. laetevirens*+ diets) vs. (*S. chordalis*- and *S. chordalis*+ diets), Enzyme: (*U. laetevirens*- and *S. chordalis*- diets) vs. (*U. laetevirens*+ and *S. chordalis*+ diets).

Over the entire experimental period, seaweed diets compared to the basal diet resulted in a higher FI (1176 vs. 1094 g; P=0.020), FCR (1.46 vs. 1.42; P<0.001) and water intake (2641 vs. 2380 mL; P=0.047) of birds. The FCR was higher when fed the *S. chordalis* compared to the *U. laetevirens* diets (1.49 vs. 1.44; P<0.001), and also higher for birds fed the enzymatically treated compared to untreated seaweed co-products (1.47 vs. 1.46; P=0.004). The water intake was higher for birds fed the *S. chordalis* compared to the *U. laetevirens* diets (2787 vs. 2495 mL; P=0.023).

Nutrient digestibility

For all nutrients, the apparent pre-caecal digestibility of the basal diet was higher compared to that of the seaweed containing diets (P<0.001; Table 4.5). Several Seaweed×Enzyme effects were observed which in the majority of cases showed a lower apparent pre-caecal digestibility coefficient for birds fed the enzymatically treated *U. laetevirens* diet compared to birds fed the untreated *U. laetevirens* and both *S. chordalis* diets. The type of seaweed affected the digestibility for most nutrients (including AAs), where birds fed the *U. laetevirens* diets showed lower apparent pre-caecal digestibility values compared to birds fed the *S. chordalis* diets, except for arginine. When an enzyme effect was observed, birds fed the enzymatically treated seaweed containing diets showed lower digestibility values compared to birds fed the untreated seaweed diets. No differences in apparent total tract digestibility were observed between basal and seaweed diets. The apparent total tract digestibility of crude fibre and crude fat were increased in birds fed the *S. chordalis* vs. *U. laetevirens* diets.

The seaweed digestibility data calculated by the difference method showed large variations (Table 4.6), mainly in the ash fraction and some of the AAs (e.g. cysteine, threonine and phenylalanine). For almost all nutrients, the apparent pre-caecal digestibility of *U. laetevirens* was higher (P<0.05) than that of *S. chordalis* co-products. In addition, the enzyme treatment reduced (P<0.05) the digestibility of most nutrients. The apparent total tract digestibility of crude fibre in *S. chordalis* was higher than that of *U. laetevirens*.

Health-related parameters

No significant differences were observed in empty or full gizzard weight and gizzard content between treatments (Table 4.7). Numerically, empty gizzard weight, full gizzard weight and gizzard content were 7, 12 and 24% lower in birds fed the enzymatically treated seaweed diets compared to birds fed the untreated seaweed containing diets, respectively. Compared to birds fed the *S. chordalis* diets, birds fed the *U. laetevirens* diets had an 11% lower (P<0.001) villus length and a 10% lower (P=0.006) villus length to crypt depth ratio (Table 4.7). Birds fed the treated *U. laetevirens* diet had an 8% larger crypt depth compared to birds fed the untreated *U. laetevirens* diet, whereas the opposite was observed for the treated and untreated *S. chordalis* diets (-4%; Seaweed×Enzyme effect P=0.035). No significant differences were observed in jejunal

Table 4.5. Effects of 10% inclusion of untreated (-) and enzymatically[1] treated (+) seaweed (*Ulva laetevirens* and *Solieria chordalis*) co-products in a broiler diet (basal) on apparent pre-caecal and total tract nutrient digestibility.

Digestibility[2]	Basal diet (B)	Seaweed diets (S)				SEM	P-value[3]			
Nutrient		*U. laetevirens*		*S. chordalis*			B vs. S	S	Enzyme (E)	S x E
		-	+	-	+					
Apparent pre-caecal (%)										
Dry matter	81.4	75.8	74.9	76.2	75.9	0.46	<0.001	0.047	0.065	0.335
Organic matter	82.9	78.1	77.2	78.5	78.4	0.40	<0.001	0.018	0.096	0.263
Ash	54.3	39.8	38.3	46.4	44.4	1.11	<0.001	<0.001	0.009	0.648
Nitrogen	84.0	74.4	71.4	74.8	74.6	0.85	<0.001	0.002	0.006	0.016
Lysine	89.7	83.2	81.6	84.0	83.5	0.56	<0.001	0.003	0.012	0.200
Methionine	94.6	92.2	90.2	91.9	91.8	0.28	<0.001	0.002	<0.001	<0.001
Cysteine	65.4	52.1	47.3	44.6	47.2	1.45	<0.001	<0.001	0.165	<0.001
Threonine	81.1	69.2	65.6	70.3	70.7	1.03	<0.001	<0.001	0.028	0.012
Isoleucine	87.2	78.6	75.5	79.3	78.4	0.78	<0.001	0.005	0.002	0.070
Arginine	88.1	81.5	80.2	78.1	77.8	0.74	<0.001	<0.001	0.113	0.331
Phenylalanine	88.8	76.8	73.5	77.2	76.9	1.04	<0.001	0.009	0.009	0.037
Histidine	85.5	73.2	72.9	74.5	73.8	0.97	<0.001	0.170	0.542	0.739
Leucine	89.0	81.0	77.8	81.7	81.0	0.82	<0.001	0.003	0.002	0.048
Valine	87.0	79.3	76.9	80.1	79.8	0.67	<0.001	0.002	0.012	0.044
Alanine	85.0	69.6	66.8	74.4	72.8	1.22	<0.001	<0.001	0.002	0.348
Asparagine + aspartic acid	84.9	70.7	67.2	73.5	72.1	1.19	<0.001	<0.001	0.003	0.178
Glutamine + glutamic acid	90.9	83.4	80.9	84.0	83.9	0.67	<0.001	0.002	0.016	0.033
Glycine	78.5	63.4	60.3	63.2	60.9	1.34	<0.001	0.968	0.005	0.666
Serine	83.2	69.6	64.6	72.2	74.3	1.21	<0.001	<0.001	0.015	<0.001
Tyrosine	89.9	81.0	79.5	80.6	79.9	0.78	<0.001	0.932	0.051	0.444
Proline	90.7	83.0	81.6	81.5	82.0	0.69	<0.001	0.210	0.299	0.055
Tryptophan	84.8	75.7	70.8	76.3	75.3	0.91	<0.001	<0.001	<0.001	0.002

Table 4.5 (continued). Effects of 10% inclusion of untreated (-) and enzymatically[1] treated (+) seaweed (*Ulva laetevirens* and *Solieria chordalis*) co-products in a broiler diet (basal) on apparent pre-caecal and total tract nutrient digestibility.

Digestibility[2]	Basal diet (B)	Seaweed diets (S)				SEM	P-value[3]			
Nutrient		*U. laetevirens*		*S. chordalis*			B vs. S	S	Enzyme (E)	S x E
		-	+	-	+					
Apparent total tract (%)										
Crude fibre	8.5	10.7	9.1	12.7	11.8	1.05	0.348	0.005	0.096	0.667
Crude fat	83.5	80.0	77.1	82.3	84.4	0.69	0.053	<0.001	0.648	0.050
Starch	98.9	98.6	98.6	99.0	98.6	0.06	0.296	0.417	0.218	0.181
Sugar	86.4	86.9	84.0	86.9	84.0	0.46	0.365	0.893	0.008	0.996

[1]Alcalase (Soufflet Biotechnologies, ≥3000 U/g) and Neutrase (Novozymes; 0.8 AU-N/g).
[2]Each value is based on 6 pens with 12 birds each (Basal diet, *U. laetevirens*-, *U. laetevirens*+, *S. chordalis*+ diet) or 5 pens (n=5 based on outlier test) with 7 birds each (*S. chordalis*- diet). [3]Statistical contrasts: Basal vs. seaweed: Basal diet vs. (*U. laetevirens*-, *U. laetevirens*+, *S. chordalis*- and *S. chordalis*+ diets), Seaweed: (*U. laetevirens*- and *U. laetevirens*+ diets) vs. (*S. chordalis*- and *S. chordalis*+ diets), Enzyme: (*U. laetevirens*- and *S. chordalis*- diets) vs. (*U. laetevirens*+ and *S. chordalis*+ diets).

Table 4.6. Effects of 10% inclusion of untreated (-) and enzymatically1 treated (+) seaweed (*Ulva laetevirens* and *Solieria chordalis*) co-products in a broiler diet (basal) on seaweed co-product apparent pre-caecal and total tract nutrient digestibility.

Digestibility[2]	*U. laetevirens*		*S. chordalis*		SEM	P-value3		
Nutrient	-	+	-	+		Seaweed (S)	Enzyme (E)	S×E
Apparent pre-caecal (%)								
Dry matter	30.2	21.1	33.6	29.0	1.73	0.096	0.036	0.484
Ash	-16.3	-31.3	139.8	116.5	16.43	<0.001	0.005	0.493
Organic matter	31.0	22.1	20.1	17.7	1.80	0.022	0.075	0.312
Nitrogen	63.2	34.2	38.2	29.0	3.76	0.008	0.002	0.077
Lysine	65.4	47.8	27.3	21.1	4.20	<0.001	0.006	0.169
Methionine	66.0	43.0	31.9	21.1	3.69	<0.001	<0.001	0.003
Cysteine	63.5	12.6	33.5	49.9	5.77	0.615	0.043	0.001
Threonine	70.9	33.4	18.9	7.9	6.11	<0.001	0.002	0.077
Isoleucine	56.0	23.3	23.1	2.2	4.91	<0.001	<0.001	0.300
Arginine	52.8	37.8	37.9	24.6	3.24	0.010	0.012	0.863
Phenylalanine	61.1	26.3	9.4	-6.9	6.23	<0.001	0.001	0.169
Histidine	2.90	1.80	1.00	-18.2	4.02	0.140	0.220	0.255
Leucine	64.2	28.7	20.5	-2.2	5.84	<0.001	<0.001	0.279
Valine	81.7	55.4	41.6	27.4	4.92	<0.001	<0.001	0.241
Alanine	147.4	116.0	68.7	38.1	9.59	<0.001	<0.001	0.949
Asparagine + aspartic acid	53.4	17.5	17.1	-6.7	5.78	<0.001	<0.001	0.417
Glutamine + glutamic acid	24.6	-1.0	1.0	-7.2	3.53	0.007	0.003	0.105
Glycine	110.3	77.3	63.7	25.0	7.73	<0.001	<0.001	0.737
Serine	29.8	-21.9	-13.8	-4.1	5.14	0.059	0.002	<0.001
Tyrosine	47.6	31.1	28.3	13.7	3.62	0.002	0.009	0.857
Proline	31.7	19.1	2.9	3.4	3.36	<0.001	0.194	0.182
Tryptophan	85.8	38.3	51.3	12.7	6.27	<0.001	<0.001	0.423
Apparent total tract (%)								
Crude fibre	43.9	29.6	62.0	53.4	4.17	0.010	0.120	0.695
Crude fat	27.4	8.7	10.3	31.5	5.94	0.754	0.980	0.111
Starch	1.3	1.6	4.9	-1.4	0.84	0.991	0.078	0.039
Sugar	11.0	-16.4	29.6	-3.4	5.70	0.155	0.006	0.776

[1], [2], [3]See Table 4.5.

Table 4.7. Effects of 10% inclusion of untreated (-) or enzymatically[1] treated (+) seaweed (*Ulva laetevirens* and *Solieria chordalis*) co-products in broiler diet (basal) on gastrointestinal tract (GIT) characteristics and plasma cytokine levels.

Tissue	Basal diet (B)	Seaweed diets (S)				SEM	P-value[4]			
Parameter		*U. laetevirens*		*S. chordalis*			B vs. S	S	Enzyme (E)	S×E
		-	+	-	+					
Gizzard[2] (g/kg BW)										
Weight empty	15.6	15.4	14.4	15.5	14.4	0.13	0.332	0.897	0.135	0.966
Weight full	22.2	21.7	18.6	22.5	21.0	0.13	0.394	0.293	0.128	0.615
Content	6.6	6.3	4.2	7.0	6.5	0.13	0.531	0.130	0.190	0.425
Duodenum[2]										
Villus length (µm)	1818	1628	1624	1782	1820	24.2	0.071	<0.001	0.715	0.659
Crypt depth (µm)	84	76	82	80	77	1.1	0.033	0.871	0.592	0.035
Villus length:crypt depth	22	22	20	22	24	0.4	0.792	0.006	0.948	0.063
Muscularis thickness (µm)	132	124	129	130	117	3.0	0.452	0.634	0.600	0.181
Jejunum[3]										
Digesta pH	6.20	6.10	6.10	6.10	6.10	0.03	0.097	0.782	0.963	0.872
Blood plasma[2]										
Interleukine-13 (pg/ml)	22.4	9.1	19.6	18.0	23.8	2.57	0.921	0.267	0.035	0.723
Haptoglobin (ng/ml)	2.0	2.1	1.3	1.7	1.5	0.15	0.438	0.928	0.097	0.521

[1]Alcalase (Soufflet Biotechnologies, ≥3000 U/g) and Neutrase (Novozymes; 0.8 AU-N/g).

[2]Each value is based on 6 replicate pens with 2 birds per pen.

[3]Each value is based on 6 replicate pens with 1 pooled sample of 2 birds per pen.

[4]Statistical contrasts: Basal vs seaweed: Basal diet vs. (*U. laetevirens*-, *U. laetevirens*+, *S. chordalis* and *S. chordalis*+ diets), Seaweed: (*U. laetevirens*- and *U. laetevirens*+ diets) vs. (*S. chordalis*- and *S, chordalis*+ diets), Enzyme: (*U. laetevirens*- and *S. chordalis*- diets) vs. (*U. laetevirens*+ and *S. chordalis*+ diets).

digesta pH (Table 4.7) or plasma IL-13 and haptoglobin levels (Table 4.7) between the basal and seaweed diets or between the seaweed diets. An enzyme effect (P=0.035) was observed for plasma IL-13 concentration with the enzyme treatment leading to higher values.

Discussion

Nutritional composition

As expected, the enzymatic treatment of the seaweed co-products did not cause large differences in nutritional composition, whereas large differences between the two seaweed species were observed in ash, N, fat, fibre and sugar content. The high mineral content of *S. chordalis* co-products and the concomitant effect on water intake might have affected other observed parameters. For example, the higher water intake could lead to diarrhoea, suboptimal bird health and reduced performance (Guiry and Blunden, 1991; Koreleski *et al.*, 2010), although signs of diarrhoea were not observed in our study. The iron content exceeded the maximum dietary level of European regulations for animal diets, while no separate regulations are in place for maximum iron content in dietary ingredients. With an inclusion level of 5 and 10%, the iron content of the seaweed co-products is diluted to dietary levels within the European regulation limits. Due to large variation in mineral content, depending amongst others on seaweed species and environmental factors (Boderskov *et al.*, 2016; Sharma *et al.*, 2018), the mineral level of seaweed to be included in animal diets needs to be determined carefully. Furthermore, the ratio between (trace) minerals is important to take into account (Bao and Choct, 2009). For example, iron impairs zinc absorption (Solomons and Jacob, 1981), potentially leading to a zinc deficiency and consequently a depressed growth performance and animal welfare, while it met dietary specification for practical diets (CVB, 2019). The N-content varied considerably between the co-products of the two species, although due to the low inclusion level of 5 and 10%, dietary N (and AA) intake per kg DM only differed 1.5-3%.

Based on their gross nutritional composition, *U. laetevirens* co-products are considered more valuable feed ingredients for broiler nutrition compared to *S. chordalis* co-products due to the higher AA and true protein, and lower ash content.

Performance

The higher FI of birds fed the seaweed containing diets compared to birds fed the basal diet, and of birds fed *U. laetevirens* vs. *S. chordalis* diets might be explained by differences in calculated ME content and nutrient digestibilities between the diets. The latter may have led to a compensatory FI which consequently led to an increased protein intake of 14.7-15.9 vs. 12.1 g N in the third experimental week of birds fed the seaweed vs. basal diets. Gous *et al.* (2018) found an inverse relationship between

protein content and FI of a diet, depending on the ME to digestible protein ratio. Taking into account the N digestibility, digestible N intake was 11.0-11.6 vs. 10.2 g N per bird of broilers fed the seaweed vs. basal diets in the third experimental week, respectively, indicating the ME and digestible protein intake were similar between treatment groups.

Based on a higher FCR in the first week and a lower FCR in the third week of birds fed *U. laetevirens* co-products compared to the other dietary treatments, it appears that these birds had to adjust to these seaweed co-products. Remarkably, the best FCR in the third week was observed for the untreated *U. laetevirens*, although this is not reflected in the digestibility coefficients. Contrary to the birds fed the *S. chordalis* diets, water intake of birds fed the *U. laetevirens* diets was not increased with FI.

The relatively large water intake of birds fed the seaweed diets might have been caused by the dietary electrolyte balance. Water intake was indeed correlated with ash content of the diets, with especially the birds fed *S. chordalis* diets having a higher water intake. Koreleski *et al.* (2010) also observed changes in water intake and DM content of excreta as a response to changing dietary levels of specific minerals. In the current study, the excreta moisture content of birds fed the basal diet was 743 g/kg, similar to 768 and 724 g/kg of birds fed the *S. chordalis* diets. Lower moisture levels of 683 and 662 g/kg were observed in excreta of birds fed the *U. laetevirens* diets. This is contrary to their water intake, which was similar for birds fed the *U. laetevirens* diets compared to the basal diet in the third experimental week. It must be mentioned that the collection method of excreta was not designed for precise excreta moisture determination, and these data reflect differences between treatments rather than precise absolute values. Differences in water intake and excreta moisture content in the current study might also be related to changes in digesta viscosity caused by differences in soluble NSP (Francesch and Brufau, 2004). Viscoelastic properties of digesta were, however, not analysed in this experiment.

Literature on the effects of seaweed (co-product) inclusion in broiler diets at nutritionally significant levels (>5%) on broiler performance is scarce and results are inconsistent. In one study, 1-3% green seaweed *U. lactuca* was added to broiler diets from day 12 to day 33 at the expense of corn (Abudabos *et al.*, 2013). Performance parameters were not influenced by the seaweed inclusion, although another study reported severe negative effects on performance after inclusion of 10, 20 and 30% *U. lactuca* seaweed in broiler diets and conclude that this intact seaweed is not suitable as dietary ingredient at levels of 10% or higher (Ventura *et al.*, 1994). These differences can at least partly be attributed to the different inclusion levels and the use of different seaweed species, since large differences in chemical composition exist between and within seaweed species (Biancarosa *et al.*, 2017, Sharma *et al.*, 2018).

Digestibility

The enzymatic treatment reduced nutrient digestibility in the diets and bird performance. Nutrient digestibility may have been reduced, for example, by complex forming and precipitation of free AAs with heavy metals (Ashmead, 1992). Furthermore,

the enzyme treatment might have altered the dietary and consequently intestinal content viscosity, which is known to by itself lead to reduced nutrient digestibility and impaired growth performance (Smits *et al.*, 1997). The enzyme treatment might additionally have led to more hydrophobic interactions in the enzyme treated seaweed co-products, which are known to reduce protein digestibility in proso millet flour and rice (Gulati *et al.*, 2017; Liu *et al.*, 2019). Based on this experiment, we cannot conclude what mechanism(s) has/have caused the reduced digestibility as a result of the enzyme treatment.

Digestibility values of the individual seaweed co-products were calculated using the difference method, which assumes an absence of interactions between the feed ingredient of interest and the basal diet (Kong and Adeola, 2014). Regarding the high variation in the digestibility values of these seaweed co-products, this assumption may not be valid as digesta viscosity and microbiota composition may be different due to the diets. When using the difference method, a high inclusion level of the seaweed co-product in the basal diet is desirable as this will lead to a more precise determination of actual digestibility values of the ingredient. Lower ingredient inclusion levels increase the error of the digestibility estimate and a potential greater deviation from the actual digestibility value if the ingredient is included in diets as the sole protein source. It, however, remains important to evaluate the effect of the seaweed (co-)products on nutrient digestibility when included in a practical poultry diet.

Health-related parameters

The lack of differences observed in gizzard characteristics and jejunal pH was unexpected, since gizzard weight and the pH in most parts of the digestive tract change with a change in fibre source (Jiménez-Moreno *et al.*, 2009a). Differences in gizzard development are most often ascribed to diet structure instead of composition (Svihus, 2011; Hamungalu *et al.*, 2020), although these factors did not differ among treatment groups in such a way that it affected gizzard characteristics in the present study. Haptoglobin is associated with iron binding and oxygen transport by red blood cells. The high iron levels in the seaweed diets, especially in the *S. chordalis* diets, did not translate into differences in plasma haptoglobin levels.

Basal vs. seaweed ~ Increased villi length corresponds to a higher nutrient uptake capacity (Cañedo-Castro *et al.*, 2019), whereas deeper crypts are indicative of a higher villi cell turnover rate, associated with a reduced digestion and uptake capacity (Pluske *et al.*, 1996a). Consequently, a higher villus length to crypt depth ratio indicates a slower turnover rate of intestinal cells leading to lower maintenance requirements and potentially increased efficiency of animals. Villi length tended to decrease by including seaweed in broiler diets. Combined with the decreased crypt depths of birds fed the seaweed diets vs. the basal diet, this indicates that less energy is spent on maintenance of the intestinal lining by the birds fed seaweed diets. However, this did not result in a better performance. In contrast, when broilers were fed 2, 4 or 6% *U. rigida* (currently named *U. laetevirens*; washed with fresh water, sun-dried and ground), longer villi were

observed compared to a diet without seaweed, with the longest villi observed in the chickens fed the 2% diet (Cañedo-Castro *et al.*, 2019). These authors combined their finding with lower serum cholesterol and triglyceride levels, without negative effects on performance. They suggested this was potentially caused by either sulphated polysaccharides or fatty acids from the seaweed product and conclude that *U. rigida* would be a good pre-biotic for enhancement of broiler health.

U. laetevirens *vs.* S. chordalis ~ Birds fed the *U. laetevirens* vs. *S. chordalis* diets had shorter villi and a decreased villus length:crypt depth, which does not reflect the better performance results of birds fed *U. laetevirens* diets. In the literature, an increase in villi length between treatments is explained by a need for an increased absorption area in order to digest and absorb nutrients from more viscous intestinal contents in a diet with higher NSP levels (Van Krimpen *et al.*, 2015). Indeed, in our study the NSP content of the *S. chordalis* co-products was higher compared to that of the *U. laetevirens* co-products, and the villi length was increased in the former compared to the latter.

***Enzyme effect* ~** The higher IL-13 levels in the birds fed enzymatically treated vs. untreated seaweed diets indicate a stronger anti-inflammatory response to extracellular pathogens. This effect was twice as large in birds fed *U. laetevirens* diets (54% reduction) compared to birds fed *S. chordalis* diets (24% reduction). Common dietary ingredients like soy contain a relatively large amount of NSP which are mostly indigestible for poultry. Part of this NSP fraction like mannans and galactomannans, have membrane components similar to that of pathogens, triggering a feed induced immune response (a.o. Kogut, 2017). Hence, the observed IL-13 level in the control and enzymatically treated diets might indicate an increased inflammatory response. Moreover, the lower IL-13 levels in the birds fed untreated seaweed diets might indicate that these untreated products improve gut health, but that the enzymatic treatment diminishes this positive effect. Potentially, proteins or peptide-carbohydrate complexes cause this positive effect, while the proteolytic enzymes reduce these bioactive complexes to ineffective building blocks.

Recommendations

To unravel the working mechanism of seaweed products and the effects on broiler health, further studies need to be conducted. A suggested focus is towards the effects of those products on viscous characteristics of the diets and chyme. Furthermore, the effects of the polysaccharides and NSP on broilers, for example by analyses of microbiota in the ceca, are of interest. An enzyme treatment with a carbohydrase targeting specific seaweed polysaccharides is suggested to improve digestibility and nutritional value of seaweed for broilers, although this enzyme should be tailored to the seaweed species of focus as different carbohydrates are present among seaweed species.

Conclusion

This study confirms the high mineral content of *U. laetevirens* and *S. chordalis* co-products and their relatively poor nutrient (especially protein and amino acid) digestibility in broilers. The inclusion of *U. laetevirens* and *S. chordalis* co-products in experimental broiler diets reduced the overall nutrient digestibility of the diet, with the proteolytic enzyme treatment of seaweed co-products reducing rather than improving performance. Addition of the *U. laetevirens* co-products to a basal diet improved performance based on growth and FCR. The enzyme treatment did not improve the studied health-related parameters, whereas the untreated seaweed products might improve broiler health.

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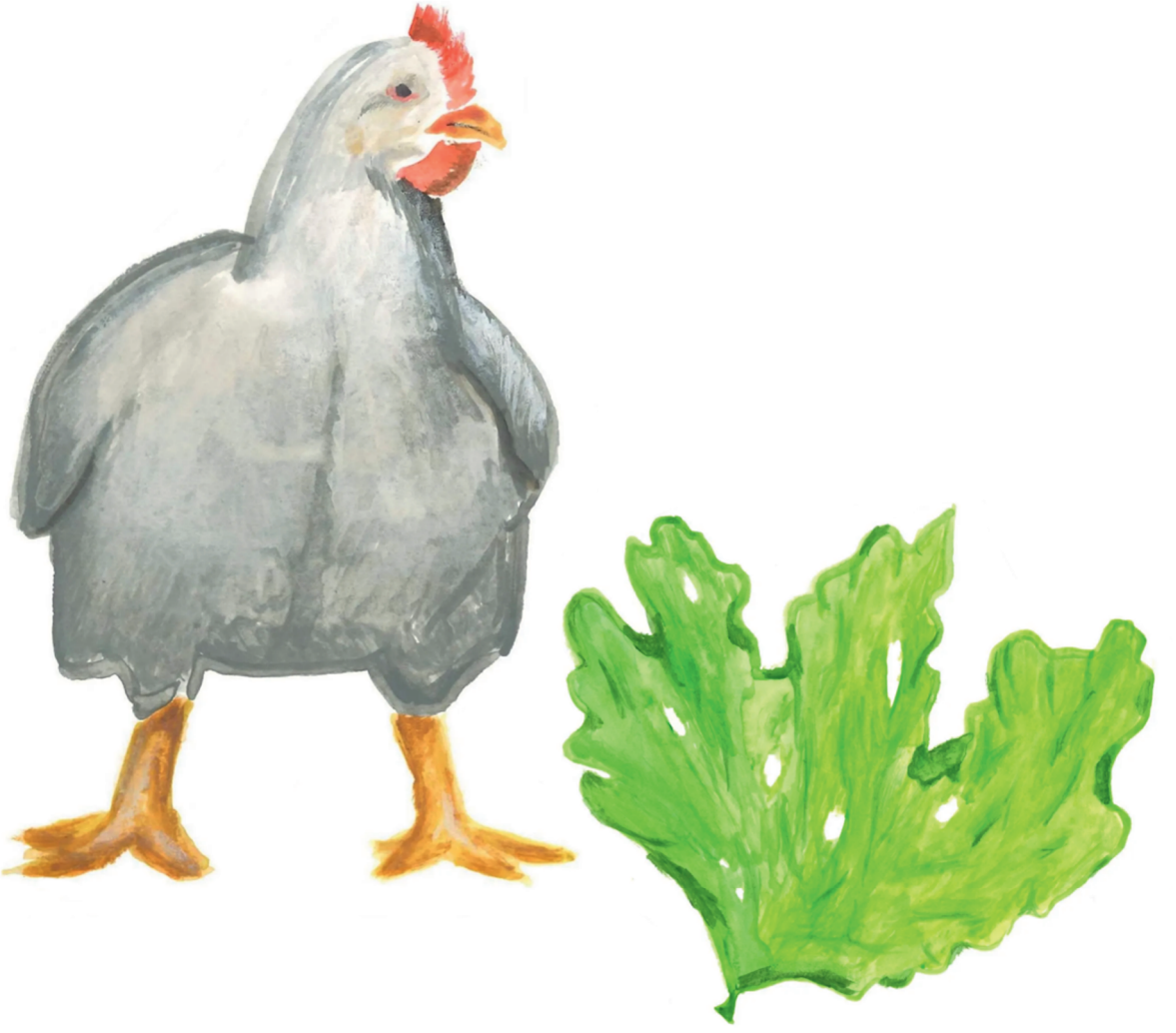

Chapter 5

Proteolytic enzyme-treated seaweed co-product (*Ulva laetevirens*) inclusion in corn-soybean and European broiler diets to improve digestibility, health and performance

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Abstract

The impact of addition of an *Ulva laetevirens* (previously *Ulva rigida*) co-product treated with a broad-spectrum endo-protease when added to a standard corn-soy (S) based diet and a diet based on European protein sources (EU) on performance, *in vivo* digestibility and multiple gastro-intestinal characteristics was investigated in broilers. In total, 624 Ross 308 one day old male broilers were fed one of six experimental diets consisting of a basal diet (S or EU), or a basal diet including the *U. laetevirens* co-product (U) treated without (U-) or with (U+) a proteolytic enzyme. Starter diets contained 0 (wk 1) and 2.5 (wk 2), and the grower diets (wk 3 and 4) 5% seaweed co-product. In the last two weeks, birds fed the S vs. EU grower diets showed a higher body weight, body weight gain and feed intake, as well as a lower feed conversion ratio (-0.05 g/g) in wk 3. Heavier gizzards (+13%) and higher gizzard contents (+92%) were observed in birds fed the EU vs. S diets, as well as longer villi (+8%). U diets had a higher water holding capacity than the basal diets (+19%). In wk 4 U inclusion resulted in increased feed conversion ratio (+0.06 g/g), water intake (+7%) and duodenal cross section (+5%). Enzyme treatment did not affect digestibility of any nutrients, except for ash which was increased in birds fed U+ vs. U- diets (+60%). U in S diets led to higher, and U in EU diets led to lower apparent pre-caecal digestibility of all nutrients ($P<0.001$ for all nutrients). Although for both diet types performance was decreased, dietary *U. laetevirens* inclusion had different effects when added to a standard corn-soy diet and a diet based on European protein sources. No obvious health effects were observed, leading to a conclusion of the absence of performance of health promoting bioactive components in the *U. laetevirens* co-product or due to the proteolytic enzyme treatment.

Keywords: seaweed, broiler nutrition, novel feed source, digestibility, enzyme

Introduction

Novel and existing feed ingredients for broiler diets are routinely investigated to create and sustain a future-proof poultry production. The seaweed *Ulva laetevirens* might contribute to the latter goal as arable land or fresh water are not needed for their production and the nutritional composition is potentially favourable, as *U. laetevirens* can have a protein content of up to 38% on a DM (dry matter) basis (Biancarosa *et al.*, 2017; Øverland *et al.*, 2019). In addition, health-promoting properties have been attributed to *Ulva spp.* when included in diets for simple-stomached animal species (a.o. Cañedo-Castro *et al.*, 2019; Øverland *et al.*, 2019).

Besides beneficial attributes, challenges to include *U. laetevirens* in broiler diets are present such as a high mineral content (Biancarosa *et al.*, 2017; Øverland *et al.*, 2019) which can induced diarrhoea (Koreleski *et al.*, 2010) or lead to lower inclusion levels due to formulation constraints (maximum nutrient level). Furthermore, seaweeds including *Ulva spp.* are poorly digested by broilers (Bikker *et al.*, 2020; Stokvis *et al.*, 2021b under review), leading to a low nutrient availability and poor performance. In conjunction with these nutritional challenges, the quantities of seaweed produced and processed are currently still relatively low leading to challenges regarding economic viability.

Potential solutions for the high mineral content can be washing using fresh water (Neveux *et al.*, 2014) while the economic feasibility can be improved by implementing a biorefinery approach (a.o. Bikker *et al.*, 2016; Torres *et al.*, 2019; Bikker *et al.*, 2020). By creating multiple fractions through biorefinery, valuable components can be extracted for use in the pharmaceutical, chemical or food industry (e.g. Holdt and Kraan, 2011). The then more cost-effective biorefined co-products are potential feed ingredients (Torres *et al.*, 2019). In the biorefinery concept, the high water content of seaweed facilitates fractionation by pressing, yielding a liquid fraction containing most soluble components including minerals, and a solid fraction containing mostly insoluble components including cell wall material. Additional treatment of the latter fraction by for example enzymes has been suggested (Stokvis *et al.* 2021a; Van Krimpen and Hendriks, 2019; Bikker *et al.*, 2016) to be a potential strategy to improve nutrient availability for broilers.

However, in a recent study (Matshogo *et al.*, 2021a), pre-treatment of seaweed meal with an exogenous fibrolytic enzyme mixture did not improve growth performance, a number of physiological parameters, and meat quality traits in broiler chickens. Recently in our laboratory using 5 (wk 1 and 2) and 10% (wk 3) *U. laetevirens* co-products in diets for broilers, a proteolytic enzyme treatment reduced nutrient digestibility and led to a higher feed conversion ratio (FCR), whereas untreated *U. laetevirens* inclusion led to a lower FCR compared to a basal diet. The differences in FCR were only observed in the third (last) week of the trial (Stokvis *et al.*, 2021b under review). Furthermore, a reduced crypt dept and villus length in the duodenum, as well as a lower blood plasma interleukin-13 level were observed in birds fed the diet enriched with untreated vs. treated *U. laetevirens*.

The current study aimed to confirm the results of our previous trial, and to further investigate the effects of a proteolytic enzyme treatment of *U. laetevirens* co-product (fraction after washing and pressing) on digestibility and health-related parameters when included in a standard corn-soy diet and a diet based on protein sources derived from European countries.

Material and methods

The animal experiment was conducted at the facility of Wageningen University & Research in Wageningen, the Netherlands. All experimental procedures were approved by the Animal Care and Use Committee of Wageningen University & Research, the Netherlands (AVD40100202010104).

Seaweed harvesting and processing

Ulva laetevirens was obtained from and processed by Olmix S.A. (Olmix Group, Bréhan, France) and harvested from the beach in France near Guisseny on September 30th, 2014 and immediately washed with fresh water and then frozen until further processing. After thawing, *U. laetevirens* was ground to 50-1000 nm particles (Inotec I175CDI-75D) and pressed twice using a belt press (Flottweg BFRU 800, Vilsbiburg, Germany) at 6 bar with intermediate rehydration (dry matter (DM)=196 g/kg) using fresh water. The enzymatic treatment consisted of the addition of broad-spectrum endo-protease (0.5% Neutrase, Novozymes; 0.8 AU-N/g) to the *U. laetevirens* rehydrated cake (co-product) on a dry weight basis at 50°C for a duration of 5 h under low agitation, followed by a 10 min enzymatic inactivation step at 80°C. Both untreated (U-) and treated (U+) *U. laetevirens* co-products were air-dried at 60°C for 48 and 30 h, respectively followed by 60 h at 50°C up to 90% DM. Finally, all *U. laetevirens* products were ground to pass a 1 mm sieve before inclusion in the experimental diets. The composition and density of both *U. laetevirens* co-products are listed in Table 5.1.

Table 5.1. Analysed nutrient content of the untreated and enzymatically[1] treated seaweed (*Ulva laetevirens*) co-products.

Item Component	Untreated	Treated
Gross nutrient content (g/kg dry matter)		
Dry matter (g/kg)	896.0	888.0
Ash	272.3	274.8
Nitrogen (N)	21.4	22.3
Crude protein[2]	107.1	111.7
Crude fibre	97.1	83.3
Crude fat	5.6	6.8
Calcium	29.6	31.2
Phosphorous	1.3	1.3
Density (g/cm^3)	0.692	0.653

[1]Neutrase (Novozymes; 0.8 AU-N/g).
[2]Calculated as N×5.0 as per Angell *et al.* (2016b).

Animals and housing

A total of 624 one-day-old male broilers (Ross 308, Morren, Lunteren, The Netherlands) with an average body weight (BW) of 42.8±1.28 g were randomly assigned to one of 48 pens with 13 birds per pen with pen weight being kept within a 3% difference from the average pen weight. Each pen (1.85×1.10 m) had a solid floor covered with wood shavings. At arrival, all birds were vaccinated against infectious bronchitis and against Newcastle disease at d15. Five days prior to the dissection of the birds of a pen (d29, 30, or 31), bedding material and solid floors were replaced by slatted floors to enable excreta collection. Each pen was assigned to one of six treatments in a completely randomized block design with 8 replicate pens per treatment. Ambient temperature was maintained at 34°C for the first two days and, thereafter, gradually reduced to 20°C on d27 and maintained at this temperature until the end of the experiment. A 23L:1D photoperiod was applied during the first three days, whereafter the dark period was increased by 1 h every day until a 16L:8D schedule was achieved. Birds had ad libitum access to feed and water. At the end of the experiment, either at 30, 31 or 32 days of age, birds were euthanized per replicate, with an intracranial sodium pentobarbital injection before sample collection.

Experimental diets

All starter (d0-13) and grower (d14-end of experiment) diets were formulated to meet or exceed requirements of all nutrients for broilers (CVB, 2019). The grower diet was supplemented with 5 g/kg titanium (Ti) dioxide and 1 g/kg cobalt-ethylenediamine tetraacetic acid (Co-EDTA) as indigestible solid and liquid phase markers to allow determination of digestibility values. All diets were produced by Research Diet Services (Wijk bij Duurstede, The Netherlands), and fed as pellets (starter: 2.5 mm, grower: 3.2 mm). In total six diets were formulated, based on two diet types: a corn-soy based diet (S) and a diet based mainly on European protein sources (EU). For either diet type, a basal diet (B: SB and EUB) and two seaweed diets with (SU+, EUU+) or without (SU-, EUU-) the enzyme pre-treatment were formulated. The SU-, SU+, EUU- and EUU+ diets during d0-6, d7-13 and d14-end of the experiment consisted of 100, 97.5 and 95% (w/w) basal diet with 0, 2.5 and 5% (w/w) U- or U+ seaweed, respectively. The ingredients of the diets and analysed nutrient composition are presented in Tables 5.2 and 5.3, respectively. The water holding capacity (WHC; Table 5.3) of the diets was determined (AACC International Method 56-11-02) by soaking 1.0±0.05 g feed pellets in deionized water in a 50 mL falcon tube for 60 min. After centrifugation (10 min at 4000×g), samples were left to drain for 15 min by placing the tubes at a 45° angle. The WHC was calculated as initial sample weight minus drained sample weight.

Performance measurements

Feed intake (FI) and water intake (WI) were recorded weekly per pen. Average BW per pen was determined upon arrival at the experimental facility, and again at d7, 14, 21 and 28. The FCR was calculated as: total pen FI over the

Table 5.2. Composition of the basal and untreated (-) and enzymatically[1] treated (+) seaweed (*Ulva laetevirens*) co-product containing starter (d0 to 13) and grower (d14 to end of experiment) diets for broilers.

Ingredient (g/kg)	Starter diet						Grower diet					
	Soy-based			European based protein			Soy-based			European based		
	Basal[2]	*U. laetevirens*		Basal[2]	*U. laetevirens*		Basal	*U. laetevirens*		Basal	*U. laetevirens*	
		-	+		-	+		-	+		-	+
Corn	479.5	449.4	449.2	290.1	264.2	264.4	400.0	400.0	400.0	200.0	200.0	200.0
Wheat	150.0	150.0	150.0	200.0	200.0	200.0	299.0	232.1	232.6	315.7	250.6	251.1
Soybean meal	227.3	226.1	226.1	60.0	60.0	60.0	161.0	167.0	166.0	50.0	50.0	50.0
U. laetevirens-	-	25.0	-	-	25.0	-	-	50.0	-	-	50.0	-
U. laetevirens+	-	-	25.0	-	-	25.0	-	-	50.0	-	-	50.0
Rapeseed meal	-	-	-	50.0	45.0	45.0	-	-	-	50.0	50.0	50.0
Sunflower meal	80.8	80.4	80.4	80.0	80.0	80.0	75.0	75.0	75.0	80.0	80.0	80.0
Peas	-	-	-	160.0	160.0	160.0	-	-	-	160.0	160.0	160.0
Corngluten feed	-	-	-	40.0	40.0	40.0	-	-	-	40.0	40.0	40.0
Potato starch	-	-	-	40.0	41.0	40.8	-	-	-	10.0	13.0	12.0
Palm fat (34.0 MJ)	-	-	-	-	-	-	-	-	-	24.0	33.5	33.5
Soybean oil (37.5 MJ)	16.5	26.0	26.3	35.0	43.0	43.0	17.0	35.0	35.0	24.0	33.5	33.5
Premix[3] (5 g/kg)	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Finely ground lime	13.5	11.8	11.7	14.2	12.5	12.3	10.8	7.1	7.0	11.1	7.4	7.2
Monocalcium phosphate	13.6	13.6	13.6	13.4	13.4	13.5	9.2	9.2	9.2	8.2	8.5	8.6
Salt	2.5	1.3	1.3	1.5	0.8	0.8	2.0	0.0	0.0	1.5	0.0	0.0
Sodium bicarbonate	1.8	1.8	1.8	2.6	2.0	2.0	2.4	1.9	1.9	2.6	1.4	1.4
L-Lysine HCl	4.2	4.2	4.2	3.5	3.5	3.5	5.1	4.8	4.9	4.6	4.3	4.5
DL-Methionine	2.8	2.8	2.8	2.7	2.7	2.7	2.7	2.7	2.8	2.7	2.7	2.8
L-Threonine	1.2	1.2	1.2	0.8	0.7	0.8	1.6	1.4	1.5	1.5	1.3	1.4
L-Valine	0.6	0.6	0.6	0.1	0.1	0.1	1.1	0.9	1.0	1.0	0.8	0.9
L-Arginine	0.7	0.8	0.8	1.1	1.1	1.1	1.6	1.5	1.6	1.4	1.3	1.4
L-Isoleucine	-	-	-	-	-	-	0.5	0.4	0.5	0.7	0.7	0.7
Titanium dioxide	-	-	-	-	-	-	5.0	5.0	5.0	5.0	5.0	5.0
Cobalt-EDTA	-	-	-	-	-	-	1.0	1.0	1.0	1.0	1.0	1.0

[1]Neutrase (Novozymes; 0.8 AU-N/g).

[2]All birds were fed their respective basal diet from d0-6.

[3]Provided per kg of diet: vitamin A, 10,000 IU; vitamin D_3, 2,500 IU; vitamin E, 50 mg; vitamin K_3, 1.5 mg; vitamin B_1, 2.0 mg; vitamin B_2, 7.5 mg; vitamin B_6, 3.5 mg, vitamin B_{12}, 20 µg; niacin, 35 mg; D-pantothenic acid, 12 mg; folic acid, 1.0 mg; biotin, 0.2 mg; Fe, 80 mg; Cu, 12 mg; Mn, 85 mg; Zn, 60 mg; I, 0.8 mg; Se, 0.15 mg.

period/(Pen BW end of period–pen BW start of period+pen BW of dead or culled birds) with FI per bird corrected for mortality calculated as: FCR×BW gain.

Sample collection and chemical analyses

Excreta were collected qualitatively during two days before dissections, after which the birds of the corresponding pens were euthanized. Ileal contents were collected from the distal 40 cm of the ileum, anterior to the ileo-caecal valve of birds and pooled per pen. Excreta and ileal chyme were stored at -20°C until further processing. Before chemical analyses, excreta and ileal chyme were freeze dried, and all samples were ground to pass a 1 mm diameter screen. The U- and U+ were analysed for DM (ISO 6496, 1999), ash, (ISO 5984, 2002), nitrogen (N; ISO 5983, 2005), crude fat (ISO 6492,

Table 5.3. Analysed nutrient content of the basal and untreated (-) and enzymatically[1] treated (+) seaweed (*Ulva laetevirens*) co-product containing starter (d0-13) and grower (d14-end of experiment) diets as fed to the broilers.

Item	Starter diet						Grower diet					
Component	Soy-based			European protein based			Soy-based			European protein based		
	Basal[2]	*U. laetevirens*		Basal[2]	*U. laetevirens*		Basal	*U. laetevirens*		Basal	*U. laetevirens*	
		-	+		-	+		-	+		-	+
Gross nutrient content (g/kg dry matter (DM)												
Dry matter (DM; g/kg)	888.7	893.6	893.7	893.1	896.9	897.2	883.8	886.7	884.6	889.3	889.7	888.8
Ash	60.2	63.1	63.8	59.3	62.2	61.8	60.0	63.4	63.9	57.7	64.7	64.2
Nitrogen	36.6	37.1	35.9	35.7	36.4	36.1	33.5	34.0	34.4	33.8	33.8	33.2
Crude protein[3]	228.6	231.6	224.3	222.9	227.2	225.7	209.6	212.7	215.0	211.5	211.0	207.7
Neutral detergent fibre	91.9	102.2	102.3	127.3	130.1	127.1	100.9	116.3	111.4	126.7	138.9	138.7
Acid detergent fibre	40.7	44.8	43.9	56.2	57.2	57.3	42.9	49.2	49.7	58.5	66.3	65.7
Acid detergent lignin	1.4	4.1	3.4	7.7	9.3	8.8	4.2	4.5	6.0	8.3	8.2	9.3
Crude fat	47.2	54.5	55.8	65.5	72.0	73.7	43.6	56.3	54.9	79.5	94.6	95.2
Sugar	51.0	49.3	48.7	43.3	40.9	42.6	44.6	44.0	43.7	42.9	40.4	40.3
Starch	430.3	431.8	411.3	426.0	405.5	388.8	470.1	444.2	440.5	434.9	396.3	387.5
Non starch polysaccharides[4]	182.7	169.8	196.2	183.0	192.3	207.4	172.1	179.5	181.9	173.5	193.0	205.2
Macro minerals (g/kg DM)												
Calcium	-	-	-	-	-	-	8.9	7.9	7.7	8.6	8.2	7.8
Phosphorus	-	-	-	-	-	-	6.8	6.4	6.5	6.9	6.8	6.9
Potassium	-	-	-	-	-	-	8.8	9.0	9.1	8.2	8.4	8.4
Sodium	-	-	-	-	-	-	1.8	1.5	1.5	1.7	1.5	1.5
Chloride	-	-	-	-	-	-	3.3	2.1	2.3	3.0	2.4	2.4
Magnesium	-	-	-	-	-	-	2.1	3.0	3.0	2.2	3.0	3.0
Sulphur							2359.1	4076.8	3967.9	2934.8	4428.7	4303.4
Micro minerals (mg/kg DM)												
Iron	-	-	-	-	-	-	195.2	326.5	331.8	211.4	348.5	361.7
Copper	-	-	-	-	-	-	17.5	22.0	24.9	20.8	19.7	21.4
Manganese	-	-	-	-	-	-	119.9	113.3	118.7	118.1	127.0	121.0
Zinc	-	-	-	-	-	-	105.2	102.1	105.7	110.8	102.9	106.9
Arsenic	-	-	-	-	-	-	0.1	0.4	0.3	0.1	0.3	0.3
Cadmium	-	-	-	-	-	-	0.2	0.2	0.2	0.2	0.17	0.2

Table 5.3 (continued). Analysed nutrient content of the basal and untreated (-) and enzymatically[1] treated (+) seaweed (*Ulva laetevirens*) co-product containing starter (d0-13) and grower (d14-end of experiment) diets as fed to the broilers.

Item	Starter diet						Grower diet					
Component	Soy-based			European protein based			Soy-based			European protein based		
	Basal[2]	*U. laetevirens*		Basal[2]	*U. laetevirens*		Basal	*U. laetevirens*		Basal	*U. laetevirens*	
		-	+		-	+		-	+		-	+
Micro minerals (mg/kg DM; continued)												
Mercury	-	-	-	-	-	-	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Lead	-	-	-	-	-	-	0.2	0.4	0.4	0.2	0.4	0.4
Nickel	-	-	-	-	-	-	2.4	2.9	2.8	2.6	3.0	3.0
Selenium	-	-	-	-	-	-	0.3	0.3	0.3	0.3	0.3	0.3
Calculated AME (MJ/kg)	12.06	12.04	12.06	12.37	12.33	12.34	12.33	12.28	12.29	12.59	12.56	12.57
Water holding capacity (g/g)	-	-	-	-	-	-	1.47	1.78	1.64	1.41	1.75	1.67

- not analysed.

[1]Neutrase (Novozymes; 0.8 AU-N/g).

[2]All birds were fed their respective basal diet from d0-6.

[3]Calculated as N×6.25.

[4]Calculated as 1000–ash–crude protein–crude fat–sugar–starch.

1999), crude fibre (ISO 6865, 2000), and Ca and P (ISO 27085, 2009), and their density (g/cm^3) was determined. Starter diets were additionally analysed for Na, K and Cl (ISO 27085, 2009; ISO 6495, 2015), starch (ISO 15914, 2004), sugar (EC 152, 2009), neutral detergent fibre (NDF; ISO 16472, 2006), acid detergent fibre (ADF) and acid detergent lignin (ADL; ISO 13906, 2008). Furthermore, grower diets were additionally analysed for Fe, Mn, Mg, Zn and Cu (ISO 27085, 2009) as well as As, Cd, Pb, Hg, Co, Se, Ni and S (DIN EN 15763, 2009), in addition to Ti and Co. The markers were measured after ashing and microwave digestion using inductive coupled plasma optical emission spectrometry (ICP-OES). The ileal samples were analysed for DM, ash, N, NDF, Ti and Co, and the faecal samples were analysed for DM, ash, N, uric acid, crude fat, NDF, Ti and Co. Uric acid was extracted using saturated lithium carbonate, and measured using a uric acid kit (HUMAN Diagnostics) according to manufacturer's instructions. Non-starch polysaccharides (NSP) were calculated as 1000–ash–(N×6.25)–crude fat–starch–sugar.

Health-related parameters

From three birds per pen with a BW close to the average pen BW, additional samples were collected. After euthanasia, the gizzard was separated from the proventriculus and the duodenum, and the full gizzard weighed. Gizzard contents were removed by rinsing with tap water and gently dried using a paper towel before the empty gizzard was weighed. From two of the same three birds per pen, the duodenum was separated from the gizzard and the jejunum before the pancreas was removed from the duodenal loop. A 1 cm piece of the proximal duodenum was dissected out just before the loop before the sample was gently rinsed in a physiological salt solution (0.9% NaCl) to remove remaining digesta before being stored in a phosphate buffered 10% formalin fixative at 4°C until further analyses. Before analyses, tissue samples were rinsed twice with tap water, and once with 70% alcohol, upon storage in 70% alcohol. The samples were cut in rings of ~3 mm length, placed in histology cassettes and embedded in paraffin using the Leica TP1020 tissue processor (Leica Microsystems B.V., Amsterdam, The Netherlands). The embedded tissue samples were cut in 5 µm thin sections, stretched, and placed on glass slides. Samples were stained using Mayer's haematoxylin and eosin standard staining protocols. A Leica DM6b microscope and LASX software (Leica Microsystems B.V., Amsterdam, The Netherlands) were used to measure villi length, crypt depth, tunica muscularis thickness and cross section of the duodenal lumen. Villus length was defined as the distance from the tip of a villus to the villus-crypt junction. Crypt depth was defined as the distance from the villus-crypt junction to the circular muscle layer. The tunica muscularis thickness was defined as the distance between the start of the circular muscle layer to the serosa. The cross section was defined as the maximum distance from the start of the circular muscle layer on opposite sides of the duodenum. The villi length to crypt depth ratio and the cross section per kg BW were calculated.

Calculations and statistical analyses

Performance parameters were calculated using FI and BW measurements over time. Apparent pre-caecal digestibility and apparent total tract digestibility of nutrients in the experimental diets were calculated, using Ti and Co as markers according to the following equation:

$$DC(X) = \left(1 - \frac{[Marker]diet \times [X]sample}{[Marker]sample \times [X]diet}\right) \times 100$$

where DC(X) is the apparent digestibility coefficient of nutrient X in % and $[Marker]_{diet}$, $[Marker]_{sample}$, $[X]_{diet}$, and $[X]_{sample}$ are the concentrations of the marker and nutrient X in the diet and digesta or excreta sample in g/kg, respectively. Apparent total tract nitrogen digestibility was calculated with faecal nitrogen corrected for nitrogen originating from urine with the use of uric acid.

Data were analysed using SAS statistical software (version 9.4, SAS Institute Inc., Cary, NC). For all data, a general linear model with contrast statements was used to determine 1) differences between birds fed the S diets and those fed the EU diets (SB, SU- and SU+ vs. EUB, EUU- and EUU+), 2) effect of seaweed inclusion *per se* (SB and EUB vs. SU-, SU+, EUU- and EUU+), 3) effects of enzyme pre-treatment (SU- and EUU- vs. SU+ and EUU+) and 4) interaction effects between Diet type and seaweed inclusion *per se*, and Diet type and enzyme pre-treatment. Model assumptions and goodness of fit were evaluated through normal distribution of residuals. Outliers identified by studentized residual >3 standard deviations from the sample mean were excluded from the analyses. Data are presented as means unless stated otherwise with differences among means with a probability <0.05 considered significant.

Results

Upon dissection, 3.5% of the birds originating from different pens and treatments were found to have ascites. No significant treatment effect was found on the incidence of ascites.

Nutritional composition

The enzymatically treated seaweed had a somewhat higher crude protein content and lower density compared to the untreated counterpart (Table 5.1). Due to isonitrogenous and isoenergetic diet formulation, S and EU diets differed in levels of fibrous components, crude fat, starch and sugar (Table 5.3). The average WHC of U diets (1.71) was numerically higher than that of B diets (1.44) and the average WHC of U- diets (1.77) was somewhat higher than that of U+ diets (1.66). All analysed micro minerals in the diets were within the limits based on the European regulations for animal diets (EG 1334/2003; EC 32/2002), and were not majorly impacted by the enzymatic treatment.

Diet type effect

In wk 1 and 2 of the experiment, a higher water intake (+27, and +63 mL per bird; P=0.015 and P<0.001, respectively) and water:feed (+0.17 and +0.09 mL/g; P=0.013 and P=0.016, respectively) were observed in birds fed S vs. EU diets (Table 5.4). In wk 3 and 4, birds fed S vs. EU diets had a higher BW (+41 and +64 g/bird; P<0.001 for both), BWG (+33 and +23 g/bird; P<0.001 and P=0.008, respectively) and FI (+25 and +27 g/bird; P=0.003 and P=0.031, respectively), as well as a lower FCR in wk 3 (-0.05 g/g; P<0.001). Based on the Co-EDTA marker, pre-caecal OM digestibility of S diets was higher than that of EU diets (P<0.001; Table 5.5). Increased gizzard weight (empty: +1.3 g/kg BW and full: +4.7 g/kg BW) and contents (+3.7 g/kg BW) were observed in birds fed EU vs. S diets (P<0.001 for all; Table 5.6). Furthermore, longer villi (+150 µm; P=0.010) and increased (P=0.0281) villus height:crypt depth were observed in birds fed EU vs. S diets.

Ulva laetevirens *co-product inclusion effect*

Dietary inclusion of 5% U in wk 3 and 4 increased FCR (+0.05 and +0.06 g/g; P<0.001 for both), water intake (+83 and +129 mL/bird; P=0.002 and P<0.001, respectively) and water:feed (+0.10 and +0.10 mL/g; P<0.001 for both). Furthermore, the apparent pre-caecal ash (P<0.001), OM (P<0.001), and N (P=0.012) digestibility coefficients based on the Co-EDTA marker were all lower after U inclusion. The duodenal cross section was larger in birds fed U vs. B diets (+160 µm/kg BW; P=0.033).

Enzyme treatment effect

Water:feed in the last wk of the experiment was higher for birds fed U- vs. U+ diets (+0.06 mL/g; P=0.029), as were apparent total tract, but not pre-caecal ash digestibility based on both the Ti and Co-EDTA markers (both P<0.001). The duodenal cross section was larger in U- vs. U+ diets (+206 µm/kg BW; P=0.021).

Diet type×Ulva laetevirens *inclusion effect*

Dietary U inclusion in wk 3 led to a lower BWG in birds fed S (-27 g/bird) vs. EU diets (-1.5 g/bird; Diet type×U effect; P=0.033). For birds fed S diets, U led to higher apparent pre-caecal digestibility of all nutrients based on the Ti marker, whereas for birds fed EU diets a lower digestibility was observed after U inclusion (P<0.001 for all nutrients). Based on both the Ti and Co-EDTA markers, a stronger reduction in apparent total tract crude fat digestibility was observed after U inclusion in EU vs. S diets (Ti and Co-EDTA P<0.001) as well as a stronger increase in apparent total tract NDF digestibility after U inclusion in S vs. EU diets (Ti P<0.001; Co-EDTA P=0.001).

Table 5.4. Effect of inclusion of 2.5 (d7-13) and 5% (d14-end of experiment) untreated (-) and enzymatically[1] treated (+) seaweed (*Ulva laetevirens*) co-product in broiler diets (basal) based on soy or European protein sources on performance parameters.

Period	Soy-based diets (SD)			European protein-based diets (ED)			SEM	P-values[3]				
Parameter[2]	Basal (B)[4]	*U. laetevirens* (U)		Basal[4]	*U. laetevirens*			SD vs. ED (DT)	B vs. U	Enzyme (E)	DT×E	DT×U
		-	+		-	+						
Day 0-7 (starter diet 1)												
Body weight gain (g)	167	166	168	166	163	170	1.4	0.793	-	-	-	-
Feed intake (g)	163	164	169	164	161	167	1.2	0.662	-	-	-	-
Feed conversion ratio (g/g)	0.97	0.98	1.00	0.99	0.98	0.99	0.003	0.951	-	-	-	-
Water intake (mL)	499	492	507	464	464	488	5.7	0.015	-	-	-	-
Water:feed (mL/g)	3.07	3.01	2.99	2.74	2.89	2.92	0.037	0.013	-	-	-	-
Body weight d7	210	209	211	209	206	212	1.4	0.793	-	-	-	-
Mortality (% per pen)	0.0	0.0	3.8	1.9	1.0	0.0	0.4	0.690	-	-	-	-
Day 7-14 (starter diet 2)												
Body weight gain	381	378	380	371	371	378	2.4	0.173	0.849	0.396	0.636	0.505
Feed intake	443	447	450	438	439	448	2.8	0.351	0.251	0.314	0.557	0.940
Feed conversion ratio	1.19	1.18	1.18	1.18	1.18	1.18	0.002	0.549	0.988	0.811	0.946	0.650
Water intake	933	944	955	849	891	904	10.4	<0.001	0.155	0.605	0.964	0.360
Water:feed	2.06	2.11	2.13	1.95	2.06	2.02	0.022	0.016	0.066	0.796	0.648	0.918
Body weight d14	591	587	590	580	577	591	3.4	0.271	0.886	0.277	0.522	0.627
Mortality	0.0	1.0	3.0	1.0	0.0	1.0	0.4	0.455	0.434	0.235	0.675	0.198
Day 14-21 (grower diet)												
Body weight gain	524	498	497	474	479	466	3.9	<0.001	0.077	0.458	0.216	0.033
Feed intake	747	739	736	702	732	714	4.3	0.003	0.564	0.267	0.451	0.064
Feed conversion ratio	1.43	1.47	1.48	1.48	1.53	1.53	0.006	<0.001	<0.001	0.909	0.979	0.904
Water intake	1344	1410	1385	1284	1400	1392	12.9	0.416	0.002	0.578	0.792	0.229
Water:feed	1.80	1.91	1.88	1.83	1.91	1.95	0.014	0.242	<0.001	0.857	0.355	0.888
Body weight d21	1114	1091	1087	1054	1057	1057	5.9	<0.001	0.351	0.867	0.847	0.167
Mortality	1.9	4.9	2.1	0.0	1.9	1.0	0.6	0.072	0.195	0.226	0.524	0.970

Table 5.4 (continued). Effect of inclusion of 2.5 (d7-13) and 5% (d14-end of experiment) untreated (-) and enzymatically[1] treated (+) seaweed (*Ulva laetevirens*) co-product in broiler diets (basal) based on soy or European protein sources on performance parameters.

Period	Soy-based diets (SD)			European protein-based diets (ED)			SEM	P-values[3]				
Parameter[2]	Basal (B)[4]	*U. laetevirens* (U)		Basal[4]	*U. laetevirens*			SD vs. ED (DT)	B vs. U	Enzyme (E)	DT×E	DT×U
		-	+		-	+						
Day 21-28 (grower diet)												
Body weight gain	702	680	679	671	670	652	4.5	0.008	0.071	0.310	0.341	0.477
Feed intake	1031	1058	1034	1000	1030	1012	6.6	0.031	0.191	0.157	0.845	0.795
Feed conversion ratio	1.47	1.54	1.52	1.49	1.54	1.55	0.007	0.125	<0.001	0.732	0.398	0.987
Water intake	1813	1904	1961	1780	1927	1910	16.6	0.514	<0.001	0.435	0.151	0.717
Water:feed	1.76	1.80	1.90	1.78	1.87	1.89	0.013	0.294	<0.001	0.029	0.116	0.895
Body weight d28	1816	1771	1766	1726	1726	1709	9.0	<0.001	0.134	0.538	0.703	0.185
Mortality	2.9	3.1	4.2	5.0	0.0	1.9	0.7	0.439	0.274	0.312	0.795	0.108

- not applicable.

[1]Neutrase (Novozymes, 0.8 AU-N/g).

[2]Each value is based on 8 replicate pens of 13 birds.

[3]Statistical contrasts: Diet type (SD basal, SD *U. laetevirens*- and SD *U. laetevirens*+ diets) vs. (ED basal, ED *U. laetevirens*- and ED *U. laetevirens*+ diets), Basal vs. *U. laetevirens*: (SD basal and ED basal diets) vs. (SD *U. laetevirens*-, SD *U. laetevirens*+, ED *U. laetevirens*- and ED *U. laetevirens*+ diets), Enzyme: (SD *U. laetevirens*- and ED *U. laetevirens*- diets) vs. (SD *U. laetevirens*+ and ED *U. laetevirens*+ diets).

[4]All birds were fed their respective basal diet from d0-6.

Table 5.5. Effects of inclusion of 2.5 (d7-13) and 5% (d14-end of experiment) untreated (-) and enzymatically[1] treated (+) seaweed (*Ulva laetevirens*) co-product in a broiler grower diet (basal) based on soy or European protein sources on apparent pre-caecal and total tract nutrient digestibility in broilers based on the titanium dioxide (Ti) and cobalt-ethylenediamine tetraacetic acid (Co-EDTA) markers.

Digestibility[2]	Soy-based diets (SD)			European protein-based diets (ED)			SEM	P-values[3]				
Nutrient	Basal (B)	*U. laetevirens* (U)		Basal	*U. laetevirens*			SD vs. ED (DT)	B vs. U	Enzyme (E)	DT×E	DT×U
		-	+		-	+						
Apparent pre-caecal (%) based on Ti												
Ash	44.9	58.2	58.6	40.7	29.0	29.8	1.78	<0.001	0.865	0.948	0.835	<0.001
Organic matter	74.4	82.0	81.8	70.0	65.2	66.1	1.01	<0.001	0.473	0.916	0.347	<0.001
Nitrogen	80.7	86.3	85.8	79.5	77.1	77.5	0.60	<0.001	0.241	0.976	0.515	<0.001
Apparent total tract (%) based on Ti												
Ash	32.2	27.8	42.0	32.1	24.0	40.8	0.96	0.386	0.474	<0.001	0.009	0.597
Nitrogen	77.8	75.3	74.1	74.9	71.9	71.3	0.37	<0.001	<0.001	0.245	0.551	0.852
Crude fat	68.1	56.9	48.1	51.4	22.5	21.2	2.63	<0.001	<0.001	0.227	0.008	<0.001
Neutral detergent fibre	6.3	19.6	16.4	17.1	20.3	20.1	0.87	0.003	<0.001	0.239	0.193	<0.001
Apparent pre-caecal (%) based on Co-EDTA												
Ash	29.7	17.8	19.7	29.0	19.6	19.9	0.93	0.814	<0.001	0.382	0.553	0.437
Organic matter	67.3	64.6	64.0	64.2	60.6	61.3	0.50	<0.001	<0.001	0.950	0.455	0.856
Nitrogen	75.4	72.9	71.8	75.5	74.0	74.3	0.51	0.140	0.012	0.690	0.479	0.300
Apparent total tract (%) based on Co-EDTA												
Ash	20.1	15.1	33.3	23.2	15.6	35.0	1.20	0.455	0.216	<0.001	0.253	0.719
Nitrogen	73.9	70.9	70.3	71.4	68.8	68.5	0.33	<0.001	<0.001	0.437	0.701	0.477
Crude fat	62.5	49.4	40.4	44.6	13.9	15.3	2.66	<0.001	<0.001	0.370	<0.001	<0.001
Neutral detergent fibre	-10.4	5.4	3.9	5.4	11.5	12.3	1.27	<0.001	<0.001	0.865	0.371	0.001

[1]Neutrase (Novozymes, 0.8 AU-N/g).

[2]Each value is based on 8 replicate pens of 13 birds.

[3]Statistical contrasts: Diet type (SD basal, SD *U. laetevirens*- and SD *U. laetevirens*+ diets) vs. (ED basal, ED *U. laetevirens*- and ED *U. laetevirens*+ diets), Basal vs. *U. laetevirens*: (SD basal and ED basal diets) vs. (SD *U. laetevirens*-, SD *U. laetevirens*+, ED *U. laetevirens*- and ED *U. laetevirens*+ diets), Enzyme: (SD *U. laetevirens*- and ED *U. laetevirens*- diets) vs. (SD *U. laetevirens*+ and ED *U. laetevirens*+ diets).

Table 5.6. Effects of inclusion of 2.5 (d7-13) and 5% (d14-end of experiment) untreated (-) or enzymatically[1] treated (+) seaweed (*Ulva laetevirens*) co-products in a broiler diet (basal) based on soy or European protein sources (Diet type) on gastro-intestinal tract characteristics.

Tissue	Soy-based diets (SD)			European protein-based diets (ED)			SEM	P-values[4]				
Parameter	Basal (B)	*U. laetevirens* (U)		Basal	*U. laetevirens*			SD vs. ED (DT)	B vs. U	Enzyme (E)	DT×E	DT×U
		-	+		-	+						
Gizzard (g/kg BW)[2]												
Gizzard weight empty	9.8	9.4	9.8	10.8	11.3	10.8	0.15	<0.001	0.895	0.784	0.190	0.507
Gizzard weight full	14.5	13.2	13.1	18.2	18.3	18.5	0.46	<0.001	0.552	0.997	0.871	0.360
Gizzard content	4.6	3.8	3.7	7.4	8.0	7.8	0.39	<0.001	0.867	0.932	0.977	0.400
Duodenum (µm)[3]												
Villus length (VL)	1909	1873	1758	1967	2050	1972	0.029	0.010	0.658	0.222	0.789	0.252
Crypt depth (CD)	121	120	128	121	124	120	0.002	0.796	0.667	0.745	0.373	0.891
VL:CD	16.2	14.8	14.2	16.4	16.9	17.0	0.410	0.0281	0.482	0.838	0.737	0.183
Muscularis thickness	123	129	126	129	124	122	0.002	0.755	0.886	0.747	0.902	0.343
Cross section (µm/kg BW)	3121	3364	3237	3184	3467	3182	0.051	0.565	0.033	0.021	0.308	0.852

[1]Neutrase (Novozymes, 0.8 AU-N/g).

[2]Each value is based on 6 replicate pens of 3 birds.

[3]Each value in the table is based on 6 replicate pens of 2 birds.

[4]Statistical contrasts: Diet type (SD basal, SD *U. laetevirens*- and SD *U. laetevirens*+ diets) vs. (ED basal, ED *U. laetevirens*- and ED *U. laetevirens*+ diets), Basal vs. *U. laetevirens*: (SD basal and ED basal diets) vs. (SD *U. laetevirens*-, SD *U. laetevirens*+, ED *U. laetevirens*- and ED *U. laetevirens*+ diets), Enzyme: (SD *U. laetevirens*- and ED *U. laetevirens*- diets) vs. (SD *U. laetevirens*+ and ED *U. laetevirens*+ diets).

Diet type×enzyme treatment effect

No Diet type×Enzyme effects were observed for performance. Apparent total tract crude fat digestibility was further reduced when U+ was added to EU diets (-30% absolute) than when added to S diets (-16% absolute) based on both the Ti (P=0.008) and Co-EDTA markers (P<0.001).

Ti versus Co-EDTA marker

Generally lower digestibility values were observed based on the Co-EDTA vs. the Ti marker (Table 5.5). The apparent total tract digestibility coefficients were lower for ash (-28%), N (-4.8%), crude fat (-16%) and NDF (-72%) calculated with the Co-EDTA marker.

Discussion

Diet type effect

The observed higher water intake in wk 1 and 2 of birds fed the S vs. EU diets is not in line with observations by Jiménez-Moreno *et al.* (2016). These authors reported an increased water intake in birds fed higher insoluble NSP levels at young ages. In the current study, dietary levels of NDF and ADF were twice that of the levels of Jiménez-Moreno *et al.* (2016) although here we did not differentiate between soluble and insoluble fibres or NSP. Solubility of fibrous components strongly determines their biological effects (Mateos *et al.*, 2013). A larger WHC of a diet also increases water intake (Jiménez-Moreno *et al.*, 2016). However, WHC in our study was similar for EU vs. S diets, hence not explaining the high water intake of birds fed S diets.

Despite diet formulation aimed at similar quantities of digestible amino acids and calculated AME (based on ingredient values), small differences between diets were observed, such as a lower AME:apparent digestible protein ratio of the S (66.64-72.87 MJ/kg) vs. EU (74.86-78.15 MJ/kg) diets. Contrary to our findings, in the literature feed intake, weight gain and feed efficiency (in g BW gain /kg feed) of male broilers from 0-3 wk of age improved when birds were fed higher AME:digestible protein ratios (70.4 vs. 77.7 MJ/kg; Gous *et al.*, 2018), although the absolute AME in our diets (12.28-12.59) correspond with the AME of their 70.4 MJ/kg digestible protein diet (AME: 12.3 MJ/kg). This indicates that the EU diets, containing the higher AME:digestible protein ratio, were similar in energy but only higher in protein compared to the S diets. When Gous *et al.* (2013) investigated body composition, protein gain was not increased with increasing AME:digestible protein ratio diets, whereas they observed an increased lipid gain, meaning energy was not the limiting factor, whereas that might have been the case for the birds in our study.

Another cause for a reduced feed intake can be in response to a combination of fibre sources (sugar beet pulp and rice hulls; Sadeghi *et al.*, 2015) and in particular of soluble fibres (Rahmatnejad and Saki, 2016). Since the EU diet contained a range of fibre sources, this could also explain the observed lower feed intake. In addition, according to Annison (1993), higher soluble NSP levels of for example wheat, increase digesta viscosity, reduce the diffusion rate of digestive enzymes into digesta, hamper their interaction at the mucosal surface and hence reduce nutrient utilization. In the current experiment, indeed a reduced digestibility of the EU vs. S diet and an increased FCR of birds fed those diets was observed, although wheat inclusion in the EU vs. S diets was only slightly higher.

The EU diets contained 24-35 g/kg palm fat, whereas the S diets did not. Valencia *et al.* (1993) reported no differences in growth and efficiency due to oil source, but observed an increased BW of 21 d old broilers fed diets with increasing levels of oil from 0 to 2 and 4%, but a decrease with further increasing oil levels to 6, 8 and 10%, while maintaining constant energy levels. In our study, the EU diets contained 4.8-6.7% soybean oil+palm fat, and compared to the 1.7-3.5% in the S diets, this might also have had a negative effect on performance.

Heavier gizzards (+13%) and gizzard contents (+35%) were observed in birds fed the EU vs. S diets, which is in accordance with data in the literature. For example, 33% heavier gizzards were observed as a result of higher dietary fibre levels (ground to <2.5 mm; Jiménez-Moreno *et al.*, 2009b; 2019). Additionally, studies of Jiménez-Moreno *et al.* (2010; 2019) observed a reduced gizzard pH and increased apparent total tract retention of N in broilers of 21 d of age in response to increasing dietary fibre levels. Surprisingly, we observed the opposite: a lower apparent total tract N digestibility in the EU vs. S diets. This could be explained by differences in the level of all dietary fibre components. For example, NDF in our diets ranged from 101 to 139 g/kg DM, whereas 84 g/kg DM was the highest level in the study of Jiménez-Moreno *et al.* (2019). Potentially the optimal level of fibres had been surpassed and the surplus of fibres in the EU diets had a negative effect on nutrient digestibility. Moreover, different responses to different fibre sources are observed in various studies in the literature (Jiménez-Moreno *et al.*, 2010).

Longer villi correspond to a higher nutrient uptake capacity (Cañedo-Castro *et al.*, 2019). The longer villi in birds fed the EU vs. S diets was contrary to our hypothesis, as we expected a decrease in villus length in response to more abrasive digesta and sloughing off of cells due to higher fibre inclusion. The lack of differences in crypt depth in birds fed the EU vs. S diets indicate that the turnover rate of enterocytes was not different between birds fed the S and EU diets. Similar observations are reported for crypt depth in response to increasing fiber levels in the literature (Tejeda and Kim, 2020). Furthermore, longer villi are reported in response to diets higher in fibre (Tejeda and Kim, 2020; Rahmatnejad and Saki, 2016). The latter studies report that the longer villi are due to more stimulus of abrasive insoluble fibres, whereas soluble fibres seem to decrease villi height. This would indicate that the EU diet contained more abrasive insoluble fibres compared to the S diet. This was in line with our research setup

including higher fibre levels in the EU diet, although in this study no differentiation was made between soluble and insoluble fibres. Despite the observed histo-morphological differences between treatments, no differences were observed in nutrient absorption, nor in performance between birds fed the EU and S diets.

Ulva laetevirens *inclusion*

Macro and micro mineral levels were all within the limits for safe use in broiler diets, although the ash content of the *U. laetevirens* co-products (270 g/kg DM) will likely cause problems when these are included at higher levels than in the current experiment.

Despite the differences in ash, fibre, crude fat and starch contents in the U vs. B diets, U inclusion at 2.5% did not affect performance parameters of the broilers in wk 2. The higher water intake and water:feed observed in birds fed the U vs. B diets in wk 3 and 4, with dietary U inclusion levels of 5%, are corresponding to the higher mineral content and the higher WHC of the U diets. A high water intake could lead to diarrhoea, suboptimal bird health and reduced performance (Guiry and Blunden, 1991; Koreleski *et al.*, 2010). The higher water intake in birds fed the U diets might have induced the negative effect on FCR by flushing out nutrients, although diarrhoea was not visually observed. The higher WHC observed for the U diets might have caused more bulky feed/chyme boluses due to the greater water retention at a similar feed intake. In this study, this is reflected by a wider duodenum (calculated per kg BW) in birds fed the U vs. B diets, but not by a larger gizzard. This was also not reflected in a lower feed intake due to a fuller crop with the high water intake due to minerals and high WHC of the diets.

In the S diets, U inclusion led to an increased apparent pre-caecal digestibility of all nutrients, but to a decreased apparent total tract nutrient digestibility, although BWG was decreased in wk 3. This increase in apparent pre-caecal nutrient digestibility in birds fed the seaweed supplemented S diets might be a beneficial effect of the addition of fibres, originating from the seaweed products, in the diet. In contrast, U inclusion in the EU diets led to a decrease in apparent pre-caecal ash, OM and N digestibility, and some small changes in digestibility of the fibrous dietary components, without affecting BWG. Potentially, there was already sufficient fibrous material present in the EUB diets to optimize digestion, and the maximum degradation capacity might have already been reached, meaning the extra fibrous material merely hampered digestion capacity. Moreover, changes in the physico-chemical conditions in the gastro-intestinal tract, due to the different diet types, may have resulted in a variation in responses in nutrient digestibility and performance traits (Tejeda and Kim, 2021; Choct *et al.*, 2010).

Enzyme treatment effect

As a consequence of the enzymatic treatment, the N content in the U+ product was slightly higher (+4.2%) in U+ vs. U- products, although due to the relatively low enzyme

inclusion levels the dietary N content was only marginally higher in the U+ vs. the U- diets. In relation to nutrient digestibility, the enzyme treatment only affected apparent total tract ash digestibility, which was increased in the U+ vs. U- diets, and decreased apparent total tract crude fat digestibility in the S but not in the EU diets. The decreased WHC of U+ vs. U- diets did not result in differences in for example water intake or gizzard content, contrary to data in the literature (Jiménez-Moreno *et al.*, 2016; 2009b). The general lack of observed differences indicates that the enzyme treatment did not improve the nutritional value of the seaweed products for broilers. If the enzyme treatment did release protein, peptides or amino acids, these might have been subject to complex forming, for example with heavy metals (Ashmead, 1992), and this may have hampered nutrient digestibility.

Digestibility based on Ti and Co-EDTA markers

Generally, the recovery of Ti (Jagger *et al.*, 1992; Sales and Janssens, 2003; Kavanagh *et al.*, 2001) is higher than that of Co (Marais, 2000; De Vries *et al.*, 2014; Udén *et al.*, 1980), consequently leading to higher calculated digestibility coefficients (De Vries and Gerrits, 2018). Digestibility coefficients in our study were indeed higher when calculated based on Ti. A high correlation was observed in digestibility coefficients based on the Ti and Co markers, especially for apparent total tract nutrient digestibility, with correlation coefficients ranging between 0.932 and 0.997 ($P<0.001$ for all). It is known that Co(II)EDTA is not completely stable, which might lead to absorption of Co in the gastro-intestinal tract and violates the assumption of inertness of the markers (De Vries and Gerrits, 2018). Furthermore, the liquid phase of digesta, in which the Co-EDTA marker is present, passes through the gastro-intestinal tract at a different rate compared to the solid phase containing the Ti marker, and might accumulate in parts of the gastro-intestinal tract (illustrated by De Vries *et al.*, 2014). Reflux, in particular of the insoluble digesta fraction, might for example increase the concentration of the solid phase marker (Ti) in the ileum (Sacranie *et al.*, 2012). This might lead to higher calculated pre-caecal compared to total tract nutrient digestibility values. This was indeed observed in the present study, although only for the high fibre S diets (SU- and SU+) but not for the EU diets. One notable difference is that of the apparent pre-caecal ash and N digestibility calculated using the two markers, specifically in the S diets. Calculated based on Ti, both ash and N digestibility increase with U inclusion, whereas based on Co they decrease with U inclusion. This might be related to the fibre fraction that holds the N and increases the reflux of solid phase digest, whereas the Co follows the liquid digesta phase and increases in concentration in the ceca as mentioned before (Sacranie *et al.*, 2012).

Digestibility of NDF was low in all experimental groups, as is expected for fibres in broilers and poultry in general. Some negative NDF digestibility coefficients were observed based on calculations using the Co marker. This indicates that the Co marker, that follows the soluble, or liquid phase of the digesta is not following the insoluble NSPs. Hence, the Co marker appears to be less suitable compared to the Ti marker for calculating digestibility of insoluble fibrous fractions in broilers.

Conclusions

This study confirms the high mineral content of *U. laetevirens* and its relatively poor nutrient digestibility in broilers. Dietary *U. laetevirens* increased apparent pre-caecal digestibility of nutrients when included in a corn-soy based diet, but decreased apparent pre-caecal digestibility when included in a diet based on European protein sources, although in both diet types *U. laetevirens* reduced rather than improved performance. The proteolytic enzyme treatment of an *U. laetevirens* co-product did not affect performance, nor did it increase nutrient digestibility, and is thus not suitable to increase the nutritional value of this seaweed co-product for broilers. No effects were observed on performance, gizzard development or histo-morphological parameters, indicating that bioactive properties related to these measurements of the seaweed co-product were lacking.

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Chapter 6

General discussion

Introduction

My research attempted to provide an analysis of the use of seaweed (or its products) as a dietary ingredient for chickens, contributing to the knowledge needed to include seaweed for its nutritional value in their diets at a large scale. Livestock for animal protein production need to be fed diets, and thus require dietary ingredients, that provide animals with sufficient and balanced nutrients to live up to their potential with an efficient use of resources. In addition, there is a strong drive to include dietary ingredients that do not partake in the food-feed competition. Emphasis is placed onto the efficiency with which animals utilise dietary nutrients. To sustain the livestock industry in the future, the search for novel dietary ingredients that fit these requirements is receiving significant interest. This research contributes to that search for novel ingredients with a particular focus towards seaweed.

The potential of seaweed as a dietary ingredient for livestock diets was investigated, with a focus on broilers (Figure 6.1). For this purpose, as a first step the nutritional value of various intact seaweeds, sourced from marine waters around Northwestern Europe was investigated for their nutritional composition and *in vitro* digestibility (Chapter 2). Based on these results, four major aspects were determined that are important before seaweed can become a valuable dietary ingredient for livestock: a reduction of the high mineral content, an increase in nutrient availability for the animal, a reduction of the cost-price and an increased shelf life. The effects of methods to address these aspects, such as washing and processing, on the nutritional value of the seaweed products were studied in Chapter 3. The nutritional composition and *in vitro* digestibility were investigated, as well as the effects of diets containing seaweed products in broilers. An enzyme treatment to increase nutrient availability for broilers was applied to co-products of two potentially interesting seaweed species (Chapter 4). Next to growth performance, in this experiment several chyme and gastro-intestinal characteristics as well as health-related parameters were investigated. To validate the observed results,

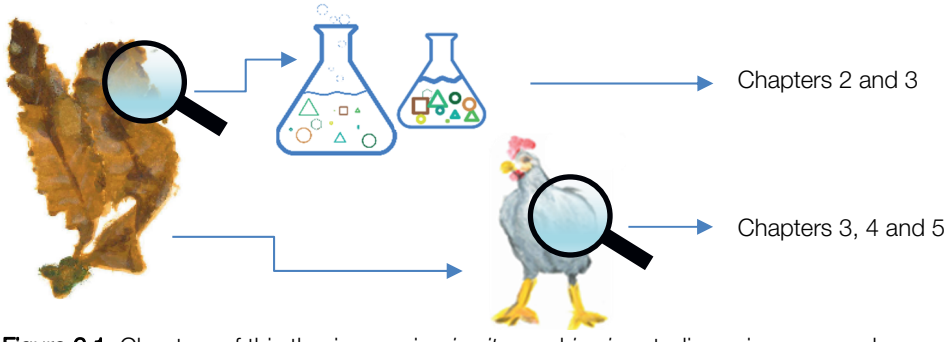

Figure 6.1. Chapters of this thesis covering *in vitro* and *in vivo* studies using seaweed.

and to further investigate working mechanisms, a co-product of the most promising seaweed species was studied with and without enzyme treatment in Chapter 5. To study the potential of these products in a more locally sourced diet, the co-products in this experiment were included in a standard corn-soy based diet as well as in a diet based on European protein sources.

In the following section, I place the findings of my PhD research in a broader perspective. I aim to discuss the applicability and the potential of seaweed for livestock diets, specifically for broilers, from data of my own research, related to observations reported in literature. Additionally, I will describe preliminary analyses related to the viscosity of seaweed diets and its effect on intestinal chyme characteristics. Limitations on the use of seaweed in broiler nutrition will be discussed before this chapter will be finalized with recommendations for the industry and the conclusions of this research.

Use of seaweed in livestock diets

Seaweed has been part of animal diets for centuries. For example, Orkney sheep have been consuming seaweed as a main part of their diet since 1832, when they were confined to the beach of their Orkney island (The Orkney sheep foundation, 2021). More commonly, seaweed has been fed as roughage to ruminants in coastal areas, especially during winter when grasslands and other feed sources were scarce (Evans and Critchley, 2014). Through the science of nutrition it became apparent that fresh or dried intact seaweed did not have a great nutritional value (Evans and Critchley, 2014). In recent times, seaweed is not often included in diets of, especially intensively farmed, livestock. Current livestock farming is based on providing nutritionally adequate diets with a high nutrient availability to increase animal performance and optimize resource use. Hence, if seaweed is to contribute to balanced livestock diets, it also needs to be studied from a nutritional point of view.

Seaweed in broiler diets

A large variation in nutritional composition of the seaweeds investigated in the present study was observed, corresponding with data of others in the literature (a.o. Belghit *et al.*, 2017; Biancarosa *et al.*, 2017). This variation was observed both between and within seaweed species as well as within seaweed co-products originating from the same seaweed species, harvest location and period of harvest (Chapter 4 vs. 5; Schiener *et al.*, 2015; Sharma *et al.,* 2018).

Macro nutrients

Mineral content ~ The mineral content of seaweed (and its co-products) is high (170-465 g/kg DM; Evans and Critchley, 2014; Chapters 2, 3, 4 and 5), which can be reduced by washing (Chapter 3). Despite a reduction in ash content of 4 – 83% due to washing with fresh water (Figure 6.2; Chapter 3; Neveux *et al.*, 2014; Milledge *et al.*, 2018), the remaining ash content is still high and only allows for inclusion of these products in broiler diets for their macronutrients at levels of <5%. Additionally, washing leads to loss of other nutrients (Chapter 3) decreasing the value of the remaining product for broiler nutrition.

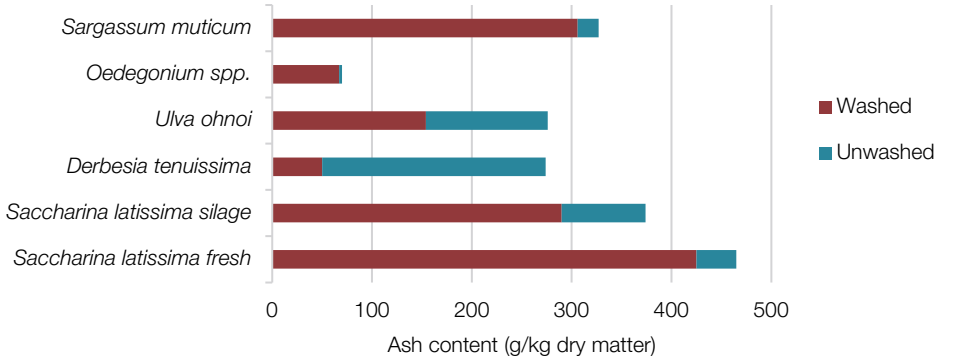

Figure 6.2. The effect of a freshwater washing treatment on reducing the ash content of seaweed in g/kg dry matter for the species *Sargassum muticum* (Milledge et al., 2018), *Oedegonium spp*., *Ulva ohnoi* and *Derbesia tenuissima* (Neveux *et al.*, 2014) and both fresh and ensiled *Saccharina latissima* (Chapter 3).

If seaweed or their co-products are included in broiler diets, the maximum inclusion level is determined to a certain extent by the mineral content, as discussed in this thesis. High mineral intake often increases water intake, and consequently enhances excreta water content (Chapter 4; Koreleski *et al.*, 2010). Furthermore, *Ulva laetevirens* co-product inclusion in broiler diets led to an increased water holding capacity (WHC) of the diets (Chapter 5), which also increases water intake (Jiménez-Moreno *et al.*, 2016). The increased WHC of the seaweed containing diets is likely caused by the soluble NSPs present in the seaweed products. These are not well digested or fermented to a large extent in poultry (Annison, 1993), and might thus also influence excreta WHC, although this was not determined in my studies.

Wet litter in poultry husbandry is a major problem. It can lead to footpad dermatitis, and consequently to a reduced welfare, performance and carcass yield in broilers (De Jong *et al.*, 2014). In the experiments performed in this thesis, footpad dermatitis was not observed, although the studies conducted in Chapter 3 to 5 are not representative for commercial broiler husbandry. The stocking density in the studies reported here ranged

from 7 to 11 broilers/m^2, whereas in intensive broiler husbandry in the European Union the maximum allowed stocking density is 42 kg/m^2 (European Commission, 2007), corresponding to a maximum of 18 broilers/m^2 (slaughter weight of 2.4 kg; Blanken *et al.*, 2021). A higher stocking density increases the amount of faeces per unit of floor space and consequently the risk for footpad dermatitis. The prevalence of severe footpad dermatitis ranges from 38 to 70% in intensive broiler husbandry (Allain *et al.*, 2009; Gouveia *et al.*, 2009; De Jong *et al.*, 2012). This has large consequences for bird health and welfare, as well as for economics. An experimentally induced footpad dermatitis in 90% of the birds led to a calculated reduction in margin of 50% per bird (De Jong *et al.*, 2014). This indicates that wet litter has severe consequences for broiler welfare and as such farmer income, and hence needs to be monitored closely when seaweed consumption increases water intake due to the mineral and soluble NSP content.

Next to the effects on performance, health and welfare, a high mineral intake leads to a high mineral excretion. In the Netherlands alone, approximately 94 million kg chicken manure is produced per year (CBS, 2019). About half of it is exported and one third is used for energy production (Billen *et al.*, 2015). During the latter process, the excreta with litter material is burned to produce electricity, and the remaining minerals like phosphorous and potassium are sold as organic fertilizer (BCMMoerwijk.nl, 2021). Specific markets are available for fertilizers high in for example potassium or phosphorous, depending on the specific needs of farmers in different areas. In general, chicken manure is a rich source of nitrogen and minerals. When the excreta would be enriched with minerals due to a high seaweed mineral intake, the mineral yield will increase, although the market for this fertilizer might be slightly different compared to that for fertilizer produced from excreta of non-seaweed-fed chickens, depending on the specific mineral composition of seaweed products and consequently of excreta. Caution is advised related to the potential of seaweed to accumulate heavy metals (Roleda *et al.*, 2019). These might be excreted and end up in the fertilizer, causing these to pollute soil and potentially vegetal material grown on these soils.

If part of a broiler seaweed diet consists of minerals, a smaller part of the diet is available for dietary ingredients that supply the animal with protein and energy. At low seaweed inclusion levels, the consequences will be limited, but with increasing inclusion levels the minerals dilute other nutrients. If for example 200 g/kg unwashed *Saccharina latissima* (ash content: 430 g/kg; Chapter 3) would be included in a broiler diet, 86 of those 200 g would consist of minerals, in total leaving only 914 g/kg in the diet to supply the animal with other nutrients. Consequently, the remaining ingredients need to be of higher nutrient density and quality to formulate a nutritionally adequate diet, or that for example more synthetic amino acids need to be included. The need to include higher quality ingredients will increase the environmental and economic impact of the diet, contrary to the idea of including seaweed in broiler diets partly to reduce the environmental impact of the broiler industry.

Protein ~ Seaweed can be high in protein (up to 376 g/kg DM; e.g. Biancarosa *et al.*, 2017; Øverland *et al.*, 2019), and the amino acid (AA) composition can be of a good

quality for animal nutrition (Angell *et al.*, 2016a). Despite this potential, the protein and AA content and quality are subject to large variation between and within seaweed species, which makes it difficult to include seaweed or its co-products in practical diets based on standardised tabulated values as is commonly done with more conventional feed ingredients. Furthermore, the average protein content is not nearly as high as that of dietary ingredients included in diets as a protein source like soybean meal (524 g/kg DM; Chapter 2). In addition, a larger part of the nitrogen present in seaweed is non-protein nitrogen, resulting in lower nitrogen-to-protein conversion factors for seaweed and their co-products compared to other feed ingredients. This leads to the conclusion that dietary inclusion of intact seaweed for its protein or AA content is at present economically not feasible. On the contrary, seaweed protein concentrates can be produced, which might be a source of good quality protein. This does require a biorefinery approach, where the sum of the value of the individual seaweed products make the use of seaweed protein for animal nutrition economically viable. However, it is highly likely that when a good quality protein extract is produced, it will be first used in human nutrition instead of in animal nutrition.

Fibre level ~ Seaweed is relatively high in fibre and NSP (on average 550 g/kg DM; Chapter 2), which are largely indigestible for broilers (Annison, 1993). Non-starch polysaccharides can be either water soluble or insoluble. The latter are not digested nor fermented in the poultry gastro-intestinal tract to a significant extent, but at moderate levels stimulate gastro-intestinal functioning though at higher inclusion levels exert opposite effects (Hetland *et al.*, 2014; Sacranie *et al.*, 2012; Mateos *et al.*, 2013; Jiménez-Moreno *et al.*, 2016). Furthermore, insoluble NSP absorbs large quantities of water, increasing the bulk of digesta. At low inclusion levels, this stimulates normal motility of the intestine and optimises digesta passage rate, in turn preventing microbial fermentation in the upper parts of the intestine (Nguyen *et al.*, 2021a). On the other hand, dietary ingredients containing insoluble NSP act as nutrient diluents in diets for high performing, highly efficient broilers, increasing the need for high quality ingredients to fulfil nutritional requirements.

Soluble NSP, on the other hand, increase digesta retention time in the gastrointestinal tract, leading to a potentially improved nutrient digestibility (Nguyen *et al.*, 2021a). At higher levels, soluble NSP can lead to strongly increased digesta viscosity and consequently reduce nutrient digestibility although their precise effect on digesta viscosity is dependent on the molecular size, presence of charged groups, and their concentration (Smits and Annison, 1996). The NSP of seaweed increases digesta viscosity and reduces nutrient digestion and absorption, although the gelling properties of seaweed are not yet fully understood (Holdt and Kraan, 2011). Hence, it is of importance to understand the viscosity inducing effects of seaweed fibrous components before including those in broiler diets.

To illustrate the different levels of these three major components; minerals, protein and NSP of seaweed, Figure 6.3 shows those components of the seaweed co-products included in Chapter 4. To illustrate, corn is included as a common energy source, whereas soybean meal is included as common protein source in boiler diets. The pie

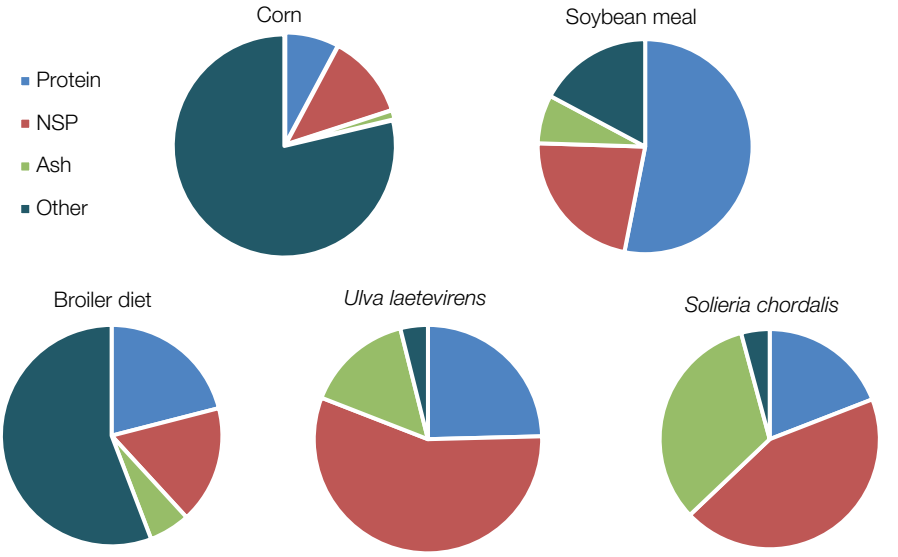

Figure 6.3. The content of three major components protein, non-starch polysaccharide (NSP) and minerals (expressed as ash) on a dry matter base of common dietary ingredients, a broiler grower diet and two seaweed co-products. Corn is included as a common energy source (CVB, 2021) and soybean meal as a protein source (Chapter 2) versus the composition of a standard corn-soybean meal broiler grower diet (Chapter 5). The seaweed co-products *Ulva laetevirens* and a *Solieria chordalis* seaweed are those included in Chapter 4 in this thesis.

charts of *U. laetevirens* and *S. chordalis* seaweed co-products show that their mineral and NSP content are still high, whereas the protein content of these seaweed co-products cannot compete yet with that of the common protein source soybean meal.

Digestibility and performance

The *in vitro* digestibility of seaweed is low (Chapters 2 and 3). In accordance to this *in vitro* data, the *in vivo* diet digestibility also decreased after inclusion of seaweed products in broiler diets (Chapters 3 and 4). The effects on *in vivo* digestibility are dependent on the diet type (corn-soy based diet vs. European protein diet; Chapter 5) and are not always consistent (Chapters 4 vs. 5). Despite a lower digestibility of the diets containing seaweed products, performance of the birds was not always detrimentally affected. The presumed link between digestibility and performance was not always evident. If the diet for example causes an inflammatory reaction, more energy and consequently nutrients are spent on the inflammatory response and less energy and nutrients are available for growth performance, as hypothesized in Chapter 5. Furthermore, growth performance might be limited by the most limiting nutrient like a specific AA or by energy availability to the animal.

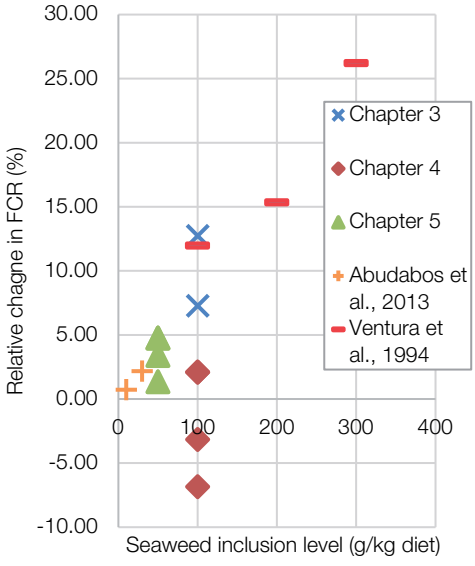

Figure 6.4. Relative change in FCR in response to different seaweed inclusion levels compared to the respective control group

When seaweed is included in broiler diets, feed efficiency generally decreases (FCR increases; Figure 6.4), although no decrease in feed intake was observed. However, the inclusion of specifically *Ulva laetevirens* co-products as fed in Chapter 4 led to an improved feed efficiency (decreased FCR) of birds fed the seaweed versus the control diets, despite the lower dietary nutrient digestibility. These results, however, could not be reproduced in Chapter 5, where similar *U. laetevirens* co-products were added to a corn-soy or European protein based diet, albeit at a lower inclusion level (in finisher diets: 5% in Chapter 5 vs. 10% in Chapter 4). Contrary to the diets in Chapter 4, which were diluted with the seaweed products, the diets in Chapter 5 were adjusted to contain equal amounts of apparent metabolizable energy (AME) and digestible AAs. These inconsistent results in Chapters 4 and 5 highlight the importance of understanding the effect of seaweed inclusion in diets on nutrient digestibility, and that a focus towards digestibility combined with nutrient and consequently resource use efficiency still needs attention. Furthermore, large differences in nutritional composition of seaweed are observed, as will be discussed later in the section 'Limitations and future of seaweed for broiler nutrition'.

Theoretically, using seaweed co-products obtained through a biorefinery approach in animal diets is an efficient way of utilizing resources. Depending on the fraction to be used in feed, different steps are necessary to improve digestibility of those products. In this thesis for example (Chapter 4 and 5), the remaining material after washing and pressing was used. This fraction mainly consists of cell wall material and insoluble components, which are generally not well digested by broilers. Chapters 4 and 5 showed that a proteolytic enzyme treatment did not improve nutrient digestibility. It was hypothesized that cell wall bound protein would become available for digestion, but no increase in N digestibility was observed. This indicates that either the cell wall bound N was not released to become available for digestion, or it made up an insignificant proportion of the entire N content, or there was simply a lack of this N in the products.

A carbohydrolytic enzyme treatment might have more potential to increase nutrient availability for broilers, by breaking down the carbohydrate components constituting this fraction (De Borba Gurpilhares *et al.*, 2019; Thygesen *et al.*, 2020). However, seaweed cell walls consist of different carbohydrates compared to those present in

land-based plants. Because of this difference, their reaction to specific chemical treatments like those used in chemical analyses to assess nutritional composition is not yet fully understood. This is highlighted in Chapter 2, where the acid detergent lignin content of specifically brown seaweed species was higher than the acid detergent fibre content, contrary to that in terrestrial plants. It might be explained by different reactions of the seaweed carbohydrates to the analytical method, compared to the carbohydrates in terrestrial plants.

For a carbohydrolytic enzyme treatment, multiple feed grade carbohydrases are already available, although the effectiveness of those on seaweed material has only been a focus in studies since recently (Matshogo *et al.*, 2021a; 2021b; Thygesen *et al.*, 2020). *In vitro* experiments treating *S. chordalis* and *U. laetevirens* co-products (fractions remaining after washing and pressing, similar to those used in Chapters 4 and 5) with multiple (mixes of) feed grade carbohydrases showed no improvement in *in vitro* digestibility (unpublished data). This indicates no breakdown of carbohydrates to any significant extent due to carbohydrases directed at the breakdown of carbohydrates present in terrestrial plants. Further identification and characterisation of seaweed carbohydrates and their bonds is required to understand and improve the digestibility of the seaweed fractions and what enzymes will target these bonds to achieve a better nutrient availability.

To increase the nutrient availability of seaweed, another method for seaweed carbohydrate breakdown could for example consist of acidic hydrolyses. This does have the disadvantage of adding minerals to facilitate pH adjustments, while the high mineral content of seaweed (co-products) is already a large drawback of using seaweed in animal diets. For this reason, this method may not be suitable unless mineral reduction technologies are subsequently developed.

It is questionable whether we should feed a seaweed fraction high in NSP, or fibre, to poultry. Ruminant animals are highly specialised and capable of utilizing these components hence be a better animal species for the use of seaweeds and seaweed co-products as a dietary ingredient. The scope of this thesis, however, was to study the suitability of seaweed as a novel ingredient for broilers. What can be stated, though, is that the seaweeds and their products included in this thesis contain nutrients suitable for broilers, but as such are challenging for inclusion in their diets yet. The research described in this thesis does contribute further to our understanding of the use of seaweed in animal diets and broiler diets in specific.

Health

Many health promoting properties are attributed to seaweed or to substances from seaweed, and in recent years multiple extensive reviews have been published (Holdt and Kraan, 2011; Øverland *et al.*, 2019; Shannon and Abu-Ghannam, 2019; Hentati *et al.*, 2020). Although certain nutraceutical properties have been ascribed to seaweed or specific seaweed compounds, it is not always clear whether these properties also remain when seaweed is included in animal diets. Questions that arise are for example:

- Do the bioactive compounds remain intact in the gastro-intestinal tract of animals during digestion to arrive at the tissue where they can be taken up and/or exert their bioactive properties?
- Are the bioactive compounds present in the seaweed co-products that are produced from such seaweed?
- Do the bioactive compounds remain intact during feed manufacturing?
- Are the bioactive compounds available to the animal if fed intact/processed seaweed, or do they become bound to other compounds creating inactive complexes?

As such, it is important to assess the effects of seaweed co-products in the target animal instead of assuming findings in *in vitro* studies or in other animal species can be extrapolated.

Ulva spp. have been reported to have, amongst others, antioxidant, anti-inflammatory and antibiotic properties (Holdt and Kraan, 2011). A wide range of parameters can be studied to assess the health status in broilers (e.g. Jeurissen *et al.*, 2002). In Chapter 4, a selection of parameters was investigated, covering immunological, histological and intestinal characteristics. The effect of fibres on gastric development was studied by measuring gizzard weight. Additionally, duodenal histo-morphology was studied, including villus length and crypt depth. The latter two and their ratio are considered to reflect intestinal functionality and integrity (Pluske *et al.*, 1996b; Cañedo-Castro *et al.*, 2019) and are widely used in the literature. Furthermore, the pH was determined of jejunal content, since pH often changes with fibre source (Jiménez-Moreno *et al.*, 2009a). Additionally, a selection of cytokines was analysed in blood plasma. Since seaweed is known to often contain high iron levels, in this chapter I investigated the effect of dietary seaweed co-products on haptoglobin, which is related to iron binding and oxygen transport by red blood cells. The second cytokine studied in Chapter 4 was Interleukine-13, which is an indicator of an anti-inflammatory response to extracellular pathogens. The results in this chapter show that feeding *U. laetevirens* led to a higher body weight gain (+11%) and a lower FCR (-7%), despite a reduced dietary nutrient digestibility. These results were combined with shorter duodenal villi of birds fed the *U. laetevirens* as compared to the *S. chordalis* diets, and the seaweed co-products without enzymatic pre-treatment led to a lower plasma Interleukin-13 level (-60%) compared to enzymatically treated co-products. It was concluded that despite the slight negative effect on villus length, the anti-inflammatory effect of non-enzymatically treated *U. laetevirens* was a positive attribute of the seaweed product, increasing the performance of the birds fed those diets compared to those fed the basal diet. For this reason, Chapter 5 further studied the effect of similar *U. laetevirens* products.

Contrary to the results observed in Chapter 4, Chapter 5 did not show potential health benefits of *U. laetevirens*. In this thesis, seaweed products were fed to broilers from day 1 of age (Chapters 3 and 4). Since the inclusion appeared to reduce broiler performance in the first week, the seaweed co-products in Chapter 5 were only

included in broiler diets at 8 days of age. The gastrointestinal tract of young broilers develops quickly during the first days of life. Arguably, the seaweed co-product inclusion after a week of feeding a basal diet, to which the gastro-intestinal tract already develops or adjusts, might not be optimal. The performance of broilers fed seaweed co-products from the second week was not impaired compared to that of broilers still fed the basal diets, although it also did not show a positive trend in performance as in the broilers fed the *U. laetevirens* co-product diet after 3 weeks (Chapter 4). It is possible that an adaptation period to the seaweed is occurring, and that with time, the broilers are better capable of dealing with their seaweed containing diet. If the seaweed is then only fed after seven days of age with increasing levels throughout the following weeks (Chapter 5), this positive influence of the seaweed might be lost in the time span of the experiment due to a prolonged adaptation period. To validate this hypothesis, a dose response study needs to be designed, including treatment groups receiving the seaweed co-products from different ages onwards and at different concentrations. With regard to the inclusion levels, attention should be paid to the mineral and heavy metal levels of the seaweed products. Although interesting, this would only be a valuable step if these seaweed products are actually to be implemented in broiler nutrition.

Another difference between the seaweed products used in Chapter 4 vs. Chapter 5 is the duration of storage before processing and inclusion in the broiler diets. Both products result from the same batch harvested in 2014 (discussed below), meaning that the storage duration was prolonged with approximately two years for the experiment described in Chapter 5. This might have influenced for example antioxidant activity, as is reported to be reduced after frozen storage for other food and feed ingredients amongst which for example a brown seaweed (Bajčan *et al.*, 2013; Obluchinskaya and Daurtseva, 2020). Despite the potential difference in for example antioxidant activity between the seaweed products used in Chapters 4 and 5, even the storage duration for the seaweed used in Chapter 4 was already long, approximately five years.

Water holding capacity and viscosity

In Chapter 4, it was hypothesized that changes in chyme viscosity might be causing the observed results. Furthermore, the WHC of the feed samples (Chapter 5) indicated that seaweed material, more specifically the fibrous components, holds more water than the other dietary constituents which potentially points to visco-elastic differences between the diets. Additionally, seaweed is known to show gelling properties (Lahaye and Axelos, 1993). Taken together, this might influence amongst other water intake as well as visco-elastic properties of feed boluses, or chyme, in the gastro-intestinal tract. Therefore, additionally diet and jejunal chyme (total and liquid fraction) viscosity were determined in samples obtained during the experiment described in Chapter 5 as an exploratory part of this research.

In the scientific literature, viscosity, the resistance of a fluid to flow due to internal friction of its components (Lentle and Janssen, 2008), of intestinal contents from the fore gut (hepato-pancreatic duct to Meckel's diverticulum) is correlated to performance parameters whereas that of the hindgut is not (Bedford *et al.*, 1991). The gelling, or viscous behaviour of ulvans (carbohydrate present in green seaweed) might be related to the highly branched structures or high proportions of short-chain polysaccharides present (Lahaye and Robic, 20017), although the gel formation mechanism is complex and not yet fully understood (Holdt and Kraan, 2011). Alginic acid is a polysaccharide found in brown seaweed which is known to show viscous behaviour when isolated. Although when included in broiler diets at 50 g/kg, intestinal viscosity was not altered (Iji *et al.*, 2001). This highlights the importance of understanding the effects of dietary seaweed products on digesta viscosity, and consequently on digestion and absorption of nutrients.

In the performed additional analyses, no differences were observed in visco-elastic behaviour of diets or total jejunal chyme amongst treatments. This was contrary to our expectation of a higher viscosity in the EU vs. corn-soy diets, and a higher viscosity in the seaweed vs. basal diets related to their higher NSP content (Chapter 5, Table 3; Choct *et al.*, 1996; Smits and Annison, 1996). Reduction of the length of NSP polysaccharides, which occurs during grinding, might have reduced dietary viscosity (Van Craeyveld *et al.*, 2008). The absence of differences in the visco-elastic properties of the total jejunal chyme could also be related to the capacity of the gastro-intestinal tract to maintain homeostasis to prevent a decreased mixing efficiency, and consequently decreased digestion and absorption (Lentle and Janssen, 2008).

The liquid fraction of the jejunal chyme from the birds fed the seaweed diets showed a numerically higher viscosity within the corn-soy based diets (Figure 6.5). This indicates that this fraction may contain some gelling properties due to the seaweed products, for example due to the soluble seaweed polysaccharides (ulvans) in the liquid fraction. This observed higher viscosity did not coincide with an improved performance, when comparing to performance parameters in the fourth week of the experiment (Chapter 5). On the contrary, an increased FCR was observed, as well as a body weight gain that tended to be decreased. Viscosity is of course not the only factor explaining the observed performance results, as explained in Chapter 5, but it might have contributed to the decrease in digestibility of the broilers fed the seaweed diets. Other parameters that were affected (numerically) together with the higher viscosity of the corn-soybean based seaweed diets compared to the basal diets were a lower gizzard content and reduced villus length, whereas the diameter of the duodenum was numerically larger. These preliminary measurements indicate that there is a potential change in viscosity of the liquid fraction of the chyme lining the feed boluses along the gastro-intestinal wall, which might interact or interfere with nutrient digestibility and absorption throughout the gastro-intestinal tract. Further in-depth research is needed to draw conclusions on visco-elastic properties of seaweed diets and the resulting chyme.

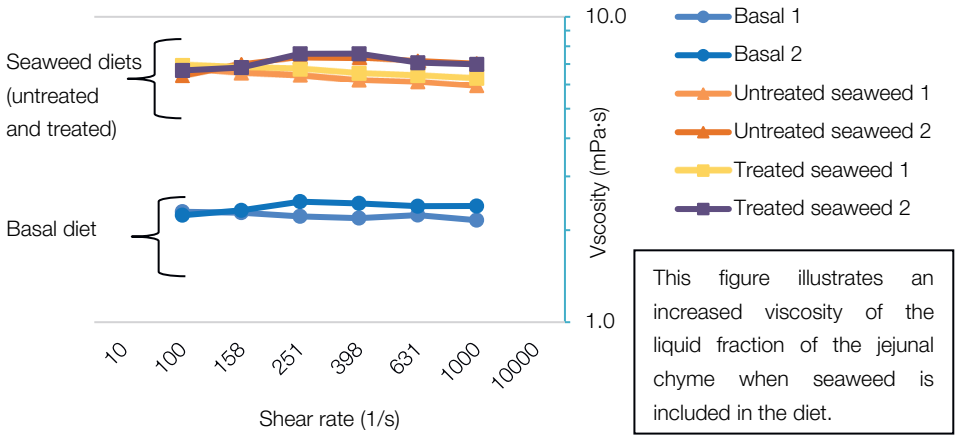

Figure 6.5. Viscosity of the liquid fraction of the jejunal chyme samples obtained from birds fed a corn-soy based basal diet or basal diet including 5% (w/w) untreated or enzymatically treated *Ulva laetevirens* co-products as included in Chapter 5.

Limitations and future of seaweed for broiler nutrition

Variation in nutritional composition

This thesis highlights the large variation in the nutritional composition of seaweed, both between and within seaweed species. The *U. laetevirens* products included in Chapters 4 and 5 originated from the same batch, but even their chemical composition was largely different. Batch, in this case, was defined as harvested from the same beach within the timeframe of one week. Separate sub-samples of this batch were used to produce the needed *U. laetevirens* products for the individual research chapters in this thesis. Despite the variation in nutritional composition between sub-samples, homogenisation steps during processing removed this variation within the sub-samples.

This underlines the importance of taking into account the natural variation in the nutritional composition of seaweed. For a more consistent seaweed product the seaweed has to be thoroughly mixed and homogenized, although due to the large variations it might be practically impossible. Or, if fractions are created or specific components extracted, it should be taken into account that the quantity and quality of the extracted product might not be consistent throughout batches and over time. This can easily be implemented in the programs used for feed formulation, although the nutritional composition of each batch then needs to be analysed. The latter includes the AA composition instead of only N content, since also the AA and the AA per unit of N highly fluctuate between and within species (Chapters 2 and 4; Angell *et al.*, 2016b; Biancarosa *et al.*, 2017).

Heavy metals

As previously discussed in the research chapters of this thesis, seaweed can accumulate heavy metals, and there is a difference in the effects of organic and inorganic heavy metals like arsenic. Seaweed accumulating heavy metals is a serious issue when seaweed is included in animal diets. Next to surpassing the allowed dietary levels and compromising bird health, the heavy metals will end up in the chicken carcass and excreta, posing problems related to the consumption of the broilers and for the application of the excreta. Consequently, this has to be dealt with before seaweed can be included in animal diets.

Economic feasibility

Seaweed production and processing requires significant energy, for example related to the drying of seaweed with its high moisture content (Chapter 2 and 3). This increases the environmental impact of broiler diets as well as the production costs. The economic feasibility of seaweed co-products originating from the *S. chordalis* and *U. laetevirens* as used in the current thesis depends on the purpose and economics of the other fraction(s). Since these products originate from an experimental and not a standardized commercial setup at the producing company, it is difficult to estimate the economic aspects for this specific process.

Based on documentations in literature on cost estimates of growing *Saccharina latissima* in the North Sea, Table 6.1 shows multiple cost estimates of digestible seaweed protein compared to that of soybean meal. It should be noted that in the cost price of seaweed and seaweed protein only costs related to the growth, harvest and transport to the shore are taken into account, but not the costs for further processing needed to extract the protein or dry the products. The calculations based on the three scenario's from literature show large variations in costs of digestible seaweed protein, although all are still at least 37 times as expensive as soybean meal protein (Burg *et al.*, 2016; Hasselström *et al.*, 2020; Mes *et al.*, 2021). Based on scenarios 4 and 5, it becomes clear that even a 10% improved seaweed N digestibility or a 10% reduction in seaweed cost price would not reduce the price of digestible seaweed protein to a level that is competitive with that of soybean meal protein. Scenario six shows that if work is done on different aspects related to seaweed, in this case resulting in a 10% higher digestibility plus a 10% reduction in costs of seaweed protein, better results are already obtained, though the cost price of digestible seaweed is still far from that of digestible soybean protein. If not only seaweed protein but also other fractions would be marketed like suggested when implementing a biorefinery approach, the value of seaweed would increase. The purpose of the produced fractions would highly influence the price. For example, a bioactive compound extracted for human nutrition or the pharmaceutical industry would be more valuable than a solid fibre fraction to be fed to ruminants as roughage. If seaweed protein could be sold as functional protein for human consumption, the price for this protein might go up to €8,- per kg functional

Table 6.1. Simulated cost of *Saccharina latissima* seaweed protein versus soybean meal protein for inclusion in animal feed, based on a seaweed farm yielding seaweed that consists per kg DM of 10% protein, 35% hydrocolloids like alginate, 40% other carbohydrates and 15% minerals. Scenarios based on literature (1-3) and a sensitivity analyses based on potential improvements in various aspects of the seaweed industry for broiler nutrition (4-5).

Scenario	1	2	3	44	55	66
Seaweed yield (kg DM)	10 ton[1]	3.35 ton[2]	20 ton[3]	10 ton	10 ton	10 ton
Seaweed costs (€/kg DM)	€ 3.16	€ 10.95	€ 1.58	€ 3.16	€ 2.84	€ 2.84
Seaweed protein costs (€/kg)	€ 31.60	€ 109.50	€ 15.80	€ 31.60	€ 28.40	€ 28.40
Seaweed protein digestibility (%)[7]	0.68	0.68	0.68	0.75	0.68	0.75
Seaweed protein costs (€/kg digestible protein)	€ 46.81	€ 162.22	€ 23.41	€ 42.25	€ 41.76	€ 37.87
Costs soybean meal (€/ton kg)[8]	€ 281.13	€ 281.13	€ 281.13	€ 281.13	€ 281.13	€ 281.13
Costs of soybean meal protein (45% protein; €/kg protein)	€ 0.62	€ 0.62	€ 0.62	€ 0.62	€ 0.62	€ 0.62
Soybean meal protein digestibility (%)[9]	0.85	0.85	0.85	0.85	0.85	0.85
Soybean meal protein costs (€/kg digestible protein)	€ 0.73	€ 0.73	€ 0.73	€ 0.73	€ 0.73	€ 0.73
Cost ratio (digestible seaweed:soybean meal protein)	64	222	32	58	57	52

[1]Based on Mes *et al.*, (2021) (Based on scenario 3 by Burg *et al.* (2016) with a reduced seaweed yield (-50%).
[2]Based on (Hasselström *et al.*, 2020).
[3]Based on Burg *et al.* (2016).
[4]Scenario based on scenario 1, with an increased seaweed N digestibility (+10%).
[5]Scenario based on scenario 1, with a reduced cost price of seaweed (-10%).
[6]Scenario based on scenario 1, with an increased seaweed N digestibility (+10%) and a reduced cost price of seaweed (-10%).
[7]Seaweed protein digestibility based on results obtained in the *in vivo* experiment described in Chapter 2, average value of apparent pre-caecal *Saccharina latissima* silage and silage residue N digestibility.
[8]Price per ton soybean meal at 4 October 2021: USD 326.90 (nasdaq.com/market-activity/commodities/zm). Currency rate: 1 USD = €0.86 (5-10-2021).
[9]Based on soybean meal crude protein digestibility values from CVB, 2021.

protein, although the needed extraction processes are currently not existing and information on the amount of functional protein in seaweed is lacking (Mes *et al.*, 2021).

Chapter 4 highlights interesting effects of *U. laetevirens* on broiler health, like a decreased inflammatory response. Such health-related effects can also contribute to the justification of including expensive seaweed products in animal diets. For example by increasing health and welfare, and consequently reducing costs for veterinary care or the need for antibiotics. The in Chapter 4 observed reduced inflammatory response was related to an increased performance. The latter was not observed in Chapter 5, indicating that the potential bioactive effect might have been lost during the slightly different production process or during the longer storage of the seaweed before processing.

Potential of seaweed as dietary ingredient for broilers

The potential of seaweed inclusion in broiler diets is determined by multiple factors. Based on the protein content and digestibility, seaweed is yet a challenging protein source in the diet of broilers. Furthermore, it is currently not economically viable to include intact seaweed in broiler diets for its nutritional value. For an optimal use of seaweed components, and to be economically viable, a biorefinery approach is suggested (This thesis; Bikker *et al.*, 2016; Magnusson *et al.*, 2016).

Biorefinery

Biorefinery is a general term and includes all technologies for sustainable use of the complete biomass of natural resources. A schematic overview of the use of seaweed in a biorefinery approach is presented in Figure 6.6. Biorefinery processes include, for example, pressing of fresh seaweed leading to a liquid and a solid fraction as used in

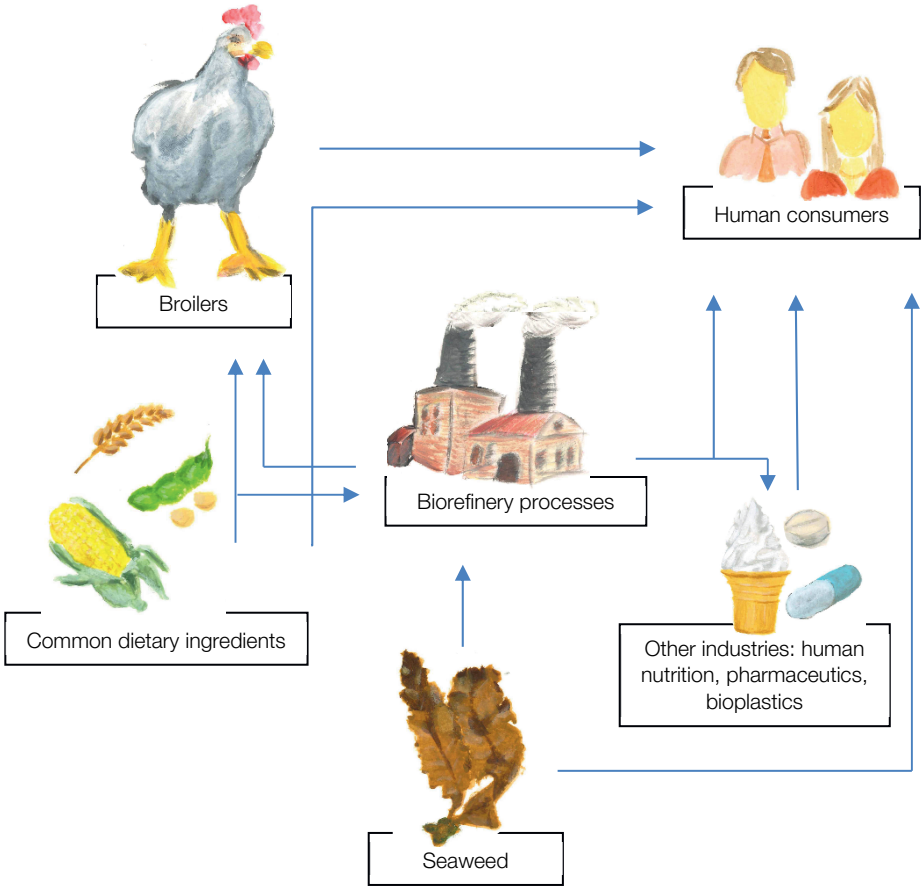

Figure 6.6. Schematic overview of the use of resources applying a biorefinery approach.

Chapters 4 and 5, but also includes washing seaweed with fresh water to in turn extract the seaweed salt from the washing water (Magnusson *et al.*, 2016), and numerous other processes. Important is to keep a focus towards the optimal use of all biomass, or fractions extracted, working towards a circular economy (Torres *et al.*, 2019).

Biorefinery processes currently ongoing in the seaweed industry are, for example, directed at extraction of agars, carrageenans and alginates for the food industry and sugar alcohols (for example mannitol) as sweetener with a lower glycaemic index as replacement of glucose for diabetics (Msomi *et al.* 2021). The suitable seaweed species for such a biorefinery approach depend on the value creation steps that are present in the specific biorefinery setup. It is highly likely that co-products from such a process will be a more cost effective feed ingredient for broiler diets or animal diets in general, although this has to be calculated separately for the individual situations.

Seaweed availability

Seaweed availability is another factor limiting the use of seaweed products in broiler diets. The production in open waters, either from wild populations as well as farmed seaweed, is not constant and depends on environmental factors (Buschmann *et al.*, 2017). Seaweed farming is currently mostly done at a large scale in Asia, where the production yields are almost completely used in human nutrition (Buschmann *et al.*, 2017). Seaweed farming in the North Sea is also investigated by multiple institutes, either solely dedicated to seaweed production or integrated in North Sea windmill parks, although the first farm setup is still in its infancy and will be an experiment to investigate all aspects related to seaweed farming on the North Sea (northseafarmers.org, 2021). Further steps need to be taken with regard to seaweed farming to be able to generate a larger and more constant supply of seaweed which products can be included in animal diets. Another possibility to increase availability could be to use 'unwanted' seaweed from algal blooms in biorefinery processes (Messyasz *et al.*, 2018). Currently, policies are active that direct towards preventing these blooms, though it could be a valuable, although not constant, source of seaweed.

Further research

Next to a more intensive study of visco-elastic characteristics, studying the effects of seaweed on the microbiota in the gastro-intestinal tract would be contributing to the knowledge of the effects of seaweed in and on the gastro-intestinal tract of broilers. It is known that the microbiota composition changes with different fibre sources (Mahmood and Guo, 2020; Nguyen *et al.*, 2021b), thus it can be expected that the caecal bacteria adapt to different soluble fibres present in seaweed products. This increases the efficiency of fibre degradation and nutrient utilisation (Nguyen *et al.*, 2021a).

Furthermore, gene expression of intestinal lining could be studied, determining for example the pathway leading to the decreased inflammatory response observed after

inclusion of *U. laetevirens* (Chapter 4). For example in salmon, a more efficient and thus less energy consuming immune response was observed in fish fed a seaweed vs. basal diet based on gene expression after an immune challenge (Palstra *et al.*, 2018). These effects would be interesting to study in broilers, related to the previously discussed changes in inflammatory response of broilers fed a seaweed diet. Preliminary results of an *in vitro* study using IPEC-J2 porcine cell lines stimulated with *U. laetevirens* products under normal conditions and under *E. coli* presence show promising results (unpublished data). It resulted in upregulated genes representing pathways related to immune response, cell development and survival and energy metabolism. These results need more in-depth investigation to be able to draw conclusions on potential positive effects of seaweed. These kinds of gene expression analyses would increase understanding of the adaptation of the gastro-intestinal tract to the seaweed product, and help to explain potential health beneficial effects.

If in the future more locally sourced ingredients will be included in animal diets, it needs to be carefully determined what the effects of seaweed products are in combination with different ingredients. Additionally, before including seaweed in commercial broiler diets it should be carefully reviewed or studied whether seaweed affects meat quality or other carcass traits.

As indicated in this chapter, progress needs to be made regarding multiple aspects of seaweed processing before it can be included in animal diets. Some of these aspects include seaweed farming, harvesting and processing including biorefinery processes. Furthermore, additional knowledge is needed related to the components that compose seaweed and the effects of those on, in this case, broilers. This also applies to seaweed co-products resulting from a biorefinery approach before those can be included in broiler diets.

Implications for the industry

Including seaweed in broiler diets for its protein contribution alone is not advisable, yet. If seaweed is to be included in broiler diets, the focus should be towards seaweed products resulting from a biorefinery approach as summarized in Figure 3, both from a nutritional and functional as well as from a circularity and economic point of view. It is advisable to share knowledge and explain limitations between the seaweed industry and the broiler feed industry. This will result in a better understanding of which products would be suitable for broiler nutrition, and which processes could contribute to mutual benefit. Such seaweed products resulting from a biorefinery approach could contribute to the mineral, protein, soluble non-starch polysaccharide and energy content of a diet, as well as contribute to broiler health, although this will depend on the specific product and seaweed species.

Conclusions

This thesis aimed to evaluate the nutritional value of seaweed and seaweed derived products for broiler nutrition. The studies conducted have contributed to the gaps in knowledge surrounding the effects of treatments to improve seaweed and the derived products on nutrient availability for broilers, broiler growth performance and health. Based on the work presented here, the following conclusions can be drawn:

- Intact seaweed is currently a challenging ingredient for providing digestible macronutrients in broilers diets.
- Washing intact seaweed with fresh water once reduces the mineral content without improving the nutritional value as determined by nutrient digestibility and broiler growth performance.
- Intact seaweed and seaweed co-product inclusion in broiler diets decreases apparent total tract digestibility of all nutrients analysed.
- Dietary supplementation of co-products of *Ulva laetevirens*, as a result of a biorefinery approach applying pressing and washing, can improve performance and health characteristics in broilers.
- A proteolytic enzyme pre-treatment with Alcalase and/or Neutrase does not increase the digestibility of seaweed in broilers.
- The effects of dietary seaweed supplementation on digestibility and performance are different for corn-soy diets and those based on European protein sources.

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Summary

By 2050, the world population is expected to have grown to 9.7 billion people (United Nations, 2017). Furthermore, the improving living standards and urbanisation are driving factors in the global increase in meat, egg and milk consumption (Boland *et al.*, 2013). Poultry production is a key factor in providing the growing world population with sufficient animal protein. The production of poultry meat is very efficient in terms of unit of feed provided versus unit of edible animal protein produced, compared to the production of for example beef (Brameld and Parr, 2016). In order to achieve this efficient poultry production, highly digestible diets, often based on cereals (corn, wheat) and soybeans, need to be fed. This often results in a competition for resources also used for human consumption (the food-feed competition), and more recently for biofuel production. Hence, this intensifies the search for novel dietary ingredients that neither compete for resources with other sectors, nor for arable land- and freshwater use. Seaweed is such a novel dietary ingredient of interest. Neither arable land, nor fresh water is needed for production, and additionally, there is currently no significant competition as a food source. Furthermore, high yields in terms of biomass per unit of surface area can be achieved (Buschmann *et al.*, 2017). Therefore, I present in this thesis a study of the nutritional value of seaweed for livestock, and explore the potential of seaweed as an ingredient in poultry diets.

To determine the nutritional value of seaweeds for livestock, in **Chapter 2** the composition and the variation therein of intact seaweeds obtained from coastal waters in Northwestern Europe were studied as source of macronutrients for livestock. Towards this aim, the chemical composition, *in vitro* digestibility and *in vitro* gas production were determined. The seaweed species included in this study were brown: *Saccharina latissima*, *Laminaria digitata* and *Ascophyllum nodossum*, red: *Palmaria palmata* and *Chondrus crispus*, and green: *Ulva lactuca*. The nutrient content was highly variable, both between and within species. Overall, the brown seaweeds contain the highest proportion of non-starch polysaccharides (up to 691 g/kg dry matter), whereas samples of the red and green seaweeds contained the highest proportion of amino acids (up to 265 g/kg dry matter). All samples had a substantial non-protein nitrogen fraction, varying from 0.12–0.29 of nitrogen. *In vitro* digestibility of all nutrients in seaweed was lower than that of soybean meal. *S. latissima, L digitata, P. palmata* and *U. lactuca* had a higher maximum gas production than alfalfa, but lower than sugar beet pulp. This leads to the conclusion that based on the protein content and amino acid pattern, intact *P. palmata* and *U. lactuca* could be a valuable protein source for farm animals, with the high non-starch polysaccharides and non-protein nitrogen contents and a low *in vitro* digestibility potentially limiting their use as a feed ingredient for monogastric species. Furthermore, the fermentability of *L. digitata, S. latissima* and *P. palmata* indicate that these intact seaweeds may have a higher nutritional value in ruminants. The high ash content in all seaweed species hampers the use of intact seaweed for both ruminants and monogastrics.

In **Chapter 3**, the effects of different treatments to improve seaweed as dietary ingredient for broilers were studied. To be able to include seaweed in broiler diets, the ash content needs to be reduced and the digestibility and the shelf life increased.

Treatments to achieve this are available, although the effect on the nutritional value of the seaweed products for broilers remained unclear. This chapter therefore investigated the effect of washing, ensiling and extraction processes on the nutritional value of seaweed products for broilers, based on nutrient content and *in vitro* and *in vivo* digestibility. The effects of ensiling, washing and extraction processes were evaluated using *S. latissima, L. digitata* and *U. lactuca*, with 2, 4, and 6 hour incubations in an *in vitro* simulated digestibility model, to obtain insight into the kinetics of digestion. In an *in vivo* study, 160 Ross 308 male broilers were fed (day 14 to 22) a basal grower diet, or the basal grower diet diluted with 100 g/kg of *S. latissima* silage or silage residue. Performance and apparent ileal and total tract nutrient digestibility were determined. Washing and ensiling reduced the ash content, but also the *in vitro* organic matter digestibility. Washing reduced *in vitro* nitrogen digestibility. Extraction of valuable sugars from seaweed decreased *in vitro* organic matter and nitrogen digestibility. Feeding seaweed diets to broilers resulted in a higher feed conversion ratio (FCR; 1.62, 1.86 and 1.77 for broilers fed the basal, silage and silage residue diets respectively), without an increase in final body weight (BW). Feeding *S. latissima* silage residue compared to silage resulted in a slightly better broiler performance and a higher pre-caecal amino acid digestibility. In conclusion, washing, ensiling and extraction processes reduced the nutritional value of the seaweed products, and did not make seaweed suitable for inclusion in broiler diets. To create suitable seaweed products for inclusion in broiler diets, a further reduction in the ash content and increase in digestibility are necessary.

In order to increase the economic viability of the use of seaweed in livestock diets, products resulting from a biorefinery approach can be included in broiler diets. To investigate methods to improve seaweed digestibility, **Chapter 4** discusses the effects of a proteolytic enzyme pre-treatment of green seaweed species *U. laetevirens* and red seaweed species *Solieria chordalis* co-products, resulting from a biorefinery process, before inclusion in a broiler diet. The effects were studied based on performance, *in vivo* digestibility of diets and seaweed co-products, and health-related parameters. The latter included analyses of intestinal content pH, histo-morphological parameters of the small intestine and plasma cytokine levels. In total, 360 Ross 308 male broilers were fed one of five experimental diets: a basal diet, or a basal diet including the *U. laetevirens* or *S. chordalis* co-product, with or without proteolytic enzyme pre-treatment of the seaweed. The starter (d0-13) and grower (d14-21) diet contained 5 and 10% (w/w) seaweed product, respectively. Inclusion of seaweed in the broiler diets increased BW gain (+14%), and feed intake (+12%) in the third week of the experiment. Birds fed the *U. laetevirens* compared to the *S. chordalis* diets had a higher BW gain (+11%), and a lower FCR (-7%). Seaweed inclusion reduced apparent faecal and ileal diet digestibility. Birds fed *U. laetevirens* vs. *S. chordalis* diets had 10% shorter villi. The enzymatic treatment reduced the digestibility of most nutrients, and increased crypt depth in birds fed the *U. laetevirens* diets, whereas the opposite was observed for the birds fed the *S. chordalis* diets. Untreated versus treated seaweed in the diets led to lower (-60%) plasma Interleukin-13 levels. In conclusion, the proteolytic enzyme treatment of the seaweed co-products did not improve performance or health-related parameters, and even reduced digestibility of the diets. Dietary inclusion of *U.*

laetevirens co-products did improve performance based on growth and FCR. Inclusion of *U. laetevirens* in broiler diets had a slight negative effect on duodenal villus length, and a positive effect on crypt depth. The inflammation response was strongly reduced in birds fed the untreated *U. laetevirens* diet, making the *U. laetevirens* co-product of interest for further research.

The experiment described in **Chapter 5** investigated a proteolytic enzyme pre-treatment to improve digestibility of *U. laetevirens* seaweed co-products when included in a standard corn-soy-based and a more challenging diet based on European protein sources. Additionally, the effects on health and performance were further investigated. In total, 624 Ross 308 one day old male broilers were fed one of six experimental diets consisting of a basal diet (soy or EU-based), or a basal diet including the *U. laetevirens* co-product treated without or with a proteolytic enzyme. Starter diets contained 0 (wk 1) and 2.5 (wk 2), and the grower diets 5% (wk 3 and 4) seaweed co-product. In the last two weeks, birds fed the soy vs. EU-based grower diets showed a higher BW, BW gain and feed intake, as well as a lower FCR (-0.05 g/g) in wk 3. Heavier gizzards (+13%) and more gizzard contents (+92%) were observed in birds fed the EU vs. soy-based diets, as well as longer villi (+8%). *U. laetevirens* supplemented diets had a higher water holding capacity than the basal diets (+19%). In wk 4 *U. laetevirens* inclusion resulted in increased FCR (+0.06 g/g), water intake (+7%) and duodenal cross section (+5%). Enzyme treatment did not affect digestibility of any nutrients, except for ash which was increased in birds fed treated vs. untreated seaweed diets (+60%). *U. laetevirens* inclusion in soy-based diets led to higher, and in EU-based diets to lower apparent pre-caecal digestibility of all nutrients ($P<0.001$ for all nutrients). Although for both diet types performance was decreased, dietary *U. laetevirens* inclusion had different effects when added to a soy or EU-based diet. No obvious health effects were observed, leading to a conclusion of the absence of performance of health promoting bioactive components in the *U. laetevirens* co-product, or of diminishing of these effects due to the proteolytic enzyme pre-treatment.

The final **Chapter 6** presents a synthesis of the results of the previous chapters and combines those results with existing literature. The potential of seaweed for broiler nutrition is discussed based on its nutritional composition and the effects on digestibility, performance and health. The water holding capacity and viscosity of seaweed are discussed in the light of their effect on diets, digesta, digestibility and performance. Afterwards, the limitations and future of seaweed for broiler nutrition is discussed, based on the variation in the nutritional composition of seaweed, the heavy metal content and the economic feasibility of seaweed inclusion in broiler diets. From this perspective, the potential of seaweed as dietary ingredient for broilers is discussed, in light of a biorefinery approach and the seaweed availability. Recommendations for further research are presented, as well as implications of this research for the industry, finalized by the conclusions drawn from this thesis.

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It is said that working towards a PhD degree feels like walking alone. To be honest, sometimes it does, but I also got a lot of support along the way. Since I have now indeed completed my studies for my PhD, there are many people to thank for walking with me along the way towards today.

First, I would like to express my gratitude to my late supervisor Dr. Marinus van Krimpen, who walked with me during the first two and a half years of my PhD studies. He inspired me to be myself and to express my curiosity on and off topic. He taught me his working mentality without losing focus of the bigger picture: life. I miss him dearly, even now, while finalizing my PhD thesis, and I will keep him in mind forever.

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About the author

Curriculum vitae – List of publications – Training and supervision plan

Curriculum Vitae

Lotte Stokvis was born on July 17th, 1990 in Zwolle, the Netherlands. After finishing secondary school, Lotte followed the BSc program Animal Sciences at Wageningen University. In 2013 she obtained her BSc degree In Animal Sciences with a major in terrestrial animals and a major in animal behaviour and welfare. During her BSc she followed courses at the Swedish Agricultural University in Uppsala, Sweden, related to animal behaviour and welfare and the design of animal experiments. After her BSc she continued her studies with a MSc in Animal Sciences at the department of Adaptation Physiology at Wageningen University. During her first MSc thesis Lotte investigated facial expressions in pigs in supposed positive and negative emotional states, as a developmental step for creating a new, easy to use and low cost welfare assessment tool. During her second MSc thesis she investigated the effects of high and low levels of dietary fibre in creep feed on feed and water intake, growth and behaviour before weaning, partly performed at Trouw Nutrition and Research in Boxmeer and Sint Anthonis. After graduating for her MSc degree in Animal Sciences, she took on a temporary position at the Adaptation Physiology Group of Wageningen University, developing a dislocation strength test for humeri and their cartilage heads in poultry. After this position she became a PhD scholar at the Animal Nutrition Group of Wageningen University, of which the results are presented in this thesis. Lotte has already started a position at Schothorst Feed Research as researcher poultry and swine nutrition.

List of publications

Clouard, C., L. Stokvis, J.E. Bolhuis and H.M.J. van Hees, 2018. Short communication: insoluble fibers in supplemental pre-weaning diets affect behavior of suckling piglets. Animal, Vol. 12, Issue 2, pp 329-333.

Bikker, P., L. Stokvis, M.M. van Krimpen, P.G. van Wikselaar, J.W. Cone, 2020. Evaluation of seaweed from marine water in Northwestern Europe for application in animal nutrition. *Animal Feed Science and Technology, 263:e114460.*

Stokvis, L., M.M. van Krimpen, R.P. Kwakkel, P. Bikker. 2021. Evaluation of the nutritional value of seaweed products for broiler chickens' nutrition. *Animal Feed Science and Technology. 280:e115061.*

Stokvis, L., C. Rayner, M.M. van Krimpen, J. Kals, W.H. Hendriks, R.P. Kwakkel, 2022. A proteolytic enzyme treatment to improve *Ulva laetevirens* and *Solieria chordalis* seaweed co-product digestibility, performance and health in broilers. *Manuscript submitted for publication.*

Stokvis, L., R.P. Kwakkel, W.H. Hendriks, J. Kals, 2022. Proteolytic enzyme-treated seaweed co-product (*Ulva laetevirens*) inclusion in corn-soybean and European broiler diets to improve digestibility, health and performance. *Manuscript submitted for publication.*

Training and supervision plan[1]

	Year
The basic package (2.9 ECTS[1])	
WIAS Introduction day	2018
WGS Scientific integrity course	2019
WGS Ethics in animal science	2019
Introduction course on personal effectiveness	2019
Disciplinary competences (12.8 ECTS)	
WIAS project proposal	2019
Course Animal nutrition and physiology	2019
Course WIAS/PE&RC advanced statistics design of experiment	2019
Professional competences (8.4 ECTS)	
Brain friendly working and writing	2019
Stress identification and management	2019
Project and time management	2020
Research data management	2020
Scientific writing	2020
Presenting with impact	2020
Course Supervising BSc & MSc thesis students	2020
Career orientation	2020
Last stretch of the PhD program	2020
Writing propositions	2020
Stratego, the unwritten rules	2020
Samenwerken in projecten	2020
Grip op je werk	2021
The final touch	2021
Presentation skills (3 ECTS)	
WIAS Science Day, Lunteren, the Netherlands, poster presentation	2019
ESPN European Symposium on Poultry Nutrition, Gdansk, Poland, oral presentation	2019
EAAP Annual Meeting, Ghent, Belgium, oral presentation	2019
Teaching competences (4 ECTS)	
Supervising course practicals of Principle of Animal Nutrition	2019
Supervising course practicals of Principle of Animal Nutrition	2020
Supervising course practicals Animal Nutrition and Physiology	2019
Supervising MSc students	2019-2021

Total 33 ECTS

[1]Completed in fulfilment of the requirements for the education certificate of the Wageningen Institute of Animal Sciences (WIAS)

[2]One ECTS equals a study load of 28 hours

Colophon

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