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Biorefining of liquid insect fractions by microfiltration to increase functionality

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ABSTRACT

Insects are gaining attention as sustainable protein sources, necessitating the evaluation of gentle processing techniques for decontamination and fractionation of edible insects. Microfiltration was shown to be an effective alternative to thermal treatments for microbial decontamination of soluble insect protein. In this study, mealworm and cricket fractions extracted at pH 3 and 8, obtained through a microfiltration (0.2 μm polyethersulphone membrane) process, were assessed. This microfiltration process yielded a cream layer, a retentate containing protein aggregates, and a decontaminated permeate containing smaller-sized proteins (mostly <75 kDa). Microfiltration improved the permeates' foamability to values higher than whey protein isolate (215–317% vs. 163%), while the retentates retained the gelling properties of up to ≈5000 Pa. Additionally, stable lipid droplets resembling plant-based oleosomes were recovered from the cream layer. This study shows that microfiltration is promising for simultaneous decontamination and fractionation, providing distinct fractions for diverse food applications.

1. Introduction

To answer the growing demand for protein, novel sources like insects can contribute to this need (Henchion et al., 2017). Insects provide excellent proteins and are considered more sustainable compared to conventional meat sources (Smetana et al., 2016; van Huis and Oonincx, 2017). The current industrial processing of insects often uses blanching to decontaminate the insects microbiologically and inactivate the enzymes that are responsible for browning and proteolysis. However, blanching severely impairs the functional properties (e.g. solubility) of the insect proteins, limiting their use in high-quality food products (Lee et al., 2019). Alternative processing methods to blanching are required to better retain the physicochemical and functional properties of insects fractions, while still achieving microbiological and enzymatic inactivation. Microfiltration is a milder decontamination method compared to blanching and delivers different fractions at the same time.

Membrane separations have already been used in the food industry for decades and may be used in insect protein isolation as well. Ultrafiltration (30 kDa) of defatted black soldier fly (*Hermetia illucens*) and yellow mealworm (*Tenebrio molitor*) protein concentrates was previously performed to fractionate the concentrates into two distinct protein fractions, but did not improve the emulsification and foaming abilities of both the permeate and the retentate (Ranasinghe et al., 2023). However, ultrafiltration retains all proteins in the retentate while the permeate will only contain lower molecular weight constituents such as salts. In contrast, microfiltration uses membranes with much larger pore sizes (0.1–10 μm) and allows the permeation of dissolved proteins, but will retain larger components like aggregates and micro-organisms. It can therefore be used to physically separate microorganisms from soluble proteins, without affecting the protein structure. Microfiltration is already commonly used in the dairy industry (Fernández García et al., 2012), but is not yet common in the nascent insect industry. Our recent study showed that microfiltration with a pore size of 0.2 μm could indeed achieve microbiological stability in soluble fractions of lesser mealworms (*Alphitobius diaperinus*) and house crickets (*Acheta domesticus*) (Sweers et al., 2023). However, the techno-functional properties of soluble insect proteins obtained in this way are still unknown.

Since microfiltration does not involve heating or other forms of denaturation, it does not affect the protein structure, so endogenous enzymes such as polyphenoloxidases (PPOs) and proteases are unaffected. Enzymatic browning and protein hydrolysis can therefore still take place, but could be controlled by using different pH values such as a

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low pH value (<4) to prevent enzymatic browning (Janssen et al., 2017a; Verruck et al., 2019). In previous research, we found that fractions with a higher degree of enzymatic hydrolysis by endogenous proteases also had a higher protein recovery in the permeate. The addition of the exogenous protease Alcalase increased the protein recovery in the permeate even more, showing that protein hydrolysis is a critical factor for the protein recovery during microfiltration (Sweers et al., 2023). It is also expected that protein hydrolysis causes altered functional properties (Gravel and Doyen, 2019).

Previous research further showed that, next to the permeate, several fractions containing valuable nutrients were obtained from the microfiltration process of insects. The retentates contain a significant amount of aggregated protein (Sweers et al., 2023). Furthermore, in the preparation of the feed for microfiltration, a cream layer is separated, containing both lipids and proteins. Given their different composition and structure, we expect that each of the obtained fractions (i.e. sterile permeate, microbiologically decontaminated retentate and cream layer) have different properties and may be suitable for different types of food applications based on their composition and functionality.

This paper aimed to evaluate the technical functionalities of the distinct fractions derived from a microfiltration process and thereby finding potential (food) applications for these fractions. The microfiltration process was performed on larvae of the lesser mealworm and adult house crickets at pH values of 3 and 8: enzymatic reactions such as enzymatic browning are strongly influenced by the pH during microfiltration and can be suppressed at lower pH (Yi et al., 2017). Finally, the microfiltration process was also performed on larvae of the lesser mealworm at a pH value of 8 with the addition of the exogenous protease Alcalase, to study the effects of strong protein hydrolysis on the protein properties.

2. Materials and methods

2.1. Insects

Larvae from the lesser mealworm (*Alphitobius diaperinus*) and adult house crickets (*Acheta domesticus*) were purchased from the commercial supplier Kreca Ento-Feed BV (The Netherlands). Before processing, all insects were starved at room temperature for about one day to remove the leftover feed and partly empty their gastrointestinal tract. The insects were frozen with liquid nitrogen and stored at −80 °C.

2.2. Preparation of the pre-filtrate

Whole insects were mixed in a 1:5 ratio with Milli-Q water (Merck Millipore, Germany), whereafter the mixture was ground using a Thermomix blender (Vorwerk & Co. KG, Germany). The pH of the obtained slurry was set to either 3 or 8 by using respectively 5M HCl (Actu-All Chemicals, the Netherlands) or 5M NaOH (Sigma-Aldrich, U.S.). The slurries were centrifuged (ThermoFisher Scientific, U.S.) at 10,000 *g* for 20 min at 4 °C in 1 L centrifuge bottles (Nalgene, U.S.). The supernatant was separated from the pellet and the cream/top layer and used directly as pre-filtrate for microfiltration or freeze-dried (Christ Epsilon 2-10D LSCplus, Germany) and subsequently stored for further analysis. To also investigate the effect of proteases on protein properties, Alcalase 2.4L (Novozymes, Denmark) was added to the pre-filtrate of lesser mealworm pH 8.

2.3. Cream washing

After separating the cream/top layer, this layer needed to be washed to remove impurities such as trace amounts of chitin. Therefore, the cream/top layer was washed by adding Milli-Q water. Subsequently, it was adjusted to pH values of 3 or 8 using either 5M HCl or 5M NaOH, respectively, followed by the same centrifugation step as described in section 2.2. After centrifuging, the cream/top layer was separated again, and considered as washed cream. The washed cream was analysed directly.

2.4. One-step microfiltration

Microfiltration was performed using a lab-scale set-up according to Sweers et al. (2023). In short, a circular, flat-sheet polyethersulphone (PES) membrane with a nominal pore size of 0.2 μm and a diameter of 90 mm was put in a stirred cell (UHP-90K, Advantec, U.S.). PES was amongst others chosen because of its usability at both pH 3 and 8, its low protein binding nature, and because it is a very common membrane material (Kumar et al., 2015). The pore size is commonly used for retaining microorganisms as microorganisms are generally larger than 0.2 μm (Fernández García et al., 2012), and this pore size was also by us found to be successful in microbiological retention (Sweers et al., 2023). The cell was filled with 350 mL of pre-filtrate. Microfiltration took place for 20 h in an incubator (Sanyo Electric, Japan) under cooling conditions (0.5–4 °C) to limit enzymatic and microbiological activity. A constant pressure of 0.3 bar and a constant stirring rate of 430 rpm were used. After the 20 h, the permeate and retentate were collected, freeze-dried, and stored until further analysis. Microfiltration was performed in triplicate.

2.5. Dry matter, protein, and lipid content

The dry matter content was estimated by weighing before and after freeze-drying. Dumas (Elementar Rapid N exceed, Germany) was used to determine the protein content. As a reference for Dumas, L-aspartic acid (Sigma-Aldrich, U.S.) was used. Following Janssen et al. (2017b), the nitrogen-to-protein conversion factors of 4.76 for whole insects and 5.60 for soluble protein fractions were used. A Soxhlet extraction system (Gerhardt Soxtherm, Germany) with hexane (Actu-All Chemicals, The Netherlands) was used to determine the lipid content. The extraction took 4 h. Sankey diagrams of the protein and lipid streams in the microfiltration process were made using e!Sankey 5 (iPoint-systems, Germany).

2.6. Molecular weight distribution

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to assess the protein molecular weight distribution of freeze-dried whole insects and freeze-dried insect fractions. Freeze-dried material was dissolved in sample buffer (Bio-Rad Laboratories Inc., U.S.) to obtain a 2 mg/mL protein solution (8 mg/mL for the permeates). The solution was mixed with Milli-Q water in a ratio of 1:1 for SDS-PAGE under non-reducing conditions, or with Milli-Q water and β-mercaptoethanol (Sigma-Aldrich, U.S.) in a ratio of 19:20:1 for SDS-PAGE under reducing conditions. Solutions were heated at 95 °C for 10 min in a Thermomixer (Eppendorf, Germany), and centrifuged at 14,600 *g* for 10 min (Eppendorf, Germany). The solutions (20 μL) and Precision Plus Protein Dual Xtra Standards (Bio-Rad Laboratories Inc., U.S.) were loaded in the wells of 4–20 % Mini-Protean TGX gels (Bio-Rad Laboratories Inc., U.S.) and ran at 180 V (Bio-Rad Laboratories Inc., U.S.) until the bottom of the gel was reached. Gels were washed with Milli-Q water, stained for 1 h with Bio-Safe Coomassie (Bio-Rad Laboratories Inc., U.S.), and then destained overnight with Milli-Q water. The gels were scanned with the Bio-Rad GS-900 Calibrated Densitometer and analysed with Bio-Rad Image Lab 6.0.1.

2.7. Browning index

Colour measurements of the liquid permeates obtained after microfiltration were performed in a Hach DR6000 UV/VIS spectrophotometer (Germany). The obtained L*, a*, and b* values were converted to the browning index (BI) using equation (1) (Maskan, 2001).

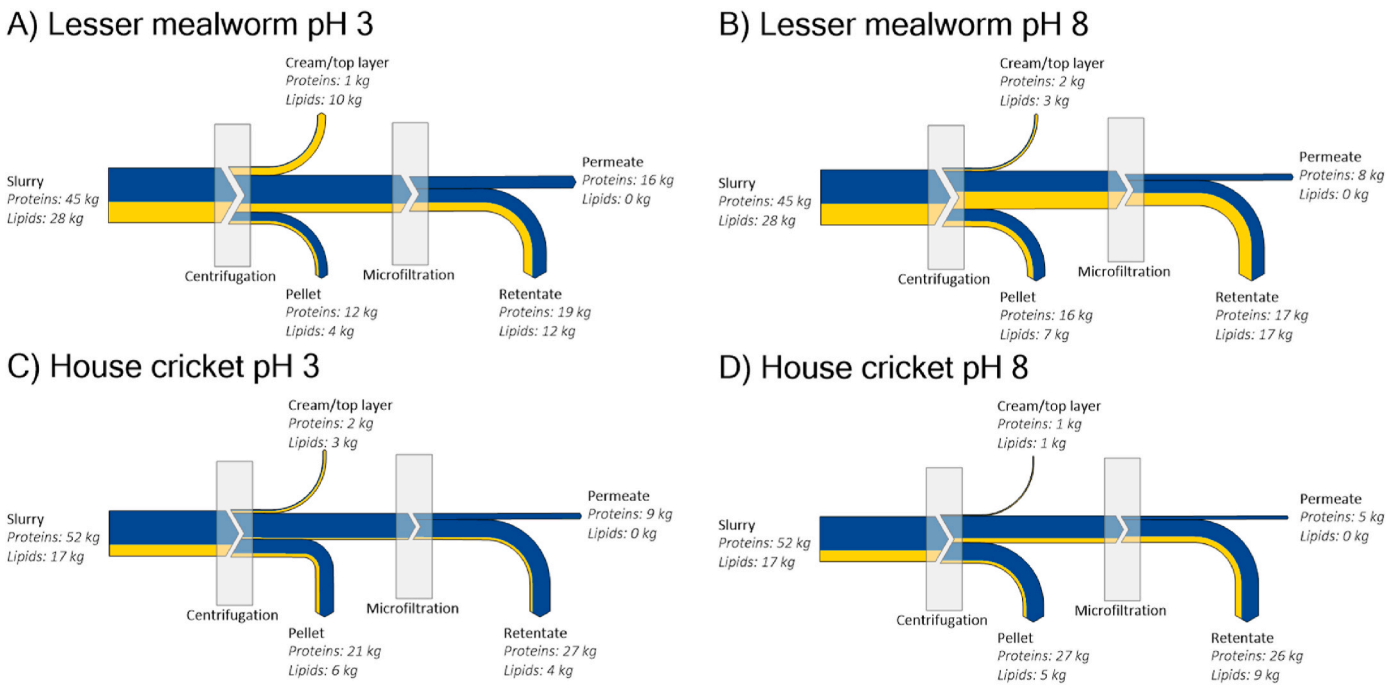

Fig. 1. Protein (blue) and lipid (yellow) streams for the microfiltration processes of lesser mealworms at a pH of 3 (A) and a pH of 8 (B), and of house crickets at a pH of 3 (C) and a pH of 8 (D). All streams start with the slurry containing an arbitrary amount of 100 kg dry matter.

$$BI = 100 * \frac{\frac{a+1.75L*}{5.645L*+a*-3.012b*} - 0.31}{0.17} \quad \text{(Eq. 1)}$$

2.8. Zeta potential

The zeta potential (mV) was measured by using the Malvern Instruments Zetasizer Ultra (Malvern Panalytical, U.K.). A folded capillary cell (DTS1080) was rinsed with ethanol and Milli-Q water, and dried with pressurised air. Fresh permeates were diluted with Milli-Q water until their protein content was 0.1% by wet weight. All permeates were measured in triplicate at 25 °C. The refractive index used was 1.45.

2.9. Protein surface hydrophobicity

The protein surface hydrophobicity was measured using a PRODAN (2-dimethylamino-6-propionylnaphtalene) probe (Tokyo Chemical Industry, Japan). A PRODAN stock solution of $1.41*10^{-3}$ M was prepared in methanol, and stored on ice until further use. Microfiltration fractions were diluted to obtain solutions with protein concentrations of 0.01%, 0.005%, and 0.0025%. One half of the solutions (1 mL) was mixed with 2.5 μL PRODAN, while the other half was used as a blank measurement. Fluorescence intensity was measured in a black 96 well plate with a clear bottom (Greiner Bio-One, Germany) at 365 nm (excitation) and 465 nm (emission) using a multi-mode microplate reader Spectramax ID3 (Molecular Devices, U.S.). The net relative fluorescence intensity (RFI) was obtained by subtracting the RFI of the solutions without the PRODAN probe from the RFI of the same solutions containing the PRODAN probe. The net RFI was plotted against the protein concentration, and the protein hydrophobicity (S_0) was obtained by determining the initial slope of the net RFI versus the protein concentration.

2.10. Foaming behaviour

The foamability was determined based on the method from Peng et al. (2020) with slight modifications. Briefly, 10 mL of 1% w/v protein dispersion was prepared in a transparent cylinder with a diameter of 33.5 mm. Freeze-dried pre-filtrate and permeates were dissolved in 0.01 M phosphate buffer (pH 7) and further adjusted to pH 7 if necessary. Whey protein isolate (BiPro, U.S.) was used as a benchmark and was prepared in the same way. Foams were formed at room temperature by whisking the solution for 2 min at 2000 rpm using an aerolatte head connected to an Ultra Turrax (IKA®, Germany). The foamability was calculated with equations (2) and (3), in which h is the height, r is the radius of the cylinder (1.7 cm), V0 is the volume of the starting solution, and Vi is the volume of the obtained foam:

$$V = \pi r^2 h \quad \text{(Eq. 2)}$$

$$FA(\%) = \frac{Vi}{V0} \bullet 100\% \quad \text{(Eq. 3)}$$

To characterise the foam stability and the average bubble size, the Foamscan (Teclis IT-Concept, France) was used. Solutions were prepared in the same way as for the foamability experiments. A foam was produced by sparging nitrogen gas through a metal frit (27 μm pore size, 100 μm distance between centres of pores, square lattice). A total volume of 60 mL was sparged in a cylinder with a diameter of 60 mm at a gas flow rate of 400 mL/min to a maximum foam volume of 400 cm^3. The foam decay and the bubble size were monitored by cameras and the Foamscan software. Foam stability was defined as the time it took for the foam to reach half of the initial foam volume. The average bubble size was obtained by using a custom MATLAB script that ran the DIPlip and DIPimage image analysis software (TU Delft, The Netherlands).

2.11. Gelation properties

Freeze-dried pre-filtrate and retentate fractions were directly dissolved in Milli-Q to contain 5.0% (w/v) protein, and tested at the same pH value (3 or 8) as the fractions were obtained. Additionally, pH 3 fractions were also tested at a neutral pH of 7. To determine the rheological properties, oscillatory strain tests were performed on a stress-controlled rheometer (Anton Paar MCR301, Austria) with a CC17 concentric cylinder geometry. When the geometry was filled with the solution, the solution was covered with a thin layer of silicone oil to prevent evaporation. Solutions were heated from 20 to 90 °C (1 °C/min), held for 5 min at 90 °C, and cooled to 20 °C (3 °C/min). During the

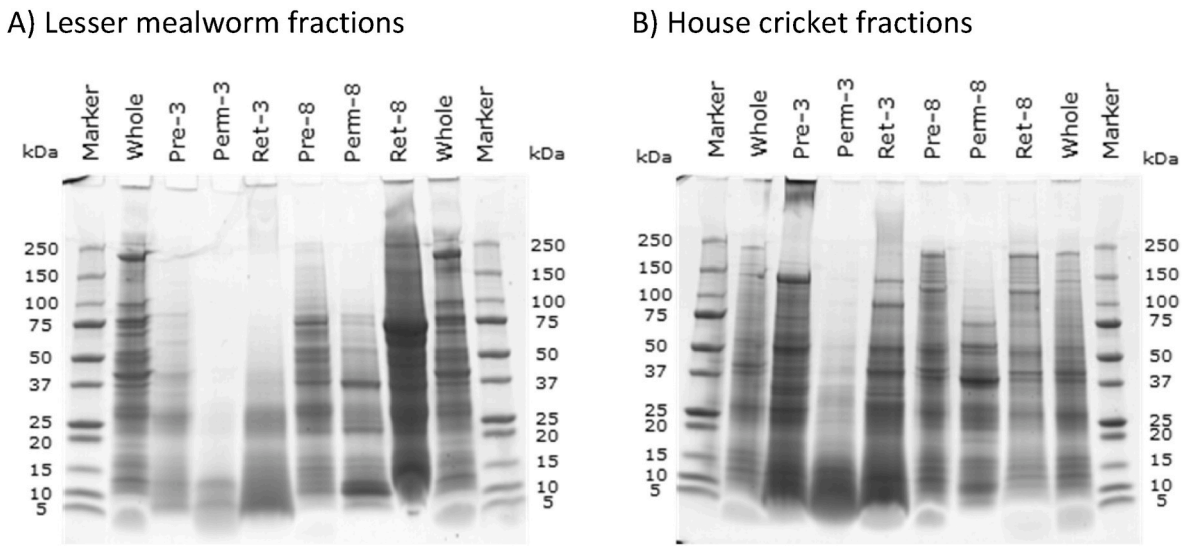

Fig. 2. Molecular weight distribution of the proteins of lesser mealworm (A) and house cricket (B) fractions measured under reducing conditions shown by SDS-PAGE profiles. The lanes are from left to right: whole, pre-filtrate pH 3 (Pre-3), permeate pH 3 (Perm-3), retentate pH 3 (Ret-3), pre-filtrate pH 8 (Pre-8), permeate pH 8 (Perm-8), retentate pH 8 (Ret-8), whole.

temperature ramp, the storage modulus (G′) and loss modulus (G″) were determined by applying oscillatory deformations with a strain amplitude of 0.5 and a frequency of 1 Hz. After the formation of the gel, an oscillatory strain sweep with strains ranging from 0.01 to 1000% and a frequency of 1 Hz was performed.

2.12. Confocal laser scanning microscopy

The distribution of protein, lipids, and chitin in the cream/top layer and the morphology of these particles were visualised using confocal laser scanning microscopy (CLSM). Images were made directly after washing the cream. The cream was diluted with demineralised water in a ratio 2:1. Rhodamine B (0.002%) was used as a stain for proteins, bodipy 505/515 (0.0005 mg/L) was used as a stain for lipids, and calcofluor white (0.01%) was used as a stain for chitin. Imaging spacers with a thickness of 0.12 mm (Grace Bio-Labs, U.S.) were used to prevent compressing of the sample on the glass slides. A Confocal Laser Scanning Microscope (Stellaris 5 Confocal LSM Leica, The Netherlands) was used with a 20× magnification. The images were analysed with the software LAS X (Leica Microsystems, The Netherlands).

2.13. Statistical analysis

Results were expressed as mean ± standard deviation (SD) and statistically evaluated through one-way analysis of variance (ANOVA) using SPSS 28.0.1.1 (IBM, U.S.). Tukey tests were used to analyse statistical differences ($p < 0.05$). All analyses were performed in triplicate.

3. Results and discussion

3.1. Overview of process and composition of the obtained fractions

Fig. 1 shows an overview of the protein and lipid quantities of the different fractions obtained during the microfiltration process. Whole insects and Milli-Q water (1:5) were blended into a slurry, for both lesser mealworms (LM) and house crickets (HC) at pH values of 3 and 8. After microfiltration, the permeate is the fraction of main interest as it was decontaminated (Sweers et al., 2023). Clear differences with respect to the permeate protein recovery can be seen between insect species (higher for LM as compared to HC) and between pH values (higher for pH 3 as compared to pH 8). Higher protein recoveries are correlated with higher endogenous protease activity, as shown by a higher degree of hydrolysis (Sweers et al., 2023). The permeate protein recovery of lesser mealworms at a pH of 3 was 35.4%, while the permeate protein recovery of house crickets at a pH of 8 was 9.4%. This means that a significant part of the protein (64.6–90.6%) ended up in the other fractions (i.e. cream/top layer, retentate, and pellet). The significant amount of proteins still present in the retentates (17.1–26.6%) are explained by membrane fouling due to protein aggregation, as discussed in Sweers et al. (2023). Next to the proteins, almost all lipids ended up in other fractions than the permeate, as the lipids were retained by the membrane. To not lose these nutrients, potential applications for other fractions than the permeate were investigated. The cream/top layer contained valuable lipids (6.1–35.6% of the total lipids) and was explored in section 3.7. The retentates still contained 37–52% of the proteins and are explored further in this article. The pellet contained insoluble proteins, and also chitin that can be extracted, but was not further investigated in this article. Chitin can be used for various applications in the food industry (e.g. thickener), but also amongst others in agriculture and the plastic industry (Rahman et al., 2023; Rehman et al., 2023).

3.2. Protein molecular weight of the fractions

The SDS-PAGE profiles under reducing conditions of the proteins of whole insects, pre-filtrates, permeates, and retentates from different treatments, are shown in Fig. 2. Protein molecular weight distribution differs between lesser mealworms and house crickets, whole lesser mealworms for example have a clear band around 75 kDa, which is less noticeable in whole house crickets. Most protein bands obtained after filtration at pH 3 have overall smaller molecular weights than the proteins obtained after filtration at pH 8. For example, the relative intensity of protein bands higher than ~40 kDa is much higher for the lesser mealworm pre-filtrate at pH 8 than at pH 3. Above 100 kDa the protein bands at pH 3 are even visually absent. Further, the permeates show protein bands with the smallest molecular weights (e.g. <15 kDa for lesser mealworm pH 3). The fractions mainly differ based on the filtration fraction (i.e. permeate vs. retentate), followed by the insect species and the pH value. This indicates that the microfiltration process fractionates the pre-filtrates in distinctively different fractions, which will also have different techno-functional properties. Similarly, the different insect species and pH values also have different protein bands, so these

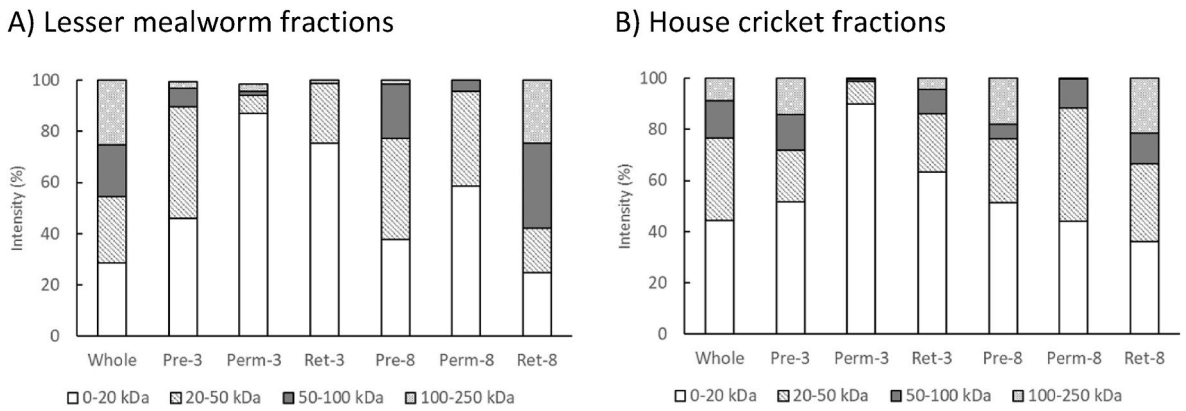

Fig. 3. Percentage of intensity of molecular weight size categories (0–20 kDa, 20–50 kDa, 50–100 kDa, 100–250 kDa) of the proteins of lesser mealworm (A) and house cricket (B) fractions, where the total intensity is 100%. The bars are from left to right: whole, pre-filtrate pH 3 (Pre-3), permeate pH 3 (Perm-3), retentate pH 3 (Ret-3), pre-filtrate pH 8 (Pre-8), permeate pH 8 (Perm-8), retentate pH 8 (Ret-8).

different fractions are expected to have different techno-functional properties.

Estimation of the Stokes radius based on the molecular weights (Erickson, 2009), indicates that even proteins of up to 250 kDa are expected to be able to cross the membrane as they are smaller than the membrane pore size (0.2 μm). Thus, proteins retained by the membrane should have higher molecular weights or are aggregated or insoluble. SDS breaks down protein aggregates that cohere through hydrophobic interactions and hydrogen bonds, so these aggregates will not appear as aggregates in the SDS-PAGE gels. The molecular weight distribution was also tested under non-reducing conditions (data not shown). The reducing and non-reducing gels were very similar, indicating that disulphide bonds play no major role in these specific proteins. Therefore, the retained proteins may be aggregated by hydrophobic interactions.

The protein bands of the retentates and pre-filtrates contain proteins that are quite similar in size, but the pre-filtrate contains also protein bands with larger molecular weights (e.g. 50–100 kDa for LM pH 3) than the retentate. This is especially the case at a pH of 3 and more for lesser mealworms than for house crickets. This indicates that proteins are broken down during the microfiltration process, which may indicate endogenous protease activity. Higher protease activity at a pH of 3 could indicate higher activity of acidic endoproteases such as aspartic proteases (Jagdale et al., 2017). This is in line with Sweers et al. (2023), in which a higher degree of hydrolysis (DH) was found for pH 3 as compared to pH 8, and a higher DH was found for lesser mealworms as compared to house crickets. Dividing the protein bands into molecular weight categories and plotting the intensities in bars (Fig. 3) illustrates more clearly that the permeates are hydrolysed into smaller molecular weight proteins. This effect is especially clear in the pH 3 fractions, where hydrolysis can also be seen in the retentates.

Enzymes, including proteases and polyphenoloxidases (PPOs) that cause browning, are expected to pass through the membrane as they are in general smaller than 100 kDa (Jagdale et al., 2017; Janssen et al., 2017a; Meriño-Cabrera and de Almeida Oliveira, 2022; Yi et al., 2013). This indicates that hydrolysis will likely happen in all fractions; in the permeate once it has passed the membrane and also in the pre-filtrate/retentate before the enzymes have permeated. However, the enzymes themselves are also slowly auto-hydrolysed (Smolin et al., 2020). For example, trypsin-like proteinases have a molecular weight of around 59 kDa (Yi et al., 2013), and this band is not visible in the lesser mealworm pH 3 permeate (Fig. 2). It should be noted that this does not automatically mean that the enzyme is not present, but the

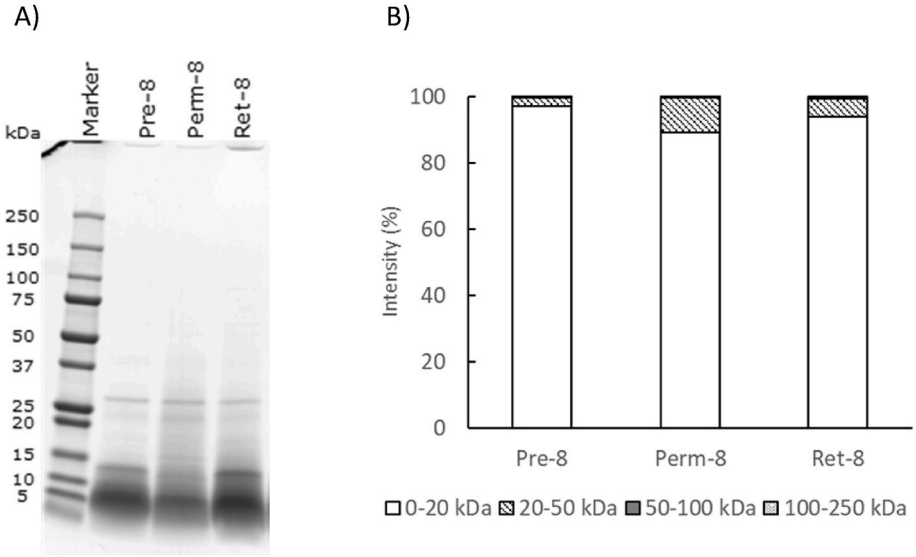

Fig. 4. Molecular weight distribution (A) of the proteins of lesser mealworm fractions hydrolysed with Alcalase measured under reducing conditions shown by SDS-PAGE profiles, and percentage of intensity (B) of molecular weight size categories (0–20 kDa, 20–50 kDa, 50–100 kDa, 100–250 kDa) of the proteins of lesser mealworm (A) and house cricket (B) fractions, where the total intensity is 100%. The lanes are from left to right: pre-filtrate pH 8 (Pre-8), permeate pH 8 (Perm-8), and retentate pH 8 (Ret-8).

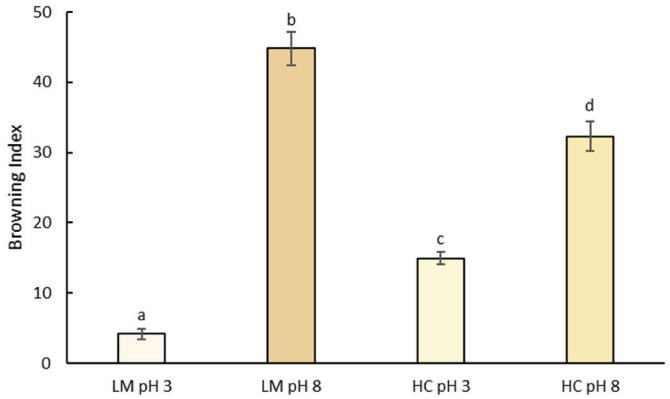

Fig. 5. *Browning index of the different permeates obtained after microfiltration (i.e. lesser mealworm [LM]* pH *3, lesser mealworm* pH *8, house cricket [HC]* pH *3, and house cricket* pH *8). The colour of the bars qualitatively indicates the colour of the permeate. Different lowercase letters indicate mean values with significant differences (*$p < 0.05$*).*

concentration is too low to be visible on the SDS-PAGE profile.

To exemplify the effect of high protease activity on the protein molecular weight distribution, the result of the addition of the exogenous protease Alcalase to lesser mealworm pH 8 pre-filtrate on the molecular weight distribution is shown in Fig. 4. Alcalase reduced the molecular weight of the protein bands for all three fractions (pre-filtrate, permeate and retentate), and no clear differences were seen between reducing and non-reducing conditions (data not shown). The molecular weight distribution of Alcalase-treated fractions is similar to the molecular weight distribution of the lesser mealworm permeate at a pH value of 3 (Fig. 2), no protein bands with high molecular weights are detected, indicating that Alcalase actively hydrolysed the proteins. All three fractions looked similar, although the permeate has a less clear band around 12 kDa. Notably, the pre-filtrate also appears to be hydrolysed, while this fraction was frozen and freeze-dried directly after the addition of Alcalase. In previous research, it was shown that protein hydrolysis still occurs at −20 °C and is already noticeable in one day (Wessels et al., 2020). It could thus be that the hydrolysis of proteins in the pre-filtrate by Alcalase progressed quickly and continued during the freezing step and the initial stages of the freeze-drying process.

3.3. Enzymatic browning of the fractions

Fig. 5 displays the browning index for the four different permeate fractions. The browning index after filtration for 20 h at pH 8 is significantly higher than after filtration at pH 3 for both insect species. This is consistent with earlier research on yellow mealworms, which showed that browning primarily occurs above a pH of 6.5 (Yi et al., 2017). The higher browning index after filtration at pH values of 8 (i.e. 44.8 ± 2.3 for lesser mealworm permeate and 32.3 ± 2.1 for house cricket permeate) is caused by the higher activity of the responsible enzymes (polyphenoloxidases) at a pH of 8 as compared to 3 (Janssen et al., 2017a; Yi et al., 2017). Other causes for browning include the possible presence of autoxidation and the formation of iron-polyphenol complexes (Janssen et al., 2019), but these are less relevant than polyphenoloxidases (PPOs) in explaining the browning differences between the different pH values.

House cricket permeate at pH 3 has a significantly higher browning index (15.0 ± 0.9) than lesser mealworm permeate at pH 3 (4.2 ± 0.7). This can be explained by more yellow components initially present in house crickets, which results in a higher b* value. Retentates followed a similar trend as permeates (results not shown). Since a lower browning index is usually desired (Janssen et al., 2017a), a pH of 3 during the entire extraction process is preferred. The microfiltration could also be performed with pressurised nitrogen instead of pressurised air to remove the oxygen exposure that is necessary for PPO to function. As PPO has a molecular weight of around 75–80 kDa (Janssen et al., 2017a), it will most likely end up in the permeate. Therefore, it should be noted that after increasing the pH of the permeate, browning can still occur.

After microfiltration of lesser mealworm pre-filtrates at a pH of 8 with the addition of Alcalase, a high browning index of 606.7 ± 26.9 is obtained in the permeate, and the permeate visually looks black. Previous research showed that endogenous serine proteases are involved in activating PPOs when this is needed for e.g. healing wounds (Lu et al., 2014; Wu et al., 2018). Furthermore, protein hydrolysis can provide substrates for active PPOs (Mesquita and Queiroz, 2013). Alcalase is a serine protease, so it could be that it is able to activate PPOs and/or provide substrate for the PPOs, so enzymatic browning can occur faster and to a greater degree before the PPOs themselves are hydrolysed and thus inactivated.

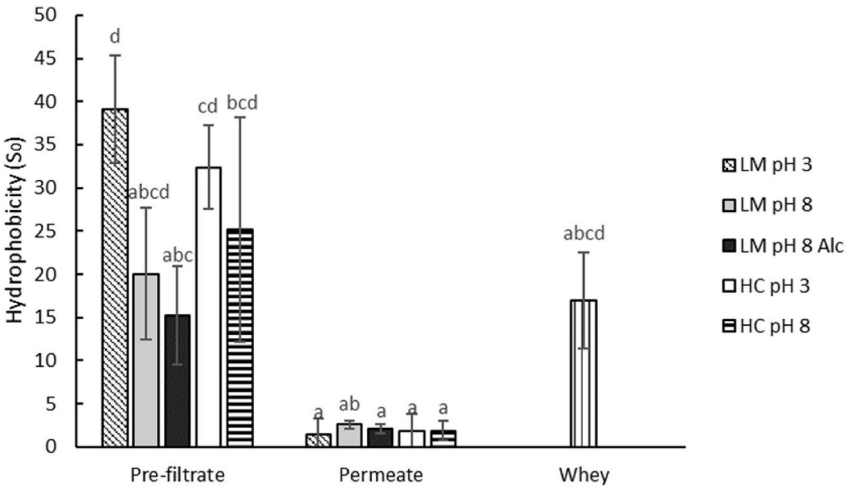

Fig. 6. *Protein surface hydrophobicity (S_0) of the pre-filtrate and permeate of the different treatments (i.e. lesser mealworm [LM]* pH *3, lesser mealworm* pH *8, lesser mealworm* pH *8 with Alcalase [Alc] addition, house cricket [HC]* pH *3, and house cricket* pH *8). Different lowercase letters indicate mean values with significant differences (*$p < 0.05$*).*

Table 1
Foamability, foam stability (expressed as foam half-life), and bubble size of foams generated from the pre-filtrate, permeate, and retentate of the different treatments. All foams were formed at pH *7, with a concentration of 1% (w/v) protein. For each parameter, different lowercase letters indicate mean values with significant differences ($p < 0.05$). N/A = not applicable.*

		Foamability (%)	Foam stability (min)	Average bubble diameter (mm)
Lesser mealworm pH 3	Pre-filtrate	0^{a}	N/A	N/A
	Permeate	215 ± 66^{bc}	11 ± 9^{a}	0.33 ± 0.11^{ab}
Lesser mealworm pH 8	Pre-filtrate	0^{a}	N/A	N/A
	Permeate	284 ± 8^{bd}	33 ± 31^{ab}	0.24 ± 0.04^{ac}
House cricket pH 3	Pre-filtrate	277 ± 6^{bd}	99 ± 14^{cde}	0.20 ± 0.01^{c}
	Permeate	269 ± 21^{bd}	149 ± 4^{c}	0.18 ± 0.01^{c}
House cricket pH 8	Pre-filtrate	89 ± 5^{e}	51 ± 19^{abd}	0.25 ± 0.01^{ac}
	Permeate	317 ± 44^{d}	79 ± 25^{bde}	0.26 ± 0.04^{ac}
Whey protein isolate		163 ± 47^{ce}	134 ± 26^{ce}	0.40 ± 0.03^{b}

3.4. Physicochemical and functional properties of the fractions

3.4.1. Zeta potential

The zeta potential was measured to gain information about the surface charge of colloidal particles, which are in this case protein aggregates. The zeta potential of all permeates was between |20| and |28| mV. The permeates at pH 3 had a positive zeta potential, while the permeates at pH 8 had a negative zeta potential. The magnitude for house cricket permeates was slightly larger than that of lesser mealworm permeates for both pH values (e.g. 27 vs. 21 mV respectively at pH 3). This is in line with previous literature and may be caused by differences in protein composition and ionic strength (Sipponen et al., 2018). An absolute zeta potential below |25| mV is considered to induce instability and can lead to protein aggregation (Berghout et al., 2015). As our values are close to that limit, the proteins in the permeate may not be completely stable and thus form aggregates. As mentioned in Sweers et al. (2023), small aggregates of up to 4 μm were indeed found in the permeate.

3.4.2. Protein surface hydrophobicity

Fig. 6 displays the protein surface hydrophobicity (S_0) of the pre-filtrates and the permeates. The surface hydrophobicity of proteins is known to affect the functional properties. For example, foam formation is enhanced with higher protein surface hydrophobicity at lower protein concentrations, though at higher protein concentrations the foaming capacities are independent of the protein surface hydrophobicity (Delahaije and Wierenga, 2022). While some fluorescent probes are affected by electrostatic interactions, PRODAN is primarily unaffected and thus allows comparison between different pH values (Alizadeh-Pasdar and Li-Chan, 2000). Pre-filtrates with a pH value of 3 showed 1.2-2.0x higher average protein surface hydrophobicity than pre-filtrates with a pH value of 8. Lower surface hydrophobicity values for higher pH values were also seen for edible honey bee brood (*Apis mellifera*), which was hypothesised to be caused by partial protein denaturation at lower pH values by protonation (Mishyna et al., 2019). Another reason is that the pre-filtrates/supernatants contain different proteins (Fig. 2) because of the extraction process. These proteins can have different surface hydrophobicity values. Interestingly, the surface hydrophobicity of the permeates is lower (factor 7–29 depending on the treatment) than the pre-filtrates and is mostly similar. The proteins that end up in the permeates are expected to be highly soluble, which could explain the low surface hydrophobicity. This supports the earlier statement that hydrophobic interactions are responsible for the aggregation in the feed.

Next to this, the lesser mealworm pH 8 fraction treated with Alcalase has a lower surface hydrophobicity than the lesser mealworm pH 8 fraction not treated with Alcalase, though this difference was not significant. Even though hydrolysis was expected to expose hydrophobic groups (Tavano, 2013), hydrolysis thus decreased the surface hydrophobicity.

3.5. Foaming of pre-filtrates and permeates

The foaming behaviour of decontaminated permeates was studied and compared to the foaming behaviour of the pre-filtrate (Table 1). Even though the pre-filtrate was not considered for actual industrial foaming applications due to its microbial load, it was used as a comparison to investigate the effect of the microfiltration process on the foaming behaviour. The retentate stream was not studied as it consisted primarily of protein aggregates and lipids (i.e. were unlikely to foam) and also contained the retained microbial contaminants. All foams were formed at pH 7, with a concentration of 1% (w/v) protein. For lesser mealworm, no foam could be formed with the pre-filtrates, probably due to the presence of lipids, while after microfiltration a foam is formed with both permeates. This inability of lesser mealworm pre-filtrate to form a foam is in line with literature, where fractions from the closely related lesser mealworm and yellow mealworm were found to be unable to form a foam (Mishyna et al., 2021; Stone et al., 2019; Yi et al., 2013). In our study, house cricket pre-filtrates (both extracted at pH 3 and 8) were able to form a foam (215 and 284% respectively). The available literature on foams made from house cricket proteins shows varying outcomes, Yi et al. (2013) found that house cricket supernatant did not foam at pH 5, 7 and 10, while a foam was formed at pH 3 with a half-time of 4 min. Freeze-dried hexane-extracted house cricket powders had a low foam capacity (<10%) and stability (<5%) (Ndiritu et al., 2019) and air-dried (60 °C) house cricket powders had a foam capacity of 26%, with a higher foam stability of 87% after 1.5 h (Udomsil et al., 2019).

The lesser mealworm permeates and the house cricket pH 8 permeate show a significantly improved foamability compared to the corresponding pre-filtrates, even better than whey protein isolate. The improvement in foaming of the permeates in comparison to the pre-filtrates could be caused by the reduction in lipids in the permeates to <1% (Fig. 1), as lipids generally are detrimental for foaming (Huppertz, 2010). Next to the low lipid contents, the improvement in foamability could also be caused by the partial hydrolysis of the proteins due to endogenous protease, as also seen in the molecular weight distribution of the permeates (Fig. 2). Partial hydrolysis was previously found to enhance the foamability of insect proteins, as smaller peptides are faster in adsorbing and stabilising the air-water interface (Leni et al., 2020; Purschke et al., 2018). However, the surface hydrophobicity (Fig. 6) of the permeates was lower than that of the pre-filtrates, which would impart a lower foamability. We conclude that the reduced lipid content is the main factor responsible for the increased foamability.

House cricket permeate separated at pH 3 had the highest foam stability (half-life of 149 min), which was similar to that of whey protein (134 min). Other fractions had a quite low foam stability (<100 min). For the house cricket permeates, the average foam stability was somewhat higher for permeates (149 and 79 min for pH 3 and 8 respectively) than for pre-filtrates (99 and 51 min for pH 3 and 8 respectively), but these differences were not statistically significant. For average bubble size, microfiltration did not appear to have a clear and consistent effect, although the bubbles of whey foam are significantly larger as compared to all insect fractions studied except for lesser mealworm pH 3. All in all, we show that microfiltration can be used to improve the foaming properties of insect proteins.

3.6. Gelation of pre-filtrates and retentates

After microfiltration, most of the protein ends up in the retentate (Fig. 1), together with the microorganisms. Therefore, it is of great

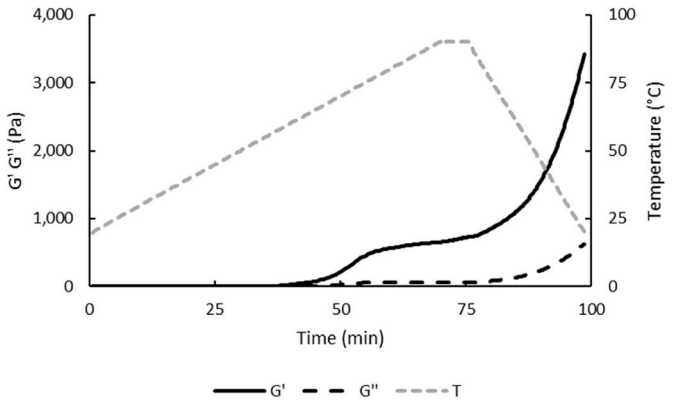

Fig. 7. Storage (G′) and loss (G″) modulus of 5% (w/v) protein suspension of lesser mealworm pH 8 retentate as a function of time during a subsequent heating, stabilisation, and cooling period. The temperature is plotted against the time as a secondary y-axis.

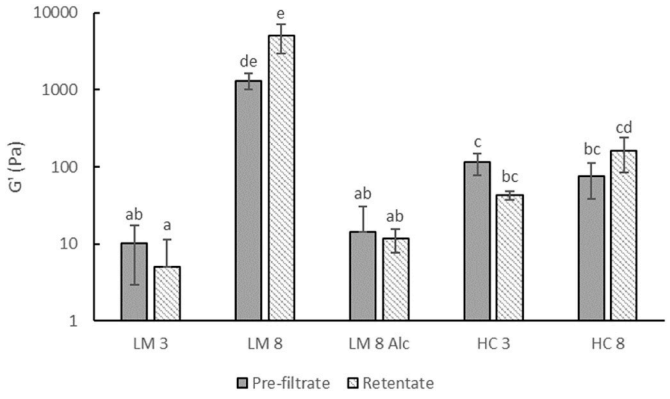

Fig. 8. *Storage modulus (G′) of the linear region during a strain sweep at 20 °C for pre-filtrates and retentates at* pH *values of 3 and 8 with 5% (w/v) protein suspensions. LM = lesser mealworm, HC = house cricket, Alc = Alcalase. Different lowercase letters indicate mean values with significant differences* ($p < 0.05$).

interest to find a potential application for this fraction. One way to make use of these proteins in terms of functionality while simultaneously inactivating the microorganisms is to form a heat-induced gel from these proteins. The thermal treatment that is needed for gelation can, if the temperature is high enough, also inactivate the microorganisms still present in the retentate. From each pre-filtrate and retentate, solutions of 5.0% (w/v) protein were prepared, which corresponds to 7–10% (w/v) of powder depending on the treatment. Depending on the fraction, the pH was either 3 or 8. At the used protein concentration for all of the pre-filtrates and retentates, a gel could be made by heating.

The temperature ramp of the lesser mealworm retentate at pH 8 is displayed exemplary in Fig. 7. The storage modulus (G′) can be referred to as the gel strength, while the loss modulus (G″) reflects the viscous response. During the heating phase, G′ gradually increased, especially after a temperature of approximately 55 °C. During the second phase, where the temperature was kept constant at 90 °C, G′ kept increasing, indicating that the gel had not fully set. During the cooling phase, both G′ and G″ increased sharply, indicating strengthening of the gel. This gel strengthening is a result of attractive forces like hydrogen bonding and van der Waals interactions between protein particles within the gel network (Ould Eleya et al., 2004). Similar profiles were obtained for the pH 8 pre-filtrate of lesser mealworms, and all house cricket fractions. However, the pH 3 fractions of lesser mealworms showed a different profile. There, the G″ and especially the G′ decreased after reaching its peak at 50 °C during the heating phase, implying that at a concentration of 5.0% protein, a temperature increase above 50 °C is destructive for the gelation properties of these fractions. This peak was at 219 ± 15 Pa for supernatants while it was only 13 ± 3 Pa for retentates. The increased amount of proteolysis could have decreased the gelling capacity of pH 3 retentates for lesser mealworms. An increased level of hydrolysis causing reduction in gelation properties at temperatures around 40–60 °C was previously observed for Bombay duck (*Harpadon nehereus*) surimi gels (Li et al., 2022).

Fig. 8 shows the final storage modulus (G′) obtained within the linear region of a strain sweep experiment that was performed at the end of the cooling phase. Especially for lesser mealworms, stronger gels (i.e. higher average G′ values) were obtained at pH 8, which is in line with the results of Yi et al. (2013). They reported that no gel formed at pH 3 with a 30% (w/v) supernatant fraction concentration for five insect species including lesser mealworms and house crickets, while at pH 7 a gel could be formed. The authors hypothesised this inability to gel to be due to an increased charge on the proteins at pH 3, preventing the proteins from aggregating (Yi et al., 2013). In our work, the strongest gel was obtained for lesser mealworm pH 8 retentates, where the G′ was around 5000 Pa in the linear region. This is significantly higher than the other retentates, as the second strongest retentate gel (house cricket pH 8) had a G′ of about 160 Pa. Lesser mealworm retentate at pH 8 also had the highest lipid content (35.2%). These lipids could even strengthen gels through protein-lipid interactions (Ningtyas et al., 2021). Gels formed at pH 3 were softer than gels formed at pH 8; increasing the pH of pH 3 fractions to 7 before gelation did not improve the gel strength (results not shown). Alcalase addition during lesser mealworm microfiltration at pH 8 reduced the gel strength considerably, as the G' reduced from around 5000 Pa to around 12 Pa. This indicates that extended hydrolysis of insect proteins is detrimental to their gelation capacity. Since the proteins in the pH 3 fractions were more hydrolysed and therefore on average smaller than the proteins in pH 8 fractions (Fig. 2), this could be one of the reasons for the lower gelation capacity of pH 3 fractions, together with the higher lipid contents of pH 8 fractions. The pre-filtrates showed similar gel strengths as the corresponding retentates and differences between pre-filtrates and retentates are statistically not significantly different. All in all, our results show that the retentates can be used to form heat-induced gels. The retentates obtained at pH 8 can form strong gels, while the gels obtained from the pH 3 retentates are soft, making microfiltration at a pH of 8 more interesting for gel formation of the retentate than microfiltration at a pH of 3. However, the acidic pH of 3 could be a beneficial factor in inhibiting microbial growth or even inactivating microorganisms, as most bacteria exhibit optimal growth at a pH between 6 and 7 (Dillon and Dillon, 2004; Lund et al., 2014). If the temperature is high enough, microbial inactivation could simultaneously be achieved. In the opinion of the European Food Safety Authority (EFSA) on frozen and dried formulations from whole house crickets, a blanching step of 10 min for >90 °C was mentioned as sufficient (Turck et al., 2021). However, in this case, just a protein extract instead of whole insects needs to be heated, which may reduce the time needed as the protein extracts are more homogeneous. If the used temperature is not high enough, another decontamination method should still be used.

3.7. Lipid droplets in the cream/top layer

Depending on the type of insect and the pH (Fig. 1), the cream/top layer that was obtained after centrifugation to obtain the pre-filtrate, contains up to 36% of the insect's lipids. Before washing, the cream layer accounted for 1.0–2.6% (w/w) of the centrifuged slurry on a wet weight basis (Fig. 1), with this percentage reducing to 0.2–1.0% (w/w) after washing. Washing reduces the cream yield due to the removal of impurities, and redispersion of some lipids in the liquid fraction. These percentages are relatively low because of the high amount of water added before centrifuging (5 parts water for 1 part insects).

Fig. 8 shows a CLSM image of the washed cream layer of lesser

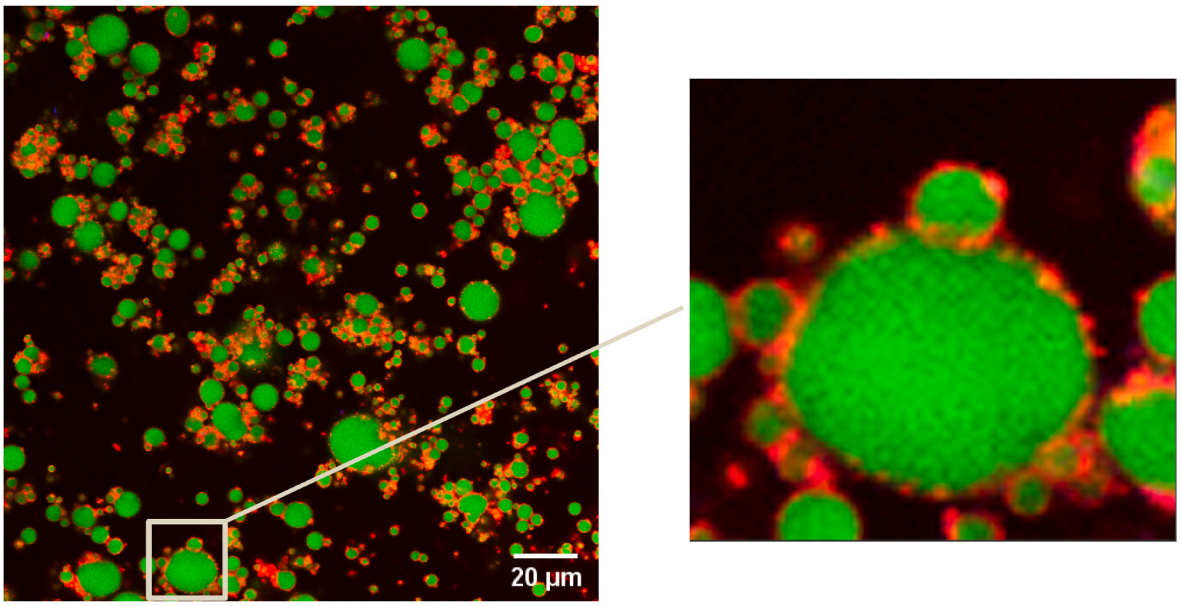

Fig. 9. CLSM pictures of washed lesser mealworm cream at a pH value of 3. Red indicates the presence of proteins, green indicates the presence of lipids, and dark blue indicates the presence of chitin, which is barely present. The images are made with a magnification of 63×.

mealworm obtained at a pH value of 3. The lipid droplets (in green) exhibit a protein layer (in red) around them. For added clarity, an enlargement of a droplet is shown. The distribution was less clearly seen for the cream layers obtained from house crickets at both pH values and lesser mealworms at a pH value of 8 (data not shown). In insects, lipids are generally distributed in fat bodies, the haemolymph, and in the cuticle (Downer and Matthews, 1976). The fat bodies mainly consist of adipocytes, which in their turn consist of lipid droplets (Arrese and Soulages, 2009). It is possible that the structures shown in Fig. 9 could be such lipid droplets. These lipid droplets have a similar structure as plant oleosomes, with a core of neutral lipids surrounded by a layer containing proteins and phospholipids (Arrese and Soulages, 2009; Nikiforidis, 2019). Oleosomes have a high potential to be used as functional ingredients in the food industry, such as natural emulsions, natural emulsifiers, imitation milk products, and edible films (Abdullah et al., 2020; Nikiforidis, 2019). Since insect oil is liquid-like at room temperature (Tzompa-Sosa et al., 2019), we speculate that these lipid droplets may yield similar functionality.

It is also possible to extract the oil from insects, which could for example be used as an ingredient in bakery products (Delicato et al., 2020; Tzompa-Sosa et al., 2019). The cream layer can thus be a high-value product of the microfiltration process by itself, for example to create emulsions.

4. Conclusion

The goal of this study was to demonstrate the effects of a microfiltration process on the techno-functional properties of insect protein and thereby finding potential food applications for the distinct fractions.

By centrifugation followed by microfiltration (0.2 µm polyethersulphone membrane), several potential applications were identified for different fractions. The sterile permeate was found to have an enhanced foamability compared to the pre-filtrates. Microbiologically contaminated retentates could undergo heat-induced gelation, enabling simultaneous inactivation of micro-organisms and gel formation if the temperature is high enough. This shows that unlike blanching, which denatures proteins, microfiltration preserves and even improves the protein functionality, as shown by the improved foamability.

Furthermore, oleosome-like lipid droplets could be obtained from the cream layer. These lipid droplets could have potential in the food industry, for example for emulsion formation.

Microfiltration at a pH of 3 led to significantly less browning as compared to microfiltration at a pH of 8. Although the addition of the exogenous protease Alcalase increased the protein recovery in the permeate, it caused a reduction in the gelation capacity and a significant increase in brown colour formation, both of which are often undesirable.

The advised pH during microfiltration depends on the preferred final product. A processing pH of 3 is advised to obtain a higher protein recovery and less enzymatic browning in the permeate during the filtration process, while a processing pH of 8 is advised if the goal is to make gels from the retentates.

All in all, this study shows the potential of a microfiltration process for soluble insect fractions as an alternative to blanching and highlights the industrial potential of products obtained during the microfiltration process. While the current lab-scale experiments are not optimised for efficiency, large-scale microfiltration systems are mature, and can employ large crossflows that significantly reduce the environmental impact of the process and the fouling of the membranes. Depending on the type of membranes, fouling can be reversed by using well-established enzymatic and acid-alkaline cleaning procedures, and for ceramic membranes also extensive heating. Furthermore, it is not unreasonable to expect that (rapid) backflushing techniques will further contribute to more efficient operation.

CRediT authorship contribution statement

L.J.H. Sweers: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft. **C.M.M. Lakemond:** Conceptualization, Writing – review & editing, Supervision. **V. Fogliano:** Conceptualization, Writing – review & editing, Supervision. **R.M. Boom:** Conceptualization, Writing – review & editing, Supervision. **M. Mishyna:** Conceptualization, Writing – review & editing, Supervision. **J.K. Keppler:** Conceptualization, Writing – review & editing, Supervision.

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.

Data availability

Data will be made available on request.

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