Journal of Functional Foods 73 (2020) 104101

Contents lists available at ScienceDirect

Journal of Functional Foods

journal homepage: www.elsevier.com/locate/jff

Twice daily oral administration of *Palmaria palmata* protein hydrolysate reduces food intake in streptozotocin induced diabetic mice, improving glycaemic control and lipid profiles

Christopher M. McLaughlin[a], Shaun J. Sharkey[a], Pádraigín Harnedy-Rothwell[b], Vadivel Parthsarathy[a], Philip J. Allsopp[a], Emeir M. McSorley[a], Richard J. FitzGerald[b], Finbarr P.M. O'Harte[a,*]

[a] School of Biomedical Sciences, Ulster University, Cromore Road, Coleraine, Northern Ireland, United Kingdom
[b] Department of Biological Sciences, University of Limerick, Castletroy, Limerick, Ireland

ARTICLE INFO

Keywords:
Streptozotocin induced diabetes
Insulin secretion
Palmaria palmata
Protein hydrolysate
GLP-1
Blood glucose

ABSTRACT

This study investigated the antihyperglycaemic effectiveness of an oral *Palmaria palmata* protein hydrolysate (PPPH), versus metformin, upon metabolic control in streptozotocin (STZ)-induced diabetic mice. Mice were administered PPPH (50 mg/kg bodyweight) or metformin (200 mg/kg bodyweight) by oral gavage twice-daily for 18 days. Blood glucose and plasma insulin were measured every third day. PPPH caused a significant reduction in blood glucose ($p < 0.001$) and a significant increase in plasma insulin ($p < 0.001$) versus STZ-treated saline controls. PPPH treatment reduced energy intake ($p < 0.05$), bodyweight ($p < 0.01$) and total plasma glucagon-like peptide-1 ($p < 0.01$) after 18 days. Terminal oral glucose tolerance (Day 18, $p < 0.05$), fasting blood glucose ($p < 0.001$), HbA1C ($p < 0.01$), plasma cholesterol ($p < 0.01$) and plasma triglycerides ($p < 0.05$) were significantly improved versus STZ-treated saline controls. All groups showed significant increases in pancreatic islet area, β-cell area, and β:α cell ratio. PPPH demonstrated potent antidiabetic potential *in vivo* through reduced food intake and improved beta-cell function.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a progressive disease that results in dysregulation of glycaemic control attributable in part to a loss of insulin secretion and/or insulin sensitivity. The therapeutics available range from sulphonylureas and glucagon-like peptide-1 (GLP-1) receptor agonists which directly enhance insulin secretion to gliptins and glifozins which enhance insulin stability through the direct inhibition of endogenous DPP-IV proteolytic enzymes and renal re-absorption of glucose via the SGLT-2 transporter, respectively (Tahrani, Barnett, & Bailey, 2016). Despite the diverse range of preventative strategies and pharmaceutical approaches available to treat diabetes the incidence and number of debilitating complications, including cardiovascular disease, neuropathy, nephropathy and retinopathy continues to increase (Dal Canto et al., 2019). The role of dietary monitoring in ameliorating and limiting the progression of diabetes has been extensively reported with an emphasis on restricting carbohydrate intake without increasing caloric intake (Snorgaard, Poulsen, Andersen, & Astrup, 2017). Patient compliance with such dietary approaches is a considerable challenge, however there is increasing interest in dietary components that may contribute to modulation of satiety and/or glycaemic control through reducing postprandial glycaemic response and reducing the glycaemic surges that contribute to disease complications.

Protein has previously demonstrated efficacy in enhancing satiation and reducing food intake as well as modulating the glycaemic response through delayed gastric emptying, enhanced insulin release through induction of incretin hormone production as well as extending insulin half-life through DPP-IV inhibition. The efficacy appears to be influenced by protein source and the degree to which a protein is processed and there is increasing interest in the use of commercial proteolytic enzymes to produce novel functional peptide hydrolysate products. The efficacy of collagen, casein, whey, soy proteins and their associated hydrolysate products have been shown to exert beneficial effects on satiety and glycaemic control however the efficacy varies greatly and has been proposed to be due to the compositional differences between them. There is growing interest in alternate protein sources given the increasing food security pressures that are emerging due to increasing global demand for sustainable sources of protein. However, there is also

* Corresponding author at: School of Biomedical Sciences, Ulster University, Cromore Road, Coleraine BT52 1SA, Co. Derry, Northern Ireland, United Kingdom.
E-mail address: fpm.oharte@ulster.ac.uk (F.P.M. O'Harte).

https://doi.org/10.1016/j.jff.2020.104101
Received 20 December 2019; Received in revised form 29 May 2020; Accepted 30 June 2020

interest in the physiological benefits of alternate protein sources particularly in relation to their potential as functional food ingredients. Seaweeds have emerged as a potential and sustainable source of protein, particularly some red seaweeds including *Palmaria palmata* which has been reported to have a protein content of > 30% (w/w) dry weight (Fleurence, Morançais, & Dumay, 2017) along with a favourable essential amino acid composition that will provide fundamental nutritional needs required from dietary protein (Bleakley & Hayes, 2017). Whilst the initial interest in *Palmaria palmata* was based on nutritional value, attention has turned to the potential benefits that *Palmaria palmata*-derived protein hydrolysates may have on appetite and glycaemic control (Drummond et al., 2018; Nobile et al., 2016; Patel, 2015; Wang et al., 2015). Constituent peptides from *Palmaria palmata* protein digestion have been previously reported to possess DPP-IV inhibitory activity *in vitro* (Harnedy, O'Keeffe, & Fitzgerald, 2015). Furthermore, the outcome of preliminary studies indicates that a *Palmaria palmata* protein hydrolysate generated with Alcalase and Flavourzyme mediate significantly higher DPP-IV inhibitory activity and insulin and GLP-1 secretory activity from cultured pancreatic BRIN-BD11 and enteroendocrine GLUTag cells, respectively compared to hydrolysates generated with other food-grade proteolytic enzyme preparations (McLaughlin et al., 2016). The objective of the present study was to determine if the anti-diabetic activity observed with the *Palmaria palmata* protein isolate hydrolysate *in vitro* translated to *in vivo*. The study aimed to compare the efficacy of repeated administration (twice daily for 18 days) of the hydrolysate versus metformin, upon glycaemic control and lipid profiles in an insulin deficient mouse model of diabetes.

2. Materials and methods

2.1. Materials

All materials, reagents, and consumables were supplied by Sigma Aldrich unless otherwise stated.

2.2. Sample preparation

A sample of air-dried milled (5 mm) *Palmaria palmata* (which was harvested off the Northeast coast of Ireland in January 2014) was purchased from Irish Seaweeds Ltd., Belfast, Co. Antrim, Northern Ireland. The macroalgae was further milled with a Cyclotec™ Mill (1 mm screen, FOSS Tecator AB, Hoganas, Sweden) and subsequently stored at ambient temperature in an opaque airtight container for 1 month.

2.3. Extraction and quantification of aqueous and alkaline soluble proteins

Crude aqueous and alkaline soluble protein extracts were prepared using the method described by Harnedy and FitzGerald (2013) with some modifications. In brief, the milled *Palmaria palmata* powder was suspended in water at a ratio of 1:20 (w/v), and gently stirred at room temperature for 3 h. The supernatant containing the aqueous soluble protein was obtained following centrifugation at 4190*g* (Sorvall RC6 Plus, Fisher Scientific, Dublin, Ireland) for 15 min at room temperature. The pellet was resuspended in 0.12 M NaOH (1:15 (w/v)) and gently stirred for 1 h at room temperature and the supernatant containing the alkaline soluble protein was obtained following centrifugation as described above. The pellet from the above was subjected to a second alkaline extraction using the same conditions and both supernatants were combined. The aqueous and alkaline soluble protein components were semi-purified and concentrated by a double isoelectric precipitation step at pH 2.5 (aqueous) and 4.0 (alkaline), respectively using 1.0 M HCl (Harnedy et al., 2015). The precipitated protein pellets obtained following the second isoelectric precipitation were resuspended in distilled water (dH_20) to a protein concentration of ~2.4% (w/v) and combined. The concentration of protein in the extracts was determined by the modified Lowry protein quantification method as described previously (Harnedy & FitzGerald, 2013). All samples were analysed in triplicate.

2.4. Enzymatic hydrolysis of macroalgal proteins

Macroalgal protein was hydrolysed by the method described by Harnedy and FitzGerald (2013). A 2% (w/v) protein solution was preheated to 50 °C and adjusted to pH 7.0 and hydrolysed with Alcalase 2.4L® and Flavourzyme 500L™, at an enzyme:substrate (E:S) ratio of 1:100 (w/v) for 4 h at 50 °C. The hydrolysis reaction was maintained at pH 7.0 using a pH-stat (842 Titrando, Metrohm, Switzerland) and terminated by heating at 90 °C for 20 min. All samples were subsequently freeze-dried (FreeZone 18L, Labconco, MO, USA).

2.5. Kjeldahl nitrogen quantification

The nitrogen content of the macroalgal sample, non-protein nitrogen (NPN) and protein nitrogen fractions generated therefrom as described by Stack et al. (2017) and the PPPH were quantified using the macro-Kjeldahl procedure as described previously (Connolly, Piggott, & FitzGerald, 2013). The nitrogen to protein conversion factor used was 4.70 (Bjarnadóttir et al., 2018). All samples were analysed in triplicate (n = 3).

2.6. Physicochemical characterisation of the protein hydrolysate

The molecular mass distribution profile of PPPH was determined by analytical gel permeation-high performance liquid chromatography (GP-HPLC) as described by Spellman, Kenny, O'Cuinn, and FitzGerald (2005). The peptide profile of the hydrolysate was determined by reverse-phase ultra-performance liquid chromatography (RP-UPLC) (Nongonierma & FitzGerald, 2012) as described previously by Nongonierma and FitzGerald (2012, 2019). The amino nitrogen content of PPPH was estimated by the TNBS method as described by Le Maux, Nongonierma, Barre, and FitzGerald (2016) with absorbance readings taken at 350 nm. All samples were analysed in triplicate (n = 3).

2.7. Induction of diabetes using low dose streptozotocin and acclimatization prior to treatment

All animal experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and EU Directive 2010/63EU for animal experiments and were approved by Ulster University Animal Welfare and Ethical Review Board. All necessary steps were taken to prevent any potential animal suffering. To induce diabetes, *HsdOla:TO* mice (8–10 weeks; Envigo Ltd, UK) were administered STZ (40 mg/kg bodyweight) once every three days over a 9-day period (i.e. −21, −18 and −15 with daily glucose monitoring post injection). This multiple low dose STZ regime represents a model of Type 1 diabetes with associated hyperglycaemia (Luo et al., 2019). Mice were given nine further days to acclimatize after the final STZ injection, until −6. After acclimatization, animals were grouped (n = 7–8 mice) according to their non-fasting blood glucose concentration (if above 12 mmol/L) and bodyweight. In total, there were 4 groups; STZ-treated animals receiving saline (STZ saline), STZ-treated animals receiving PPPH (STZ PPPH), STZ-treated animals receiving metformin (STZ Metformin), and healthy mice (no STZ) receiving saline (saline controls). From −6 to 0, each group was given twice daily oral saline (0.9% w/v) mimicking treatment pattern which commenced from day 0. A dose of 50 mg/kg was chosen based on the efficacy of PPPH in a dose–response pilot study examining glucose tolerance in mice. On day 0, twice daily treatments of PPPH (50 mg/kg bodyweight up to a maximum volume of 200 μl) or metformin (200 mg/kg bodyweight), by oral gavage (09.00 h and 17.00 h) began and were maintained throughout the remainder of the

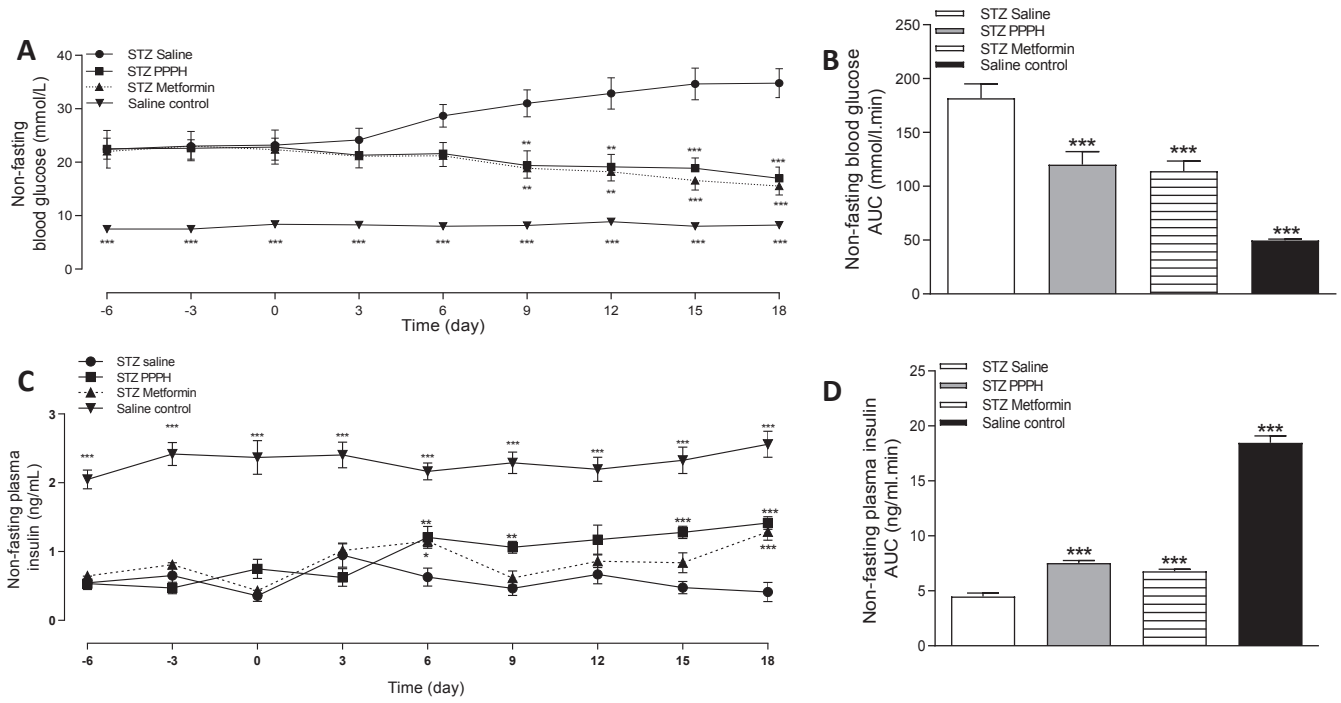

Fig. 1. Effect of twice-daily oral administration of Palmaria palmata protein hydrolysate (PPPH) (50 mg/kg BW) or metformin (200 mg/kg BW) on non-fasting blood glucose (A) and non-fasting plasma insulin (C) in streptozotocin (STZ)-induced diabetic mice, along with corresponding area under the curve (AUC) data (B & D). Parameters were measured 6 days prior to commencing treatment on day 0 running to day 18. Values represented as mean ± S.E.M. (n = 7–8). **p < 0.01, ***p < 0.001 vs STZ saline control.

study (up to day 18). Throughout the study (day 0 to day 18) the mice were assessed at 3-day intervals where the blood was removed from the tail for both non-fasting blood glucose and insulin determination. Blood glucose levels were measured using a handheld glucometer (Bayer Contour, Leverkusen, Germany). Blood was centrifuged at 12,000 rpm for 10 min and plasma stored in low-bind Eppendorf tubes at −20 °C prior to insulin analysis by a radioimmunoassay (RIA) (Flatt & Bailey, 1981). Furthermore, animal bodyweight and food intake were measured throughout the study.

2.8. Terminal analyses

To examine oral glucose tolerance after 18 days treatment, blood samples were measured following tail venepuncture in 8 h fasted mice. Blood glucose was measured prior to (0 min) administration of an oral glucose challenge (18.8 mmol/kg bodyweight). Once administered, whole blood glucose was measured from the tail vein at 15, 30, 60, 90 and 120 min. Blood glucose and plasma insulin was measured as described in Section 2.5. The lipid profile of terminal plasma samples (day 18) was determined using an I-Lab 650 clinical chemistry system (Instrumentation Laboratory, Warrington, UK), including triglycerides and cholesterol. Reagents for triglyceride and cholesterol analysis were also obtained from Instrumentation Laboratory. For analysis of bone mineral density (BMD) and bone mineral content (BMC), animals were anesthetized using Isoflurane and parameters were measured using DXA scanning (Piximus Densitometer, Inside Outside sales, USA). Following euthanasia, pancreata were immediately excised and stored in 4% paraformaldehyde until processing for histology.

2.9. Pancreatic histology

Pancreatic histology was conducted using mouse anti-insulin (1:500 dilution; Abcam, ab6995) and guinea-pig anti-glucagon (PCA2/4, 1:400 dilution; raised in-house) primary antibodies alongside Alexa Fluor 594 goat anti-mouse IgG (1:400 dilution) and Alexa Fluor 488 goat anti-guinea pig IgG (1:400 dilution) secondary antibodies (both Abcam). Slides were viewed under a FITC (488 nm) or TRITC (594 nm) filter using a fluorescent microscope (Olympus system microscope, model BX51) and photographed using a DP70 camera adapter system. Islet parameters were analysed using Cell^F image analysis software (Olympus Soft Imaging Solutions, GmbH, Germany).

2.10. Statistical analyses

All results were analysed using GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, CA, USA). Where appropriate, data were compared using one-way and two-way analysis of variance (ANOVA), followed by Newman-Keul's *post hoc* test. Incremental area under the curve (AUC) for plasma glucose and insulin were calculated using GraphPad Prism. Groups of data were considered to be significantly different if $p < 0.05$. All data is presented as mean ± S.E.M.

3. Results

3.1. Characterisation of Palmaria palmata biomass and PPPH

The total, non-protein and protein nitrogen content of the macroalgal sample was determined to be 2.87 ± 0.02%, 0.85 ± 0.08% and 2.02 ± 0.10% (w/w), respectively. The protein content of the seaweed was estimated to be 9.50% (w/w) when calculated from experimentally obtained % PN value. Proximate analysis showed the hydrolysate had 93.22% (w/w) protein, 3.10% (w/w) carbohydrate, 1.64% (w/w) ash and 2.04% (w/w) moisture. The amino nitrogen content of the hydrolysate was determined to be 21.37 ± 0.37 mg N/g protein. Molecular mass distribution results for PPPH indicate that 54.80% (w/w) of peptides were < 1 kDa, 33.10% (w/w) of peptides were in the range 1–5 kDa, 7.70% (w/w) of peptides were in the range 5–10 kDa, and 4.40% (w/w) of peptides were > 10 kDa. The RP-UPLC profile of the hydrolysate, which is included as a Supplementary Figure (Fig. S1),

indicates that the majority of peptides are hydrophilic in nature.

3.2. Effect of PPPH on non-fasting blood glucose and plasma insulin

Mice showed a significant improvement in non-fasting blood glucose of the PPPH group (50 mg/kg bodyweight) versus the STZ-treated saline group, which was comparable to oral metformin (200 mg/kg bodyweight) by day 9 ($p < 0.01$; Fig. 1A). This improvement persisted until the end of the study period (day 18) and an overall reduction in blood glucose (by 35–37%) from the area under the curve (AUC) data ($p < 0.001$) was observed, when compared to the STZ-treated saline controls (Fig. 1B). PPPH also resulted in an increased insulinotropic response (Fig. 1C), reflected in the 1.5- to 1.7-fold increase in the integrated AUC values, which was similar to the increase observed with metformin when compared with the STZ saline controls ($p < 0.001$; Fig. 1D).

3.3. The effects of 18-day treatment with PPPH on food intake, bodyweight and plasma total GLP-1

In STZ-induced diabetic mice, PPPH and saline treatment (no STZ) groups displayed a 24% ($p < 0.05$) and 31% reduction ($P < 0.001$) in cumulative energy intake after 18 days of treatment, respectively (Fig. 2A). PPPH and metformin treated mice showed a 7% and 5% reduction in bodyweight when compared to STZ saline controls (Fig. 2B), respectively. This contrasts with healthy control mice, which gained 9% bodyweight over this period. The cumulative energy intake was higher for the STZ-treated saline group and this might explain why the weight loss in this group was not as marked as in the PPPH and metformin treated mice (Fig. 2B). We did not measure energy expenditure or the activity of these mice. One other potential explanation of the weight loss could be that PPPH and metformin treated STZ mice might display an increase in physical activity and/or energy expenditure could possibly explain this unexpected finding in relation to weight loss. In addition, there was a significant reduction in plasma triglycerides (Fig. 4B), which could lead to additional weight loss if these were being used as an energy source. PPPH treatment, unlike metformin, also resulted in a ~50% reduction in total plasma GLP-1 when compared to the STZ saline controls ($p < 0.01$), reaching a value close to the lean saline controls (Fig. 2C).

3.4. The effects of 18-day treatment with PPPH on glycaemic control, fasting blood glucose, HbA_{1C}, cholesterol and triglycerides

Both PPPH and metformin treatment resulted in a significant reduction in fasting blood glucose concentrations after 18 days (55% $p < 0.05$ and 38% $p < 0.01$, respectively) when compared to the STZ saline controls (Fig. 3C). However, treatment with PPPH and metformin did bring the postprandial blood glucose concentrations down to those of the healthy control mice (81% lower than STZ saline mice). Furthermore, both PPPH and metformin significantly reduced ($p < 0.05$, $p < 0.01$, respectively) postprandial blood glucose following an oral glucose challenge (Fig. 3A & B). These improvements in glycaemic control were also reflected in an approximately 3.5% and 4.0% reduction in HbA_{1C} in PPPH and metformin treated groups, respectively, versus STZ-treated controls (Fig. 3D).

Both the PPPH-treated group and the lean control group exhibited a lower concentration of plasma cholesterol at the end of the treatment period (day 18) ($p < 0.01$ and $p < 0.05$ versus STZ saline controls, respectively). However, metformin treatment led to no significant change in plasma cholesterol (Fig. 4A). Only the PPPH treated mice showed a significant reduction in plasma triglyceride concentration at the end of the study ($p < 0.05$ versus STZ saline controls; Fig. 4B).

3.5. The effect of 18-day treatment with PPPH treatment on islet morphology

PPPH treatment resulted in a 2-fold increase in average islet size (Fig. 5A; $p < 0.05$ versus STZ saline control). This was supported by a 3-fold increase in β-cell area ($p < 0.05$; Fig. 5B) with no significant change in α-cell area (Fig. 5C). Both PPPH and metformin showed significant improvement in β:α cell ratio ($p < 0.001$; Fig. 5D), However, these improvements were still notably lower than that of the saline treated healthy control group ($p < 0.05$).

3.6. The effects of 18-day treatment with PPPH on total and femur region of interest bone mineral density and bone mineral content

Neither PPPH nor metformin caused a significant change in total or femur region of interest bone mineral density (Fig. 6A & C). PPPH did however cause a significant increase in total bone mineral content versus STZ saline controls ($p < 0.01$) and lean controls ($p < 0.05$), whereas metformin only led to a significant increase in BMC versus STZ saline controls ($p < 0.01$) (Fig. 6B).

4. Discussion

This study demonstrated that a *Palmaria palmata* protein hydrolysate generated with Alcalase and Flavourzyme from a highly pure protein isolate elicits significant beneficial anti-hyperglycaemic and anorexigenic effects through improvements in blood glucose, plasma insulin, energy intake, plasma triglycerides and bone mineral content and density. The proteolytic enzyme preparations, Alcalase and Flavourzyme, employed herein were selected based on preliminary *in vitro* data. This data indicated that the *Palmaria palmata* hydrolysate generated with this enzyme combination mediated higher DPP-IV inhibitory activity and insulin and GLP-1 secretory activity from cultured pancreatic BRIN-BD11 and enteroendocrine GLUTag cells, respectively

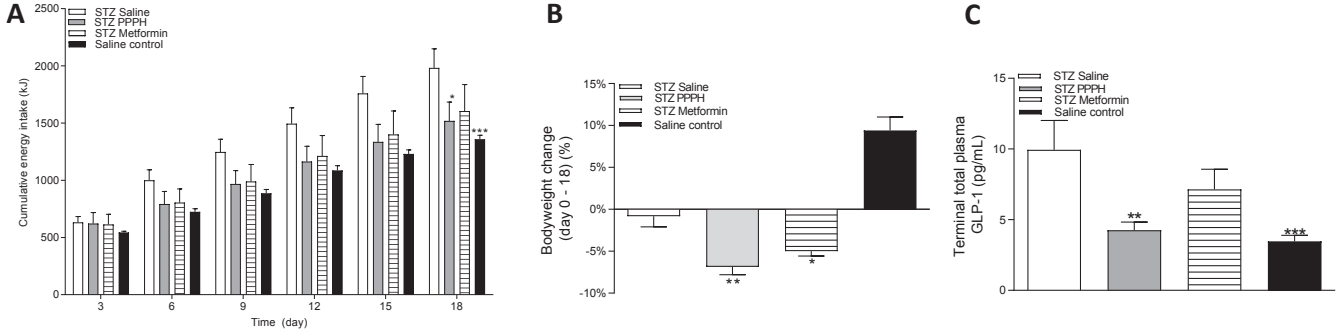

Fig. 2. Effect of twice-daily oral administration of Palmaria palmata protein hydrolysate (PPPH) (50 mg/kg BW) or metformin (200 mg/kg BW) on (A) energy intake, (B) % bodyweight change and (C) plasma total GLP-1 in streptozotocin (STZ)-induced diabetic mice, treated for 18 days. Food intake was measured at 3 day intervals up to day 18. Values represent mean ± S.E.M. ($n = 7$ or 8). *$p < 0.05$,**$p < 0.01$ and ***$p < 0.001$ versus STZ saline controls.

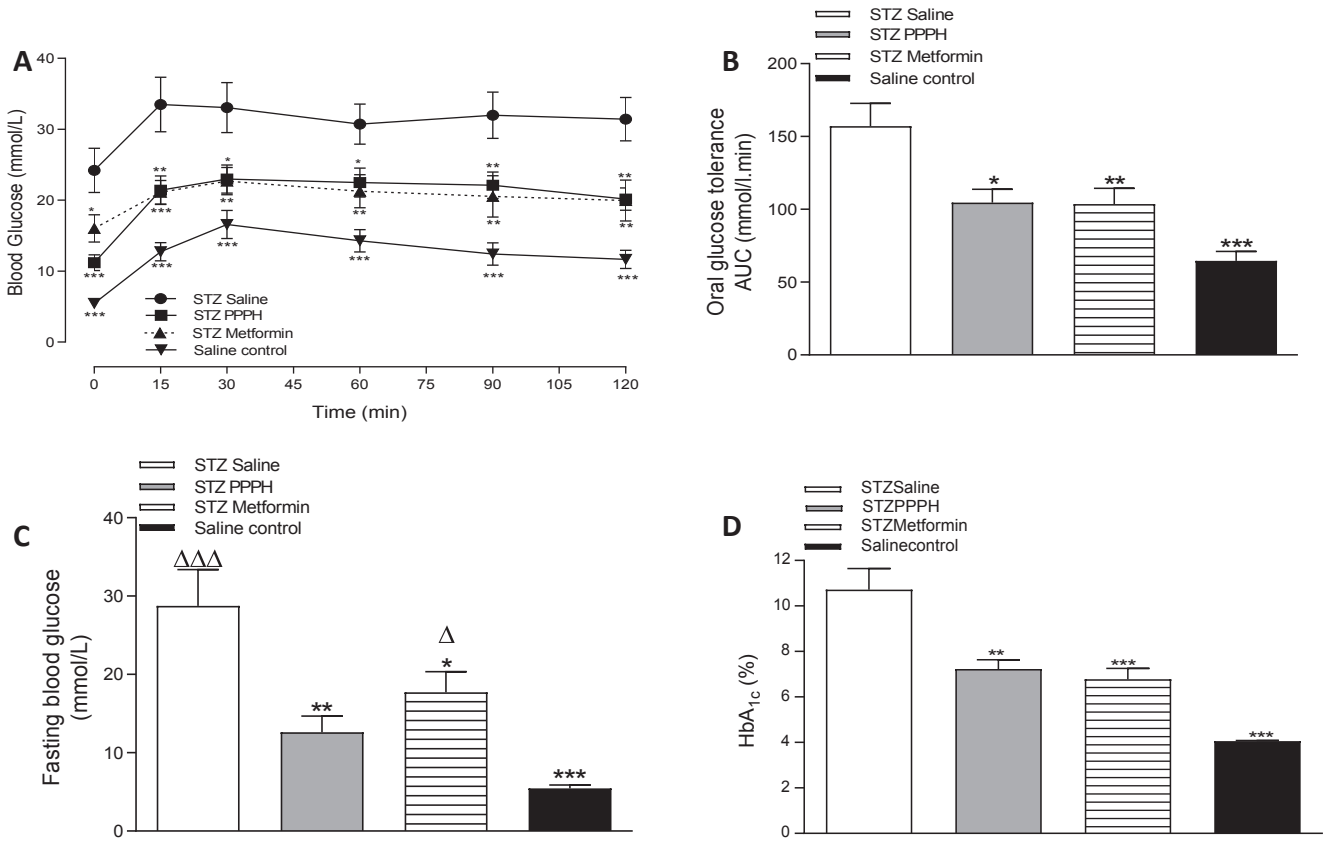

Fig. 3. Effect of twice-daily oral administration of Palmaria palmata protein hydrolysate (PPPH, 50 mg/kg BW) or metformin (200 mg/kg BW) for 18 days on (A) terminal oral glucose tolerance and (B) associated area under the curve data, (C) fasting blood glucose and (D) glycated haemoglobin (HbA_{1C}) in STZ-induced diabetic mice. Values are expressed as mean ± S.E.M. (n = 7–8). *p < 0.05, **p < 0.01, ***p < 0.001 compared to STZ saline controls and $^{\Delta}p < 0.05$, $^{\Delta\Delta\Delta}p < 0.001$ versus heathy controls.

compared to hydrolysates generated with other proteolytic enzyme preparations (McLaughlin et al., 2016). The study demonstrates that the biological activity observed *in vitro* with PPPH translated to *in vivo*. The underlying mechanisms behind the action identified herein within the digestive tract is poorly understood (Caron, Domenger, Dhulster, Ravallec, & Cudennec, 2017). However, it is generally accepted that weight loss strategies involving reductions in carbohydrates or fats, have shown improved dietary compliance with increasing intake of proteins (Galbreath et al., 2018). This is often attributed to protein acting upon metabolic targets, which control satiety, such as alterations in leptin or ghrelin (Klok, Jakobsdottir, & Drent, 2007). Gut hormones such as GLP-1, glucose-dependent insulinotropic polypeptide (GIP), cholecystokinin (CCK), and peptide-YY (PYY) also have potential roles in appetite control (Hameed, Dhillo, & Bloom, 2009; Holst, 2013; Lafferty, Flatt, & Irwin, 2018). Each of these hormones are involved in satiety via specific cellular receptor activation and thus a peptide hydrolysate from *Palmaria palmata* may be able to target multiple hormonal pathways. While it may be possible that the other minor/low level carbohydrate and ash components within the PPPH contribute to the overall observed activity, it is assumed that the active components are proteinaceous in nature as the PPPH contained 93.22% (w/w) protein. Furthermore, it is likely that the results observed herein are as a result of synergistic effects arising from a number of peptides within the unfractionated/complex hydrolysate. Crude protein hydrolysates rarely exert their bioactive effect through a singular mechanism of action, and more likely to have several modes of action. Work by

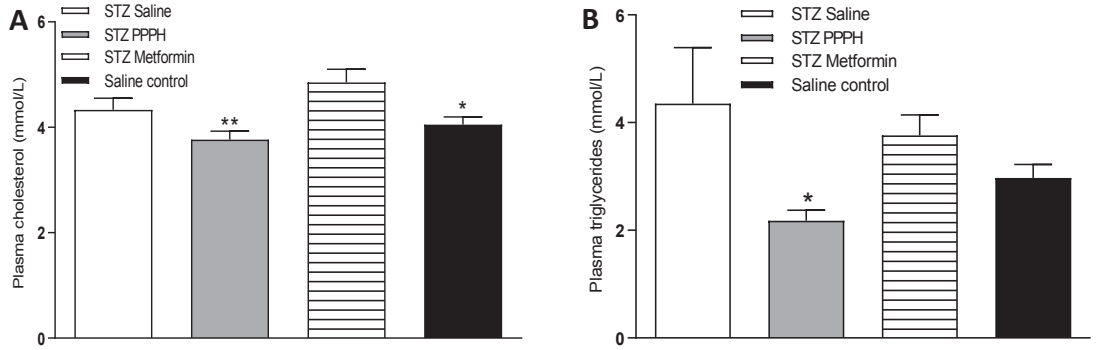

Fig. 4. Effect of twice-daily oral administration of Palmaria palmata protein hydrolysate (PPPH, 50 mg/kg BW) and metformin (200 mg/kg BW) for 18 days on (A) plasma cholesterol and (B) plasma triglycerides in streptozotocin (STZ)-induced diabetic mice. Values are expressed as mean ± S.E.M. (n = 7 or 8). *p < 0.05, **p < 0.01, compared to STZ saline controls.

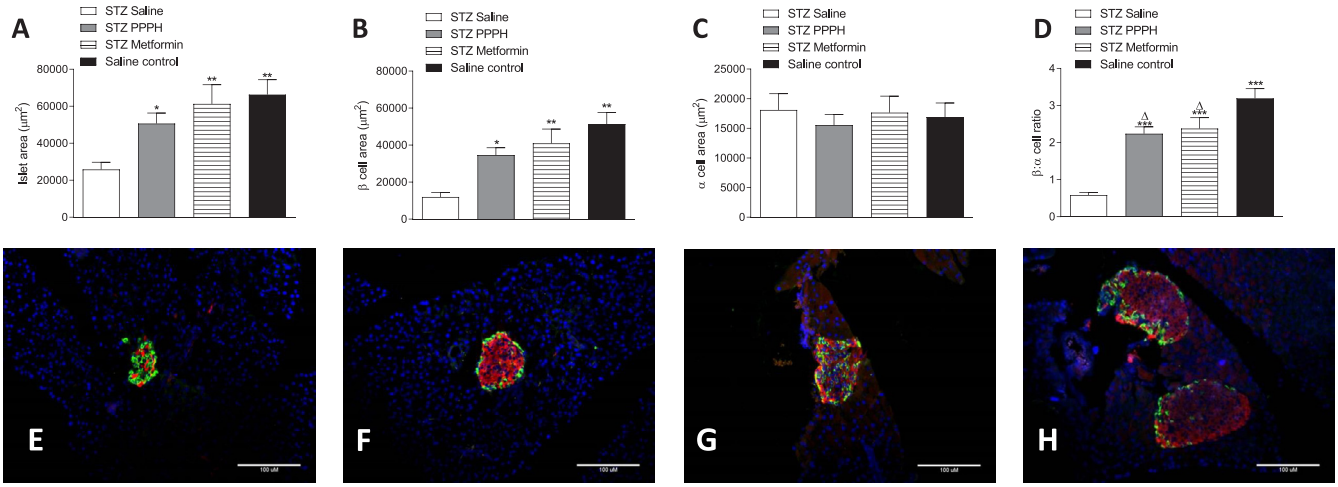

Fig. 5. Effect of twice-daily oral administration of Palmaria palmata protein hydrolysate (PPPH, 50 mg/kg BW) or metformin (200 mg/kg BW) on (A) islet area, (B) β-cell area, (C) α-cell area and (D) β:α cell ratio in STZ-induced diabetic mice. Representative images of islets from (E) STZ saline control group, (F) PPPH-treated group, (G) metformin-treated group and (H) healthy control group. Images were stained for insulin (red), glucagon (green) and 4′,6-diamidino-2-phenylindole(DAPI; blue) and shown at 20x magnification. Values are represented as mean ± S.E.M. (n = 7 or 8). *p < 0.05, **p < 0.01, ***p < 0.001 compared to STZ saline controls and $^{\Delta}$p < 0.05 compared with healthy control mice. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Diepvens, Häberer, and Westerterp-Plantenga (2008) showed exogenous protein intake produced differential release of GLP-1, ghrelin, PYY and CCK, which could affect satiety. Furthermore, Caron et al. (2017) found that protein, or protein hydrolysates can promote significant secretion of CCK, which in turn may be able to attenuate food intake via a reduction of gastric emptying or neuronal activation to hindbrain appetite centres (Carreiro et al., 2016; Simpson, Parker, Plumer, & Bloom, 2012). There are many avenues for investigating how PPPH reduces food intake in mice. However, not all circulating satiety-related hormones were measured due to the low plasma volumes available at timed intervals. The caloric reduction shown in Fig. 2A is thought to be the leading contributor to improved non-fasting blood glucose (Moebus, Göres, Lösch, & Jöckel, 2011) through a reduction in overall energy intake.

Metformin treatment initially appears more effective than PPPH in reducing non-fasting glucose parameters, which could be attributed to the satiating potential of metformin (Adeyemo et al., 2015), but it is also used as a higher dose than PPPH in this study. Metformin is also effective in reducing hepatic glucose output, which is a characteristic trait of the current STZ-induced insulin deficient animal model (Burcelin et al., 1995; Zafar, Naeem-ul-Hassan Naqvi, Ahmed, & Kaimkhani, 2009). The observed increase in circulating plasma insulin (Fig. 1C) could be due to direct agonism of the beta cells by peptides or amino acid induced insulin secretion (Newsholme, Brennan, & Bender, 2006). Alternatively, GLP-1-stimulated insulin release or increased beta cell mass could contribute to the observed raised circulating insulin and data from Fig. 5 appear to suggest that the latter may be more likely. Furthermore, it's relatively unlikely that the significant increase of circulating insulin seen within the PPPH treated group from day 6 to 9 (p < 0.01) and day 15 to 18 (p < 0.001) (Fig. 1C) was solely amino

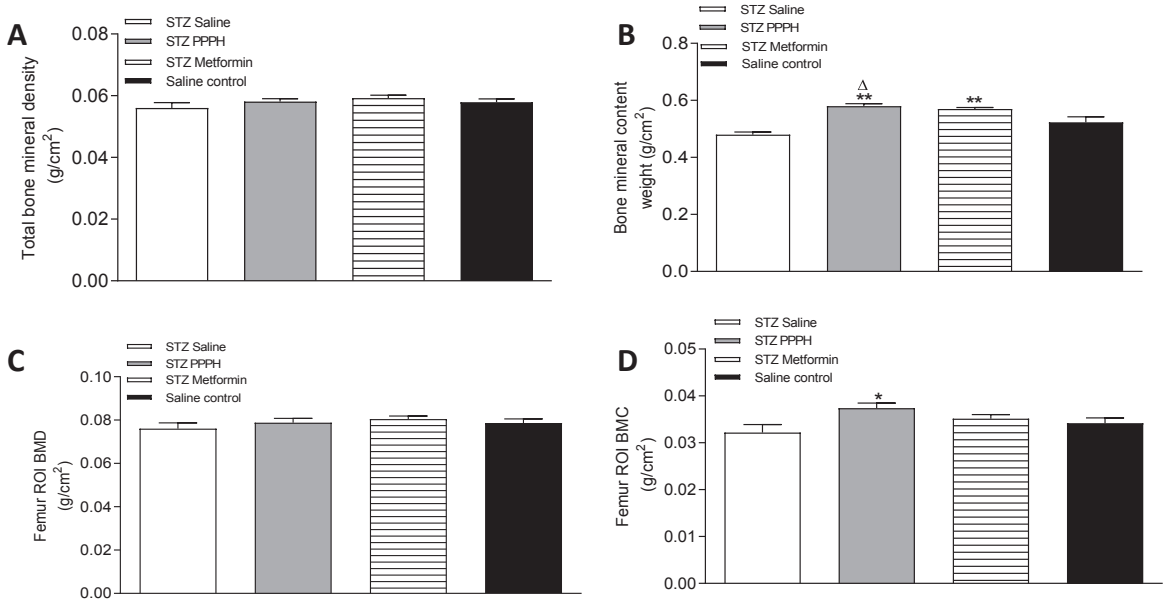

Fig. 6. Effect of twice-daily oral administration of Palmaria palmata protein hydrolysate (PPPH, 50 mg/kg BW) and metformin (200 mg/kg BW) for 18 days on (A) total bone mineral density (BMD), (B) bone mineral content (BMC) and femur region of interest (ROI), (C) BMD and (D) BMC, assessed by PIXImus DEXA in STZ-induced diabetic mice. Values represent mean ± S.E.M. (n = 7 or 8). *p < 0.05, **p < 0.01, versus STZ saline controls. $^{\Delta}$p < 0.05 versus healthy controls.

acid derived due to their expected short circulating half-life after gavage. Amino acids responses are expected to mediate a response that is relatively short-lived and therefore the observed insulinotropic improvements may be attributed to peptide-derived components within the PPPH or synergistic interactions arising from different peptides within the hydrolysate. While the amino acid composition of the hydrolysate was not determined our previous analyses on *Palmaria palmata* protein extracts demonstrated an amino acid composition which was similar to that reported previously by Mæhre, Jensen, and Eilertsen (2016) and Bjarnadóttir et al. (2018), where there was a predominance of aspartic acid, glutamic acid, leucine and alanine residues. Another possibility is that the increase in plasma insulin could also in part be indirectly linked to inhibition of plasma DPP-4, resulting in increased circulating endogenous GLP-1. While the inhibition of plasma DPP-4 was not assessed in the present study, PPPH was shown to inhibit DPP-4 (IC_{50}: 0.97 ± 0.03 mg/mL) *in vitro* (Harnedy et al., 2015). Protein hydrolysates from various biological sources such as dairy, soy and marine are being extensively mined for their ability to inhibit DPP-4 activity in an attempt to mimic current oral small molecule pharmacological treatments (Power, Nongonierma, Jakeman, & Fitzgerald, 2014; Song, Wang, Du, Ji, & Mao, 2017).

Long-term biomarkers of glycaemic control include assessment of the level of serum glycated haemoglobin (HbA_{1c}). Measurement of HbA_{1C} estimates the mean glycaemic control over a 3-month period in humans, or approximately 6 weeks in mice. The present study period was 39 days and chronic hyperglycaemia was identified in poorly controlled diabetes (STZ Saline) with a HbA_{1C} of 10.7% in the diabetic STZ saline administered mice. Interestingly, the PPPH and metformin treatment group mediated an improvement in HbA_{1c} value of 7.2% ($p < 0.01$) and 6.7% ($p < 0.001$) (Fig. 3D), respectively, indicative of an improved glycaemic status (Woo, Shestakova, Ørskov, & Ceriello, 2008). Protein intake has only recently been positively associated with reductions in HbA_{1c}, and this could be due to offsetting the effects of other macronutrients such as carbohydrates which directly affect HbA_{1C}, or through general reduction in carbohydrate intake by increased protein intake. Improvement in blood glucose concentrations could arise due to reductions in food intake, but increased insulinotropic responses within the PPPH group may also contribute to the improved HbA_{1C} seen over the treatment period. Thus, establishing HbA_{1C} levels both pre- and post-treatment could be useful to accurately compare the change within each treatment group. A significantly reduced fasting blood glucose concentration was noted within both PPPH treated ($p < 0.01$) and metformin treated ($p < 0.05$) mice compared to the STZ saline treated control group (Fig. 3C). Interestingly, 12 h fasting blood glucose fell by nearly 16 mmol/L and 11 mmol/L following PPPH and metformin treatment, respectively. This improvement could be due to increased pancreatic insulin content or perhaps through increased cellular glucose uptake upon treatment (Dale et al., 2018). Furthermore, this improvement in glycaemic control was mirrored by the oral glucose tolerance results after 18 days of treatment (Fig. 3A & B). Here PPPH was more effective than metformin, and this may reflect a reduction in immune response noted following extensive hydrolysis of proteins (Kiewiet, Faas, & de Vos, 2018). This may have indirectly assisted in pancreatic recovery/reduction in initial pancreatic damage after STZ treatment and an associated moderate increase in beta cell mass and circulating insulin.

The digestibility of seaweed proteins is not well documented and studies on their bioavailability in humans are scarce (Holdt & Kraan, 2011)). It has been suggested that small peptides from seaweed may possess bioactivity, for example, of relevance for blood pressure regulation (Seca & Pinto, 2018). Others have shown that seaweed supplementation for 4 weeks in Type 2 diabetic subjects showed reduced postprandial glucose, accompanied by reduced circulating triglycerides plus significantly increased HDL cholesterol and increased antioxidant enzyme activity (catalase, GPX and SOD) (Kim, Kim, Choi, & Lee, 2008). These various benefits have been attributed to peptide and other components such as fibre. A more recent study by Sørensen, Jeppesen, Christiansen, Hermansen, and Gregersen (2019) showed benefit of three different dietary seaweeds including *Palmaria palmata* in reducing glycated haemoglobin in mice.

Terminal analysis of total plasma GLP-1 was performed in this study (Fig. 2C). Metformin treatment would have been expected to inhibit DPP-4 activity and thus improve circulating GLP-1 concentration (Cuthbertson, Patterson, O'Harte, & Bell, 2009, 2011). Despite this, the results showed that STZ saline control mice has the highest concentration of circulating GLP-1. This may be explained in part by hyperphagia and heightened GLP-1 release from intestinal L-cells. However, it has previously been suggested that elevated levels of GLP-1 arise as a compensatory mechanism in the diabetic state in order to counteract beta-cell loss and hyperglycaemia (Rydgren, Börjesson, Carlsson, & Sandler, 2012). Furthermore, these findings in combination with the histology results (Fig. 5) suggest that pancreatic health was potentially returning to near normal for the PPPH treatment group, as the concentration of total GLP-1 were at similar levels to those of the non-STZ saline treated group. Thus, in future work GLP-1 responses as a marker of dysfunctional β-cells and hyperglycaemia could be determined in STZ-induced diabetes models. However, in the first instance it would be necessary to determine how total GLP-1 translates to active GLP-1 and indirectly how this could be related to DPP-4 inhibition. Furthermore, characterisation studies are required to identify the peptide(s) mediating the observed response.

5. Conclusion

The results presented herein is the first report showing the beneficial effect of *Palmaria palmata* protein hydrolysates (PPPH) upon satiety, fasting and non-fasting glucose parameters, lipid profile and indirect markers of pancreatic β-cell function in a multiple low-dose STZ-induced diabetes mouse model. This study found treatment with PPPH to be at least as effective or more effective than metformin upon metabolic parameters. However, further studies are required to confirm the antidiabetic efficacy of PPPH in a human study before food claims can be considered. There is also further *in vitro* and *in vivo* research required to identify and characterise specific bioactive peptides within the crude hydrolysate. In conclusion, PPPH, a format which is representative of its ultimate application as a functional food ingredient, has potent anti-diabetic and satiating bioactivities and has significant potential as a functional food ingredient for the management of T2DM and food intake. Different vehicles have already been described in the literature that have been used to deliver biofunctional hydrolysates. However, further studies are required to assess the impact, if any, of the delivery matrix and the processing conditions associated with utilising this hydrolysate on the retention and ultimate release of the bioactivity. Furthermore, human studies are required to confirm retention of bioactivity when delivered as a constituent of a meal.

6. Ethics statement

All animal protocols were approved by the Ulster University, Animal Welfare and Ethical Review (AWERB) Committee and performed according to the UK Animals (Scientific Procedures) Act 1986 and EU Directive 2010/63EU. All necessary steps were taken to prevent any potential animal suffering.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This research was funded under the National Development Plan, through the Food Institutional Research Measure, administered by the Department of Agriculture, Food and the Marine, Ireland under grant number 13/F/467 and a Northern Ireland Department for Employment & Learning N.I. (DEL) PhD scholarship to Christopher M. McLaughlin.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2020.104101.

References

Adeyemo, M. A., Mcduffie, J. R., Kozlosky, M., Krakoff, J., Calis, K. A., Brady, S. M., & Yanovski, J. A. (2015). Effects of metformin on energy intake and satiety in obese children. *Diabetes, Obesity and Metabolism, 17*(4), 363–370. https://doi.org/10.1111/dom.12426.

Bjarnadóttir, M., Aðalbjörnsson, B. V., Nilsson, A., Slizyte, R., Roleda, M. Y., Hreggviðsson, G.Ó., ... Jónsdóttir, R., et al. (2018). *Palmaria palmata* as an alternative protein source: Enzymatic protein extraction, amino acid composition, and nitrogen-to-protein conversion factor. *Journal of Applied Phycology, 30*, 2061–2070.

Bleakley, S., & Hayes, M. (2017). Algal proteins: Extraction, application, and challenges concerning production. *Foods, 6*(5), 33. https://doi.org/10.3390/foods6050033.

Burcelin, R., Eddouks, M., Maury, J., Kande, J., Assan, R., & Girard, J. (1995). Excessive glucose production, rather than insulin resistance, accounts for hyperglycaemia in recent-onset streptozotocin-diabetic rats. *Diabetologia, 38*(3), 283–290. https://doi.org/10.1007/BF00400632.

Caron, J., Domenger, D., Dhulster, P., Ravallec, R., & Cudennec, B. (2017). Protein digestion-derived peptides and the peripheral regulation of food intake. *Frontiers in Endocrinology, 8*(APR), https://doi.org/10.3389/fendo.2017.00085.

Carreiro, A. L., Dhillon, J., Gordon, S., Higgins, K. A., Jacobs, A. G., McArthur, B. M., et al. (2016). The macronutrients, appetite, and energy intake. *Annual Review of Nutrition, 36*(1), 73–103. https://doi.org/10.1146/annurev-nutr-121415-112624.

Connolly, A., Piggott, C. O., & FitzGerald, R. J. (2013). Characterisation of protein-rich isolates and antioxidative phenolic extracts from pale and black brewers' spent grain. *International Journal of Food Science and Technology. 48*, 1670–1681.

Cuthbertson, J., Patterson, S., O'Harte, F. P. M., & Bell, P. M. (2009). Investigation of the effect of oral metformin on dipeptidylpeptidase-4 (DPP-4) activity in Type 2 diabetes. *Diabetic Medicine, 26*(6), 649–654. https://doi.org/10.1111/j.1464-5491.2009.02748.x.

Cuthbertson, Joy, Patterson, S., O'Harte, F. P., & Bell, P. M. (2011). Addition of metformin to exogenous glucagon-like peptide-1 results in increased serum glucagon-like peptide-1 concentrations and greater glucose lowering in type 2 diabetes mellitus. *Metabolism: Clinical and Experimental, 60*(1), 52–56. https://doi.org/10.1016/j.metabol.2010.01.001.

Dal Canto, E., Ceriello, A., Rydén, L., Ferrini, M., Hansen, T. B., Schnell, O., ... Beulens, J. W. J. (2019). Diabetes as a cardiovascular risk factor: An overview of global trends of macro and micro vascular complications. *European Journal of Preventive Cardiology, 26*(2S), 25–32. https://doi.org/10.1177/2047487319878371.

Dale, H. F., Jensen, C., Hausken, T., Lied, E., Hatlebakk, J. G., Brønstad, I., ... Lied, G. A. (2018). Effect of a cod protein hydrolysate on postprandial glucose metabolism in healthy subjects: A double-blind cross-over trial. *Journal of Nutritional Science, 7*(e33), 1–9. https://doi.org/10.1017/jns.2018.23.

Diepvens, K., Häberer, D., & Westerterp-Plantenga, M. (2008). Different proteins and biopeptides differently affect satiety and anorexigenic/orexigenic hormones in healthy humans. *International Journal of Obesity, 32*(3), 510–518. https://doi.org/10.1038/sj.ijo.0803758.

Drummond, E., Flynn, S., Whelan, H., Nongonierma, A. B., Holton, T. A., Robinson, A., & Brennan, L. (2018). Casein hydrolysate with glycemic control properties: Evidence from cells, animal models, and humans. *Journal of Agricultural and Food Chemistry, 66*(17), 4352–4363. https://doi.org/10.1021/acs.jafc.7b05550.

Flatt, P. R., & Bailey, C. J. (1981). Abnormal plasma glucose and insulin responses in heterozygous lean (ob/+) mice. *Diabetologia, 20*(5), 573–577.

Fleurence, J., Morançais, M, & Dumay, J. (2017). Seaweed proteins. In Proteins in food processing (2nd ed., pp 197–211). Cambridge, UK: Woodhead.

Galbreath, M., Campbell, B., Labounty, P., Bunn, J., Dove, J., Harvey, T., et al. (2018). Effects of adherence to a higher protein diet on weight loss, markers of health, and functional capacity in older women participating in a resistance-based exercise program. *Nutrients, 10*(8), https://doi.org/10.3390/nu10081070.

Hameed, S., Dhillo, W. S., & Bloom, S. R. (2009). Gut hormones and appetite control. *Oral Diseases, 15*(1), 18–26. https://doi.org/10.1111/j.1601-0825.2008.01492.x.

Harnedy, P. A., & FitzGerald, R. J. (2013). Extraction of protein from the macroalga Palmaria palmata. *LWT - Food Science and Technology, 51*(1), 375–382. https://doi.org/10.1016/j.lwt.2012.09.023.

Harnedy, P. A., O'Keeffe, M. B., & Fitzgerald, R. J. (2015). Purification and identification of dipeptidyl peptidase (DPP) IV inhibitory peptides from the macroalga Palmaria palmata. *Food Chemistry, 172*(2015), https://doi.org/10.1016/j.foodchem.2014.09.083.

Holdt, S. L., & Kraan, S. (2011). Bioactive compounds in seaweed: Functional food applications and legislation. *Journal of Applied Phycology, 23*(3), 543–597. https://doi.org/10.1007/s10811-010-9632-5.

Holst, J. J. (2013). Incretin hormones and the satiation signal. *International Journal of Obesity, 37*(9), 1161–1168. https://doi.org/10.1038/ijo.2012.208.

Kiewiet, M. B. G., Faas, M. M., & de Vos, P. (2018). Immunomodulatory protein hydrolysates and their application. *Nutrients, 10*(7), 1–22. https://doi.org/10.3390/nu10070904.

Kim, M. S., Kim, J. Y., Choi, W. H., & Lee, S. S. (2008). Effects of seaweed supplementation on blood glucose concentration, lipid profile, and antioxidant enzyme activities in patients with type 2 diabetes mellitus. *Nutrition Research and Practice, 2*(2), 62. https://doi.org/10.4162/nrp.2008.2.2.62.

Klok, M. D., Jakobsdottir, S., & Drent, M. L. (2007). The role of leptin and ghrelin in the regulation of food intake and body weight in humans: A review. *Obesity Reviews, 8*(1), 21–34. https://doi.org/10.1111/j.1467-789X.2006.00270.x.

Lafferty, R. A., Flatt, P. R., & Irwin, N. (2018). Emerging therapeutic potential for peptide YY for obesity-diabetes. *Peptides, 100*(2018), 269–274. https://doi.org/10.1016/j.peptides.2017.11.005.

Le Maux, S., Nongonierma, A. B., Barre, C., & FitzGerald, R. J. (2016). Enzymatic generation of whey protein hydrolysates under pH-controlled and non pH-controlled conditions: Impact on physicochemical and bioactive properties. *Food Chemistry, 199*, 246–251.

Luo, Z., Soläng, C., Mejia-Cordova, M., Thorvaldson, L., Blixt, M., Sandler, S., & Singh, K. (2019). Kinetics of immune cell responses in the multiple low dose streptozotocin mouse model of type 1 diabetes. *FASEB BioAdvances, 1*(9), 538–549.

Mæhre, H. K., Jensen, I. J., & Eilertsen, K. E. (2016). Enzymatic pre-treatment increases the protein bioaccessibility and extractability in dulse (*Palmaria palmata*). *Marine Drugs, 14*(11), 196.

Moebus, S., Göres, L., Lösch, C., & Jöckel, K. H. (2011). Impact of time since last caloric intake on blood glucose levels. *European Journal of Epidemiology, 26*(9), 719–728. https://doi.org/10.1007/s10654-011-9608-z.

McLaughlin, C. M., Parthsarathy, V., Allsopp, P. J., McSorley, E. M., FitzGerald, R. J., Harnedy, P. A., & O'Harte, F. P. M. (2016). Protein hydrolysates from the red seaweed Palmaria palmata enhanced both insulin and GLP-1 secretion in cultured cells and improved acute glucose tolerance in mice. *Diabetic Medicine, 33*, 82.

Newsholme, P., Brennan, L., & Bender, K. (2006). Amino acid metabolism, β-cell function, and diabetes. *Diabetes, 55*(SUPPL. 2), 39–47. https://doi.org/10.2337/db06-S006.

Nobile, V., Duclos, E., Michelotti, A., Bizzaro, G., Negro, M., & Soisson, F. (2016). Supplementation with a fish protein hydrolysate (Micromesistius poutassou): Effects on body weight, body composition, and CCK/GLP-1 secretion. *Food and Nutrition Research, 60*, 1–9. https://doi.org/10.3402/fnr.v60.29857.

Nongonierma, A. B., & FitzGerald, R. J. (2012). Tryptophan-containing milk protein-derived dipeptides inhibit xanthine oxidase. *Peptides, 37*(2), 263–272.

Nongonierma, A. B., & FitzGerald, R. J. (2019). Features of dipeptidyl peptidase IV (DPP-IV) inhibitory peptides from dietary proteins. *Journal of Food Biochemistry, 43*(1), 1–11. https://doi.org/10.1111/jfbc.12451.

Patel, S. (2015). Functional food relevance of whey protein: A review of recent findings and scopes ahead. *Journal of Functional Foods, 19*, 308–319. https://doi.org/10.1016/j.jff.2015.09.040.

Power, O., Nongonierma, A. B., Jakeman, P., & Fitzgerald, R. J. (2014). Food protein hydrolysates as a source of dipeptidyl peptidase IV inhibitory peptides for the management of type 2 diabetes. *Proceedings of the Nutrition Society, 73*(1), 34–46. https://doi.org/10.1017/S0029665113003601.

Rydgren, T., Börjesson, A., Carlsson, A., & Sandler, S. (2012). Elevated glucagon-like peptide-1 plasma levels, as a possible adaptive response, in diabetic NOD mice. *Biochemical and Biophysical Research Communications, 423*(3), 583–587. https://doi.org/10.1016/j.bbrc.2012.06.011.

Seca, A. M. L., & Pinto, D. C. G. A. (2018). Overview on the antihypertensive and anti-obesity effects of secondary metabolites from seaweeds. *Marine Drugs, 16*(7), https://doi.org/10.3390/md16070237.

Simpson, K., Parker, J., Plumer, J., & Bloom, S. (2012). CCK, PYY and PP: The control of energy balance. *Handbook of Experimental Pharmacology, 209*, 209–230. https://doi.org/10.1007/978-3-642-24716-3_9.

Snorgaard, O., Poulsen, G. M., Andersen, H. K., & Astrup, A. (2017). Systematic review and meta-analysis of dietary carbohydrate restriction in patients with type 2 diabetes. *BMJ Open Diabetes Research and Care, 5*(1), https://doi.org/10.1136/bmjdrc-2016-000354.

Song, J. J., Wang, Q., Du, M., Ji, X. M., & Mao, X. Y. (2017). Identification of dipeptidyl peptidase-IV inhibitory peptides from mare whey protein hydrolysates. *Journal of Dairy Science, 100*(9), 6885–6894. https://doi.org/10.3168/jds.2016-11828.

Sørensen, L. E., Jeppesen, P. B., Christiansen, C. B., Hermansen, K., & Gregersen, S. (2019). Nordic seaweed and diabetes prevention: Exploratory studies in KK-Ay mice. *Nutrients, 11*(6), https://doi.org/10.3390/nu11061435.

Spellman, D., Kenny, P., O'Cuinn, G., & FitzGerald, R. J. (2005). Aggregation properties of whey protein hydrolysates generated with *Bacillus licheniformis* proteinase activities. *Journal of Agricultural and Food Chemistry, 53*(4), 1258–1265.

Stack, J., Tobin, P. R., Gietl, A., Harnedy, P. A., Stengel, D. B., & FitzGerald, R. J. (2017). Seasonal variation in nitrogenous components and bioactivity of protein hydrolysates from *Porphyra dioica*. *Journal of Applied Phycology. 29*, 2439–2450.

Tahrani, A. A., Barnett, A. H., & Bailey, C. J. (2016). Pharmacology and therapeutic implications of current drugs for type 2 diabetes mellitus. *Nature Reviews Endocrinology, 12*(10), 566–592. https://doi.org/10.1038/nrendo.2016.86.

Wang, T. Y., Hsieh, C. H., Hung, C. C., Jao, C. L., Chen, M. C., & Hsu, K. C. (2015). Fish skin gelatin hydrolysates as dipeptidyl peptidase IV inhibitors and glucagon-like peptide-1 stimulators improve glycaemic control in diabetic rats: A comparison between warm- and cold-water fish. *Journal of Functional Foods, 19*, 330–340. https://doi.org/10.1016/j.jff.2015.09.037.

Woo, V., Shestakova, M. V., Ørskov, C., & Ceriello, A. (2008). Targets and tactics: The relative importance of HbA1c, fasting and postprandial plasma glucose levels to glycaemic control in type 2 diabetes. *International Journal of Clinical Practice, 62*(12), 1935–1942. https://doi.org/10.1111/j.1742-1241.2008.01941.x.

Zafar, M., Naeem-ul-Hassan Naqvi, S., Ahmed, M., & Kaimkhani, Z. A. (2009). Altered liver morphology and enzymes in streptozotocin induced diabetic rats. *International Journal of Morphology, 27*(3), 719–725. https://doi.org/10.4067/s0717-95022009000300024.