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Research Paper

Characterising the efficacy and bioavailability of bioactive peptides identified for attenuating muscle atrophy within a *Vicia faba*-derived functional ingredient

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ABSTRACT

Characterising key components within functional ingredients as well as assessing efficacy and bioavailability is an important step in validating nutritional interventions. Machine learning can assess large and complex data sets, such as proteomic data from plants sources, and so offers a prime opportunity to predict key bioactive components within a larger matrix. Using machine learning, we identified two potentially bioactive peptides within a *Vicia faba* derived hydrolysate, NPN_1, an ingredient which was previously identified for preventing muscle loss in a murine disuse model. We investigated the predicted efficacy of these peptides *in vitro* and observed that HLPSYSPSPQ and TIKIPAGT were capable of increasing protein synthesis and reducing TNF-α secretion, respectively. Following confirmation of efficacy, we assessed bioavailability and stability of these predicted peptides and found that as part of NPN_1, both HLPSYSPSPQ and TIKIPAGT survived upper gut digestion, were transported across the intestinal barrier and exhibited notable stability in human plasma. This work is a first step in utilising machine learning to untangle the complex nature of functional ingredients to predict active components, followed by subsequent assessment of their efficacy, bioavailability and human plasma stability in an effort to assist in the characterisation of nutritional interventions.

Introduction

In recent years, a number of functional ingredients, such as hydrolysates, have been evaluated in a wide range of health areas (Patel, 2015; Udenigwe and Aluko, 2012; Wu et al., 2019). Key active components of hydrolysates, bioactive peptides, are known to exert functional effects beyond nutrition alone and present a prime opportunity for prevention and treatment of chronic disorders (Cicero et al., 2017; Moughan et al., 2014). For key components of functional ingredients to modulate physiological pathways downstream they must survive gut transit and depending on their target, they may need to be absorbed through the intestinal barrier (Rein et al., 2013). There have been some promising results in identifying key components within functional ingredients, for example, the antioxidant peptides TY and SGGY were identified after simulated gastrointestinal digestion of walnut extracts (Feng et al., 2019) and some bioavailable whey peptides have been demonstrated to improve oxidative status in muscle cells, albeit their stability in biological fluids or their benefits *in vivo* are still to be shown (Corrochano et al., 2019). Therefore, a further step in characterising key molecules within a larger matrix would be to move beyond *in vitro* simulated gastrointestinal digestion (SGID) and intestinal barrier transport, by investigating stability of molecules in a relevant physiological target which would help to progress a functional ingredient to clinical studies. However, due to inconsistent findings, there is little consensus on functional ingredient strategies that can be used as interventions to prolong both life- and health-span and reduce the expensive reliance on drugs to much later years in life (Keehan et al., 2017; Li-Chan, 2015; Doherty et al., 2020). By not only identifying active components of functional ingredients but also subsequently assessing their efficacy, bioavailability and stability may be key to enhancing the adoption of functional ingredients as both prevention and intervention nutritional strategies.

Traditionally, bioactive peptides have been discovered by generating peptide libraries from known bioactive sequences or by randomly testing peptides within hydrolysates (Xu et al., 2014). The latter approach generally is time consuming and involves expensive purification steps such as ultrafiltration to produce peptide-rich fractions. Although these

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fractions are tested for bioactivity, further validation is needed to attribute biological effect to specific sequences, due to the large number of peptides within fractions, this step is highly time and resource consuming (Hernández-Ledesma et al., 2005; Tao et al., 2018; Sánchez and Vázquez, 2017). In recent years, bioinformatic approaches for the characterisation of key bioactive peptides from natural sources have been increasingly adopted, yet these strategies often involve time consuming screening of proteomic data from plant and animal sources for bioactive peptides (Capriotti et al., 2015; Holton et al., 2013). Therefore, the discovery of active components would benefit from technology integration to untangle the very complex nature of food-derived products. Artificial Intelligence (AI) is comprised of a number of techniques which have been utilised in recent years in life science discovery (ÁlvarezMachancosesÓFernández-Martínez, 2019; Yang et al., 2019). Of note, in the area of peptide discovery, a machine learning approach has been used for both prediction of bioactivity and prediction of specific properties. Due to the high volume of known active peptides that can be used for training of models, areas where AI-aided peptide discovery had been particularly prolific are anti-microbial (Fjell et al., 2009; Su et al., 2019; Yoshida et al., 2018), anti-viral (Chang and Yang, 2013), anti-inflammation (Khatun et al., 2019; Manavalan et al., 2018a), anti-cancer (Manavalan et al., 2017). Interestingly, machine learning methods have been also proposed for not just discovery of novel molecules but also the prediction of properties that are of crucial interest in discovery pipelines, such us cell penetrability (Manavalan et al., 2018b) or toxicity (Gupta et al., 2013). Despite these advances, functional ingredient discovery using AI has only recently been described successfully, where a machine learning approach was shown to be capable of predicting a characterised bioactive functional ingredient sourced from the *Oryza sativa* proteome which effectively modulated circulating cytokines and improved physical performance in human (Rein et al., 2019; Kennedy et al., 2020a). Additionally, a similar approach identified two peptides within the *Pisum sativum* proteome with significant anti-aging properties (Kennedy et al., 2020b, 2020c). To that end, computational approaches and more specifically, machine learning, holds significant promise to characterise bioactive elements within functional ingredients in an effort to validate their efficacy and stability.

Recently, a functional ingredient, NPN_1, derived from the *V. faba* was shown to address skeletal muscle loss (Cal et al., 2020). In this study, Cal et al. (2020) reported beneficial effects on protein synthesis, protein degradation and TNF-α secretion *in vitro*. In a murine disease model, daily administration of NPN_1, for 18 days, attenuated muscle atrophy in the soleus hindlimb muscle, increased integrated density of Type I and Type II fibres and significantly up-regulated protein synthesis-related genes. This highlights the potential to address muscle atrophy by simultaneously addressing the balance of protein synthesis, protein degradation and inflammation in skeletal muscle (Schiaffino et al., 2013; Cai et al., 2004).

The aim of this research is to bring forward the characterisation of natural functional ingredients, by identifying constituent active molecules, using machine learning, and subsequently validating efficacy, assessing bioavailability and stability in a biological matrix. In this study, we use a machine learning approach to characterise bioactive peptides within NPN_1. We synthetically reproduce and validate the effects of two predicted bioactive peptides, found within the proteome of NPN_1, on muscle protein synthesis and inflammation *in vitro*. We also assess the bioaccessibility and bioavailability of NPN_1 by mimicking the upper gut transit and assessing the predicted peptides ability to be transported across the intestinal barrier *in vitro*. Finally, we examine the stability of both peptides within NPN_1 in human plasma.

Methods

Materials

Phorbol-12-myristate-13-acetate (PMA, P8139), trifluoroacetic acid (TFA), 10 kDa MWCO centrifugal concentrators (Vivaspin 500, Z614025), thiazolyl Blue Tetrazolium Bromide (MTT, M5655), lipopolysaccharide (LPS) from *Escherichia coli* O127:B8, pepsin (P6887), pancreatin (P7545), porcine bile extract (B8631), penicillin-streptomycin solution (P4333), human plasma (P9523) were obtained from Sigma-Aldrich (MO, USA). Human monocytic leukaemia THP-1 cells (88081201), C2C12 muscle cells (91031101) and Caco-2 intestinal cells (86010202) were from European Collection of Authenticated Cell Cultures and purchased from Sigma-Aldrich (MO, USA). HT-29 intestinal cells were obtained from American Type Culture Collection (ATCC HTB-38). Optima grade formic acid, water and acetonitrile, used for sample and mobile phase preparation, Pierce BCA protein concentration kit used to determine protein concentration, Pierce Peptide Retention Time Calibration Mixture, LC-MS/MS system, RSLCnano Ultimate 3000 system coupled to Q Exactive Orbitrap mass spectrometer through electrospray interface, 0.22 μm filter PES syringe filter and S6 In-Cell ELISA Kit were obtained from Thermo Fisher Scientific (CA, USA). The TNF-α ELISA kit was purchased from BioLegend (CA, USA). Desalting cartridges, Oasis HLB, with 10 mg sorbent, were obtained from Waters Corporation (MA, USA). Horse serum (26050–088) and Hanks' Balanced Salt solution (HBSS, 14025) were from Gibco Life Technologies (CA, USA). Roswell Park Memorial Institute medium (RPMI 1640) and Dulbecco's Modified Eagle Medium (DMEM) were from Lonza (Basel, Switzerland). PEAKS software, used for peptide identification, was obtained from Bioinformatics Solutions Inc. (ON, Canada).

Characterisation of NPN_1

A predictive machine learning approach was used to predict peptides with two different activities, anti-inflammatory and protein synthesis, both these activities were chosen based on the activity of the natural hydrolysate, NPN_1 (PeptiStrong™) that was created to reduce inflammation and improve protein synthesis (Cal et al., 2020). To predict anti-inflammatory activity, we focused on TNF-α reduction and used an untargeted approach (Fig. 1). Structured and unstructured data sources such as scientific literature, patents and public databases were interrogated for anti-inflammatory peptides, specifically, for peptides capable of attenuating TNF-α secretion. The data collected was then manually curated to ensure high quality standards. Once a reliable non redundant dataset of labelled anti-inflammatory peptides was attained ($\sim 10^4$ data points), a neural network predictive architecture consisting of stacked recurrent and dense layers was trained in 10-fold cross validation. No explicit features were extracted for the input sequences, rather, latent features were automatically computed by a first embedding layer in the architecture. Each of the best models for the validation sets were then fine-tuned on a set of peptides specifically labelled for TNF-α inhibition. This smaller set ($\sim 2 \times 10^2$ peptides) included a set of proprietary peptides that were previously validated for activity in-house from multiple natural sources. These peptides were tested mostly on THP-1 cells at various concentrations (typically ranging from 0.005 μg/mL to 50 μg/mL) and exhibited different levels of activity, including no activity (therefore used as negative examples for the training purpose). The ensemble of the 10 resulting neural network models was used to predict on another set of proprietary peptides with experimentally determined effect on TNF-α secretion and an accuracy higher than 85% was measured.

Predictive models (in this specific case based on neural networks) are initially trained with datasets built and curated from literature, patents or public databases and subsequently refined with internally validated peptides. The predict-test-refine loop can be iterated multiple times before achieving the desired accuracy. The library of unlabeled peptides activity is assigned to generally consist of peptides characterised from natural sources through Mass Spectrometry.

A targeted approach was employed to identify peptides with muscle protein synthesis bioactivity. Specifically, proteins involved in the myogenesis and hypertrophy mechanisms were targeted (Table 1). Known protein-protein and protein-peptide interactions of those proteins

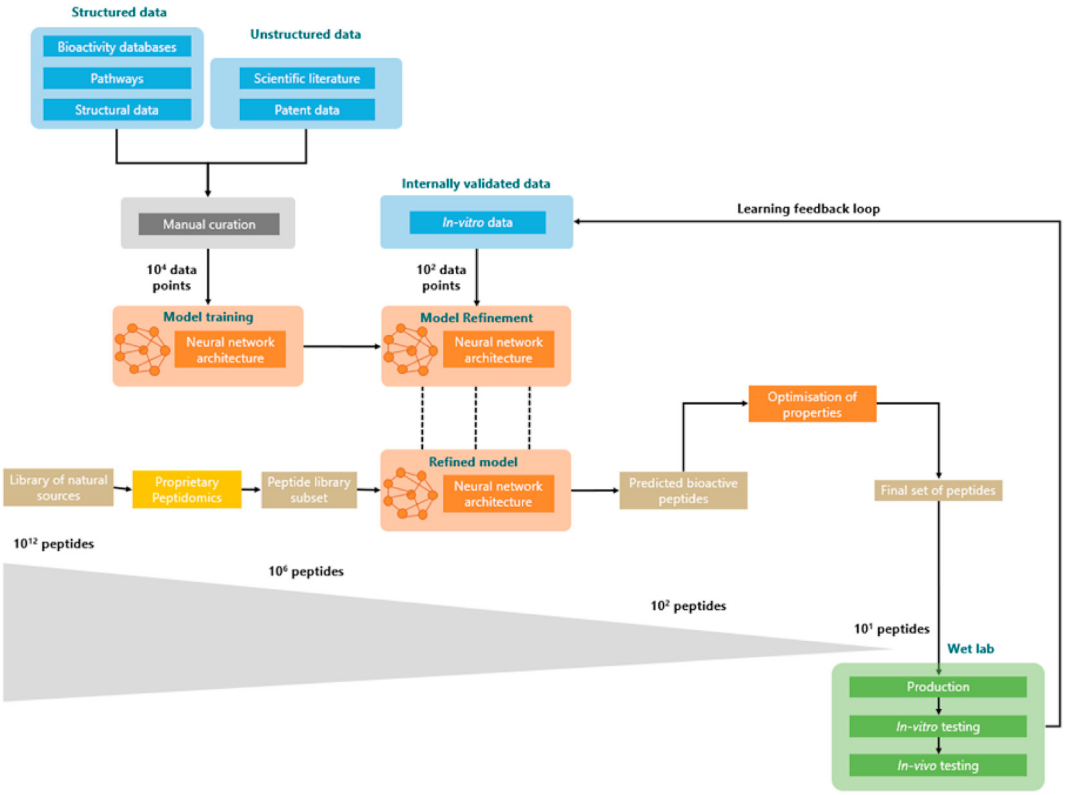

Fig. 1. Workflow for peptide prediction.

Table 1
Examples of Uniprot entries used for targeted approach to identify proteins of interest for prediction of muscle synthesis bioactive peptides.

Uniprot ID	Gene	Protein	Organism
P23443	KS6B1	Ribosomal protein S6 kinase beta-1	Human
Q9UBS0	KS6B2	Ribosomal protein S6 kinase beta-2	Human
Q13541	4EBP1	Eukaryotic translation initiation factor 4E-binding protein 1	Human
P42345	MTOR	Serine/threonine-protein kinase mTOR	Human
P62753	RS6	40S ribosomal protein S6	Human
P31749	AKT1	RAC-alpha serine/threonine-protein kinase	Human
P15172	MYOD1	Myoblast determination protein 1	Human
P13349	MYF5	Myogenic factor 5	Human
P15173	MYOG	Myogenin	Human
P23760	PAX3	Paired box protein Pax-3	Human
P23759	PAX7	Paired box protein Pax-7	Human
Q06413	MEF2C	Myocyte-specific enhancer factor 2C	Human

were analysed to identify binding regions of note. Then peptide profiles likely to mimic those interactions were built to be used to scan unlabeled peptides within our database of MS profiles.

The two approaches were finally combined to carry out the final prediction and identify the most efficacious peptides within the proteome of *V. faba* derived NPN_1. BLASTP (Camacho et al., 2009) (word size = 2; matrix = PAM30, e-value = 10000) was used to filter out peptides exhibiting homology to known bioactive peptides. As a result of these targeted and untargeted approaches, two highly ranked novel peptides were selected for further investigation.

Peptide synthesis and ingredient production

All predicted peptides used in this study were produced by GenScript (NJ, USA), where peptide sequence and purity (95–99%) were validated by HPLC–MS/MS. All peptides were solubilised in ultrapure H_2O and aliquoted for single use.

NPN_1 was prepared as previously described by Cal et al. (2020) (Cal et al., 2020). Briefly, commercially obtained protein powder from *V. faba* (60–63% protein content; AGT Foods Europe, The Netherlands) was solubilised in an alkalising solution and homogenised by agitation. A food-grade endoprotease catalysed the hydrolysis reaction under pre-defined enzyme-specific conditions, such as temperature and a value of pH 6, for several hours. Following hydrolysis, the hydrolysate underwent a thermal enzyme deactivation by raising temperature (85 °C) and a pasteurisation step. Finally, it was spray dried to a fine powder with an inlet temperature of >160 °C.

Cell culture

C2C12 cells are an immortalized mouse skeletal muscle cell line used in biomedical research to study the differentiation of myoblasts and to explore skeletal muscle biochemical pathways. C2C12 cells were maintained at 37 °C and 5% CO_2 in complete growth medium (DMEM, 1% L-glutamine solution, 1% penicillin-streptomycin and 10% sterile filtered foetal bovine serum (FBS) previously heated at 55 °C for 30 min. THP-1 cells were maintained in RPMI 1640 supplemented with 1% L-glutamine, 10% heat-inactivated FBS and 1% penicillin–streptomycin. Intestinal cell lines Caco-2 and HT-29 were cultured and differentiated as previously described by Ferraretto et al. (2018) (Ferraretto et al., 2018).

Determination of S6 phosphorylation

Phosphorylation of S6 was measured as described by Cal et al. (2020) (Cal et al., 2020). Briefly, C2C12 cells (8000 cells/cm^2) were seeded in growth medium in a 96-well plate. After 48 h, cells were differentiated in differentiation medium (DMEM, 1% L-glutamine, 1% penicillin-streptomycin and 2% heat inactivated horse serum) for 7 days. Before treatment, cells were starved for 3 h in growth medium without serum. Additionally, cells were incubated in HBSS for 1 h to deprive amino acids. Finally, differentiated myoblasts were treated for 30 min

with the peptide HLPSYSPSPQ (0.005–5 μg/mL), insulin (positive control, 0.58 μg/mL) or HBSS (untreated control). Experiments were performed in triplicate on three different days. Phosphorylation of S6 was assessed by using S6 In-Cell ELISA Kit according to manufacturer's instructions.

Determination of TNF-α secretion

The anti-inflammatory activity of the peptide TIKIPAGT was assessed using differentiated THP-1 cells (Daigneault et al., 2010). In brief, THP-1 undifferentiated monocytes cells were seeded in 24-well plates (1.0×10^6 cells/well) and maintained in culture medium with 100 nM PMA for 72 h to induce their differentiation into macrophages. After differentiation, macrophages were treated with TIKIPAGT (0.05–5 μg/mL) for 24 h, subsequently washed and stimulated with 100 ng/mL of LPS for further 24 h. Cell supernatants were collected and frozen at −80 °C. TNF-α was quantified in supernatants by using a TNF-α ELISA kit following manufacturer's instructions.

Simulated in vitro gastrointestinal digestion

To assess the stability of HLPSYSPSPQ and TIKIPAGT within NPN_1, SGID was performed. Firstly, NPN_1 was reconstituted in ultrapure H_2O at a final concentration of 37.5 mg/mL. Then, it was centrifuged at 4000 rpm for 20 min, the supernatant was filtered through 0.22 μm filter PES syringe filter and its protein content was determined using BCA assay. SGID was performed on NPN_1 supernatant as described by Minekus et al. (2014) with some modifications (Minekus et al., 2014). As suggested by Minekus et al. (2014), oral phase was not performed as NPN_1 was digested as a liquid formulation (Minekus et al., 2014). Gastric phase was performed by mixing NPN_1 with porcine pepsin (100 U of pepsin per mg of protein) for 2 h at 37 °C with continuous shaking. An aliquot was taken for further LC-MS/MS analysis. Intestinal phase was performed by mixing gastric chyme with pancreatin (2 U of enzyme per mg of protein) and bile extract (10 μmol of bile per mg of protein) for 2 h at 37 °C. Intestinal digestion was stopped by adding protease inhibitor 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (1 mM). Digestates were sterilised filtered using 0.22 μm PES syringe filter. Samples were stored at −80 °C for further LC-MS/MS analysis and cell exposure. SGID and LC-MS/MS analysis were performed on three different days.

LC-MS/MS analysis

Before LC-MS/MS analysis, SGID samples were filtered using a 10 kDa MWCO centrifugal concentrators. Samples were acidified with 0.2% formic acid, desalted, lyophilized, and reconstituted in Optima grade H_2O. The protein content of an aliquot was determined by BCA assay and a fraction containing 5 μg of peptides, was lyophilized and reconstituted in 5 μL Optima grade H_2O containing 0.1% TFA and 5 μg of Pierce Peptide Retention Time Calibration Mixture.

LC-MS/MS analysis was performed on RSLCnano Ultimate 3000 system coupled to Q Exactive Orbitrap mass spectrometer using electrospray interface (ThermoFisher Scientific Inc., CA, USA). NPN_1 samples were separated utilising 60 min gradient from 5 to 75% of Optima grade acetonitrile in Optima grade H_2O containing 0.1% formic acid on 15 cm reverse-phase C18 column at a flow rate of 300 nL min^{-1}.

Mass spectrometer was operated in data-dependent mode, with MS and MS/MS at 70000 FWHM and 17500 FWHM resolution, respectively. From the MS scan, the 15 most intense ions were selected for MS/MS. Fragmentation spectra from putative peptides were used for peptide identification using PEAKS software (Bioinformatics Solutions Inc., ON, Canada) with following parameters: enzyme, none; peptide mass tolerance, 10 ppm; fragment mass tolerance 0.05 Da; variable modifications, oxidation (M), Deamidation (NQ), Pyro-glu from Q; false discovery rate (FDR) 1%; activation method CID; PEAKS "PTM finder" module for the full PTM profile of all peptides in the sample.

Intestinal transport of peptides

Intestinal co-culture model was performed as previously described by Ferraretto et al. (2018) by seeding 70% differentiated Caco-2 cells together with 30% differentiated HT-29 cells at a density of 4×10^4 cells/cm^2 (Ferraretto et al., 2018). Co-cultures were seeded in Transwell® Millicell® 24 insert plates (1.0 μm) assembled to a Millicell® 24 well receiver tray (EMD Millipore, MA, USA) and kept in complete RPMI 1460 medium. Transepithelial electrical resistance (TEER) was monitored on 0-, 3- and 6-days post-confluence using a Millicell®-ERS voltohmmeter (EMD Millipore, MA, USA).

Co-culture treatments were performed as previously described by Corrochano et al. (2019) (Corrochano et al., 2019). Before treatment, co-cultures were washed 2 times with HBSS and incubated for 30 min in HBSS at 37 °C. Then, 200 or 100 μg of SGID NPN_1 was mixed together with HBSS up to 400 μL (final concentration 0.5 and 0.25 μg/μL) were added to the apical side of the inserts and 800 μL of HBSS were added to the basolateral compartments. Samples were not lyophilized. TEER values were monitored immediately and then again at 2 h. After the 2 h treatment, apical and basolateral solutions were collected and kept at −80 °C prior to LC-MS/MS analysis. Intestinal transport was performed in triplicate for each concentration and on three different days.

Human plasma stability

The stability of lead peptides was assessed in human plasma over 24 h (Di et al., 2005). Commercially available human plasma was reconstituted in 5 mL of ultrapure H_2O. Peptides were mixed with human plasma to a final concentration of 0.1 mg/mL and incubated at 37 °C in a Dynex AM89B heater (Dynex Technologies, NY, USA) with continuous movement. Aliquots (50 μL) were taken at the time points 0 min, 5 min, 30 min, 1 h, 2 h, 4 h, 8 h, 16 h and 24 h and mixed with 200 μL of ice-cold methanol containing 0.1% formic acid. Then, tubes were vortexed for 30 s and left the on ice for at least 3 min. After centrifugation (15,000 rpm, 10 min, 4 °C), supernatants were collected and dried in a SpeedVac Savant SPD1010 (Thermo Scientific) overnight. Dried samples were stored at −80 °C until LC-MS/MS analysis was performed. Human plasma stability was tested in triplicate on three experimental occasions. MS data was processed by PEAKS Online X build February 1, 1010.85, Bioinformatics Solutions Inc.

Cell viability assay

The 3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl- 2H-tetrazolium bromide (MTT) colorimetric method was used to determine cell viability after treatments. Mitochondrial respiration, an indicator of cell viability, was assessed by the mitochondrial-dependent reduction of MTT to formazan (Mosmann, 1983). THP-1 and C2C12 cells were seeded in 96–well plates and cultured as mentioned above. Both cell lines were treated with increasing concentrations of peptide (0.005 μg/mL – 50 μg/mL) for 24 h. Thereafter, medium was replaced with 0.5 mg/mL MTT dissolved in culture medium and cells were incubated at 37 °C for 2 h. The MTT solution was removed and 100 μL DMSO solution was added to each well. The plate was vortexed for 5 min and absorbance of the solution was measured at 570 nm. The viability of differentiated THP-1 cells and C2C12 cells in response to treatment with peptides was calculated as % cell viability = (OD treated/OD control) x 100.

Statistics and visualisation

All statistical analyses were performed using the statistical computing software R (R Core Team. R: A languag, 2018). For *in vitro* experiments, significant differences between treatment groups and untreated controls were determined by one-way ANOVA followed by a Dunnett's test. Data are presented as a percentage of untreated controls (mean ± SEM of at least 3 independent experiments). Estimated decay rate was established

using y = e^{a + bx} for half-life calculations, for these graphs, each data point represented the area of the MS peak as reported by PEAKS at the corresponding timepoint. Data points for each peptide were scaled as a percentage of the maximum area for that peptide in any replicate. Data points equal to zero were dropped from the analysis. All time points were represented by 8 or 9 datapoints from experimental triplicates. Before fitting the model, data was dropped after the first time point where the limit of detection was reached. For all analyses, P value < 0.05 was considered significant. Graphs were generated using the 'ggplot2' R package (Wickham, 2009) and the exponential decay model was fit by a custom Python script using the NumPy v1.18 (van der Walt et al., 2011) and SciPy v.1.4.1 (Virtanen et al., 2020) libraries.

Results & discussion

This study utilised machine learning to identify two constituent bioactive peptides in a functional ingredient (NPN_1) which was previously shown to reduce muscle wastage *in vivo* (Cal et al., 2020). The efficacy and oral bioavailability of predicted constituent peptides within a larger matrix was assessed *in vitro*, as well as their ability to resist degradation in human plasma.

Prediction of constituent peptides within of NPN_1

This study employed machine learning to characterise a functional ingredient derived from *V. faba.* Following LC-MS/MS analysis of NPN_1, constituent peptides were predicted with selective targets of the phospho-S6 pathway in skeletal muscle cells and TNF-α secretion in macrophages, respectively. In addition, we show that predicted peptides consistently survive SGID, cross the Caco-2:HT29 intestinal barrier and exhibit a favourable *in vitro* stability profile.

Using a machine learning approach to characterise the peptide hydrolysate NPN_1, we identified constituent peptides: HLPSYSPSPQ (Fig. 2A) with predicted bioactivity for S6 phosphorylation and TIKIPAGT (Fig. 2B) predicted with anti-TNF-α activity (Table 2). HLPSYSPSPQ, contains 10 AA residues with a molecular weight of 1112.19 Da with a neutral net charge while TIKIPAGT, consists of 8 AA with a molecular weight of 799.95 Da and displayed a net charge of 1. TIKIPAGT was identified within parent protein, B0BCL7. While HLPSYSPSPQ was distributed across three parent proteins: Q43674, Q43673 and P05190.

Previously, traditional wet lab isolation and testing methods have faced the challenge to characterising functional ingredients (Capriotti et al., 2015). However, deciphering the complexity of functional ingredients and being able to identify active compounds is key in helping understand the kinetics of the extract, the likelihood of it performing *in vivo* and specifically in human (Udenigwe and Aluko, 2012; Lafarga and Hayes, 2017; Craik et al., 2013). Until recently, the use of AI and machine learning for discovery in life science had been theorised but not fully realised (Kadurin et al., 2017; Sanchez-Lengeling and Aspuru-Guzik, 2018; Segler et al., 2017). However, there are some recent exceptions, Rein et al. (2019) described a novel anti-inflammatory rice functional ingredient characterised with four anti-inflammatory AI discovered bioactive peptides (Rein et al., 2019). A further three constituent peptides within the same ingredient were described by Kennedy et al. (2020), using machine learning methods (Kennedy et al., 2020a). Additionally, AI and machine learning was recently utilised to predict a novel bioactive peptide, discovered in *P. sativum*, that reduced cellular aging in a double blind placebo clinical trial (Kennedy et al., 2020b). Combined, these results not only establish the intelligent approach to discovery and characterisation but also highlights the specificity of this process resulting in measurable characterised functional ingredients. Although further clinical testing is required, these results are an early indication that by adopting machine learning techniques, there is an opportunity to identify the key active compounds within a functional ingredient and predict associated bioactivities in a wide range of different health benefitting areas.

Bioactivity of predicted peptides

To validate the effects of HLPSYSPSPQ and TIKIPAGT, their activity was assessed *in vitro*. The effect of HLPSYSPSPQ on protein synthesis was measured using S6 phosphorylation. The treatment of differentiated myoblasts with 0.05 μg/mL of predicted constituent bioactive peptide HLPSYSPSPQ, significantly increased the phosphorylation of S6 by 50% compared to untreated cells ($P < 0.01$, Fig. 3). The *in-silico* predictions were further validated *in vitro* as TIKIPAGT significantly decreased TNF-α secretion by 55% in LPS-stimulated macrophages at 0.05 μg/mL compared to untreated cells ($P < 0.001$, Fig. 4). Surprisingly, higher concentrations (0.5 and 5 μg/mL) exerted equivalent efficacy which may be indicative of a saturation effect. In addition, none of the peptides induced cell toxicity compared to untreated control (Fig. S1).

A recent study demonstrated that NPN_1, a *V. faba* derived hydrolysate, increased phosphorylation of S6 in skeletal muscle cells and reduced TNF-α in macrophages (Cal et al., 2020). In addition, NPN_1 mitigated soleus muscle loss and boosted Type I and Type IIa fibre density in mice. Phosphorylation of S6 induces the translation of mRNA transcripts for ribosomal proteins and elongation factors which ultimately leads to muscle protein synthesis (Peterson and Schreiber, 1998; Gordon et al., 2013). Cells treated with the peptide HLPSYSPSPQ exhibited a significant increase in S6 phosphorylation, these results are in alignment with previous findings where NPN_1 treatment, also significantly increased phosphorylation of S6 in differentiated myoblasts ($P < 0.05$) (Cal et al., 2020). As HLPSYSPSPQ cannot be the sole peptide responsible for the S6 phosphorylation activity of NPN_1, further study is required to assess the effects on efficacy and bioavailability from other constituents within this

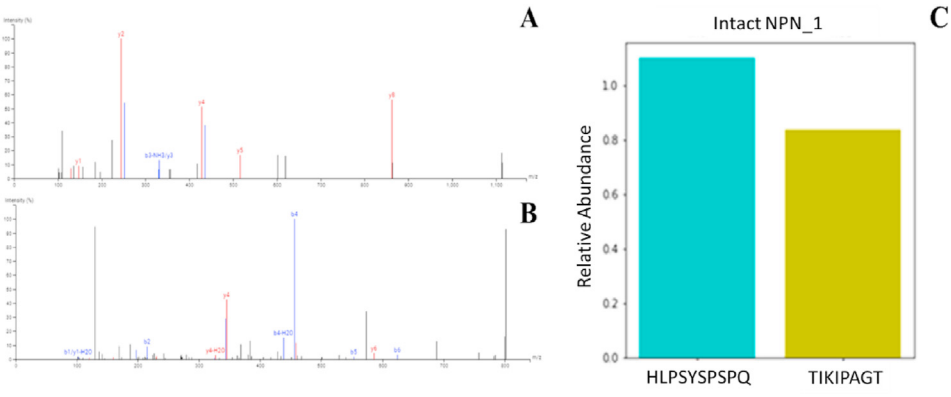

Fig. 2. MS/MS ionization spectra of the peptides found in the functional ingredient, NPN_1. MS/MS ionization spectra of (A) HLPSYSPSPQ, (B) TIKIPAGT and (C) relative abundance of HLPSYSPSPQ and TIKIPAGT found in the functional ingredient, NPN_1.

Table 2
Characterisation of bioactive peptides.

Sequence	Length	Molecular Weight	Charge	Uniprot ID		
				Protein 1	Protein 2	Protein 3
TIKIPAGT	8	799.95	1	B0BCL7		
HLPSYSPSPQ	10	1112.19	0	Q43674	Q43673	P05190

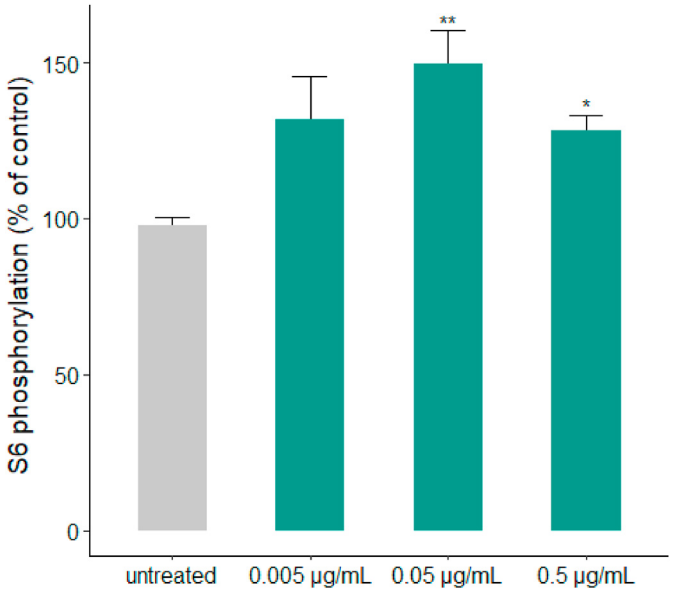

Fig. 3. Effect of HLPSYSPSPQ treatment on S6 phosphorylation. C2C12 cells were treated with HLPSYSPSPQ (0.05 µg/mL – 5 µg/mL) for 30 min following a starvation protocol and compared to untreated cells. (One-way ANOVA analysis; *P < 0.05, **P < 0.01; at least 3 independent replicates).

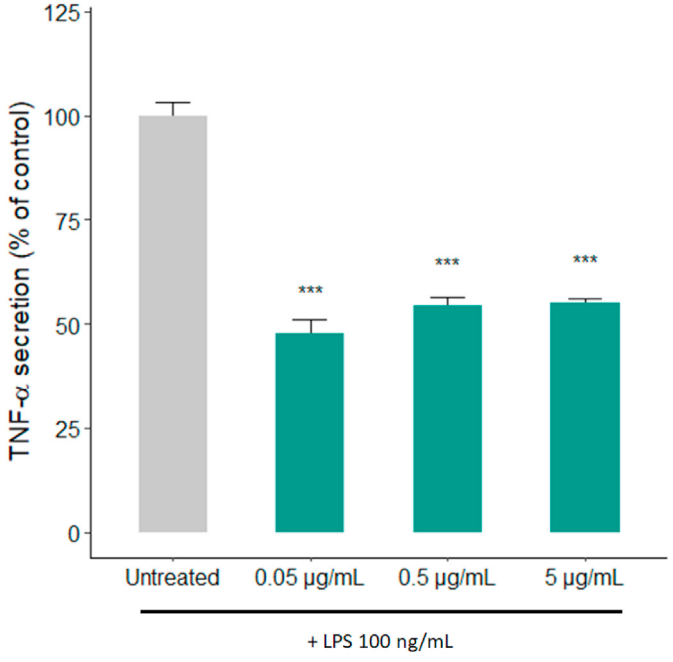

Fig. 4. Anti-inflammatory activity of TIKIPAGT. Effect of TIKIPAGT on TNF-α secretion in THP-1 differentiated macrophages. THP-1 macrophages were treated with TIKIPAGT (0.05–5 µg/mL) for 24 h before treating with 100 ng/mL of LPS for 24 h. The secretion of TNF-α was quantified by ELISA. (One-way ANOVA analysis; ***P < 0.001; at least 3 independent replicates).

hydrolysate. Of note, Cal et al., 2020 observed no effect on S6 phosphorylation in the same cell line with unhydrolysed *V.faba*, indicating a possible peptide network specific effect. Additionally, the same study demonstrated that the functional ingredient, NPN_1, decreased LPS-induced TNF-α release in human macrophages. The cytokine, TNF-α, is responsible for producing chronic inflammation, which is implicated in skeletal muscle dysfunction (Londhe and Guttridge, 2015). Moreover, TNF-α has been shown to inhibit the regeneration of satellite cells, precursors to skeletal muscle cells, in dystrophic muscle (Acharyya et al., 2010). Similar to NPN_1, the application of constituent peptide, TIKIPAGT, resulted in a significant reduction of TNF-α in human macrophages. Corrochano et al. (2019) used macrophages to test the anti-inflammatory effect of 6 bioavailable natural peptides found in whey proteins through traditional screening methods, however, none of the treatments altered TNF-α secretion (Corrochano et al., 2019). The anti-inflammatory ingredient described by Rein et al. (2019) contained four bioactive peptides discovered through AI and was tested *in vivo*, where 20 g of a rice protein hydrolysate significantly reduced TNF-α in healthy subjects in a 24-h kinetic study which suggests that bioactive peptides within the product are able to reach their target organs (Rein et al., 2019). This further highlights the opportunity of AI and machine learning on successful discovery whereby bioaccessible constituent bioactives can be described and validated.

Bioaccessibility and bioavailability of predicted peptides

Certainly, for bioactive peptides to exert their therapeutic effect in a specific target downstream, they need to be bioaccessible and bioavailable. Therefore, the stability of HLPSYSPSPQ and TIKIPAGT, within NPN_1, was assessed through the upper gut tract *in vitro*. Initially, SGID consisting in a 2-h gastric phase followed by a 2-h intestinal phase was performed on NPN_1. It was shown that the lead peptides HLPSYSPSPQ and TIKIPAGT survived to the proteolytic effect of pepsin, the acidic conditions of the stomach and, subsequently, to the 2-h simulated intestinal digestion (Fig. 5 A and B). Although this study did not aim to absolute quantify peptide content, the abundance of HLPSYSPSPQ and TIKIPAGT at the different gut stages, was estimated by comparison with other peptides within the NPN_1. Median peak area value of 71 peptides present in all sample sets was used for normalisation, yielding median normalised relative abundance only of the peptides which survive all gut stages. This normalised relative abundance allows for the closer look at relative ratios of peptides at different phases. As shown in Figs. 2C and 5C, the two peptides of interest, HLPSYSPSPQ and TIKIPAGT, show similar ratio in intact NPN_1 and after the SGID process. They are both found at around the median of the surviving peptides. The bioavailability of HLPSYSPSPQ and TIKIPAGT was tested by treating a Caco-2:HT29 intestinal barrier with SGID NPN_1 using an FDA approved technique to measure human intestinal permeability (Li et al., 2016; Larregieu and Benet, 2013). The integrity of the intestinal barrier was maintained across the study as the TEER values were not significantly altered pre- and post-treatment and did not differ from negative control (cells with medium, data not represented). Of note, both peptides were able to cross the intestinal co-culture and, therefore, likely to reach target organs downstream (Fig. 6A and B). This also underlies that HLPSYSPSPQ and TIKIPAGT are not only resistant to stomach and intestinal proteases but also to the metabolic activity of brush border enzymes such aminopeptidases, endopeptidases and carboxypeptidases. Interestingly, after intestinal absorption, the peptide TIKIPAGT is third of the median, whereas

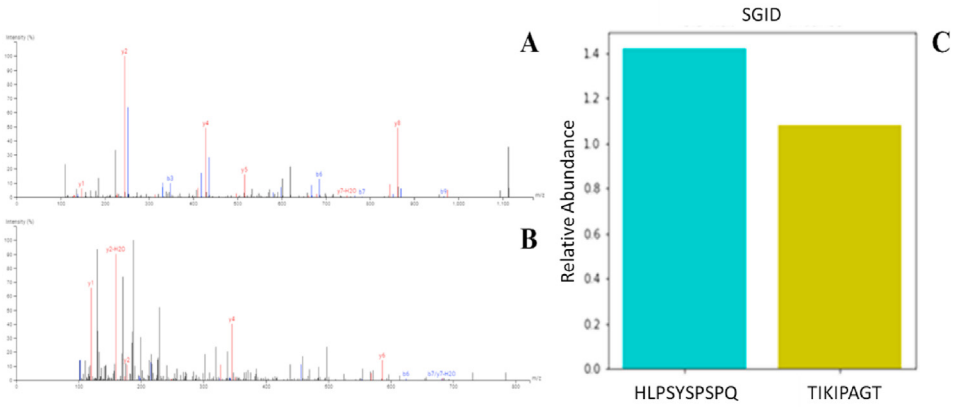

Fig. 5. MS/MS ionization spectra of the peptides found in the simulated gastrointestinal digested functional ingredient, NPN_1. MS/MS ionization spectra of (A) HLPSYSPSPQ, (B) TIKIPAGT and (C) relative abundance of HLPSYSPSPQ and TIKIPAGT found in the full simulated gastrointestinal digested (SGID) functional ingredient, NPN_1.

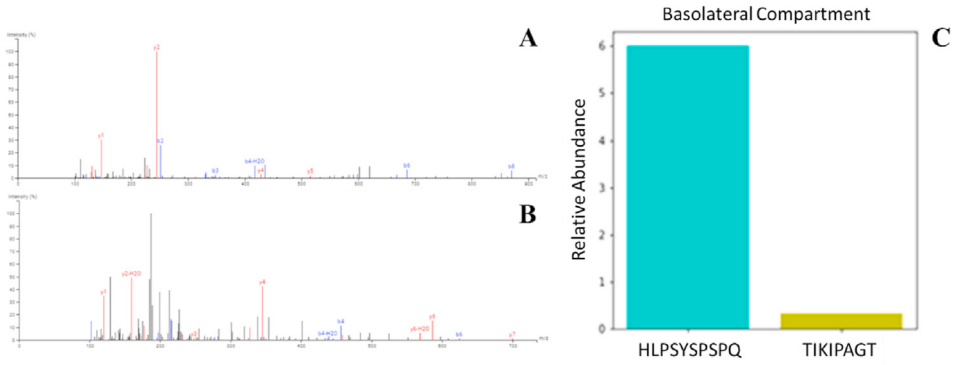

Fig. 6. MS/MS ionization spectra of the peptides in the basolateral compartment after 2-h intestinal co-culture treatment with the simulated gastrointestinal digested functional ingredient, NPN_1. MS/MS ionization spectra of (A) HLPSYSPSPQ, (B) TIKIPAGT and (C) relative abundance of HLPSYSPSPQ and TIKIPAGT found in the basolateral compartment after 2-h intestinal co-culture treatment with the simulated gastrointestinal digested functional ingredient, NPN_1.

HLPSYSPSPQ is 6 times the median (Fig. 6C).

The resistance of predicted peptides to proteolytic digestion was assessed using simulated *in vitro* digestion. Both peptides, HLPSYSPSPQ and TIKIPAGT, survived not only gastric but also intestinal digestion. Similarly, a recent study also identified several parent sequences of HLPSYSPSPQ and TIKIPAGT, after hydrolysis of *V. faba* with pepsin followed by trypsin, however, their bioavailability was not tested (Samaei et al., 2020). HLPSYSPSPQ and TIKIPAGT both crossed the intestinal co-culture barrier, indicative of a promising enhanced bioavailability, with HLPSYSPSPQ exhibiting greater relative abundance than TIKIPAGT. This difference could be addressed by examining their amino acid composition. The presence of prolines embedded within the peptide sequences has been reported to provide peptide stability within the gut transit (Savoie et al., 2005). HLPSYSPSPQ contains 3 prolines, rendering it less susceptible to proteolytic degradation than TIKIPAGT. Furthermore, HLPSYSPSPQ is more hydrophobic compared to TIKIPAGT which would suggest easier passage through the intestinal layer. Additionally, degradation of longer parent peptides might result in release of HLPSYSPSPQ which could explain the increase in its abundance relative to median. Namely, the HLPSYSPSPQ motif is found to be contained in 30 longer sequences of peptides in intact NPN_1, 11 longer sequences of peptides after SGID and 8 longer sequences of peptides in basolateral compartment, indicating the robustness of this sequence. In comparison, TIKIPAGT is found to be contained in 8 longer sequences of peptides in intact NPN_1, 2 longer sequences of peptides after SGID and doesn't feature in any longer sequences of peptides in basolateral samples, leaving a smaller pool for replenishment of degraded peptide.

There have been noteworthy efforts to enhance oral delivery of peptides by creating improved formulations (i.e. enzyme inhibitors, surfactants and nanoparticles) or performing peptide chemical modifications (i.e. cyclization or unnatural amino acids) (Ghosh et al., 2018). However, we demonstrated that the unmodified linear peptides, HLPSYSPSPQ and TIKIPAGT, can resist the upper gut transit and be transported across the *in vitro* intestinal barrier while they are embedded within a peptide network. Certainly, HLPSYSPSPQ and TIKIPAGT were transported across the intestinal barrier with other constituent peptides of NPN_1 that were mostly neutral and hydrophilic residues, and whose length was mainly ranged between 7 and 11 amino acids (Fig. S2). In agreement, the passive diffusion transport of bioactive oligopeptides through the intestinal tight junctions have been previously reported (Shen and Matsui, 2017) although the optimum charge for peptide paracellular transport is not well stablished yet (Wang and Li, 2018).

Human plasma stability of predicted peptides

In addition to resisting gut transit and having good intestinal permeation, peptides need also to possess enough stability in blood to exert their health benefits in target tissues. Fig. 6 shows the estimated decay rate of the peptides HLPSYSPSPQ (Fig. 7A) and TIKIPAGT (Fig. 7B) in human plasma. Model parameters were made for HLPSYSPSPQ ($a = -0.63$, $b = 3.99$) and TIKIPAGT ($a = -2.47$, $b = 4.27$). HLPSYSPSPQ showed a half-life of 65.79 ± 3.79 min and reached the limit of detection at 8 h. TIKIPAGT presented a half-life of 16.85 ± 0.40 min with a limit of detection reached at 2 h.

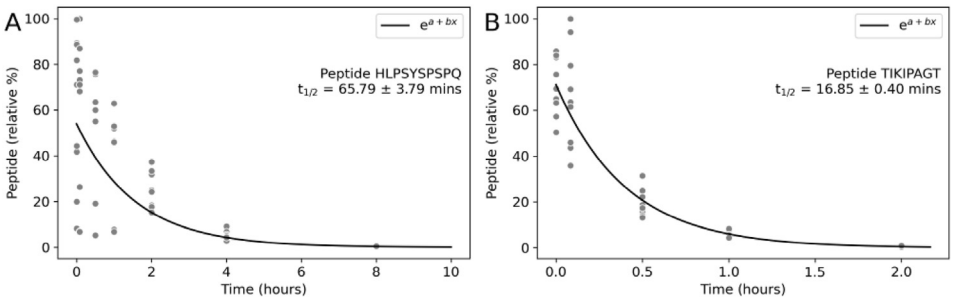

Fig. 7. Human plasma stability of predicted peptides. Human plasma stability of (A) HLPSYSPSPQ and (B) TIKIPAGT4.

Evaluation of bioaccessibility and bioavailability of bioactive components in functional ingredients is important for the development of these ingredients. However, it is noteworthy that there is a lack of extended research where the behaviour of food components is tested in other biological matrices such as plasma (Rein et al., 2013). Both peptides exhibited good stability in human plasma, notably HLPSYSPSPQ showed a half-life value of 65.79 min. These results agree with previous findings where peptides containing threonine at the N-terminus exhibited a prolonged half-life in biological matrices (Di, 2015). Although HLPSYSPSPQ and TIKIPAGT were not measured in animals receiving hydrolysate NPN_1 treatment in a disuse murine model described by Cal et al., 2020), the half-life exhibited by both peptides especially that of HLPSYSPSPQ may potentially have contributed to the *in vivo* efficacy observed in this previous study (Cal et al., 2020), however, it would be necessary to quantify these peptides in any subsequent studies. While both peptides featured here represent lead predicted peptides for bioactivity, other peptides were characterised though machine learning, and as the efficacy of NPN_1 cannot be attributed to HLPSYSPSPQ and TIKIPAGT alone, further work is required to validate efficacy and investigate bioactivity, bioaccessibility, bioavailability and stability of these other constituent peptides.

Conclusions

Here, we provide evidence for the use of machine learning to identify efficacious bioactive peptides within a functional ingredient, NPN_1. By characterising constituent peptides, we can better evaluate the bioaccessibility and bioavailability of an ingredient and indicate if the ingredient should be selected for further development. As both HLPSYSPSPQ and TIKIPAGT were among peptides which remained intact, demonstrated good intestinal barrier transport properties and stability *in vitro*, these results indicate NPN_1 should progress to investigation in human. Ultimately, we demonstrate that machine learning is an advanced powerful tool capable of characterising functional ingredients and identifying peptides which can target multiple physiological pathways and warrants further *in vivo* investigation to determine peptide quantification and pharmacokinetic profiles following oral administration.

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CRediT authorship contribution statement

Alberto R. Corrochano: Methodology, Validation, Formal analysis, Investigation, Project administration, Writing – original draft, Writing – review & editing. **Roi Cal:** Methodology, Validation, Investigation, Writing – review & editing. **Kathy Kennedy:** Methodology, Validation, Investigation. **Audrey Wall:** Formal analysis, Visualization, Project administration, Writing – original draft, Writing – review & editing. **Niall Murphy:** Methodology, Visualization. **Sanja Trajkovic:** Methodology, Investigation, Visualization. **Sean O'Callaghan:** Formal analysis, Visualization. **Alessandro Adelfio:** Methodology, Software, Data curation, Visualization. **Nora Khaldi:** Supervision, Conceptualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

All authors are employees of Nuritas Limited and declare no competing interests. The design of the study; the collection, analyses, and interpretation of data; the writing of the manuscript and the decision to publish the results was performed by Nuritas.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2021.03.008.

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