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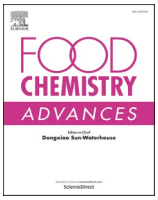

The effects of processing steps on avenanthramides, avenacosides and β-glucan content during the production of oat-based milk alternatives

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ABSTRACT

Oat-Based Milk Alternatives (OMAs) may provide health benefits resulting from oat nutritional compounds; avenanthramides, which are polyphenols providing anti-inflammatory and antioxidant effects; avenacosides - saponins with anti-bacterial and anti-fungal properties; and β-glucans, which may assist in lowering blood cholesterol and prevention of diabetes and cardiovascular diseases. Oats undergo multiple processing steps to ensure a sensory appealing and safe product, however, little research has been carried out on the specific effects on these compounds. This study aimed to determine concentration of avenanthramides, avenacoside A and β-glucan in OMA samples throughout 12 stages of production. Avenanthramides and avenacosides were measured using liquid chromatography-mass spectrometry, with β-glucan determined spectrophotometrically using a modified enzymatic assay.

An overall reduction of 42 % was observed in measured β-glucan, with a decanting stage a main contributing factor. Measured β-glucan was negatively impacted by glucoamylase treatment, yet increased upon α-amylase, 90 °C and high shear treatments. Avenanthramides and avenacoside A significantly increased after initial enzymatic treatment with α-amylase, whilst avenanthramides increased again upon 90 °C heat treatment and decanting. However, avenanthramide concentration decreased after UHT and prolonged heat treatments, suggesting a susceptibility to degradation at temperatures above 120 °C. With this information, future production may be optimised to better preserve potential health benefits of OMAs.

1. Introduction

1.1. Oat-based milk alternatives

Plant-based alternatives for milk are becoming increasingly popular due to factors such as environmental and animal welfare concerns, as well as veganism - which has increased by 360 % within the past ten years leading to an estimate of over 500,000 vegans in the UK (Aydar et al., 2020). Oat based milk alternatives (OMAs) have generated consumer interest, with unit sales rising nearly 700 % between 2018 and 2019 (Ramsing et al., 2023). This surge in popularity may be partly due to a rise in nut allergies (Brough et al., 2015) which limits the potential consumption of nut-based milks, and soy allergies (Jeske et al., 2017) which may prevent consumption of soy milk. With nut-based milks being such a broad category, including; almond milk, coconut milk, hazelnut milk, pistachio milk, and walnut milk (Sethi et al., 2016), and with soy ranked second in popularity for plant-based milk alternatives (PBMAs) worldwide (Olías et al., 2023), it is possible the increase in allergies may have a substantial impact on PBMA options for many consumers. Also, despite an increase in soy milk sales throughout the 1990s and early 2000s, sales have slowed after research suggested soy may be an endocrine disruptor as it contains isoflavones that are structurally similar to oestrogen (Ramsing et al., 2023). These limitations may have led the way for OMAs, which have become an important substitute for other PBMAs, and a successful commercial cereal milk (Yu et al., 2023). OMAs have been reported to be the most widely consumed PBMA globally (Olías et al., 2023). OMAs may also provide health benefits arising from oats unique bioactives, as well as dietary fibre β-glucan.

OMAs are a rich source of functional proteins, lipids, vitamins and

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minerals (Sethi et al., 2016). With protein ranging from <1–4 (g/100 mL), carbohydrates 8–25 (g/100 mL), iron, calcium and potassium at 0–1, 0–300 and 40–400 (mg/mL) respectively, and vitamin B12 at 0–0.9 (mcg/100 mL) (Cui, 2023). Processing treatments potentially result in the loss of nutrients, however vitamins and minerals can be replenished by fortification of OMAs (Sethi et al., 2016). Yet little research has taken place investigating the possibility to replenish oat bioactives and β-glucan content. Therefore, this study focuses specifically on the effects on β-glucan, avenanthramides, and avenacoside A.

1.2. Oat bioactives

Oats contain phytochemicals concentrated in the outer layers of the grain (Alrahmany et al., 2013), including a number of compounds with free-radical scavenging capability, thus exhibiting antioxidant properties (Liu et al., 2004), such as phenolics and sterols (Peterson et al., 2001). Antioxidants are known to protect the body from disease (Chen et al., 2016) and reduce oxidative damage (Redaelli et al., 2016).

Phenolic compounds are secondary products of plant metabolism (Multari et al., 2018), which may help prevent cancer and heart disease, and even provide some protection to the food products they are in from rancidity - improving shelf life, stability and taste (Redaelli et al., 2016). Phenolic compounds in oats are composed of flavonoids, phenolic acids, and avenanthramides (Li et al., 2017). Avenanthramides are novel substituted N-cinnamoyl anthranilate alkaloids that are unique to oats (Peterson et al., 2001). With an estimation of over 25 different avenanthramides (Multari et al., 2018), and both genetic and environmental factors affecting the content, a range as wide as 25–407 μg/g has been found in oats (Piironen et al., 2023). Avenanthramides have been demonstrated in-vitro to provide anti-inflammatory and antiatherogenic effects, due to inhibition of adhesion molecule expression and pro-inflammatory cytokines and chemokines (Liu et al., 2004). The most abundant forms of avenanthramides include those comprising of 5-hydroxyanthranilic acid conjugated with *p*-coumaric acid (known as avenanthramide-A, or 2p), ferulic acid (known as avenanthramide-B, or 2f), and caffeic acid (known as avenanthramide-C, or 2c) (Collins, 1989; Piironen et al., 2023).

Sterols are abundant in cereals (Maatta, 1999), however oats contain the sterol saponin, and are the only saponin-containing cereal (Martínez-Villaluenga & Peñas, 2017). Saponins are a class of bioorganic compounds found in plants, described by their soap-like foaming. They are glycosides of triterpenes and steroids, with multiple medicinal properties (Ashour et al., 2019). Saponins have been shown to have anti-fungal and anti-cytotoxic actions (Abbas et al., 2015), as well as anti-inflammatory, anti-bacterial, anti-viral, and anti-cancer properties (Ashour et al., 2019). There are two distinct classes of saponins in oats – avenacins, and avenacosides (Osbourn, 2003). Avenacoside A and B, both unique to oats (Pecio et al., 2013), are bisdesmosidic steroid saponins, meaning they have two sugar residues bound at different ends of the aglycone as opposed to monodesmosidic saponins which have one sugar residue bound to the aglycone (Önning & Asp, 1993).

1.3. β-Glucans

β-Glucan, a linear polysaccharide (Deswal et al., 2014) found in the internal aleurone and sub aleurone cell walls of oat (Holtekjølen et al., 2006), is a soluble fibre which has gained interest due to its many functional and bioactive properties (El Khoury et al., 2012). Soluble fibres slow glucose absorption through delaying gastric emptying and increasing total intestinal transit time (Bashir & Choi, 2017). Oat β-glucan intake may be beneficial in controlling and preventing diabetes due to a reduction in the effect of postprandial glycaemic and insulinemic response in blood (Daou & Zhang, 2012), providing a beneficial role in insulin resistance (El Khoury et al., 2012). β-Glucan has been shown to lower total blood cholesterol (Daou & Zhang, 2012), with the cholesterol reducing properties shown to be retained in the oats even after being processed into oat milk, with a decreased plasma cholesterol and low-density lipoprotein cholesterol level shown in participants when 0.75–1 L of OMA was consumed per day over a 4-week period (Onning et al., 1998). Studies, including in-vitro, animal and clinical, have shown that β-glucan may have anti-tumour, immunomodulating, and bone injury healing effects (Bashir & Choi, 2017). β-glucan has even been found effective against infectious diseases and cancer (El Khoury et al., 2012). β-Glucan may also improve stability in OMAs as products with intact β-glucan have increased stability through storage. A loss of the β-glucan network has been found to lead to a thin and unstable beverage (Patra et al., 2022).

A health claim relating to reduction in plasma cholesterol concentrations at oat β-glucan intakes of at least 3 g per day was authorised by the European Food and Safety Authority (EFSA) in 2010 (Liutkevičius et al., 2015). The soluble β-glucan content in oats has been measured at 3.9–7.5 g per 100 g dry mass (Gajdošová et al., 2007), and the highest reported content being 8 g (Bashir & Choi, 2017). This would suggest an intake of 37.5–77 g of naked oats may meet the intake required for the health benefits acknowledge by EFSA. However, it is yet unclear how much OMA would be required in order to meet these recommendations, depending on solid content, oat source, and how much β-glucan is retained throughout processing.

1.4. Processing stages

Production of OMAs is unique; the processing steps have been developed to create a similar appearance and texture to cow's milk; with oats undergoing a series of physical, chemical and mechanical operations (Yu et al., 2023). Frequently used processing operations are described by McClements (2020) and include: hydrolyses to enzymatically degrade starch and plant materials, blanching to inactivate the enzymes by heating, separation of large insoluble matter by centrifugation, homogenisation to mechanically break down particulate insoluble matter, thermal processing to inactivate spoilage and pathogenic bacteria, and formulation to add additional ingredients such as lipids, minerals, and preservatives (McClements, 2020). Therefore, these stages were selected for use in this study, and further discussed below.

Enzymatic hydrolysis of the starch is necessary to resolve the processing challenges associated with starch gelatinisation during heating (Sethi et al., 2016). As oats consist of 50–60 % starch, with gelatinisation temperatures of 44.7–73.7 °C, this may cause issues during the heat treatment stages of OMA production (Deswal et al., 2014), with gelatinisation resulting in high viscosity (Sethi et al., 2016), and potentially technical problems in downstream processing (Mäkinen et al., 2016). Depending on the enzyme used and the conditions of use, such hydrolyses can reduce the formation of colloids with high viscosity, affect the taste by increasing sweetness, and impact the nutritional properties (Yu et al., 2023). The enzyme α-amylase catalyses the hydrolysis of starch into shorter oligosaccharides through the cleavage of α-D-(1–4) glycosidic bonds; hence treatment of oats with α-amylase has been shown to be effective in increasing the content of reducing sugars due to the breakdown of starch, increasing protein extraction, as well as phenolic content and therefore antioxidant activity due to the disruption of cell walls (Babolanimogadam et al., 2023). Glucoamylase consecutively hydrolyses the α-1,4 glycosidic bonds from the non-reducing ends of starch, which results in the production of glucose, as well as hydrolysing the α-1,6 linkages (Soccol et al., 2006). Glucoamylase is widely used in the food industry for the saccharification of partially processed starch to glucose (Kumar & Satyanarayana, 2009). Enzymatic starch hydrolysis typically requires coordinated action of different endo and exo-amylases; two of the major enzymes involved being α-amylase and glucoamylase (Douglas Crabb & Mitchinson, 1997; Kumar & Satyanarayana, 2009). This may therefore mean glucoamylase is useful in conjunction with α-amylase in order to fully breakdown starch to maximise sweetness and reduce viscosity. Previous studies have suggested that liquification enzymes are best applied during gelatinisation,

with the starch in an amorphous form being more easily digestible by the amylases (Deswal et al., 2014).

High sheer mixing is a mixing technique involving intense mechanical agitation and shearing forces, to achieve blending, dispersion of particles and emulsification. Comprised of rotary elements and fixed elements (screens), high sheer mixers can perform intense lateral mixing due to the gap of rotor and stator resulting in high shear stresses (Vashisth et al., 2021). This may be useful when applied to OMA development, with emulsification from homogenisation leading to increased stability and heterogeneity in dairy beverages (Martinez-Monteagudo et al., 2016). Decanting is a stage whereby centrifugal force separates the samples into two fractions; the liquid fraction and a slurry containing the larger particles. The grinding waste in PBMA production is separated by decanting in order to remove coarse particles from the product (Mäkinen et al., 2016). PBMAs are usually formulated with fats, thickeners, stabilizers, colours, flavours, and nutrients (McClements et al., 2019). Product formulation with additional ingredients is important to stabilise and emulsify products, whilst addition of nutrients in food substitutes may be necessary to ensure the nutritional quality (Mäkinen et al., 2016). The high-pressure homogenisation step transforms the oil and water phases into an oil-water emulsion (McClements, 2020), which maintains a high resistance to creaming, leading to an increase in stability (Yu et al., 2023). OMAs have reduced stability in comparison to other milks, partly due to the high polydispersity index (PDI) of particles within the OMA (Jeske et al., 2017). PDI is the measurement of the heterogeneity of a sample based on size and is determined by dynamic light scattering (Mudalige et al., 2019). Homogenisation results in a relatively low PDI (Hu et al., 2017), and reduced particle size (Amador-Espejo et al., 2014), leading to reduced sedimentation of particles (Xia et al., 2019). Ultra-high-pressure homogenisation can extend shelf life due to the lethality of high pressure on microorganisms, and therefore may provide a lower cost alternative to pasteurisation and conventional thermal treatments, whilst better preserving functional compounds including vitamins, polyphenols, and flavonoids, and even the texture attributes (Xia et al., 2019). Ultra Heat Treatment (UHT) is a high temperature treatment of 135–150 °C for a few seconds using direct heating methods including steam injection, steam infusion, or indirect heating methods, to ensure sterility and extend shelf life at room temperature (Sethi et al., 2016). It has been suggested that a shorter UHT holding time should be employed for beverages to highly retain vitamins during storage (Zhang et al., 2007).

However, the extent to which these processing stages may affect the nutritional properties is unclear (Yu et al., 2023), with some studies suggesting the nutritional value of oats is adversely affected by such multiple processing steps (Zhang et al., 2007). Stability of avenanthramides may be affected by pH and temperature. They have been shown to diminish in an alkaline solution, potentially due to hydrolysis of amide bonds, with instability being increased further on heat treatment. However, the effects have been shown to be more pronounced in certain types of avenanthramide than others (Dimberg et al., 2001). Enzymatic treatment has been reported to affect the phenolic content in oats; an up to three-fold increase in free phenolic acid was found after treatment of oats with cellulase, viscozyme and α-amylase (Alrahmany et al., 2013). However, the same study revealed a lower level of total free phenolic acid content of oat bran after treatment with amyloglucosidase. This suggests that the effects are enzyme dependent. β-Glucan has also been shown to be affected by enzymes, with an increased extractability of β-glucan polymers resulting from degradation of cell wall polysaccharides, which were naturally entangled with β-glucans in the grain (Henrion et al., 2019). Studies have demonstrated the levels of soluble β-glucan activity to be affected differently depending on the type of heat processes; increasing when boiled into porridge in hot water for 10 min yet decreasing when baked into bread (Johansson et al., 2007). Therefore, the effects of the specific heat treatment during production on OMAs is yet to be investigated.

The aims of this study were to investigate the effects of each processing step on the nutritional profile of OMAs; by quantifying the levels of avenanthramides, avenacoside A, and β-glucans. This project sought to determine how the bioactive compounds are affected, to support future research into potential solutions and methods of preservation of these components.

2. Materials and methods

2.1. Chemicals

HPLC grade water, methanol and hexane, were obtained from Fisher Scientific UK. LC-MS grade formic acid (98–100 %) and acetonitrile were purchased from Merck (Darmstadt, Germany). Standards of avenanthramide A (i.e. 2p), avenanthramide B (i.e. 2f), and avenanthramide C (i.e. 2c), avenanthramide D phyproof®, and avenacoside A (>95 %), were purchased from Sigma Aldrich Co. Ltd. (Gillingham, UK). Lichenase, β-glucosidase, glucose oxidase/peroxidase (GOPOD) reagent buffer, GOPOD enzymes along with D-glucose standard solution, were obtained from Megazymes, Neogen (County Wicklow, Ireland). Buffer reagents: sodium dihydrogen orthophosphate dihydrate, sodium hydroxide solution, glacial acetic acid, and sodium azide were obtained from Merck Life sciences UK Ltd. (Gillingham, UK). Oat flour was obtained from Glebe Farm foods Ltd. (Huntingdon, UK). α-Amylase and glucoamylase were purchased from Enzyme Supplies Ltd. (Oxford, UK).

2.2. β-Glucan analyses

β-Glucan concentration was determined according to methods discussed by McCleary et al. (2020), and using the Megazymes β-glucan mixed linkage assay, with slight adjustments to account for background sugars within the product. The principle involved the use of lichenase to specifically hydrolyse β-glucan to oligosaccharides, which are then cleaved to glucose by β-glucosidase. The glucose was then quantified by the use of glucose oxidase-peroxidase-buffer mixture, using a Orion AquaMante 8000 UV–Vis spectrophotometer (Thermo scientific).

The steps were as follows; 3 mL of OMA sample was added to a 25 mL falcon tube and heated in a boiling water bath for 5 min. After cooling to room temperature, 8 mL of 95 % aqueous ethanol was added to samples and mixed on a vortex mixer. Tubes were then centrifuged for 10 min at 1800 x g. The supernatant was then discarded. The pellet was suspended in 20 mM sodium phosphate buffer (pH 6.5) to a total volume to 4 mL. This was incubated for 5 min at 50 °C. Lichenase solution (0.2 mL) was added, with samples then incubated for 1 h at 50 °C, with regular vigorous stirring. Sodium acetate buffer (5 mL, 200 mM, pH 4.0) was added to the samples, mixed on a vortex mixer, and centrifuged at 1000 x g for 10 min. Aliquots of 0.05 mL were dispensed into falcon tubes, with 0.1 mL of β-glucosidase solution added, and incubated at 50 °C for 10 min. GOPOD reagent (3 mL) was added to each tube and incubated at 50 °C for 20 min. Samples were transferred to glass cuvettes, with absorbance measured on a spectrophotometer at 510 nm, against a reagent blank. To account for background glucose within the samples, the process was repeated for each sample using buffer in place of enzymes. The absorbance was then used as a sample blank and subtracted from the initial results.

A glucose standard was prepared for each repetition, using 0.1 mL D-glucose standard (1 mg / 1 mL), 0.1 mL 200 mM sodium acetate buffer

(pH 4.0), with 3 mL GOPOD reagent, incubated for 20 min alongside samples. This was then used within the following calculation (Eq. 1) to determine β-glucan concentration:

$$\beta - glucan\ \left(\%\frac{w}{w}\right)(g/100mL) = \Delta A \times F \times \frac{9.2}{3} \times\ 1000\ \times \frac{1}{1000} \times \frac{1}{1000} \times \frac{162}{180} \times D \quad (1)$$

where:

ΔA: absorbance after β-glucosidase treatment minus reaction blank absorbance

F: factor for the conversion of absorbance values to μg of glucose

9.2/3: volume correction factor

1000: volume adjustment factor

1/1000: conversion from μg to g

1/1000: conversion from mg to g

162/180: factor to convert from free D-glucose to anhydro-D-glucose, as occurs in β-glucan

D: dilution factor (further dilution prior to incubation with β-glucosidase (samples were diluted by 2)

This formula can be simplified to the following (Equation 2):

β-glucan (g/100mL) = absorbance x factor x 2 × 0.00276 (Equation 2)

The same formula was carried out on the sample blanks, to be subtracted from the initial measurement and to give the final result.

2.3. Avenanthramides and avenacosides

2.3.1. Sample preparation

Extraction was carried out based according to McCarron et al. (2024). Samples (10 mL) were placed into a separating funnel with 10 mL of hexane, shaken for 5 s and left to equilibrate for 15 min before removing the fat. They were then centrifuged at 4 °C for 10 min at 8,500 x g, with the remaining hexane and fat layer removed using a glass mini pipette. An aliquote (500 μL) of this sample was then added to 1.5 mL of acetonitrile containing 50 μL of formic acid and shaken for 1 h. The samples were then centrifuged for 10 min at 8,500 x g, filtered using a 1.4 μm filter and analysed by LC-MS/MS as described below. Each sample was analysed in triplicate.

2.3.2. LC-MS/MS analysis

Aliquots (1 μL) of the prepared samples were injected into a UPLC - triple quadrupole (QQQ) mass spectrometer (MS/MS) (LCMS 8050, Shimadzu UK, Milton Keynes), fitted with a Luna Phenyl-Hexyl column (150 × 2.0 mm inner diameter, 5 μm, Phenomenex, Aschaffenburg, Germany) equipped with a guard column of the same type. Eluent A was composed of 0.1 % formic acid in water, and Eluent B was composed of 0.1 % formic acid in acetonitrile. Using a flow rate of 300 μL/min, the system was operated at 25 °C, starting with 32 % B under isocratic conditions for 1 min, then increasing the content of B to 70 % over 3 min, followed by an increase to 100 % B over 2 min, and keeping isocratic conditions for 3 min. Eluent was pumped down again to 32 % B over 2 min and held isocratically for a further 3 min. Analysis was performed in ESI mode using the following multiple reaction monitoring (MRM) transition ions: avenanthramide A: 298 > 254.15, 298 > 133.9, 298 > 159.85; avenanthramide B: 327.8 > 284.25, 327.8 > 268.1, 327.8 > 160.85; avenanthramide C: 314 > 178.2, 314 > 134.85, 314 > 134.2; avenanthramide D: 282 > 238.2, 282 > 118.95, 282 > 144.85; avenacoside A: 1061.7 > 899.3, 1061.7 > 163. Dwell time was 10 ms for each transition and Q1, collision energy and Q3 voltages were optimised using standards of each compound.

The calibration curves were run with a linear curve fit, a weighting of 1/Cˆ2, and were not forced through the origin. A quantitative method with external standards was used; avenanthramide A, B, C, as well as avenacoside A. Avenanthramides A, B and C have been shown in literature to be the three major forms in oats (Meydani, 2009), with avenacoside A as another primary component in oats (Wang et al., 2017). Therefore, a targeted approach searched for these four compounds. Each standard was diluted with 75 % acetonitrile / 25 % water, in order to match the sample conditions for solvent composition. Quantifier ions used for identification were; avenanthramide A 298 > 254, avenanthramide B 328 > 284, avenanthramide C 314 > 178, avenanthramide D 282 > 238, and avenacoside A 1107.05 > 1061.45 respectively. Data acquisition and quantification was performed in Labsolutions Insight software (Shimadzu).

2.4. OMA production

OMA samples were produced using steps closely resembling those used in commercial manufacturing processes (Zhang et al., 2007; Yu et al., 2023). Fig. 1 demonstrates the basic steps used within the process. A ratio of 13.5/86.5 oat flour to water was combined, and mixed at room temperature at speed 2.5 in a Thermomix® (Vorwerk, Wuppertal, Germany) before being heated to 60 °C, with continuous stirring for five minutes, or until a visual change in consistency due to starch gelatinisation was observed. Enzymatic treatment was used to breakdown the starch within the oats during this gelatinisation stage, to ensure the starch was more easily digestible by the amylases and to prevent issues such as an increase in viscosity during the heat treatment stages of OMA production (Deswal et al., 2014). Enzymes included α-amylase (Thermomix® temperature lowered to 55 °C and maintained for 15 min) to reduce viscosity in starch solutions, followed by glucoamylase (55 °C for 10 min) to hydrolyse 1,4-α-glucosidic linkages in the starch and produce glucose. The activity levels of the two enzymes were 30k (activity minimum 30,000 μ/mL where 1 μ/mL is defined as the amount of enzyme in 1 mL enzyme solution to liquefy 1 mg of soluble starch / min), and 300k (activity minimum 300,000 μ/mL), respectively. After enzymatic treatment the product was heated to 95 °C (5 min) to inactivate the enzymes. Samples were high shear mixed at 8000 rpm for 5 min to reduce particle size, and decanted on a Westfalia separator decanter, type FRB 468,518 (Oelde, Germany), at speed setting C to remove the larger particles.

Samples were diluted at this stage to replace potential moisture lost as a result of evaporation (due to transferal between previous steps carried out not within a closed system) and from removed solids. The solid content of samples at each stage was calculated by measured weight before and after heating to dryness (50 °C overnight) and used to determine calculations for dilution. This ensured the total solid content was maintained at approximately 10 % (w/w) throughout.

Ingredient addition included rapeseed oil (1.5 % w/w) – as oils are required in order to create an emulsion, to improve stability of the product (McClements, 2020), salt (0.1 % w/w), and calcium carbonate (0.1 % w/w). Calcium carbonate was selected due it's insolubility, meaning it does not promote aggregation of anionic proteins due to ion binding and electrostatic screening effects, meaning it can be dispersed throughout the product (McClements, 2020). All additional ingredients were added to samples within the Thermomix, at a speed of 2.5 to combine thoroughly, after which products were heated to 70 °C to ensure an appropriate temperature for homogenisation. Products were homogenised using High pressure homogeniser (Niro Soavi, Panda, GEA Group, Germany), to micronize particles, emulsify oil, and produce

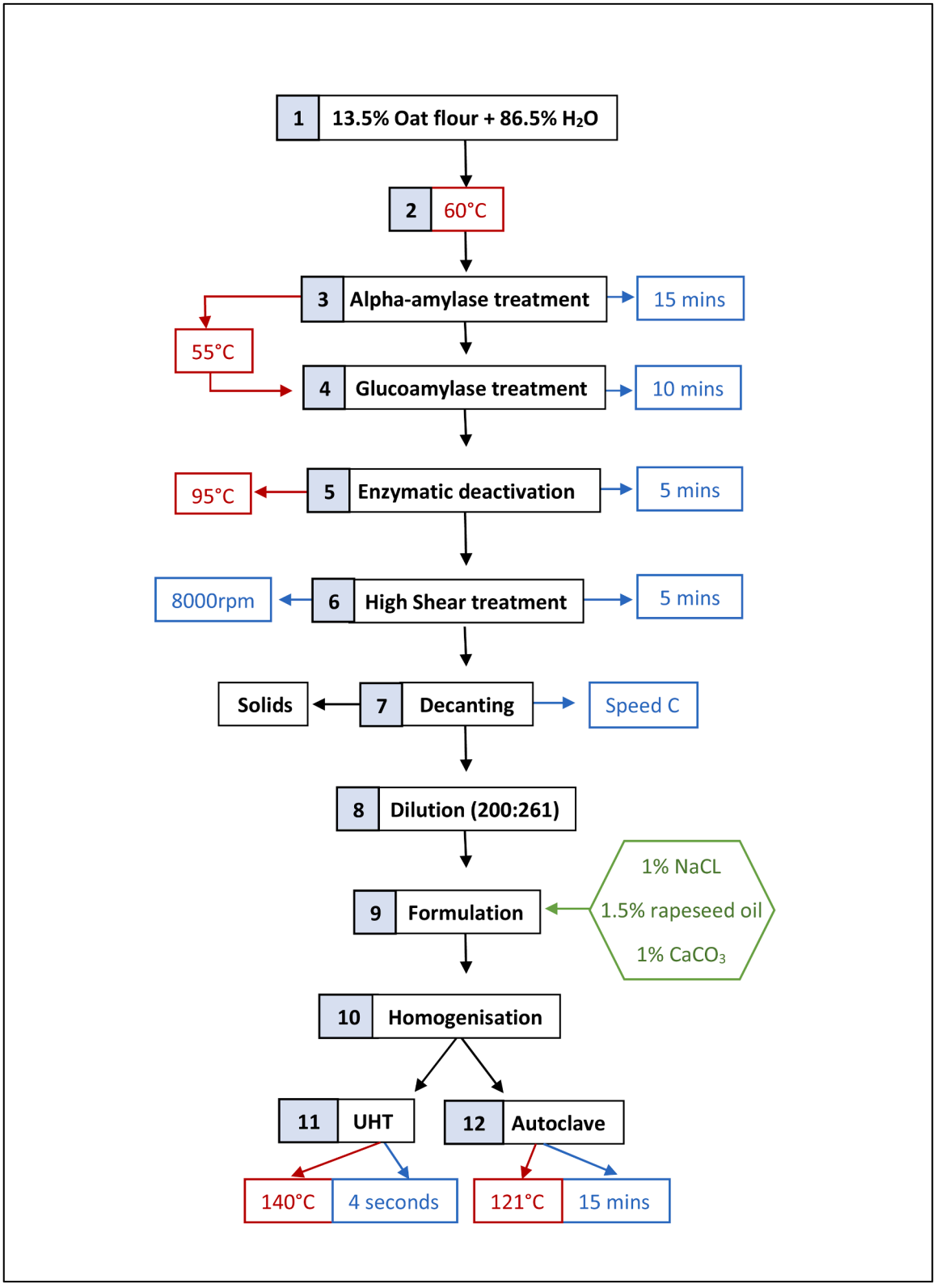

Fig. 1. Basic processing steps used to create OMA products. Numbers represent stages at which samples were taken for analyses. Process carried out a total of 3 times (one for each complete rep).

more uniform and stable emulsion (Amador-Espejo et al., 2014; Yu et al., 2023).

Finally, UHT at 140 °C was applied, for a total of 4 s using an Armfield HTST/UHT system, type FT74XTS (Armfield ltd, Hampshire, England) to ensure extend shelf life. In order to assess the potential effects of a prolonged heat treatment, in comparison to a short ultra-heat treatment, post-homogenated samples were also autoclaved in place of UHT. This was carried out in glass Duran bottles, at 121 °C for 15 min ($F_0$15).

The larger particles removed during the decanting stage were

collected within the machinery. These were then weighed and used for subsequent β-glucan analyses. This process was repeated in entirety 3 times for each complete rep, with aliquots removed for analyses at each stage of production.

Good manufacturing practice was ensured, with the same standard operating procedure strictly followed for each replicate, identical raw materials from the same source from trusted suppliers, equipment thoroughly cleaned before and between replicates, and ingredients stored in sanitary and sealed conditions (enzymes stored <5 °C).

2.5. Statistical analyses

Quantitative differences in concentration of avenanthramides, avenacosides and β-glucan were analysed by one-way analysis of variance (ANOVA) using XLSTAT (Version 2022.5.1.1388. Paris, France). For compounds exhibiting significant difference in the one-way ANOVA, Tukey's honest significant difference (HSD) test was applied for multiple pairwise comparisons. In multiple pairwise comparisons, significance was assumed at $p \leq 0.05$.

3. Results

3.1. Avenanthramides and avenacoside A

Avenanthramide b was detected in substantially higher concentrations in all samples in comparison to avenanthramide a and c (Fig. 2) as previously reported (McCarron et al., 2024). All avenanthramides were found to significantly increase post enzymatic treatment with α-amylase in comparison to the initial oat flour/water mixture. Similarly, a significant increase of avenanthramides a, b and c was seen after the product was heated to 90 °C for enzymatic inactivation. It is also clear from Fig. 2 that concentration of avenanthramides significantly decreased during the dilution stage, however, this is obviously due to the addition of water to the product during this stage of processing. A decrease in avenanthramides was seen after UHT treatment, with a significant decrease after autoclaving, suggesting these compounds are susceptible to higher degradation at temperatures above 120 °C, significantly so with prolong heat treatments.

Avenacoside A was measured at higher levels than avenanthramides a, b and c at all sampling points, with the pattern of change over processing somewhat different to the avenanthramide measurements (Fig. 3). Avenacoside A was observed to decrease significantly after the initial step of heating the oat/water mixture to 60 °C, whereby gelatinisation would have occurred. Despite a significant increase after enzymatic treatment with α-amylase, a reduction in avenacoside A was observed after treatment with glucoamylase. No reduction was observed as a result of UHT or autoclaving, suggesting avenacoside A is not susceptible to heat degradation at the temperatures of 140 °C for 4 s, or at 121 °C for 15 min.

3.2. β-Glucan analyses

Similarly to the pattern observed in avenacoside A, β-glucan was found to significantly decrease after heating to 60 °C, with an increase after treatment with α-amylase, with a reduction of β-glucan seen post glucoamylase treatment (Fig. 4). β-Glucan increased after samples were heated to 90 °C, and again after high sheer mixing.

β-Glucan significantly reduced however, between pre- and post-decanting, suggesting it was partially lost within the decanted solids. To determine this, β-glucan was measured within the collected solids for comparison. As the moisture content was substantially lower in this sample, potentially skewing the results when measured in g/100 mL, moisture analyses were carried out. Weights of samples were measured pre-and post-drying at 50 °C for 48 h, to determine moisture content (Table 1).

The solid content of the collected slurry was measured to be 21.8 % (w/w), in comparison to the pre-decanted sample at 11.1 % (w/w), and decanted sample at 10.4 % (w/w). This was then factored in to determine β-glucan per 100 g of solids within the samples. The β-glucan concentration was still found to significantly reduce in the sample post decanting; reducing from 4.95 to 3.48 g/100 g, whilst 2.12 g/100 g of β-glucan was measured within the decanted slurry samples. The weight of the total removed slurry, combined with solid content and β-glucan analyses, led to a calculation of 2 g per 27.2 g β-glucan being removed from the samples, a total of 7.4 %. No reduction was observed in β-glucan measurements after UHT or autoclaving, suggesting it is not susceptible to heat degradation at these timepoints or temperatures.

4. Discussion

The significant increase in avenanthramides and avenacoside A after α-amylase treatment suggests that these compounds may be released

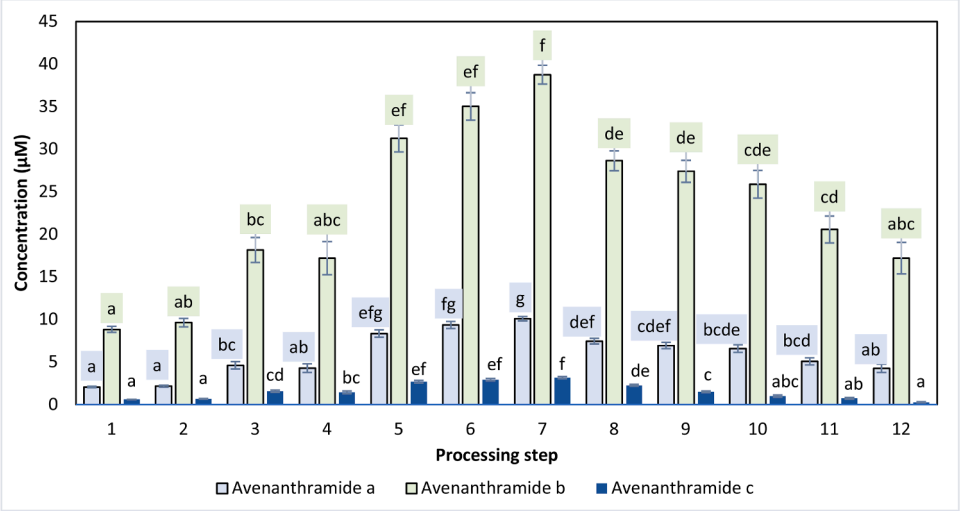

Fig. 2. Concentration of avenanthramides (μM in oat milk alternative, OMA, sample) at each stage of processing (1: oat flour in water, 2: 60 °C heated, 3: α-amylase enzymatic treated, 4: glucoamylase enzymatic treated, 5: 90 °C heated, 6: high sheer treated, 7: decanted, 8: diluted, 9: formulated, 10: homogenised, 11: UHT, 12: Autoclaved). *Data represents means of three technical replicates of three production sample replicates ± standard deviation. Sample not sharing a common small letter significantly different (Tukey honestly significant difference at $p \leq 0.05$).*

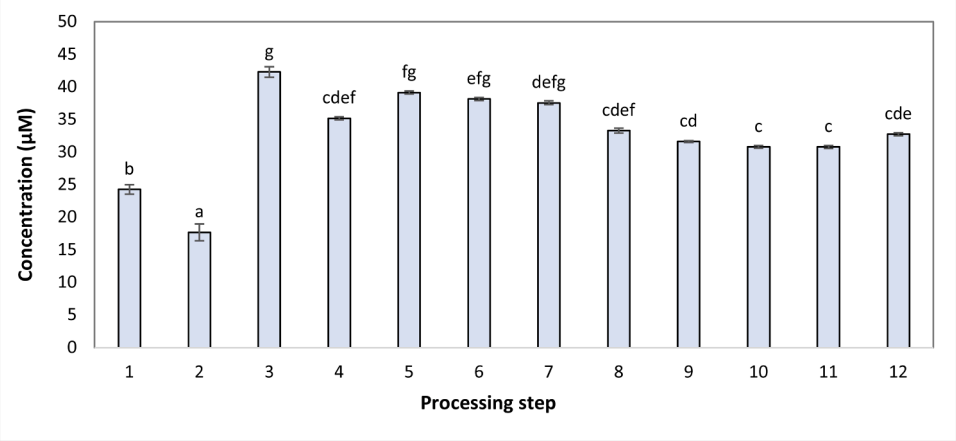

Fig. 3. Concentration of Avenacoside A at each stage of processing (1: oat flour in water, 2: 60 °C heated, 3: α-amylase enzymatic treated, 4: glucoamylase enzymatic treated, 5: 90 °C heated, 6: high sheer treated, 7: decanted, 8: diluted, 9: formulated, 10: homogenised, 11: UHT, 12: Autoclaved). *Data represents means of three instrumental replicates of three sample replicates ± standard deviation. Sample not sharing a common small letter were significantly different (Tukey honestly significant difference at $p \leq 0.05$).*

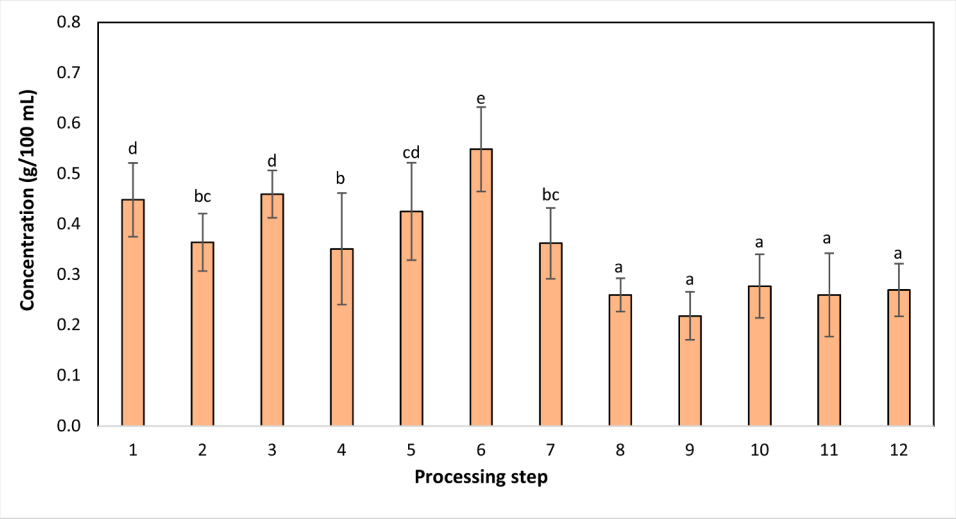

Fig. 4. Concentration of β-glucan at each stage of processing (g of β-glucan per 100 mL of OMA sample); (1: oat flour in water, 2: 60 °C heated, 3: α-amylase enzymatic treated, 4: glucoamylase enzymatic treated, 5: 90 °C heated, 6: high sheer treated, 7: decanted, 8: diluted, 9: formulated, 10: homogenised, 11: UHT, 12: Autoclaved). *Data represents means of three instrumental replicates of three sample replicates ± standard deviation. Sample not sharing a common small letter were significantly different (Tukey honestly significant difference at $p \leq 0.05$).*

Table 1
Concentration of β-glucan in decanted slurry, and samples pre and post decanting.

		β-Glucan concentration	
	Solid content (%)	g/100 mL	g/100 g
Pre-decanting	0.11	0.55 ^{c}	4.95 ^{c}
Post-decanting	0.10	0.33 ^{a}	3.48 ^{b}
Decanted Slurry	0.22	0.47 ^{b}	2.11 ^{a}

Data represents means of three instrumental replicates of three sample replicates ± standard deviations (p-value < 0.0001). Differing small letter represent sample significance from multiple comparisons as determined by Tukey honestly significant difference (at p = 0.05).

during enzymatic hydrolyses. Treatment with α-amylase has previously been demonstrated to increase the yield of antioxidant properties and phenolic content in OMAs (Babolanimogadam et al., 2023). As the majority of phenolic acids in oats have been identified to be in bound form (Multari et al., 2018) being linked to cellulose and hemicelluloses through ester linkages in the cell wall (Chen et al., 2015), it is possible that liberation of these compounds may result from the ability of α-amylase to hydrolyse the ester linkage between phenolic acids and starch, as well as the polysaccharides in cell walls where phenolic compounds are concentrated (Chen et al., 2015).

Again, a significant increase of avenanthramides a, b and c was seen after the product was heated to 90 °C for enzymatic inactivation. Heat treatments can lead to an increase in yield due to cell wall disruption (Babolanimogadam et al., 2023), potentially from a similar mechanism of releasing bound compounds. High temperatures are shown to be efficient in polyphenol extraction, achieving extremely high yields perhaps due to the breaking of lignin bonds, which are degraded at high temperatures (Antony & Farid, 2022).

The decrease in avenanthramides seen after high heat treatments may be a result of oxidation of the polyphenols (Larrauri et al., 1998). Studies have shown that despite an increase of phenolic compound extraction with high temperatures, a significant drop of phenolic content

is generally seen after exposure to temperatures above 100 °C due to thermal degradation (Larrauri et al., 1997). This may explain why concentrations dropped after exposure to 140 °C and 121 °C, after UHT and autoclaving respectively. Alternative methods of sterilisation may provide a potential solution to this reduction, for example high pressure homogenisation, which may preserve nutritional compounds whilst maintaining the safety with prevention of microbes in the product (Xia et al., 2019). However, if negative effects of these compounds, such as contributing bitterness and astringency on the sensory profile (Osakabe et al., 2024), or reduced calcium bioavailability due to the potential for polyphenols to bind to minerals and reduce absorption (Powell et al., 1994), are concluded, then research may focus on methods of removal of these compounds, if required.

The decrease in avenacoside A after the initial heat treatment of 60 °C may result from the immersion of oat flour in water during the heating stage as opposed to the heat itself; previous studies have shown that incubation of oats in water for 5 min decreased the saponins in oatmeal, potentially as a result of naturally occurring enzymes in the oats which may transform the avenacosides to 26-desglucoavenacosides (Önning & Asp, 1993). The same study showed that oat saponins seemed to survive a long heat treatment with methanol without degradation. This may explain the reduction in the initial heating and soaking stage and is supported by the fact that there was no significant decrease after UHT or autoclaving at later stages. Desglucoavenacosides are monodesmosidic with less bitterness, and thereby may have increased antibiotic and haemolytic activities (Önning & Asp, 1993), which may contribute a beneficial effect on the product. Avenacosides degradation during high heat treatment has been shown in literature to be affected by pH, with a pH of 4 leading to a 50 % reduction (Sang & Chu, 2017). The products in this study were maintained at approximately pH 7, and any effect of pH was not investigated. The use of iron and stainless-steel during processing can also dramatically increase the rate of degradation at pH 4–6 (Sang & Chu, 2017), but again was not investigated within the current study. Indeed, all equipment remained the same for each sample, aside from the final stage - whereby autoclaved samples were not exposed to the stainless-steel equipment used in the UHT treated samples. Whether this may have influenced the measurements is unclear, and further investigation would be required.

The significant decrease in β-glucan after heating to 60 °C may be associated with the gelatinisation of starch within the oats (which occurs at temperatures of 44.7–73.7 °C) (Deswal et al., 2014), as this would compete for available water, preventing the release and solubilisation of the β-glucan (Grundy et al., 2017). This was counteracted by starch hydrolyses by α-amylase, whereby the β-glucan significantly increased. This increase may be due to a reduction in the gelatinisation of the starch, enabling the release of β-glucan. The reduction of β-glucan seen post glucoamylase treatment may be due to degradation of β-glucan into glucose; β-glucans from oats, particular in flour form as was used in this study, can be hydrolysed to glucose by enzymes such as β-glucanase (Grundy et al., 2017), or through mixtures of enzymes such as 1, 3-β-d-glucanase and β-glucosidase (Danielson et al., 2010), so perhaps in the present study the gluco-amylase had a similar effect.

The increase in measured β-glucan after samples were heated to 90 °C and after high sheer mixing may result from increased soluble β-glucan with increased extractability, due to breakdown of cell walls, as β-glucans are held within the internal aleurone and sub aleurone cell walls of the oat (Holtekjølen et al., 2006). Milling has been previously shown to increase the release of β-glucan from oat (Ulmius et al., 2011) due to the alterations of the structure of the cell wall (Yiu, 1986). As high sheer mixers contain colloid mills as part of their mechanics (Vashisth et al., 2021), this may result in a similar effect. The lack of reduction of β-glucan after UHT or autoclaving steps, suggests it is not susceptible to heat degradation at the temperatures of 140 °C for 4 s, or at 121 °C for 15 min. This may be a promising indicator for the ability to preserve β-glucan throughout heat treatment stages of OMA production.

Results suggest a total amount 0.26 g/100 mL β-glucans remained in the product post UHT after a beginning concentration of 0.45 g/100 mL, a total reduction of 42 %. Whether this is significant enough for producers to seek alternative processing conditions, is not clear. To meet the 3 g/day intake that EFSA indicates would be necessary to meet their requirements for a health claim, this would mean an increased consumption from 5.7 to 11.54 L of OMA product. Both values are extremely high, and therefore aiming to reach this level of intake through OMA alone, may be unrealistic. Increasing the β-glucan content to meet these recommendations more easily may negatively alter the sensory profile. However, further investigation may be required on this matter.

5. Conclusion

The results suggest that avenanthramides, avenacoside A and β-glucan are somewhat differently affected by certain stages in the production of an oat milk alternative product, due to their differences in structure and composition.

Measured β-glucan reduced upon initial heat treatment at 60 °C as a result of starch gelatinisation, and after enzymatic treatment with glucoamylase. Yet a significant increase was observed upon treatment with α-amylase resulting from starch hydrolyses, and after high-shear treatment and 90 °C enzymatic inactivation stages - demonstrating these stages to be efficient tools in its release. Despite a reduction of 42 % in measured β-glucans from beginning to end of processing, a large proportion of this was collected within the decanted solids, leading to the possibility of re-introduction to the product. However, the effect of this on the sensory profile is yet to be concluded and may not be desirable.

Avenanthramide concentrations were negatively impacted by both short ultra-heat treatment and prolonged heat treatments suggesting a susceptibility to thermal degradation at higher temperatures. These compounds significantly increased in concentration post α-amylase treatment, 90 °C heating, Avenacoside A concentration significantly decreased post 60 °C heating, and upon enzymatic treatment with glucoamylase, yet increased post α-amylase.

The significant increase in all measured components upon treatment with α-amylase, despite a decrease in β-glucan and avenacoside A upon glucoamylase treatment, demonstrates the importance of enzymatic hydrolyses to release compounds, and suggests that selection of specific enzymes may be crucial. The susceptibility to thermal degradation of avenanthramides at higher temperatures may encourage investigation into alternative methods to heat sterilisation in order to preserve these compounds. However, despite varying stages of increase and reduction throughout, the total avenanthramide and avenacoside A concentrations were not found to significantly reduce from beginning to end of production. This could suggest that the current protocol for OMA production is not necessarily detrimental to overall compound concentration, suggesting there may be no need for a focus on preservation.

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CRediT authorship contribution statement

Roisin McCarron: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Lisa Methven:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Sameer Khalil Ghawi:** Writing – review & editing, Resources, Methodology. **Stephanie Grahl:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Ruan Elliott:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Stella Lignou:** Writing – review &

editing, Validation, Supervision, Software, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests

Stephanie Grahl was employed by the company Arla Foods amba. She participated in the conceptualisation, supervision of the study and writing – reviewing & editing. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest"

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Data availability

Data will be made available on request.

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