Journal of Stored Products Research 102 (2023) 102095

Contents lists available at ScienceDirect

Journal of Stored Products Research

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

Impact of red flour beetle infestations in wheat flour and their effects on dough and bread physical, chemical, and color properties

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ARTICLE INFO

Handling Editor: Dr Christos Athanassiou

Keywords:
Red flour beetle
Wheat flour
Bread quality
Waste

ABSTRACT

The impact of *Tribolium castaneum* on the quality and technological suitability of bread-making wheat flour was evaluated. The aim of this study is to investigate whether insect-infested flours can be used, after pest removal, avoiding the common use of insecticides and flour waste. The tests were carried out with wheat flour infested red flour beetle with 500 adults/kg (N_1), 1000 adults/kg (N_2) and 2000 adults/kg (N_3), for two weeks. Flour color, total starch, protein and water contents, mineral composition and flour acidity were studied. The technological properties of the respective doughs and of bread were characterized. The results showed that infested flours acidity was significantly higher (N_1, N_2 and N_3 = 0.3 g H_2SO_4/100 g) than the control (0.1 g H_2SO_4/100 g), the total starch content decrease, from 78 g (control) to 70 g/100 g dm (N_3), and protein content did not significantly change, 7 g/100 g (control) to 8 g/100 g (N_2); flour infested showed darker greyish tone which colour differences ΔE higher than 5, while the bread crust was lighter; the gelatinization properties of starch were slightly influenced by degree of infestation with gelatinization temperature reduction of about 5°c. There was not impact on the structure of the doughs from infested flours measured by rheology. The extensibility of the doughs, before and after fermentation, was moderately affected by the insects. For the respective breads, they kept their softness for longer, without significant volume change. These results encourage insect tolerance on grains and derivatives, avoiding chemical toxic insecticides and food waste. This pretends to be a contribution to pest management and decision support systems, prevention, and control of losses, relevant subject to stored products.

1. Introduction

Cereals are a basic ingredient in the food supply chain that should be continuously available to the world population (McKevith, 2004). However, data obtained before COVID-19, estimated that more than 690 million people are chronically malnourished (Le Mouël and CForslund, 2017; FAO and UNICEF, 2022). By 2050, a significant increase in the world's population is expected and food supply will have to increase by 50% compared to current volume to meet projected demand (Alexandratos and Bruinsma, 2012; Bond et al., 2013, FAO and UNICEF, 2022). Climate change, land and water availability and other environmental and social factors present major constraints to safeguarding global food security (Wheeler and von Braun, 2013; Willett et al., 2019). With the COVID-19 pandemic there has been a worsening of food prices and disruptions in the supply chain. The European recent conflict between two majors agricultural suppliers, Russia-Ukraine, has also international repercussions on cereals trading, namely in the Middle East and North Africa, where food shortages are common and depend on grain imports (Geng et al., 2022; Hassen and El Bilali, 2022; Jagtap et al., 2022). This "noise" is threatening the achievement of the Sustainable Development Goals (SDGs), notably SDG 1 (No poverty), SDG 2 (zero hunger) and DG 12 (Responsible Consumption and Production) (Mundial, 2012; FAO, 2017; FAO et al., 2017; UN, 2015; Save Food, 2015; Hassen and El Bilali, 2022). Measures that can be taken are to reduce livestock production and losses. As animal protein and B-complex is essential to ensure the balance of human health, alternatives have already been studied that can compensate for meat shortages such as entomophagy, introduction of insects into animal and human diets. As far as losses are concerned, increasing production, *per si,* is not enough if reduce losses and waste along the whole value chain are not reduced. Regarding the storage period of cereals, one of the main causes of losses are insect pests. To suppress insects, the most widely combat method used is the chemical insecticides. The Directive 2009/128/EC of the European Parliament and the Council of 21 October 2009 adopted on 12 February 2019 in Strasbourg established a plan for the use of sustainable pesticides and encouraged the Commission and the Member States to take a set of

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https://doi.org/10.1016/j.jspr.2023.102095
Received 3 November 2022; Received in revised form 30 January 2023; Accepted 9 February 2023
Available online 9 March 2023

preventive and innovative measures to minimize dependence on pesticides (Regulation, 2015/2283, 2015).

There are insects' pests associated with stored cereals that belong to the same Family of insects for human consumption, with special emphasis for the red flour beetle *Tribolium castaneum* Herbst. This is a coleopteran which is a Tenebrionidae as the mealworm *Tenebrio molitor* L. and the lesser mealworm *Alphitobius diaperinus* Panzer, both associated with stored cereals and derivatives, the last one classified as pest. Being of the same family these insects' species have similar physiologies favorable for entomophagy. *T. castaneum* is a pest of processed cereals and flours that easily adapts to new conditions, reproducing large populations and with the ability to be resistant to most classes of pesticides (Venkatrao et al., 1960; Smith et al., 1971; Zettler and Cuperus, 1990; Zettler 1991; Owens, 2001; Hagstrum and Subramanyam. 2009; Opit et al., 2012; Upadhyay et al., 2018). Being an insect very difficult to eradicate and familiarly close to edible insects we analyzed the impact on flour quality of its presence. The adults of the red flour beetle produce 1.4 benzoquinone, methyl-1,4-benzoquinone and ethyl-1, 4-benzoquinone and these compounds act as an external defense mechanism, used as repellents and irritants, against microorganisms, including antifungal and predator properties (Padin et al., 1997; Akbar et al., 2004; Yezerski et al., 2007 Lord, 2007; Pedrini et al., 2015; Duarte et al., 2022). Duarte et al. (2022) demonstrated that benzoquinones produced by adults of *T. castaneum* have repellent action, slowing the growth of fungi such as Aspergillus flavus, A. fumigatus, *A. niger*, Fusarium sp. and fungicide against Penicillium spp. The constrain is whether we are faced with of mycotoxigenic strains of fungi that in the presence of infestations can produce mycotoxins (Duarte et al., 2021a). The toxicity of benzoquinones is not classified as carcinogenic towards humans and requires further research (IARC, 1999). Tonk et al. (2015) reported antimicrobial activity based on, phylogenetic and structural properties of three defensive genes (Def_1, Def_2 and Def_3) and their relevance in the immunity of *T. castaneum* against the pathogens of gram-positive bacteria. On other hand Yokoi et al. (2012) found antimicrobial peptide (AMPs) genes in *T. castaneum* that play a prominent role against gram-negative species, as *Escherichia coli* and *Enterobacter cloacae*. Finally, adding also the nutritional and chemical composition of different stages of life of *Tribolium castaneum*, Duarte et al. (2021b) demonstrated that this species has a similar composition to *T. molitor*.

Although FAO encourages entomophagy using commodity pests (van Huis et al., 2013), our study does not intend to consider *T. castaneum* as edible but to study the impact of red flour beetle adults' infestation on rheology and technological properties of wheat flour for bread making, and to add information from previous experiments. Few authors have already done studies on the effects of *Tribolium* spp infestations on wheat for baking, and bread itself. They started with low populations and analyzed the flours after 1, 2 and 3 months Özkaya et al. (2009) and 5 months Smith et al. (1971). The changes damage on grains, wheat flour losses, moisture content, protein, gluten, ash contents and bread volume were related to storage time, degree of infestation and temperature. *Tribolium* spp showed an increase in reproduction rate, as after 3 months reached more than 2000 insects/kg while *T. molitor* showed no reproduction therefore its results were similar to the control (Smith et al. 1971, 1971; Özkaya et al., 2009; Lü et al., 2022).

In this work, moisture, acidity, total content of starch and protein content, mineral composition, and color of the flour, the, were studied. The viscoelastic and technological properties of the respective doughs, fermented and non-fermented, were evaluated. The volume, and specific volume, specific weight, initial firmness and hardening rate, the color, crust, and crumbs of the breads were characterized.

These experiments are a contribution to more information that complements previous studies, as some of these analysis were made for the first time. These characteristics were evaluated to support evidence for the rational use of cereals and derivatives, reusing of flour partially infested for baking as a guarantee of access to food from emerging countries or suffering chronicum famine. This is also a contribution to base-knowledge for decision-making and innovative measures to minimize pesticide dependence and loss reduction.

2. Materials and methods

2.1. Insects mass rearing

Tribolium castaneum insects, used in the trials, were obtained from wild populations caught by pitfall traps located in stores of rice mills and other stored grains, reared, and kept in cultures for less than 5 years at Entomology Laboratory of the Department of Biosystems Sciences and Engineering (DCEB) of ISA, University of Lisbon. The cultures were maintained under 30 °C ± 1 °C and 70% ± 5% r.h., in a 95:5 proportion of wheat flour and yeast (*Saccharomyces cerevisiae* Hansen), according to Haines (1991).

For mass rearing of *T. castaneum* insects, 10 glass jar were used. In each glass jar, it was introduced 50 g wheat flour for baking bread more 100 adults and maintained at the conditions previously mentioned. After two weeks, the adults were removed, from the flour, by sieving with 800 μm mesh. These ten replications of sifted flour remained five weeks and the adults of the first progeny were all removed, using the same method. These adults were used for experiments (Fig. A.1. Appendix).

2.2. Wheat flour infestation

For the bioassays, the wheat flour for baking bread (Granel Moagem de Cereais, S.A., Portugal) was selected, resulting from recent milling of healthy, ripe, and non-germinated wheat grains. According to the technical sheet, delivered by the company, maximum moisture content of 14.5% is expected, a minimum percentage of gluten in dry matter of 8%, total ash between 0.61% and 0.75%, as well as a maximum acidity of 0.100 g $H2SO_4$/100 g for the purpose of this flour *Portaria* n° 254/2003, March 19' (D.R. 2003).

Wheat flour was exposed to three levels of infestation by red flour beetle adults: N_1. 500 insects/kg; N_2. 1000 insects/kg and N_3. 2000 insects/kg and control (C) wheat flour without insects.

For each level of infestation and for the control, four replications per treatment were made, each replication containing 500 g of wheat flour and the respective number of insects, according to the categories described above. The flasks were maintained in a climatic chamber (30 °C ± 1 °C of temperature and 70% ± 5% of relative humidity) for two weeks.

At end of the two weeks of exposure, the content of the flasks was divided into two samples of 250 g and transferred to glass vials, transforming four replications of 500gr into eight replications of 250 g per degree of infestation. The samples were frozen for 24 h at −18 °C, to eliminate living insects. After this, the content of each flask was sieved using 800 μm mesh to remove all the insects and the sift flour used for characterization studies (Figure A2. Appendix).

2.3. Flour characterization

The moisture content of the samples was measured using a PMB202 moisture meter (Adam, UK). Five gram of wheat flour were used for each reading, and measurements and three replications per treatment.

The method described in NP 2967 (NP, 2008) was used to determine the acidity of the flour, through the preparation of flour extract in ethanol, which was titrated, using 0.05 N NaOH, (NP 2967. C410/CT 41 NP 2967:1991) after filtration.

To determine the amount of total starch present in the different samples (C,N_1, N_2, N_3), the Total Starch kit (Megazyme, Ireland) was used, and the procedure for the determination of the total starch content, in samples containing resistant starch, was followed.

Sample incubations were performed in a Model 2871 bath (Thermo Scientific, USA). Centrifugations were done in Espresso Centrifuge equipment (Thermo Scientific, USA). Readings of the absorbances of

glucose present in the final solutions were taken on a Cary Series UV–Vis spectrophotometer (Agilent Technologies, USA). The conversion of glucose to starch was performed using the calculations described in the kit.

For protein determination, the processes were based on the Kjeldahl method (AOAC Official Method 2001.11; 2011; Thiex and Manson, 2002), which allows the estimation of protein content from the determination of nitrogen present in a sample. For conversion of nitrogen to protein a factor of 5.7 was used (Boulos et al., 2020).

For mineral content determination, 0.3 g of flour belonging to each category, in triplicate, were digested in a solution composed of 3 mL of a solution of 65% nitric acid (Sigma-Aldrich, USA) and 9 mL of a solution of 37% hydrochloric acid (Chem-Lab, Belgium). Digestion took place in a DigiPrep, MS 50 ml 48 Pos digester (SCP Science, Canada) for 1 h at 105 °C. After digestion, the volumes of the solutions were adjusted to 50 ml. The solutions were homogenized and allowed to settle. About 10 ml were collected from each solution, to be read in the Inductively Coupled Plasma (ICP) equipment (Thermo Fisher, USA) (Figure A1, Appendix).

All these procedures were performed in triplicate for each category.

2.4. Starch properties and rheology assessment of doughs

Rheology and technological properties of the doughs produced from the infested flours, and the behavior of the starch during the application of thermomechanical conditions, were evaluated using micro-doughLAB 2800 equipment (Perten Instruments, Australia). The mixing conditions and temperature were adapted, according to the following technique: homogenization for 30 s, introduction of water, mixing at 30 °C for 330 s, heating to 90 °C for 900 s (constant rate), temperature stabilization at 95 °C for 400 s, and cooling from 95 °C to 50 °C in 600 s (constant rate), keeping the final temperature at 50 °C for 240 s. The rotation speed of the blades was 63 rpm in the first 30 s and afterwards it was 120 rpm. The torque value for the adjustment of the water absorption WA (%) parameter was 130 ± 5 mN m; the hydration of the masses considered the initial moisture of the samples. Five replications were done for each different treatment.

The parameters described were measured, as follows: WA (%), dough development time DDT (min), dough stability time DST (min), dough softening degree DSD (mN.m), and the dough consistency at different places of the graphic: C1 (mN.m; maximum consistency at 30 °C), C2 (mN.m; minimum consistency before gelatinization), C3 (mN.m; starch gelatinization point), TG (°C; gelatinization temperature), C4 (mN.m; minimum consistency after gelatinization) and C5 (mN.m; final consistency) (Figure A3 Appendix).

2.5. Method for dough preparation

To obtain doughs, from infested flour (N1–N3) and non-infested (C), the formulation used was: 100 g of flour (for each treatment and control, with three replications), 1.7 g of salt and, in the case of fermented doughs, 4.0 g yeast. Water was added according to the results determined in the micro-dough LAB 2800 (PerkinElmer, USA) for water absorption: about 52.0 ± 1.0 g, except for N1 flour that needed 54.0 g of water.

For the fermented doughs, water, and yeast were placed in the cup of the Thermomix TM31 equipment (Vorwerk and Co., Germany), to stimulate yeast activity at 37 °C, using speed three for 30 s and then adding the 100 g flour sample and the salt. After mixing each dough was placed in an XFT133 fermentation oven (Unox, Italy), for 1 h, at a temperature of approximately 35 °C (Fig.A.3 Appendix).

For the unfermented doughs, water, salt, and wheat flour were added at the same time. A progressive increase in speed was performed, until reaching speed six, making a mixing time of 60 s. Later, the spike mode was used for 120 s.

The doughs intended for the extension tests were given the form of strips, using a press and oiled mold. The doughs used in the rheometer tests were shaped into balls about 1–2 cm in diameter.

Unfermented doughs were identified as C (control dough), N_1, N_2, and N_3 (levels 1, 2 and 3 of infestation, as previously described). The fermented doughs were identified as FC (control fermented dough), FN_1, FN_2, and FN_3. Each dough was produced in duplicate.

2.6. Dough extension properties

The extension properties of the previously prepared doughs (non-fermented: CD, DN1, DN2, DN3; and fermented: CFD, FDN1, FDN2, FDN3) were evaluated through uniaxial extension tests, using a Kieffer Dough and Gluten Extensibility Rig probe, coupled to a TA.XTplus texturometer (StableMicroSystems, UK) with a 5 kg load cell, and according to the method described in Bureova et al. (2014). Five replications were performed, for each treatment. The test conditions limited the test speed to 1.0 mm/s, and the maximum distance the probe could travel to 35 mm. The parameters evaluated were: ME - maximum extension (mm), EMR - extension at the point of maximum resistance (mm), MR-maximum resistance (N) and DE - deformation energy (N.s).

2.7. Analysis of the fundamental rheology properties of the doughs

The fundamental viscoelastic behavior of the doughs (CD, DN1, DN2, DN3, CFD, FDN1, FDN2 and, FDN3) was evaluated by small-amplitude oscillatory shear (SAOS) measurements using a Haake Mars III

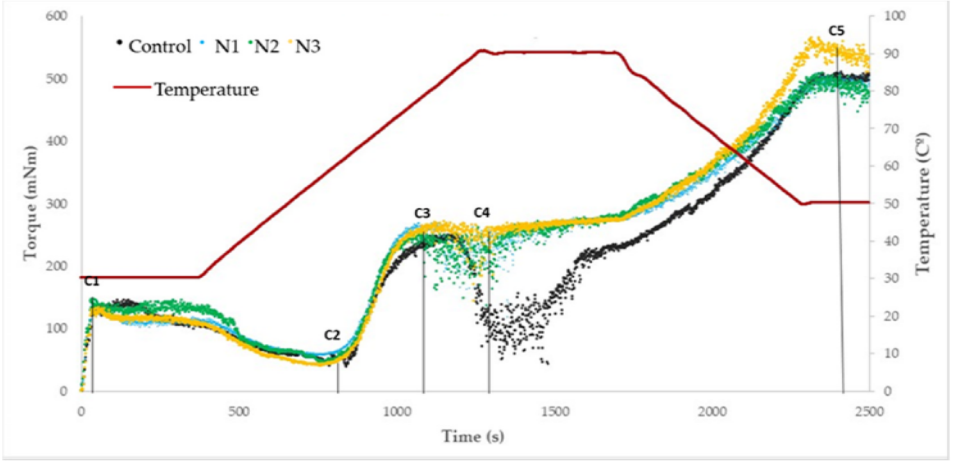

Fig. 1. Kneading and pasting profiles of the flours infested with *Tribolium castaneum* (N1 = 500 insect/kg; N2 = 1000insect/kg; N3 = 2000 insects/kg) and control sample (C) and from the control, in a MicrodoughLAB (C1 (mN.m; maximum consistency at 30 °C), C2 (mN.m; minimum consistency before gelatinization), C3 (mN.m; starch gelatinization point), C4 (mN.m; minimum consistency after gelatinization) and C5 (mN.m; final consistency)).+ Torque is the output of the equipment directly related to dough consistency; N1 = 0.5 insect/g; N2 = 1 insect/g; N3 = 2 insects/g.

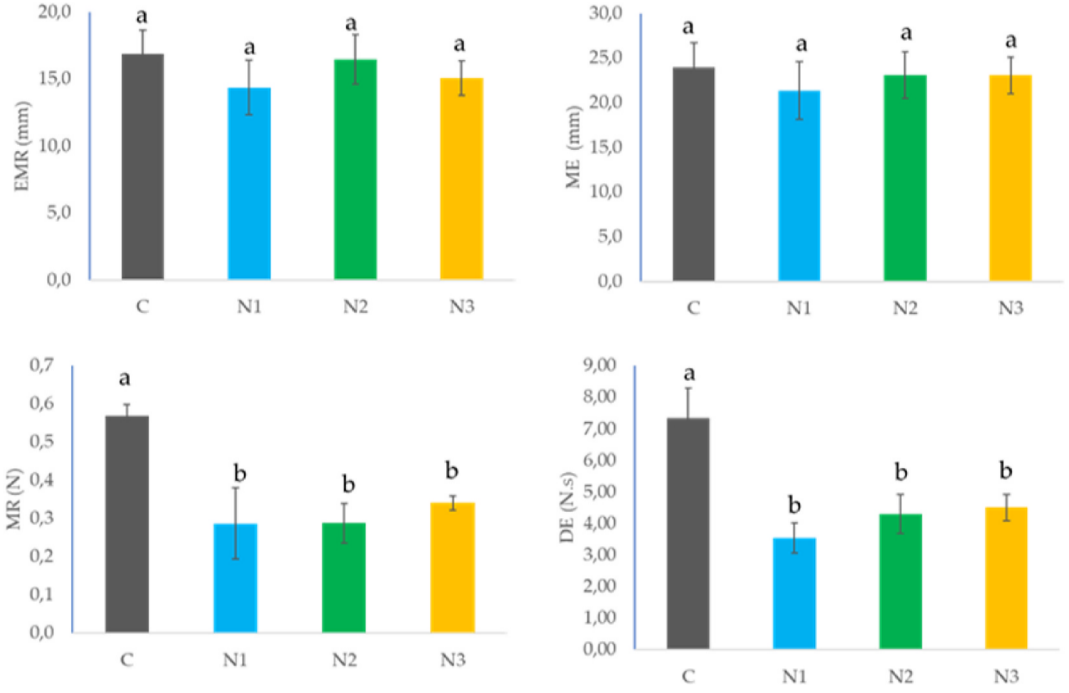

Fig. 2. Extension properties of the doughs produced with flours infested with *Tribolium castaneum* (N1 = 500 insect/kg; N2 = 1000insect/kg; N3 = 2000 insects/kg) and control sample (C) and of the control dough (C): EMR - extension at the point of maximum resistance (mm); ME - maximum extension (mm); MR-maximum resistance (N); DE - deformation energy (N.s). *Different letters above the columns correspond to significantly different values ($p < 0.05$) between samples.

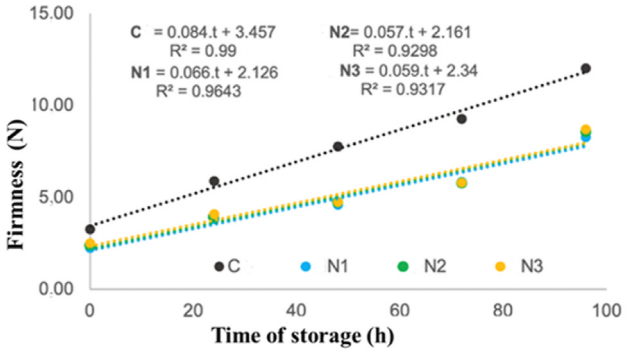

Fig. 3. Linear regressions relative to hardening over 96 h, of breads produced with flours infested with *Tribolium castaneum* (N1 = 500 insect/kg; N2 = 1000insect/kg; N3 = 2000 insects/kg) and control sample (C).

rheometer (Thermo Scientific, USA) with a universal Peltier temperature controller. A parallel plate sensor system (PP20), and 1 mm gap, were used. Stress sweeps were carried out to determine the linear viscoelastic zone of the doughs and decide the stress to be used in the frequency sweep tests. The frequency scanning tests were performed after the dough had stabilized for 5 min, and a temperature of 5 °C was maintained to inhibit the fermentative activity in the doughs during the testing. Stress sweeps were performed in duplicate, and frequency sweeps were in triplicate. After being placed in the equipment, the dough portions were covered with liquid paraffin, to avoid dehydration.

2.8. Doughs' pH

The pH was determined using pH Basic 20 equipment, CRISON (Crison Instruments, SA, Spain) for the fermented, and non-fermented doughs. The determination was carried out by perforating the dough with the direct measuring electrode. Five replications per dough were performed. The equipment calibration was conducted with the solid body electrode, and with the respective pH 4 and pH 7 buffer solutions.

2.9. Breadmaking process and bread characterization

Loaves of bread were prepared from the infested (N_1, N_2, N_3) and non-infested (C) flours previously obtained following a standardized receipt commonly used in our laboratory. The impact of *T. castaneum* infestations on the flours' baking quality was evaluated through the characterization of its hardening, the quantification of its total and resistant starch values. Instrumental evaluation of the color of the crumb and crust of the loaves of bread and the measurement of its volume and density were also conducted.

For bread production to 300 g of different treated flours, water, according to WA (water absorption) previously determined, 12.0 g of baker's yeast, and 3.0 g of sugar were placed in the Thermomix TM31 cup and stirred at speed three for 30 s at 37 °C to activate the yeast. The flour sample and salt were added, and the mixing speed was progressively increased to speed six, for the homogenization to proceed for 60 s. The spike mode was used for 120 s.

A rectangular mold with the dimensions of 25.5 × 12.0 × 6.5 cm3 was oiled and floured. The dough was placed inside the mold for fermentation, at 35 °C for 1 h.

After fermentation, the dough was placed in an XFT133 convection oven (UNOX, Italy), at 160 °C, for 30 min. After baking, the bread was removed from the oven and was allowed to cool for 2 h. This procedure was replicated for each level of infestation.

2.10. Bread volume

The volume of the loaves of breads was measured according to the international standard Baking Quality AACC International Method 10–05.01 (AACC 10–05.01, 2001). A metal box (52 × 20 × 10 cm3) was used, where the bread was placed and filled to the top with rapeseed. Triplicate measurements were taken for each bread. The loaves of bread were weighed, and the density and the specific volume were calculated.

2.11. Firmness of bread

The firmness of the loaves of bread was assessed using a TA. XTplus texturometer (StableMicroSystems, UK) in the penetration mode according to the procedure described by (Graça et al., 2018).

The loaves of bread were cut into slices 20 mm thick, allowing each slice to rest for 15 min before testing. In the tests, a 10 mm diameter cylindrical acrylic probe was attached to the texturometer. Penetration into the bread slices took place to a depth of 5 mm, at a speed of 1 mm/s, using a 5 kg load cell. The firmness (N) of each reading corresponded to the point of highest resistance offered by the sample to the probe movement.

Measurements were taken on the day of bread making, as well as on the following four days, at the same hour. Per day, two slices of each of the loaves were used, and three readings were taken per slice. This entire procedure was performed in duplicate.

The hardening of each loaf was characterized by the linear equation (1)

$$\text{Hardness} = A \times t + B \quad (1)$$

where A is to be interpreted as the hardening rate (N/h), t as the time (h), and B as the initial firmness (N).

2.12. Instrumental evaluation of the color of flour and bread

The instrumental evaluation of the flour, and the crumb and crust of the loaves of bread was conducted using a CR-400 colorimeter (Konica Minolta, Japan) using the Lab* CIELab coordinates.

The luminosity L* ranges from 0 (dark) to 100 (light); the a* coordinate ranges from 60 (red) to −60 (green); the b* coordinate varies between 60 (yellow) and −60 (blue). The colorimeter was calibrated with a white standard (L* = 97.21; b* = 0.14; a* = 1.99).

The total color difference between flours (ΔE) is relative to the difference between the color of the control sample (C) and the other samples (N_1, N_2, N_3), and was calculated by equation (2)

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

2.13. Statistical analyses

The statistical analyses of the results were performed using the SPSS program (IBM, USA), using an analysis of variance (ANOVA), and applying the Tukey's Honest Significant Difference test, for a significance level of 95% ($p < 0.05$), to verify the existence of significant differences between the values obtained.

3. Results

3.1. Flour characterization

The moisture, acidity, total starch, and protein contents of the infested wheat flours and control are shown in Table 1. Wheat flour with the highest infestation by *T. castaneum* (N_3) showed a significantly higher moisture content ($p = 0.007$).

When we observe the flours' acidity, infested flours showed, significantly, higher acidity compared to the control ($p < 0.001$), all exceeding the maximum limit defined for quality flour for bread making, 0.1 g H_2SO_4/100 g - The control flour also showed a slightly higher acidity to this limit (Table 1). As insect infestation trials were conducted at 30 °C ± 1 °C and 70% ± 5% RH, for 2 weeks, for insect grow under optimal conditions, this may explain the high levels of acidity, due to lipid oxidation. Total starch in the control flours was significantly higher than in the infested flours ($p = 0.01$) as starch is the preferred source of food for these insects. A slight tendency in protein content increase in infested flours was noticed although it was not statistically significant, this can be explained, first instance, by the presence of residual larvae or eggs left after sieving the insects off the flour.

In Table 2, one can see that there was no variation in mineral composition of the flour with different degree of infestation. In fact, only at the highest level of infestation a slight increase of 5% sulfur was noticed.

The effect of *T. castaneum* infestation on the wheat flours was perceptible to the naked eye, being confirmed by ΔE values obtained by the colorimeter (Table 3), considering differences when ΔE values greater than 5 (ΔE > 5) (Sánchez-Mariñez et al., 1997), that gives the significant differences between treatments when compared with the control. There is a positive relationship between the increase in the level of insect infestation and ΔE > 5. The value of L* tends to decrease, representing a decrease in the luminosity of the flours; the value of a* and b* tend to increase, representing an intensification of colour.

3.2. Starch properties and technological evaluation of flours

Fig. 1 shows the profile of the different doughs analyzed during kneading and pasting conditions. This is the graphic output of the MicrodoughLAB device where resistance of dough against kneading is measured as torque in mN.m. This parameter is related to the dough consistency. There are visually perceptible differences at C_4 (mN.m; minimum consistency after gelatinization) and C_5 (mN.m; final consistency). The parameters evaluated in these tests, including the values of C4 (mN.m; minimum consistency point after gelatinization) and C5 (mN.m; final consistency) are shown in Table 4.

There were no significant differences in the water absorption (WA; $p < 0.001$) of the control and almost all infested flours. Only the less infested samples N1 showed a slightly different value around 4% higher (52.0% compared to 54.0%).

Table 1
Main characterization parameters of flour samples of *Tribolium castaneum*, expressed in dry matter (dm), water content (%), acidity (g H2SO4/100 g), total starch (g/100 g dry mass) and protein content (g/100 g dry mass). Different letters in the same column correspond to significantly different values ($p < 0.05$) between samples.

	Moisture (%)	Acidity (g H_2SO_4/100 g)	Total starch (g/100 g)	Protein (g/100 g)
C	12.4 ± 0.2a	0.1 ± 0.0a	78.2 ± 2.4 b	6.9 ± 0.3a
N_1	12.6 ± 0.3a	0.3 ± 0.0 b	73.0 ± 1.3 ab	7.8 ± 0.0a
N_2	12.8 ± 0.3 ab	0.3 ± 0.0 b	71.5 ± 7.6a	8.2 ± 0.8a
N_3	13.4 ± 0.1 b	0.3 ± 0.0 b	69.5 ± 2.7a	7.6 ± 0.4a

*N1 = 500 insect/kg; N2 = 1000insect/kg; N3 = 2000 insects/kg; C = control.

Table 2
Mineral content (mg/100 mg) of the flours infested by *Tribolium castaneum*. Different letters in the same row correspond to significantly different values ($p < 0.05$) between samples.

Mineral (mg/100 g)	C	N_1	N_2	N_3
Potassium	192.0 ± 2.5a	197.5 ± 4.2a	199.7 ± 1.0a	199.8 ± 2.6a
Phosphorus	112.3 ± 2.4a	112.3 ± 0.7a	112.5 ± 0.8a	114.9 ± 0.8a
Sulfur	101.7 ± 2.7a	102.8 ± 0.3a	104.1 ± 1.0a	**106.1 ± 1.4b**
Magnesium	23.8 ± 0.2a	24.0 ± 1.0a	24.3 ± 0.4a	24.7 ± 0.2a
Calcium	10.7 ± 0.9a	10.4 ± 0.8a	10.6 ± 0.3a	10.6 ± 0.6a
Sodium	2.0 ± 0.6a	1.9 ± 1.2a	1.7 ± 0.3a	1.8 ± 0.5a
Iron	0.9 ± 0.1a	0.9 ± 0.1a	0.9 ± 0.0a	0.8 ± 0.0a
Manganese	0.8 ± 0.0a	0.8 ± 0.0a	0.8 ± 0.0a	0.8 ± 0.0a
Zinc	0.5 ± 0.1a	0.5 ± 0.0a	0.4 ± 0.0a	0.4 ± 0.0a
Copper	<0.1a	<0.1a	**0.1 ± 0.0b**	**0.1 ± 0.0b**
Boron	<0.2a	<0.2a	<0.2a	<0.2a

*N1 = 500 insect/kg; N2 = 1000insect/kg; N3 = 2000 insects/kg; C = control.

Table 3
Instrumental analysis of the color (coordinates L*, a*, b*, and color difference values - ΔE) of the wheat flour samples infested with *Tribolium castaneum* Different Greek letters in the ΔE column correspond to a ΔE > 5 between samples.

	L[a]	a[a]	b[a]	ΔE
C	93.0 ± 7.4	−0.7 ± 0.1	12.2 ± 1.7	–
N_1	80.7 ± 7.0	0.6 ± 0.1	17.6 ± 1.0	12.4_{α}
N_2	72.3 ± 6.3	1.4 ± 0.4	17.5 ± 1.2	21.4_{β}
N_3	68.3 ± 7.5	1.6 ± 0.3	19.5 ± 1.1	25.8_{γ}

[a] N1 = 500 insect/kg; N2 = 1000insect/kg; N3 = 2000 insects/kg; C = control.

Table 4
MicrodoughLAB technological parameters of the wheat flour samples infested with *Tribolium castaneum*. Different letters in the same column correspond to significantly different values (p < 0.05) between samples; water absorption (WA; %), dough development time (DDT; min), dough stability (DST; min), dough softening degree (DSD; mN.m), and the dough consistency at different places of the graphic: C1 (mN.m; maximum consistency at 30 °C), C2 (mN.m; minimum consistency before gelatinization), C3 (mN.m; starch gelatinization point), TG (°C; gelatinization temperature), C4 (mN.m; minimum consistency point after gelatinization) and C5 (mN.m; final consistency).Different letters in the same column correspond to significantly different values (p < 0.05) between samples.

WA	C	N_1	N_2	N_3
	52.1 ± 0.4a	54.0 ± 0.4 b	52.0 ± 0.0a	52.0 ± 0.0a
DDT (min)	2.2 ± 0.3a	1.1 ± 0.1 b	1.7 ± 0.8c	1.0 ± 0.1 b
DST (min)	2.3 ± 0.2a	3.1 ± 1.1a	4.2 ± 0.6 b	3.0 ± 0.7 ab
DSD (mN.m)	36.0 ± 3.5a	19.0 ± 3.7 b	17.6 ± 11.5 b	16.8 ± 4.7 b
C_1 (mN.m)	128.2 ± 8.3a	123.8 ± 3.6a	133.0 ± 5.5a	133.0 ± 6.2a
C_2 (mN.m)	46.2 ± 10.1a	57.3 ± 6.2 b	60.6 ± 9.0a	50.0 ± 7.5a
C_3 (mN.m)	236.8 ± 12.1a	255.3 ± 13.1a	250.0 ± 5.5a	252.5 ± 10.0a
TG (°C)	82.0 ± 1.8a	78.2 ± 1.8 ab	78.2 ± 1.8 ab	77.2 ± 1.7 b
C_4 (mN.m)	73.6 ± 16.3a	224.8 ± 17.0 b	205.0 ± 25.1 b	211.5 ± 21.6 b
C_5 (mN.m)	442.4 ± 31.8a	473.0 ± 35.7a	504.4 ± 47.60 ab	554.0 ± 40.0 b

*N1 = 500 insect/kg; N2 = 1000insect/kg; N3 = 2000 insects/kg; C = control.

When temperature started to rise one can see on Fig. 1 a negative peak marked as C_2 (mN.m; minimum consistency before gelatinization) and no significant differences were found among the flour samples (p > 0.05). This reduction in consistency, is due to effect of temperature in viscosity with increase in molecule mobility until ±60 °C, including protein matrix links disruption. With further temperature increase starch granules start to gelatinise and consistency rises until C_3 (mN.m; starch gelatinization point). At about 90 °C the starch hydrolysis by enzymes starts to decrease the consistency again. there is the major difference in tested flours behaviour, i.e., control shows the highest decrease on consistency at C_4, meaning an important level of hydrolysis, all the infested flours showed a similar pattern, with a slight decrease. This major difference must be related to the fact of infestations, reducing the enzymatic hydrolysis. The consistency recovers and starts building up with cooling and interestingly the highest consistency was reached by the higher infested flour. The gel formed on cooling, by the lixiviated amylopectin molecules, was stronger in flours with higher exposure to insects.

From Table 4, one can be seen that the dough development time (DDT) was higher for the control, which was lower for stability. In addition, softening was higher in the control. Although differences are not dramatic, the infested flours were showing a slightly better performance on kneading. These mixing properties are ruled by gluten, a protein with glutenin and gliadin contents, building the structure of the dough.

3.3. Characterization of viscoelastic properties of doughs

The extension properties of doughs for 60 min, produced from the infested flours are shown in Fig. 2. Regarding the extensibility, at the point of maximum strength and maximum extensibility, no significant differences were identified between the different flours. A significant reduction in the maximum resistance as well as on the maximum strain energy values was detected in doughs from infested flours when compared with the control (p < 0.001) showing an impact on weakening the internal structure, i.e., on the network responsible for dough resistance (elasticity). This may be attributed to the decrease of starch filling of the gluten matrix due to the presence of insect infestation.

The analysis of the fundamental rheological properties of the doughs (fermented and non-fermented) produced from samples C, N_1, N_2, and N_3, showed that all the doughs exhibit the elastic modulus (G′) higher than the viscous one (G″) (Fig.s A.4 and A.5, Appendix), showing that they present a similar behavior to that of a poorly structured gel, typical of wheat flour doughs.

These results reveal that the effect of insects' infestation did not significantly impact the structure of the wheat flour dough, before and after fermentation, or these fundamental tests were not sensitive enough to capture changes that were previously found by technological testing during kneading and pasting conditions in another equipment.

3.4. Evaluation of the quality of bread obtained from infested wheat flours

The quality of the bread can be estimated by comparing volumes, and density (Table 5). Results showed no significant differences in these properties among the different loaves of bread.

Fig. 3 shows simple linear regressions that best represent the evolution of bread firmness over five days. The equations obtained from the linear regressions present R2 >0.90, indicating that more than 90% data fits the tendency.

Concerning the texture of the bread, the initial firmness was significantly higher in the control loaves of bread, compared to the infested ones, as well as the hardening rate, which was significantly higher in control bread, meaning that the loaves from infested flours were significantly softer. Firmness is mostly governed by amylopectin crystallization.

3.5. Evaluation of the bread cross-sections

The impact of *T. castaneum* infestations on the structure and appearance of loaves of breads produced with the infested flours (N_1, N_2, and N_3) was visible through a loss of structure in these loaves (Fig. 4)

Table 5
Parameters related to the physical characterization of breads produced from flours infested by *Tribolium castaneum*. Initial firmness (N), Hardening rate (N/h), Volume (cm3), Specific volume (cm3/g) and Specific Weight (g/cm3). Different letters in the same column correspond to significantly different values (p < 0.05) between samples.

	Quality parameters			Texture	
	Volume	Specific volume	Specific weight	Initial firmness	Hardening rate
	(cm^3)	(cm^3/g)	(g/cm^3)	(N)	(N/h)
C	3.6 ± 0.7a	0.1 ± 0.0a	800.0 ± 14.1a	2.0 ± 0.0 a	0.5 ± 0.0 a
N_1	2.3 ± 0.3 b	0.1 ± 0.0 b	815.0 ± 77.8a	2.0 ± 0.2 a	0.5 ± 0.0 a
N_2	2.6 ± 0.3 b	0.1 ± 0.0 b	860.0 ± 70.7 a	2.1 ± 0.1 a	0.5 ± 0.0 a
N_3	2.4 ± 0.3 b	0.1 ± 0.0 b	835.0 ± 35.4a	2.1 ± 0.1 a	0.5 ± 0.0 a

*N1 = 500 insect/kg; N2 = 1000insect/kg; N3 = 2000 insects/kg; C = control.

Fig. 4. Cross sections of bread produced with flour infested with *Tribolium castaneum* (N1 = 500 insect/kg; N2 = 1000insect/kg; N3 = 2000 insects/kg) and control sample (C).

revealed by bigger pores on larger holes in the crumb.

During fermentation, the N_1, N_2 and N_3 doughs increased in volume faster than the control. They finished the fermentation earlier, showing a comparable volume after 60 min of fermentation for control, 50 min of fermentation for N1, 40 min of fermentation for N2 and 30 min of fermentation for N3 (Fig. 5).

The differences in the fermentation of the loaves of bread, after baking, were enough to create different surfaces tops, with N_1, N_2 and N_3 loaves having rough flat surface and the Control-bread having a smoother and convex surface (Fig. 5). Furthermore, it is possible to observe that C dough did not grow at same rate as others, during fermentation and ended up reaching a lower height. However, after baking, differences in bread volumes were not significant.

3.6. Characterization of the color of the crumb and crust of the bread

Instrumental measurement of crumb and crust color of breads showed significant differences between crumb and crust from infested and control breads, although these differences were more evident in the crust (Table 6). The L* value coordinate increased in the crusts of breads produced from infested flours, which are lighter in color than the control bread ($p < 0.001$ for the crust; $p = 0.004$ for the crumb). The crust of the breads produced with the infested flours also presented higher values of a* and b* coordinates ($p < 0.001$; for both), being closer to the red and yellow tones, when compared to the crust of the control bread.

4. Discussion

Several authors have considered that the presence of insects increases the water content in stored cereals and derivatives (Sokoloff et al., 1966; Sokoloff, 1972; Stejskal and Hubert, 2006; Mohammad et al., 2012; Hemery et al., 2018; Duarte et al., 2021a). When comparing the control samples to the highest infested wheat flour 2000 insects/kg (N_3) there was a slight increase of 1% moisture (13.4 ± 0.1% moisture).

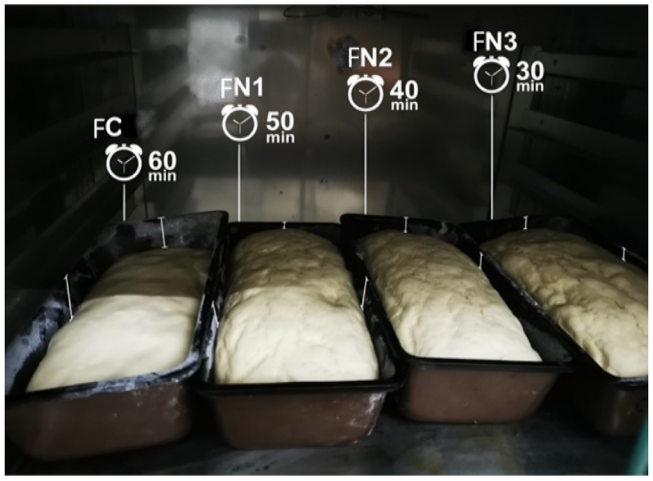

Fig. 5. Bread doughs produced with flour infested by *Tribolium castaneum* (FN1 = 500 insect/kg; FN2 = 1000insect/kg; FN3 = 2000 insects/kg) and control sample (FC) with the indication of the fermentation time of each one.

Table 6
Coordinate values for L*, a*, b*, and ΔE of the color of the crumb and crust of the breads produced from flour infested with *T. castaneum*. Different letters correspond to significantly different values ($p < 0.05$) between samples.

	L[a]	a[a]	b[a]	ΔE
C (crust)	55.8 ± 2.2	0.4 ± 0.4	2.8 ± 1.2	–
N_1 (crust)	62.0 ± 1.9	9.0 ± 0.4	28.8 ± 0.7	29.0c
N_2 (crust)	63.8 ± 2.1	8.2 ± 0.8	28.9 ± 1.0	28.4c
N_3 (crust)	60.8 ± 1.1	8.6 ± 0.7	29.2 ± 1.1	28.1c
C (crumb)	69.8 ± 1.9	−0.9 ± 0.1	17.4 ± 0.6	–
N_1 (crumb)	65.4 ± 4.1	0.5 ± 0.3	19.5 ± 0.8	5.0a
N_2 (crumb)	66.6 ± 2.8	0.8 ± 0.1	20.3 ± 0.6	4.5a
N_3 (crumb)	63.8 ± 0.7	0.9 ± 0.0	19.8 ± 0.1	6.5 b

[a] N1 = 500 insect/kg; N2 = 1000insect/kg; N3 = 2000 insects/kg; C = control.

Still within the legislation requirements that considers the maximum of 14.5% moisture.

As far as acidity is concerned, the legislation allows a maximum limit of 0.1 g H_2SO_4/100 g for flour intended for the bakery industry. All tests, except for the control, and whatever the infestation level, presented 0.3 g H2SO4/100 g, exceeding the allowed value for bread flours. The acidity of cereals and flours can increase with storage time, environmental conditions and insect infestation, assuming the presence of uric acid in flour samples and subsequently the occurrence of hydrolysis of lipids into fatty acids, and protein hydrolysis into amino acids or intermediate products of protein decomposition (Lustig et al., 1977; Sánchez-Martinez et al., 1997; Keskin and Ozkaya, 2015; Venkatrao et al., 1960; and Smith et. 1989). The environmental conditions of experiments were optimized for the development of red flour beetle, 30 °C ± 1 °C and 70% ± 5% r.h., and, consequently, the increase of insects' activity should have contributed to the recorded acidity.

As for the total starch, its decreased was expected with the increase of the insect population as well as other authors recorded in their studies (Lustig et al., 1977; Sánchez-Martinez et al., 1997). Even though significantly different, there was no proportional reduction according to the different degrees of infestation of 500 insects/kg (N_1) up to 2000 insects/kg (N_3), the difference was less than 10 g total starch/100 g (dm).

There was a slight increase tendency in protein content in infested flours, although statistically irrelevant. This can be explained by the residual presence of eggs or even larvae of the first instar that passed in the sieving of flour. But one of the characteristics of the entomophagy on human and animal diets is the fact that insects are a good alternative of animal protein (van Huis et al., 2013; Duarte et al., 2021a).

There were no differences in the mineral composition of the flours between different treatments, except sulfur content, where there was an increase of 5% sulfur in flours infested with 2000 insects/kg (N3). The copper content also changed, but with very small differences. Insects can, in fact, modify the mineral composition of flours. Keskin and Ozkaya (2013) found a significant increase in copper content in wheat flour infested with *Sitophilus granarium* and Duarte et al. (2021a) determined high copper content in adults, and high sulfur content in the larvae of red flour beetles. Higher levels of sulfur (and copper) may be related to excrement and exuviae, resulted from insect activity which also contribute to an increase in ash content (Mehmood et al., 2018).

The infestations of *T. castaneum* and *T. confusum* Duval in flours are usually followed by changes in color and odor, which are mainly attributable to cuticular secretions of adults, for example benzoquinones (Payne, 1925; Loconti and Roth 1953; Sánchez-Mariñez et al., 1997; Ali et al., 2009; Fardisi et al., 2017) corresponding to the results of our experiments.

Dough development time (DDT) and dough softening degree (DSD; mN.m) were higher and dough stability (DST; min) lower in flour control dough. Although differences are not dramatic, the infested flours showed a slightly better performance on kneading. These mixing properties are ruled by gluten, a protein with glutenin and gliadin contents, building the structure of the dough. Previous studies with other insect species, for example Heteroptera species, reported a decrease of gluten proportion by a progressive hydrolysis of the gliadin and glutenin components, altering viscoelastic properties and consequently reducing bread-making quality (Pérez et al., 2005; Yezerski et al., 2007; Mohammad et al., 2012). In these experiments, after removing red flour beetles from the flours, the protein content did not seem significantly affected by the infestation since these insects prefer starch as food.

Starch consists of amyloses and amylopectin, and according to Fredriksson et al. (1998) it is possible to establish a negative relationship between the amylose/amylopectin and starch gelatinization. Starches with a high amylopectin content have a better stable gel than gels with high amylose content. *T. castaneum* and *Sitophilus zeamais* (Motschulsky) (Coleoptera, Curculionidae) easier hydrolyze starches with higher amylopectin contents, having more difficulty to consume amylose (Applebaum and Konijn, 1965; Fredriksson et al., 1998; Antunes et al., 2016).

The significant reduction of maximum strength and maximum energy values in the doughs from infested flours, when compared with the control, showed the impact of the infestations on the weakening of the internal structure, on the network responsible for dough resistance (elasticity) (Mohammad et al., 2012). This can be attributed to the decrease on the starch in the gluten matrix due to the starch ingestion by insects.

The overall results obtained from the analysis of the fundamental rheological properties of the doughs (fermented and unfermented) produced from infested and control flours reveal that the effect of insect infestation had no significant impact on the structure of the dough before and after fermentation.

Related to the texture of the bread, the initial firmness was higher in the control breads and softer in the breads derived from infested flours. Firmness is mostly governed by the crystallization of amylopectin. As some authors have stated that because *T. castaneum* is more efficient in the digestion of amylopectin, consequently there will be a decrease in the amylopectin content in the doughs for bread, less crystallized amylopectin and consequently softer breads, especially on doughs from flours with 2000 insects/kg (N_3) (Fredriksson et al., 1998; Elisena and Eliana, 2007; Antunes et al., 2016). The infestation did not have a significant impact on the final volume, the specific volume and density of the breads, therefore not affecting the quality of the breads.

The differences observed during fermentation, in particular the loss of structure, may indicate an excessive fermentation in breads produced from infested flours. This can be explained by the increase in enzymatic activity, as previously mentioned about the behavior of infested flours. These results are in line with other published studies (Venkatrao et al., 1960; Smith et al., 1989).

Differences on crumb and crust color of breads may have connection with the acidity/pH of the doughs. Ashoor and Zent (1984) and Wolfrom et al. (1974) identified a positive relationship between food browning (a consequence of the intensification of Maillard reactions) and the increase in pH. Since the pH of the control dough is significantly higher than the pH of the doughs produced from the infested flours, it is possible that this is the cause of the lower L* values (corresponding to darker tones) of the control crust.

When excreta and exuviæ are discussed for infested flours, we need to consider that red flour beetle is an external pest and much of the excreta, exuviae and insects, in their different development stages, were sieved and separated from the wheat flours before processing. These external pests are easier to clean when compared with hidden pests as *Sitophilus* spp. and *Rhyzoperta dominica* Fabricius. Singh and Sinha (1977) evaluated nutritional properties of *Sitophilus* spp, finding the high protein content of adults (76%–78% dry weight). These weevils have hidden infestation behavior, develop their entire developmental cycle inside the grain. They come out of the grain when they emerge as adults. In a situation of large infestations of *Sitophilus* spp, fumigation or other control treatments are repeatedly used to kill insects from egg to adult. But the eggs, larvae, pupae, exuviae and excreta will always remain inside the grain. That will then be used for human or animal consumption, especially if the whole grain is consumed such as in rice. Özkaya et al. (2009) studied the debris of an external pest: *T. confusum* Jacquelin du Val, and a hidden pest: *R. dominica*. They found that *T. confusum* produced little amounts of debris (0.17–0.22%), in contrast with R. *dominica* that produced high amounts of debris (11.42–14.65%). Excreta and exuviæ are more pertinent issues for hidden infestations than for external ones.

There were few studies related with odor and taste in the breads, which were detected after four months of infestation period (Özkaya et al., 2009).

In addition, within a circular economy perspective, insects separated from flours can be used as an ingredient in animal feed or organic fertilizers.

5. Conclusions

The present study aimed to evaluate the impact on the quality and performance of wheat flour infested, with an external pest, for making bread. The insect species selected was red flour beetle, that belongs to the family of two of the edible insect' species, and is resistant to almost all classes of insecticides. All experiments used a high number of insects, at adult stage: 500 insects/kg, 1000 insects/kg, and 2000 insects/kg that remained for two weeks before sieving the wheat flour to be processed into bread.

The increase of the moisture on infested flours, is pertinent because the activity of insects enhances water content mainly due to respiration. Although these results showed an increase of moisture, under conditions of greater infestations, the wheat flour kept moisture content values bellow the values allowed by law for wheat flours for bread making. Given that the wheat flour used, already presented (0.1 g H2SO4/100 g) an acidity content close to the limit allowed by law, we consider that more studies should be developed, to analyze if the increase in acidity/pH is related to environmental conditions or to the insect activity. This higher acidity observed (0.3 g H2SO4/100 g) in the infested flours may have influenced the color of the kernels and crust of the breads, mitigating Maillard reactions.

Starch composition consists of amylose and amylopectin, and it is the food basis of red flour beetle, preferably the amylopectin. A decrease in starch supporting the gluten matrix influenced the elasticity of the doughs, and the final breads produced from infested wheat flour are softer. But infested wheat flours did not change the fundamental rheological properties of fermented and unfermented doughs. These results encourage to focus on this thematic.

Today, we face dramatic challenges and is urgent to implement innovative integrated food management and distribution, taking full advantage of commodities and by-products to appease harmful effects for current and future generations. The purpose of this study is to contribute for the knowledge that helps to mitigate the impacts of disruption to the food supply chain, reduce food waste and pesticide use. Since red flour beetle is an insect very difficult to eradicate and familiar to two edible species, more studies should be developed to complement this work and help answer questions such as: is it so important that there is no tolerance to the presence of *T. castaneum* at the end of the food

chain? Nowadays we can say that we are in the face of a paradigm between edible insects and pests.

It is a "supreme irony" that "billions of rupees are spent worldwide every year to save crops that contain no more than 14% of plant protein by killing another food source (insects) that can contain up to 75% of high-quality animal protein"Premalatha et al. (2011).

Authorship statement

All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript. Furthermore, each author certifies that this material or similar material has not been and will not be submitted to or published in any other publication before its appearance in the *Journal of Stored Products Research*.

Authorship contributions

Conceptualization ISousa and OCarvalho; Methodology ISousa OCarvalho; Investigation, SDuarte, CGraça and HGeirinhas; Data analysis HGeirinha SDuarte; Writing—original draft preparation SDuarte; Writing—review and editing OCarvalho and ISousa; Supervision OCarvalho and ISousa; Project administration OCarvalho.

All authors have read and agreed to the published version of the manuscript.

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.

Acknowledgments

This article is based on the results of a master thesis conducted within the joint master's in food engineering (MSc) in Instituto Superior de Agronomia, University of Lisbon, Portugal) within the orientation of ISousa and Ocarvalho under the project PTDC/ASP-PLA/28350/2017. Funding: This work was possible through FCT funds UIDB/04129/2020/LEAF and PTDC/ASP-PLA/28350/2017 project.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jspr.2023.102095.

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