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Stability assessment and laboratory scale fermentation of pastes produced on a pilot scale from mealworms (*Tenebrio molitor*).

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Abstract

European consumers generally still have a reluctant attitude towards the consumption of insects. One strategy to trigger the willingness to consume edible insects is to process them invisibly in familiar foodstuffs. To facilitate this, insects need to be processed to intermediates that can be incorporated readily in products by the food industry. To this end, a mealworm paste that was free of tastable exoskeleton particles was manufactured successfully using industrial equipment. Of this novel intermediate the proximate and fatty acid composition, moisture content, water activity, pH, viscosity, peroxide and *p*-anisidine values and colour was analysed. Next, the impact of storage temperature (4°C and -21°C) and presence of preservatives (sodium nitrite and sodium lactate) on the chemical and microbial stability of the paste were evaluated, as well as its fermentability to assess the most suited preservation strategy. During storage at -21°C, all tested parameters remained constant for three months, except for some fat oxidation. At 4°C, substantial microbial growth was observed during three weeks of storage, regardless of the use of preservatives. Finally, the paste was also shown to be suited for fermentation. Future research should assess whether fermentation can extend the storage time and/or improve the product quality.

Keywords

Tenebrio molitor; mealworms; paste; microbial stability; chemical stability; fermentation

1. Introduction

Edible insects are being introduced in Europe as a sustainable alternative for conventional meat because of their high nutritional value and low environmental impact (Smetana, Palanisamy, Mathys, & Heinz, 2016). Several studies conclude that European consumers still have a reluctant attitude towards the consumption of insects (Hartmann & Siegrist, 2016; Looy, Dunkel, & Wood, 2014). In Europe, eating insects is associated to being dirty and dangerous and to feelings of disgust (Looy *et al*., 2014). One of the proposed solutions to open the European market for edible insects is to process them in an invisible way in familiar foodstuffs. This strategy is suggested and also shown in market research to result in a higher willingness to eat insect-based foods (Caparros Megido *et al*., 2016; Hartmann & Siegrist, 2016; Tan, Verbaan, & Stieger, 2017). The existence of stable and well characterized insect intermediates, such as powders and pastes, may facilitate the incorporation of these ingredients in food products by the food industry and may enlarge the portfolio of insect-based foods in the market. However, today no pastes are available on the B2B market, and it is not generally known how qualitative and stable pastes can be produced at an industrial scale.

When fragmenting fresh mealworms to obtain a paste, it can be expected that the nutrient availability for the endogenous microbiota increases, hence possibly inducing microbial spoilage and safety risks (Fellows, 2009). Mealworms are known to contain a high microbial load after rearing (Stoops *et al*., 2016; Vandeweyer, Crauwels, Lievens, & Van Campenhout, 2017). A heat treatment such as blanching or microwave drying reduces the microbial load except for the bacterial spores, which are heat resistant (Vandeweyer, Lenaerts, Callens, & Van Campenhout, 2017). Fresh and blanched mealworms are characterized by a high water activity and an ideal pH for bacterial growth, making them susceptible for microbial spoilage (Vandeweyer, Crauwels, *et al*., 2017). According to Vandeweyer, Lenaerts, *et al*. (2017),

blanched mealworms can be stored at 4°C for six days without exceeding the general food spoilage level for food of 7 log cfu/g (W. Sperber & Doyle, 2009). As mealworms contain high levels of unsaturated fatty acids, they can also be susceptible for fat oxidation, causing rancidity (Jeon *et al*., 2016; Tzompa-Sosa, Yi, van Valenberg, van Boekel, & Lakemond, 2014). This study investigates these parameters to explore the storability of insect pastes.

In fact, to obtain storable pastes from mealworms, the use of a preservative may be a prerequisite, which will also be studied. Sodium nitrite and sodium lactate are currently used to extend the shelf life of meat by inhibiting microbial growth, fat oxidation and/or colour changes (Alahakoon, Jayasena, Ramachandra, & Jo, 2015). Nitrite inhibits microbial growth by inhibiting metabolic enzymes of bacteria, limiting oxygen uptake by bacteria and interfering with the proton gradient. Nitric oxide binds with iron ions, which results in a limited iron availability for microbial growth (Alahakoon *et al*., 2015). Nitrite also retards rancidity during storage by terminating the auto-oxidation of the fat and limiting the pro-oxidant activity of iron ions (Alahakoon *et al*., 2015). Driven by the consumers' demand for more natural or organic meat products, industry is searching for alternatives for sodium nitrite. According to Seydim *et al*. (2006), sodium lactate is a suitable alternative to preserve meat and poultry. Sodium lactate also inhibits microbial growth and retards lipid oxidation (Seydim, Guzel-Seydim, Acton, & Dawson, 2006).

An alternative preservation strategy to prevent unwanted microbial growth would be to ferment the mealworm paste using a starter culture. Evidence that this is feasible for a mealworm paste produced at laboratory scale has recently been published (Borremans, Lenaerts, Crauwels, Lievens, & Van Campenhout, 2018). In this study, the goal is to explore whether this starter culture could also successfully ferment a paste produced using a more industrially scalable set-up. As an additional advantage, the process of fermentation also can

lead to improved nutritional and organoleptic properties in a food product, which could also increase its value (Bourdichon *et al*., 2012).

This study is, to our knowledge, the first to report on the production process of pastes from non-defatted mealworm larvae as food ingredients. The production was done at pilot scale, using equipment that is scalable to industrial scale. Mealworms were steamed and then fragmented in a series of two cutters. All freshly produced intermediates were then characterized with respect to moisture content, water activity, pH, viscosity, proximate composition and fatty acid composition. Next, the intermediates were subjected to storage experiments under appropriate conditions to evaluate their microbial and chemical stability during storage, since a minimal storability is a requirement for intermediates to be useful in the food industry. Pastes containing no preservative, sodium nitrite or sodium lactate were included in this analysis. Since fresh and processed mealworms are prone to browning (Janssen *et al*., 2017; Van Campenhout, Lenaerts, Callens, & Van Der Borght, 2017), the colour of freshly produced and stored intermediates was measured as well. Finally, it was also investigated whether the paste produced at pilot scale can be fermented, as this can be an alternative preservation strategy, and the progress of fermentation was assessed using culture-dependent plate counts.

2. **Materials and methods**

2.1 *Production of mealworm pastes*

Living mealworms (Nusect, Ledegem, Belgium) were steamed for 5 min in a steam oven (ClimaPlus Combi CPC61, Rational GmbH, Germany) to kill the larvae and to reduce their microbial load. Then 9.7 kg mealworms were fragmented for 25 min in a cutter under vacuum (UM 12, Stephan, Belgium) followed by a second grinding step in a microcutter (MC 15, Stephan, Belgium). This procedure and these instruments were selected in preliminary

research so as to (1) be realizable at industrial scale and (2) yield a fine paste that does not contain tastable exoskeleton particles which would lead to an unwanted sensory sensation.

2.2 *Storage of the paste and analyses during storage*

An aliquot of 2.4 kg of freshly prepared paste was kept aside for fermentation. Of the remaining part, one third was produced without preservatives (control), another third was supplemented with 150 mg $NaNO_2$/kg paste (EMSURE®, ACS, Reag. Ph. Eur. Analytical reagent, Merck Millipore) and the last part with 50 g/kg paste of a 60% sodium DL-lactate solution (Syrup, 60 % w/w, synthetic, Sigma Aldrich). Each mixture was homogenized for 30 seconds in a Foss Homogenizer (2096, Hogänäs, Sweden). Aliquots of 250 g of the pastes were transferred into sterile 250 mL containers (PP transparent with HDPE cap, Corning®, New York) and stored at 4°C and -21°C. The moisture content, water activity, pH, viscosity, proximate composition and fatty acid composition were analysed immediately after processing. During the storage experiments, the moisture content, water activity, pH, viscosity, fat oxidation, colour and microbial counts were monitored. The pastes stored at 4°C were analysed weekly during three weeks after production, while those stored at -21°C were measured every month up to three months after production. Fat oxidation was measured immediately after production and after storage. All analyses were performed in triplicate (n = 3).

2.3 *Fermentation*

An amount of 1.2 kg of mealworm paste was inoculated with the commercial meat starter Bactoferm F-LC (Chr. Hansen, consisting of a mixture of *Pediococcus acidilactici*, *Lactobacillus curvatus*, and *Staphylococcus xylosus*), according to the manufacturers' instructions. This meat starter culture was chosen given the fact that the paste is a meat-type product containing animal proteins. Its moisture content (about 66%) and protein content (about 24% on fresh weight basis) is also comparable to that of traditional meat such as

chicken, pork, and beef. Mealworm paste was produced at laboratory scale and fermented at laboratory scale with this starter in previous work (Borremans *et al*., 2018). An equal mass was not inoculated to be used as control. In parallel, in order to investigate the necessity of nitrite during fermentation, identical fermentation experiments were also performed on pastes supplemented with 0.015% $NaNO_2$ (w/w). The fermentation set-up is based on the optimisation of a fermentation strategy on paste produced at lab scale described by Borremans *et al*., 2018. Based on these results, 2.8% NaCl (w/w), 0.75% d(+)-glucose (w/w) were also added to both pastes, as is a general practice in meat fermentation. After mixing, twelve 50 mL Falcon tubes were filled with the paste and incubated at 35 °C for 2 weeks. The fermentations (uninoculated and inoculated) were performed in triplicate. Sampling was carried out on days 0, 3, 7 and 14 for pH analysis and microbial counts. To avoid interruption of the course of the fermentations by sampling, three Falcon tubes were withdrawn per sampling time point.

2.4 *Proximate analysis*

For the proximate analysis, the moisture, protein, ash and fat content was determined. A more elaborate description of the methods used for this analysis was recently published (Lenaerts, Van Der Borght, Callens, & Van Campenhout, 2018). Briefly, the moisture content of the pastes was calculated after drying the sample in a forced air oven at 105°C for 17 hours.

The protein content was determined using the Kjeldahl method as described by Chang (2010). Crude protein was estimated as N x 6.25. The method was verified using acetanilide (pure, UCB) as a reference. The protein content was not corrected for chitin. The determination of the ash content was performed as described by Marshall, (2010). The samples were incinerated in a muffle furnace at 550°C until constant weight was reached.

The Soxhlet method was used to determine the fat content of the samples. Since for the Soxhlet method, the moisture content has to be below 10 %, the paste was first freeze dried for 48 hours to reduce its moisture content (Min & Ellefson, 2010).

2.5 *Fatty acid composition*

The fatty acid composition of the fat obtained after the Soxhlet extraction was determined with GC-MS as described by Kandhro *et al*. (2008). The fats were first esterified in a 0.5 M sodium methoxide solution with the addition of a 20% BF_3-methanol solution. Then, the fatty acid composition was measured using a GC-MS (GC 7820A/5977E MSD, Agilent) fitted with a SLB-IL60 capillary column (30 m x 0.25 mm, Sigma Aldrich). Initially, the present fatty acids were identified using the SCAN mode. Afterwards, the MS was used in the SIM mode under electron impact ionization of 70 eV. The data were analysed with the Agilent MassHunter Quantitative Analysis software. Methyl tricosanoate was used as the internal standard. The fatty acids were quantified by comparing the relative response of the ratio of the unknown to the internal standard with the relative response of the standard mixture (Kandhro et al., 2008). The amount of each fatty acid was expressed as a percentage of the total fatty acid content. More details on the preparation of the samples and the GC-MS parameters used can be found in Lenaerts *et al*. (2018).

2.6 *Viscosity, water activity and pH*

Viscosity measurements were performed with a rheometer (Physica MCR 301) equipped with a rough parallel plate system (PP25/P2). A gap of 2 mm between the spindle and the plate and a minimum waiting time of 30 seconds was applied. A shear rate ramp was conducted on every sample of the paste during the storage period. The shear rate ramp was performed from 0.1 s^{-1} to 100 s^{-1} in a logarithmic scale at 5°C and the viscosity was calculated and compared at a shear rate of 1 s^{-1}. The water activity of the samples was

measured as previously described (Vandeweyer, Lenaerts, *et al*., 2017). The pH was measured with a digital pH meter (Portamess 911 with SI analytics electrode).

2.7 *Fat oxidation*

The fat obtained by the Soxhlet method as described earlier was used for the determination of the fat content and the fatty acid profile. During the Soxhlet procedure, the fat was heated at 105°C for 17 h. Since fat oxidation can be influenced by heat (Choe & Min, 2006), a cold extraction method, the modified Folch method (Min & Ellefson, 2010), was used to obtain fat for the analysis of the peroxide and *p*-anisidine values. Samples were homogenized with a chloroform-methanol mixture (2:1) (ACS Reag. Ph Eur.) and subsequently filtered through a Büchner funnel fitted with a black ribbon filter (454). The remaining filter cake was extracted twice more with the chloroform-methanol mixture. All three extracts were collected and after separation of the two layers in a separating funnel, the chloroform phase was collected. Next, the chloroform phase was washed with a 0.88% sodium chloride solution to remove contaminants. Afterwards, the chloroform was evaporated at 45°C under vacuum in a rotary evaporator (R-200, Büchi). The fat obtained was used to determine the peroxide and *p*-anisidine values (Min & Ellefson, 2010). A modified method based on Wu & Mao (2008) was applied to determine peroxide values, as described in Lenaerts *et al.* (2018). Peroxide values were expressed as units of meq. O_2/kg fat sample. The *p*-anisidine values were determined according to Tenyang *et al*. (2017). More details can be found in Lenaerts *et al*. (2018).

2.8 *Colour evaluation*

Colour measurements were performed with a colorimeter (CR-5, Konica Minolta) using the CIELAB colour space, where the L* value represents the lightness, the a* value the red-green direction of the colour and the b* the yellow-blue direction. The colour was measured on five points of the petri dish containing the sample with a measuring aperture of 30 mm and

214 the specular component excluded. At each measuring point during storage, the colour was
215 compared with the initial colour and differences in a*, b* and L* were compiled into the total
216 colour difference (ΔE*). In addition, for each measuring point the browning index (BI) was
217 determined. The parameters were calculated by means of the following expressions (Pathare,
218 Opara & Al-Said, 2013):

$$\Delta E^{*} = \sqrt{\Delta a *^{2} + \Delta b *^{2} + \Delta L *^{2}}$$

$$BI = 100 \times \frac{X - 0.31}{0.17}$$

$$X = \frac{(a^{*} + 1.75\ L^{*})a}{5.645\ L^{*} + a^{*} - 3.012\ b^{*}}$$

219 2.9 *Microbial analyses*

220 Microbiological analyses during the storage experiment (total aerobic mesophilic counts,
221 total anaerobic mesophilic counts, total aerobic psychrotrophic counts, *Enterobacteriaceae*,
222 aerobic spore counts and anaerobic spore counts) were performed as previously described
223 (Vandeweyer, Lenaerts, *et al*., 2017). In addition to the analysis plan described earlier, fresh
224 and steamed mealworms as the starting material to prepare the pastes were included. For the
225 fermentation experiment, numbers of lactic acid bacteria (LAB) and sulphite reducing
226 clostridia were also determined apart from the total aerobic mesophilic and the aerobic spore
227 counts. The latter two were performed in the same way as for the storage experiment. LAB
228 were incubated on de Man, Rogosa & Sharpe agar (MRS, Biokar Diagnostics) for 72 h at
229 30 °C and sulphite reducing clostridia on Iron Sulphite Agar (ISA) for 48 h at 37 °C.

230 2.10 *Statistical analyses*

231 All analyses were performed in triplicate. Results are reported as mean values ± standard
232 deviation (SD). Statistical analyses were performed using IBM SPSS Statistics 23 software.
233 Differences were analysed by one-way ANOVA in case variances were equal, followed by
234 the Duncan post-hoc test. If variances were not equal, the Kruskal-Wallis test with the Dunn-

Bonferroni post-hoc test was performed. For all tests a significance level of 0.05 was considered.

3. **Results and discussion**

3.1 *Impact of the processing of whole mealworms into a paste on the nutrient composition*

First, the proximate analysis and the fatty acid composition of the freshly prepared paste without additive were determined (Table 1). Even though the mealworms used to produce the paste were not examined for their nutritional value and even though care must be taken when comparing nutritional results from different studies due to the impact of the origin of the mealworms as well as differences in the analysis techniques applied, it can be stated that the protein, fat and ash content of the paste was in line with results reported for whole mealworms in literature (Nowak, Persijn, Rittenschober, & Charrondiere, 2016; Payne, Scarborough, Rayner, & Nonaka, 2016). This indicates that the mechanical processing of the paste does not dramatically alter the composition of the mealworms. Compared to traditional products of animal sources (more specifically beef, pig, turkey and salmon), the mealworm paste has a higher protein as well as fat content (Table 1).

Differences can also be observed when comparing the fatty acid composition. The profile of the fatty acid composition is in accordance with results previously obtained for whole mealworms, as are the most abundant fatty acids (palmitic acid, oleic acid and linoleic acid) (Paul *et al.*, 2017; Tzompa-Sosa *et al.*, 2014). Yet differences in the actual amounts were present, for example lower amounts of palmitic and higher amounts of linoleic acid were found in this research compared to that of Paul *et al.* (2017). As in insects the fatty acid biosynthesis/accumulation is highly dependent on environmental conditions such as substrate type (Fontaneto *et al.*, 2011), these differences are most likely the result from the fact that the mealworms of each study are not reared in an identical manner. Furthermore, the linoleic acid content is also considerably higher than that in traditional products of animal sources (Table

1) and given its essential nature in mammalian nutrition, this aspect is a clear benefit of this novel insect intermediate. The same can be stated concerning its high content of unsaturated fatty acids, which can be compared to that in raw salmon. The latter does, however, contain a more favourable fatty acid composition, given its higher content of omega-3 fatty acids.

3.2 *Impact of storage temperature on the moisture content, water activity, pH and viscosity of the pastes during storage*

Next, we determined the impact of storage both at 4°C and at -21°C on a number of key parameters determining the microbial stability, being the moisture content, water activity and pH of the pastes. Although some statistical differences were observed in the moisture content and the water activity over the storage period at 4°C (Table 2), these changes are not meaningful and will not directly affect the stability of any of the studied pastes during storage. However, the water activity of the pastes was very high and their pH was near neutral, as expected based on own previous results on whole mealworms (Lenaerts *et al*., 2018). These are very favourable conditions for microbial growth (Bonazzi & Dumoulin, 2011; Fellows, 2009). Indeed, a reduction in the pH was observed during storage at 4°C. This is likely the consequence of microbial growth (acid production) in the intermediate. It can be expected that the microbial stability of this paste will be troublesome, which is discussed in paragraph 3.8.

Storage at more reduced temperatures, however, was found to be more suited to stabilize the paste. At -21 °C, the moisture content, water activity and pH values of the pastes remained constant over a period of three months (data not shown). This indicates that no microbial growth occurred in the intermediate.

3.3 *Impact of preservatives on the moisture content, water activity, pH and viscosity of the pastes during storage*

Two preservatives, sodium nitrate and sodium lactate were added to the paste as they can be used to extend the shelf life of food-products by inhibiting microbial growth. Their

addition did not affect the moisture content and water activity of the paste during storage at 4°C (Table 2), as was expected. Yet at the same time neither of the preservatives could prevent the occurrence of a reduction in the pH, though the reduction in pH was less pronounced in the presence of the preservatives, especially for sodium lactate (Table 2).

Immediately after production, the viscosity of the paste was measured and found to be 342 ± 24 Pa.s, which is comparable to the viscosity of a rather thick tomato ketchup (Bayod, Willers, & Tornberg, 2008). The viscosity of the pastes remained also stable during storage at 4°C and -21°C. More specifically, the viscosity was 340 ± 42 Pa.s after three weeks at 4°C and 303 ± 30 Pa.s after three months storage at -21 °C. Furthermore, we determined that the addition of preservatives also did not interfere with this parameter as the viscosity remained 335 ± 38 Pa.s and 283 ± 18 Pa.s for paste with sodium nitrite and sodium lactate, respectively.

3.4 *Oxidation during storage of the pastes*

An important aspect for the chemical stability of the produced paste is the fact that mealworms contain, as shown in Table 1, more unsaturated than saturated fatty acids in comparison to other traditional products of animal sources. Unsaturated fatty acids are less stable than saturated ones and are thus more susceptible to fat oxidation, which can lead to product deterioration (Tao, 2015). Fat oxidation is a time-dependent process which can be described by two parameters. The peroxide value represents primary oxidation, while secondary oxidation can be measured by means of the *p*-anisidine value. The lower the peroxide value, the better the quality of the matrix is. Since primary oxidation products of fats are not stable, secondary oxidation products are formed and concomitantly peroxide values decrease over time. Secondary oxidation products cause off-flavours and -odours, which is why it is important to determine both oxidation parameters (Choe & Min, 2006). For most oils, criteria exist for maximum values of both parameters. As insects constitute a new food matrix, no criteria for insect oil or other insect-derived products have been set up so far.

Therefore, the criteria imposed by EFSA for fish oil are considered in this research as a reference to decide whether the oxidation status is acceptable or not.

Table 3 shows the results of the fat oxidation measurements of the pastes, represented by the peroxide and *p*-anisidine values. No primary oxidation occurred in the pastes, since the peroxide value was below the detection limit of 0.5 meq. O_2/ kg fat (Murray-Brown Laboratories, 2010) immediately after production and it remained below the detection limit during storage. On the other hand, *p*-anisidine could be detected. Values were below the EFSA criterion for fish oil (AV < 20) for all samples, but secondary oxidation increased significantly during storage, regardless of preservative treatment or storage temperature.

The preservatives even had a worsening impact on secondary fat oxidation. This cannot be explained for sodium lactate, but in the case of sodium nitrite, it is postulated that nitrite can be a strong oxidant in the Fenton reaction and that this oxidizing effect can be higher than the reducing effect at high sodium nitrite doses (Doolaege *et al*., 2012; Skibsted, 2011).

3.5 *Evolution of colour during storage of the pastes*

The colour of the pastes was also monitored during storage (Table 4), as it can influence the physical properties of a product containing the novel insect intermediate. For this purpose the L*, a* and b* values were measured for the calculation of the total colour difference between a time point during storage and the initial colour (ΔE*) and for the calculation of the browning index (BI). When ΔE* is more than 3, the colour difference is assumed to be narrowly visible. When ΔE* is more than 6, the colour difference is clearly visible (Wibowo *et al*., 2015). The browning index characterizes in particular the intensity of the brown colour (Hirschler, 2012; Pathare, Opara, & Al-Said, 2013). Since mealworms are known to turn brown during processing (Janssen *et al*., 2017), it is important to focus on this colour aspect. Small ΔE* colour differences around the value of 3 (i.e. just visible) were recorded for all types of pastes throughout storage. The browning index decreased significantly during the

first week of storage at 4°C and the first month at -21°C, but afterwards it remained constant. The decrease during the first week implies a loss of the intensity of the brown colour, which is unexpected since in general mealworms are known to be susceptible to browning after killing. It appears that the sequence of treatments applied in this study to obtain a paste present a good strategy to counteract the browning process, but more research is needed to unravel the molecular mechanism(s) behind this observation.

3.6 *Impact of the processing of whole mealworms into a paste on the microbiota*

Since this is the first study to produce at pilot scale pastes from non-defatted mealworm larvae, the impact of this production process on the microbiota was followed. To this end, microbiological counts of fresh mealworms, steamed mealworms and the fresh paste made of steamed mealworms were determined (Table 5). All aerobic counts of fresh mealworms (total mesophilic count, spore count, psychrotrophic count and *Enterobacteriaceae*) are comparable to earlier research (Caparros Megido *et al*., 2017, 2018; Stoops *et al*., 2016; Vandeweyer, Crauwels, *et al*., 2017; Vandeweyer, Lenaerts, *et al*., 2017). Small differences can be explained by the different origin of the mealworms as well as variation between batches (Vandeweyer, Crauwels, *et al*., 2017).

Five minutes of steaming yielded log reductions of 5.2, 5.7, 5.4 and 5.6 for the total aerobic and anaerobic count, psychrotrophic count and *Enterobacteriaceae* respectively. Much smaller reductions of 1.2 and 0.4 log cfu/g, which were not statistically significant, were recorded for the aerobic and anaerobic endospores, respectively. The paste showed slightly but statistically not significant higher counts (total aerobic count, total anaerobic count, psychrotrophic count and *Enterobacteriaceae*) compared to the counts of steamed mealworms (resp. $p = 0.258$, $p = 0.137$, $p = 0.085$ and $p = 0.095$). This indicates that some microbial contamination occurred during the production process of the paste, which was

indeed executed in clean but not sterile conditions. The amount of aerobic and anaerobic spores was hardly influenced by the production of the paste.

3.7 *Microbial stability during storage of the pastes*

As stated previously, the microbial stability of the mealworm paste during storage seems to be the major concern for this novel intermediate, given that the pH measurements indicate the occurrence of microbial growth at 4°C but not at -21°C (Paragraph 3.2). To further investigate this observation, microbiological counts were determined for the paste made of steamed mealworms, which was either stored for 3 weeks at 4°C (Table 5) or for 3 months at -21°C (data not shown). At the same time the impact of the presence of sodium lactate or sodium nitrite as a preservative on the microbial stability during storage was also determined (Table 5).

The results of the microbial counts during storage at 4°C indeed reveal that the total aerobic and anaerobic count and the psychrotrophic count increased dramatically during the first week of storage. This increase occurred regardless of whether a preservative was added or not, and regardless of the type of the preservative. Furthermore, the microbial growth continued further on during the second and third week. At two weeks of storage at 4°C, all aforementioned numbers were above the level of 7 log cfu/g which is considered as a general spoilage level for foods (Sperber & Doyle, 2009), confirming the observations made based on the pH.

While the preservatives did not impact the rise in the total aerobic/anaerobic counts, growth of *Enterobacteriaceae* did vary among treatments (Table 5). At two weeks of storage, their number was significantly lower in the two pastes with a preservative than in the paste without addition, but at three weeks their number in the paste with sodium lactate reached the same level as in the untreated paste. The aerobic and anaerobic spore counts remained more or less constant during storage. Most likely, this can be explained by the fact that existing

spores did not germinate combined with the fact that no new spores were formed. Germination of spores was to be expected in the paste without preservative and in the paste with sodium lactate because the steaming as a heat treatment could have activated them and because the pH of the pastes was close to neutral and hence favourable for spore germination. In the paste with sodium nitrite, spore germination was likely inhibited by the nitrite (Jay, Loessner, & Golden, 2005).

From these counts it is clear that fragmenting the mealworms into a paste has the consequence that nutrients become more available for micro-organisms. Combined with a high moisture content, a water activity close to 1 and a near-neutral pH, this makes the paste an ideal environment for microbial growth. Even though steaming as a pretreatment before fragmenting involves a tremendous reduction in the microbial load, it is not enough to provide an acceptable shelf life to the paste as an intermediate. Moreover, the preservatives tested here at their maximal legislated concentration (150 mg/kg for sodium nitrite and 3% for sodium lactate) had no influence on the shelf life of the paste stored at 4°C. Processing mealworms into a paste with a certain microbial shelf life at refrigerator temperature (and being safe, which was even not been taken into account in this study) will require other and more successful conservation strategies.

Finally, we also determined the microbial counts during storage at -21°C. As expected from the observed pH stability at this temperature, the microbial load of the paste, even without preservatives, did not increase (data not shown). This provides additional evidence that storage at -21°C offers a valid preservation strategy for the insect industry. Nevertheless the need for freezing to provide a longer microbial shelf life is associated with a relative high economical cost, making it worthwhile to look for alternative conservation strategies such as fermentation.

3.8 *Fermentation as an alternative for preservatives to prolong shelf life*

Recent work from our group has shown that a paste produced at laboratory scale from blanched mealworms can be fermented using a commercial meat starter culture (Borremans *et al*., 2018). During laboratory paste production, the larvae were crushed. Crushing was essential since previous research had shown that whole mealworms could not be fermented with success, probably due to the exoskeleton preventing the starter culture to reach the protein fraction and other nutrients inside the larvae. This paste of crushed mealworms contained major exoskeleton particles, and as such it is not a suitable intermediate for the food industry. In the current study, paste was produced to contain no particles at all and using equipment that is scalable to industrial scale. To confirm that this paste can also be fermented, a fermentation was performed using the process optimised at laboratory scale with the same meat starter culture. To be able to observe the role of the starter, an uninoculated control was incubated in parallel. To investigate whether nitrite is needed during the fermentation to suppress the background microbiota, as is done in traditional meat fermentation, uninoculated and inoculated samples containing nitrite were included in the experimental set-up as well.

The pH profile (Figure 1) shows a clear acidification in function of time, both in the control and when the starter culture is supplemented, and regardless of the presence of nitrite. Overall for all inoculated samples, the pH decreased from 6.78 to 5.27, but did not reach a value below 5.10 which is necessary to provide a barrier against most foodborne pathogens (Hutkins, 2007). This is in contrast to our previous observations (Borremans *et al*., 2018), where pH was reduced to below this value. An explanation could be the fact that prior to the production of the paste, in the protocol used here the larvae were steamed and not blanched as in the previous research and that the microbial reduction caused by steaming is lower than that obtained by blanching. Log reductions obtained for blanching in previous research (Vandeweyer *et al*., 2018) were 5,6, 5,5, 6,1 and 0,8 for total aerobes, psychrotrophs, *Enterobacteriaceae* and aerobic spores, respectively. As mentioned before, here steaming

resulted in log reductions of 5,2, 5,4, 5,6 and 1,2 for the respective counts. For all types of counts mentioned except for the spores, the numbers remaining on the heat treated larvae were higher when they were steamed than when they were blanched. Care must be taken in this comparison, however, since the batch of mealworms analysed was different for the two studies. Nevertheless, a larger microbial load remaining after heat treatment can explain a lower activity of the starter culture due to a higher competition for nutrients and hence a lower pH reduction during fermentation.

Microbial counts (total aerobic count, LAB, aerobic spore counts and sulphite reducing bacteria) were monitored during fermentation. The addition of the starter appeared to be necessary to reduce sulphite reducing clostridia, which were present in non-inoculated samples (up to 7.2 log cfu/g) and which were likely responsible for the strong decomposition odour of the non-inoculated paste. However, after 14 days sulphite reducing bacteria also appear in the fermented paste without nitrite, but not in the fermentations containing nitrite. Hence, nitrite can assist in preventing growth of the unwanted background microbiota, in the same way as in traditional meat fermentations. Inoculated samples were dominated by LAB, most likely originating from the starter. Their counts almost completely coincided with the total counts and increased during fermentation from 6.4 log cfu/g at day 0 to 9.0 log cfu/g at day 14. In the paste without starter, an initial increase in the number of LAB was observed as well, but it did not coincide with the total count during the whole experiment as for inoculated samples. In general, these results reveal the potential of fermentation to control unwanted microbial growth in the insect paste. They also emphasize the importance of exploring the ideal pretreatment of the larvae to reduce the background microbiota and of the formulation of the starter mixture that may also include preservatives. Further research is necessary to determine whether the fermentation process indeed has a positive effect on the shelf life of the paste.

4. Conclusions

This study describes in the first place the pilot scale production, the characterization and the chemical and microbial stability of a paste obtained from non-defatted mealworms. The procedure for production of the paste is straightforward to be executed using standard equipment available in the food industry and leads to an intermediate with characteristics that are comparable to other foods or intermediates. Concerning the stability, the viscosity and the colour of the paste are not affected by storage at either 4 °C (chilling) or -21 °C (freezing). In contrast, microbial stability does depend on storage temperature. While freezing of the paste ensures microbial stability, chilled storage does not. Whatever temperature applied, care must be taken to provide a substantial heat treatment prior to fragmentation. Also after fragmenting the microbial quality of the paste should receive extensive attention. Chilled storage requires (the combination with) other conservation strategies than the preservatives tested in this study. In this respect, sodium nitrite and sodium lactate, which are the preservatives that can be selected in the first place to improve shelf life, do not inhibit microbial growth in the insect matrix. Further research is needed on the conservation potential of for instance sodium lactate at higher concentrations, the addition of both ascorbic acid and sodium nitrite or the addition of other preservatives, preferably in combination with vacuum or gas packaging or other technologies. Another issue that threatens the stability of the paste is the occurrence of secondary fat oxidation both in chilled or frozen mealworm slurry. However, the level of oxidation is limited and the peroxide value remains even below the level that a fresh and refined product should have (1 meq/kg) (Gunstone, 1996).

Ensuring microbial stability can thus be concluded to be the biggest challenge remaining for future work. In line with this, it can be concluded that fermentation of pastes may entail a promising strategy, since its feasibility was demonstrated in this study. In a next step, it needs to be investigated whether the microbial and chemical shelf life of the fermented paste during

chilled storage is better than that of unfermented paste. If so, on top of that fermentation may also enhance the flavour, the nutritional content and/or the functional properties of this insect intermediate and contribute to the development of innovative insect-containing foods.

Once the stability of the paste can be ensured, other physical properties, such as gel property, of this novel intermediate should be studied in follow-up research. These analyses will also help define the set of food products that could best benefit from the introduction of this intermediate.

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Figure caption:

Fig. 1. pH and the microbial counts during fermentation of the paste. Graphs show mean values of 1 to 3 replicates. **(A)** results from paste with no nitrite, but no starter culture added; **(B)** results from paste with no nitrite, but with starter culture added; **(C)** results from paste with nitrite, but no starter culture added; and **(D)** results from paste with nitrite and starter culture added.

Table 1: Proximate composition (g/100g DM) and fatty acid composition (g/100 g fatty acids) of freshly prepared paste from steamed mealworms and without additive. Values are means of 3 replicates ± standard deviation. In addition, the composition of a number of meat sources is given.

	Data current study Mealworm paste without additive	**Data from literature**[1]			
		Beef[a]	Pork[b]	Turkey[c]	Salmon[d]
Proximate composition					
Moisture (g/100g)	66.77 ± 0.34	65.3	70.3	72.0	67.8
Protein (g/100g)	33.74 ± 0.72	18.6	20.2	21.8	20.0
Fat (g/100g)	18.62 ± 0.31	14.8	9.8	6.0	11.0
Ash (g/100g)	2.36 ± 0.06	0.4	N.A.	N.A.	1.1
Fatty acid composition					
Decanoic acid (C10:0)	0.02 ± 0.00	0.2	0.2	N.A.	0.0
Lauric acid (C12:0)	0.33 ± 0.01	0.2	0.3	0.3	0.0
Myristic acid (C14:0)	2.28 ± 0.09	3.2	1.6	1.3	5.3
Pentadecanoic acid (C15:0)	0.09 ± 0.00	0.4	0.1	0.2	0.4
Palmitic acid (C16:0)	17.48 ± 0.34	25.0	24.4	23.4	14.3
Palmitoleic acid (C16:1)	1.07 ± 0.07	4.9	2.3	5.7	8.6
Heptadecanoic acid (C17:0)	0.17 ± 0.00	1.0	0.3	N.A.	0.2
Stearic acid (C18:0)	2.97 ± 0.02	13.9	13.3	8.2	2.6
Oleic acid (C18:1)	39.37 ± 0.61	40.6	41.6	34.5	21.0
Linoleic acid (C18:2)	35.62 ± 1.01	2.6	11.1	23.7	4.0
Linolenic acid (C18:3)	0.23 ± 0.00	0.5	0.0	2.3	1.2
Eicosenoic acid (C20:1)	0.23 ± 0.01	0.2	1.0	N.A.	11.6
Arachidonic acid (C20:4)	0.15 ± 0.01	0.2	0.5	N.A.	0
Total SFA	23.33 ± 0.33	44.1	40.6	33.6	22.8
Total UFA	76.67 ± 0.33	53.0	59.0	66.4	77.1
Total MUFA	40.64 ± 0.58	49.3	45.0	40.4	53.9
Total PUFA	36.00 ± 0.87	3.7	14.0	26.0	23.2

DM = dry matter
SFA = saturated fatty acids
UFA = unsaturated fatty acids
MUFA = mono-unsaturated fatty acids
PUFA = poly-unsaturated fatty acids
N.A. = Data not available

[1]Data was extracted from NEVO-online.rivm.nl, maintained by the Dutch National Institute for Public Health and the Environment. The food names used as search terms were: [a] Beef >5% fat raw; [b] Pork 5-14% fat raw; [c] Turkey raw; [d] Salmon farmed raw.

Table 2: Moisture content, water activity and pH during storage of pastes prepared from steamed mealworms without additive, with sodium nitrite (SN) or sodium lactate (SL) stored at 4 °C. Values are means of 3 replicates ± standard deviation.

Intermediate type	Storage time	Treatment	Moisture content (%)	Aw (-)	pH (-)
Paste	0 weeks	Without additive	66.77 ± 0.34^{x}	0.99 ± 0.00^{x}	7.03 ± 0.01^{x}
	1 week	Without additive	$66.82 \pm 0.41^{a,x}$	$0.99 \pm 0.00^{a,x}$	$6.94 \pm 0.02^{a,x}$
		SN	$66.74 \pm 0.45^{a,x}$	$0.98 \pm 0.00^{b,y}$	$6.96 \pm 0.00^{a,x}$
		SL	$65.57 \pm 0.14^{b,y}$	$0.98 \pm 0.00^{a,b,x}$	$7.10 \pm 0.02^{b,x}$
	2 weeks	Without additive	$67.27 \pm 0.30^{a,x}$	$0.99 \pm 0.00^{a,x}$	$6.56 \pm 0.20^{a,y}$
		SN	$66.97 \pm 0.15^{a,x}$	$0.99 \pm 0.00^{a,x}$	$6.71 \pm 0.01^{a,y}$
		SL	$65.74 \pm 0.11^{b,y}$	$0.98 \pm 0.00^{a,x}$	$7.05 \pm 0.03^{b,x}$
	3 weeks	Without additive	$67.29 \pm 0.25^{a,x}$	$0.99 \pm 0.00^{a,x}$	$6.53 \pm 0.22^{a,y}$
		SN	$67.09 \pm 0.40^{a,x}$	$0.99 \pm 0.00^{a,x}$	$6.68 \pm 0.11^{a,b,y}$
		SL	$65.68 \pm 0.26^{b,y}$	$0.99 \pm 0.00^{a,x}$	$6.91 \pm 0.05^{b,y}$

n.d.: not determined.

^{a,b} Mean values per storage time with the same superscript are not statistically different ($P > 0.05$).

^{x,y} Mean values per treatment type with the same superscript are not statistically different ($P > 0.05$).

Table 3: Peroxide and *p*-anisidine values of pastes prepared from steamed mealworms without additive, with sodium nitrite (SN) or sodium lactate (SL) stored at 4 °C for three weeks and at -21°C for three months. Values are means of 3 replicates ± standard deviation.

Intermediate	Storage time and temperature	Treatment	Peroxide value (meq.O_2/kg fat)	*p*-anisidine value (-)
Paste	0 weeks	Without additive	< LOD^{x}	1.36 ± 0.03^{x}
	3 weeks (4°C)	Without additive	< LOD^{a,x}	3.05 ± 0.91^{a,y}
		SN	< LOD^{a}	9.24 ± 0.42^{b}
		SL	< LOD^{a}	8.18 ± 0.82^{b}
	3 months (-21°C)	Without additive	< LOD^{x}	4.03 ± 0.32^{y}
		SN	n.d.	n.d.
		SL	n.d.	n.d.

LOD = Limit of Detection (0.5 meq. O_2/kg fat).

n.d.: not determined.

^{a,b} Mean values per storage time with the same superscript are not statistically different (P > 0.05).

^{x,y} Mean values per treatment with the same superscript are not statistically different (P > 0.05).

Table 4: Total colour difference with the initial value (ΔE*) and browning index (BI) of pastes prepared from steamed mealworms without additive, with sodium nitrite (SN) or sodium lactate (SL) stored at 4 °C for three weeks and at -21°C for three months. Values are means of 3 replicates ± standard deviation.

Intermediate	Storage time (and temperature for pastes)	Treatment	ΔE* (-)	BI (-)
Paste	0 weeks	Without additive	-	$52.85 \pm 0.96^{a,x}$
		SN	-	$52.85 \pm 0.96^{a,x}$
		SL	-	$52.85 \pm 0.96^{a,x}$
	1 week (4°C)	Without additive	$3.38 \pm 0.33^{a,x}$	$41.11 \pm 0.86^{a,y}$
		SN	$3.61 \pm 0.49^{a,x}$	$41.62 \pm 0.09^{a,b,y}$
		SL	$3.27 \pm 0.07^{a,x}$	$43.27 \pm 0.82^{b,y}$
	2 weeks (4°C)	Without additive	$3.19 \pm 0.53^{a,x}$	$42.58 \pm 0.61^{a,y}$
		SN	$3.78 \pm 1.40^{a,x}$	$42.81 \pm 1.05^{a,y}$
		SL	$2.84 \pm 0.10^{a,x}$	$44.96 \pm 0.34^{b,z}$
	3 weeks (4°C)	Without additive	$2.98 \pm 0.53^{a,x}$	$43.04 \pm 1.57^{a,y}$
		SN	$4.79 \pm 1.42^{a,x}$	$41.88 \pm 1.75^{a,y}$
		SL	$2.96 \pm 0.28^{a,x}$	$43.61 \pm 0.30^{a,y,z}$
	1 month (-21°C)	Without additive	3.34 ± 0.01^{x}	41.81 ± 0.50^{y}
		SN	n.d.	n.d.
		SL	n.d.	n.d.
	2 months (-21°C)	Without additive	3.87 ± 0.34^{x}	41.03 ± 0.81^{y}
		SN	n.d.	n.d.
		SL	n.d.	n.d.
	3 months (-21°C)	Without additive	3.03 ± 0.40^{x}	42.37 ± 1.17^{y}
		SN	n.d.	n.d.
		SL	n.d.	n.d.

n.d.: not determined.

^{a,b} Mean values per storage time with the same superscript are not statistically different ($P > 0.05$).

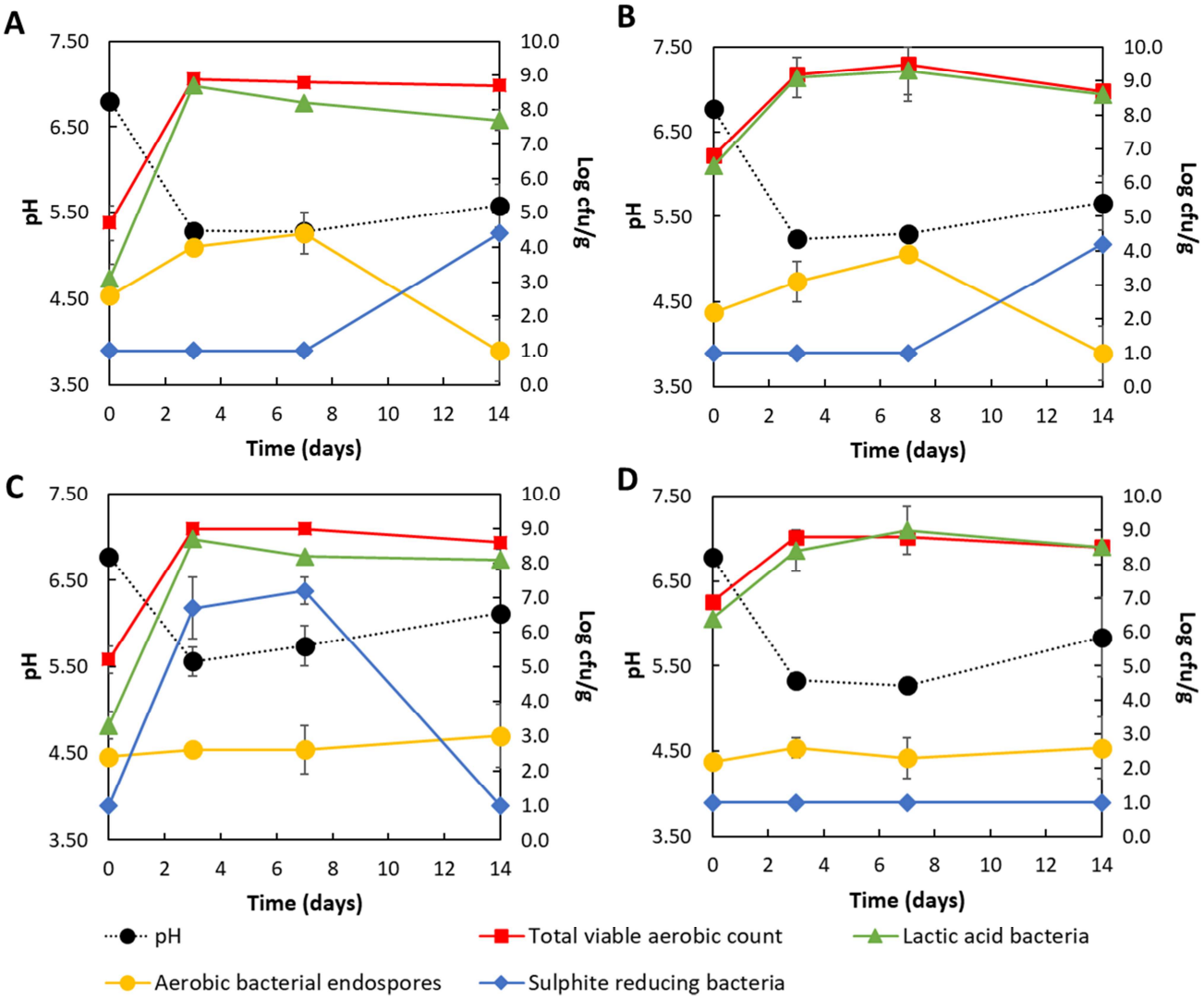
^{x,y,z} Mean values per treatment with the same superscript are not statistically different ($P > 0.05$).

Table 5: Microbiological counts of untreated mealworms, steamed mealworms and freshly prepared paste, and counts of paste without preservative, paste with sodium nitrite (SN) and paste with sodium lactate (SL) during storage at 4 °C. Values are means of 3 replicates ± standard deviation.

Storage time	Treatment	Microbial counts (log cfu/g)					
		Total aerobic count	Total anaerobic count	Psychrotrophic count	Aerobic endospores	Anaerobic endospores	*Entero-bacteriaceae*
0 weeks	None	8.1 ± 0.1^{a}	8.0 ± 0.0^{a}	6.7 ± 0.2^{a}	3.1 ± 0.5^{a}	1.5 ± 0.4^{a}	6.9 ± 0.2^{a}
	Steamed	2.9 ± 0.5^{b}	2.3 ± 0.5^{b}	1.3 ± 0.2^{b}	1.9 ± 0.3^{a}	1.1 ± 0.1^{a}	1.3 ± 0.2^{b}
	Paste without additive	$3.6 \pm 0.6^{b,x}$	$3.8 \pm 1.3^{b,x}$	$2.2 \pm 0.5^{b,x}$	$2.1 \pm 0.0^{a,x}$	$1.2 \pm 0.1^{a,x}$	$2.2 \pm 0.5^{b,x}$
1 week	Paste without additive	$6.4 \pm 0.1^{a,y}$	$5.8 \pm 0.7^{a,y}$	$7.0 \pm 0.4^{a,y}$	$1.8 \pm 0.6^{a,x}$	$1.2 \pm 0.1^{a,x}$	$3.4 \pm 0.9^{a,y}$
	Paste with SN	$6.5 \pm 0.0^{a,y}$	$6.5 \pm 0.0^{a,y}$	$6.8 \pm 0.3^{a,y}$	$1.6 \pm 0.4^{a,x}$	$1.3 \pm 0.2^{a,x}$	$1.9 \pm 1.1^{a,x}$
	Paste with SL	$5.9 \pm 0.7^{a,y}$	$5.9 \pm 0.6^{a,x}$	$6.3 \pm 1.0^{a,y}$	$2.2 \pm 0.1^{a,x}$	$1.0 \pm 0.0^{a,x}$	$4.1 \pm 1.0^{a,x}$
2 weeks	Paste without additive	$8.7 \pm 0.3^{a,z}$	$8.2 \pm 0.5^{a,z}$	$8.7 \pm 0.3^{a,z}$	$1.9 \pm 0.4^{a,x}$	$1.2 \pm 0.3^{a,x}$	$7.3 \pm 0.4^{a,z}$
	Paste with SN	$8.6 \pm 0.1^{a,z}$	$7.9 \pm 0.7^{a,y}$	$8.6 \pm 0.0^{a,z}$	$2.2 \pm 0.1^{a,x}$	$1.3 \pm 0.2^{a,x}$	$4.7 \pm 0.6^{a,y}$
	Paste with SL	$8.3 \pm 0.7^{a,z}$	$7.3 \pm 0.1^{a,y}$	$8.2 \pm 0.6^{a,z}$	$2.0 \pm 0.2^{a,x}$	$1.2 \pm 0.2^{a,x}$	$4.8 \pm 1.8^{a,x,y}$
3 weeks	Paste without additive	$9.2 \pm 0.4^{a,z}$	$8.1 \pm 0.4^{a,z}$	$9.1 \pm 0.5^{a,z}$	$2.7 \pm 0.2^{a,x}$	$1.3 \pm 0.2^{a,x}$	$6.8 \pm 0.9^{a,z}$
	Paste with SN	$8.8 \pm 0.5^{a,z}$	$8.6 \pm 0.6^{a,y}$	$8.8 \pm 0.5^{a,z}$	$2.3 \pm 0.1^{a,x}$	$1.2 \pm 0.2^{a,x}$	$2.8 \pm 0.9^{b,x}$
	Paste with SL	$8.6 \pm 0.2^{a,z}$	$8.3 \pm 0.4^{a,y}$	$8.5 \pm 0.2^{a,z}$	$2.6 \pm 0.2^{a,y}$	$1.2 \pm 0.2^{a,x}$	$6.7 \pm 0.7^{a,y}$

[a,b] Mean values per storage time with the same superscript are not statistically different ($P > 0.05$).

[x,y,z] Mean values per treatment type with the same superscript are not statistically different ($P > 0.05$).


A
B
C
D
pH
Log cfu/g
Time (days)
7.50
6.50
5.50
4.50
3.50
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
2
4
6
8
10
12
14
pH
Total viable aerobic count
Lactic acid bacteria
Aerobic bacterial endospores
Sulphite reducing bacteria

Highlights

- Mealworm paste without exoskeleton particles can be produced in industrial equipment.
- Storage at -21°C ensures colour, viscosity and microbial stability for 3 months.
- Storage at 4°C does not ensure microbial stability during 3 weeks of storage.
- The two tested preservatives did not increase the microbial stability of the paste.
- The mealworm paste can be fermented using a commercial meat starter culture.