1 Nature of the inactivation by high hydrostatic pressure of natural contaminating
2 microorganisms and inoculated *Salmonella* Typhimurium and *E. coli* O157:H7 on
3 insect protein-based gel particles.

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Abstract

In this work inactivation by high hydrostatic pressure of naturally contaminated total aerobic mesophilic (TAM) bacteria and molds and yeasts (M&Y) as well as the inactivation of intentionally inoculated pathogens (*Salmonella* Typhimurium and *Escherichia coli* O157:H7) in insect protein (*Acheta domesticus*) enriched gels as food matrix was studied, and survival data modeled. Results indicated that high hydrostatic pressure technology reduced the microbial load of both, the natural contaminating microorganisms (TAM and M&Y) and the intentionally inoculated pathogenic microorganisms. However, inactivation of the initial microbial load was lower than 5-log reductions. Kinetic inactivation parameters were deduced by using the Weibull distribution function which presented high goodness of fit. Kinetic parameters of the model showed that the most resistant microbial group was total aerobic mesophilic bacteria, followed by *E. coli* O157:H7, mold and yeast and *Salmonella* with kinetic parameters of 2.21, 1.58, 1.27, and 0.17 (min $\times$ mL/CFU) at 350 MPa, respectively. Regarding the pathogenic microorganisms inoculated in insect protein gels, *E. coli* O157:H7 may be a good candidate Gram negative target microorganism for high hydrostatic pressure process development, because it usually contaminates these products and was the one with the highest baro-resistance. These reasons support that high-pressure treatments for this type of food should be designed considering this microorganism as a target.

Keywords: High Hydrostatic Pressure, preservation, gels, *Acheta domesticus* protein, *Salmonella*, *E. coli* O157:H7

1. Introduction

Insects currently constitute part of the diet of approximately 2000 million people and their consumption is being considered as a strategy to combat famine in countries with protein deficits (FAO 2013). Insects also represent a business opportunity for industries in the first world to develop novel foods while simultaneously tackling the environmental impact of traditional livestock farming, currently required to meet protein demands. Nutritionally speaking, insects contain many important components, including vitamins, minerals and animal proteins (Wang et al., 2004). With the January 2018 adoption of the Novel Foods Regulation (EU) 2283/2015, insects and their derivatives are considered Novel Foods and are subject to the corresponding approval procedures. This represents a significant change from previous legislation by opening the door to insect consumption.

One of the insects under consideration for the production of new high-protein foods for human consumption is *Acheta domesticus* (common cricket). According to studies carried out by Rumpold and Shölter (2013), the common cricket has a protein content ranging from 64.38 to 70.75% in dry base. On comparing protein content of the common cricket with conventional protein sources, it is comparable to that of animal sources such as chicken (69.1% dry base) and fish (71.0% dry base) and higher than that of vegetable sources such as soybean (51.6% dry base) (Bosch et al. 2014; Makkar et al. 2014). Thus, it could partially or totally replace the protein from these animal sources in foodstuffs.

One of the main concerns regarding the consumption of insects and their derivatives is food safety. These insects may become contaminated by pathogenic and non-pathogenic microorganisms at levels that can alter the food or serve as a vector of food-borne

disease unless properly controlled by physical or chemical methods (natural antimicrobials). There are recent studies into the presence of different microorganisms in insects. Marin et al (2019) and Kashiri et al. (2018) characterized the natural flora in larvae of *Hermetia illucens*, finding an absence of *Listeria monocytogenes* and clostridium sulfite reducers and presence of *Salmonella* and *E. coli*. In the case of *Acheta domesticus*, *Listeria monocytogenes* has not been detected but the presence of *Salmonella* spp or *Escherichia coli* has been described, although not in all cases (Caparros et al., 2017; Grabowski and Klein, 2017; Osimani et al., 2017; Vandeweyer et al., 2017). Fernandez et al (2018) carried out a risk profile for *Acheta domesticus* for human consumption, reporting that there are currently no specific microbiological criteria in European legislation for insects or insect-derived products destined for human consumption. The authors concluded that common crickets (*Acheta domesticus*) show high concentrations of microorganisms compared to other food products and, therefore, specific hygiene measures for insects should be established in order to ensure food safety.

Edible insects are cooked, often fried or grilled, for consumption at home or in restaurants, with cooking options that can guarantee food safety. However, when considering the industrialization and production of new insect-based foods, approaches may change substantially, as manipulation, formulation, possible cross contamination or applied technologies can impact on the levels of pathogenic microorganisms (Marin et al 2018). Bearing this in mind, specific inactivation processes must be established for these new foods. According to Rumpold et al (2014), the use of high hydrostatic pressure (HHP) and cold plasma (CP) can reduce the surface microbiological load of insects. In this respect, Kashiri et al (2018) studied the effect of HHP on *E. coli* O157:H7 inoculated on the surface of *Hermtia illucens* larvae, as well as its effect on

mold and yeast and total aerobic mesophilic bacteria contaminating the larvae. The results indicated that more than 5 logarithmic reduction cycles in the microbial load at 400 MPa were achieved after 7 minutes of treatment. Likewise, Marin et al (2019) studied the effect of HHP on *Salmonella* inoculated on the surface of *Hermetia illucenes* larvae. The results indicated that at 350 MPa for 7 minutes a reduction of 5 logarithmic cycles in *Salmonella* was achieved and the microorganism was completely inactivated after 15 minutes of treatment at that same pressure.

Despite the environmental and cost efficiency benefits of insect rearing and consumption reported by experts and world organizations, in general, western consumers are still reluctant to consume insects. Consequently, insect proteins should be introduced as derived products in which the insect is not physically recognizable. However, to date, no research has been described on the effect of high pressures on more complex insect-derived food matrices.

In this work, the inactivation of *E coli*, *S. Typhimurium*, total aerobic mesophilic bacteria and molds and yeasts using high hydrostatic pressure will be studied in order to obtain suitable information to develop preservation conditions for gels containing *Acheta domesticus* protein extract as a basis for the formulation of protein-based foods using this insect.

2. Material and Methods

2.1 Protein extract

Protein was extracted by acid extraction as described by Yi et al. (2013). Briefly, two hundred grams of insect flour (*Acheta domesticus*) was weighed and 600 mL of distilled

water was added. The suspension was then acidified with 1 g of ascorbic acid. This mixture was stirred for 1 hour and then the mixture was filtered through 400 µm pore-size filter. The filtered product was stored in a freezer until use. The filtrate was subjected to centrifugation 15,000 x g for 30 minutes. The supernatant was removed and the pellet frozen at -80 ° C, until lyophilization to obtain the insect protein powder.

2.2 *Salmonella enteritidis* var. Typhimurium and *Escherichia coli* O157:H7 cultures

The strain of *Salmonella* Typhimurium inoculated in insect isolate protein gels used in HHP inactivation studies was supplied by The Spanish Type Culture Collection (CECT 443). The lyophilized strain was rehydrated in 10 mL of Tryptic Soy Broth (TSB) (Scharlab Chemie S.A., Barcelona, Spain). After 30 min of rehydration time, 5 mL of culture was incubated at 37°C in 200 mL bottles of TSB with constant stirring (200 rpm) for 6 h. After that period, 40 mL of the culture was poured into 400 mL bottles of TSB and incubated for 14 h at 37°C with constant stirring (200 rpm). After the incubation period, the cells were centrifuged twice at 4000 x g for 15 min at 4°C and re-suspended in 20 mL of TSB. The pellet was distributed into 2 mL sterile plastic cryogenic vials containing TSB supplemented with 20% glycerol. The 2 mL vials were stored at -80°C until use. The approximate concentration in each vial was 3×10⁹ CFU/mL.

Escherichia coli O157:H7 was obtained from the Spanish Type Culture Collection (CECT 4972) and inoculated in insect protein gels used in HHP inactivation studies. For rehydration, the same procedure was followed as for *Salmonella*, described above. After a rehydration period of 30 min, 5 mL of culture was inoculated in 200 mL bottles of TSB and incubated at 37°C for 6 h with constant stirring at 200 rpm. Then, 40 mL of the culture was transferred into 400 mL bottles of TSB and incubated for 12 h at 37°C

with constant stirring at 200 rpm. After incubation, cells were centrifuged twice at 4000 × g for 15 min at 4°C and suspended in 20 mL of TSB. The cells in the pellet were placed into 2 mL sterile plastic cryogenic vials containing TSB supplemented with 20% glycerol. The 2 mL samples were immediately stored at –80 °C until they needed. The concentration of each sample was approximately 3×10⁹ CFU mL⁻¹

2.3 Gel production, inoculation and High Hydrostatic Pressure treatments.

To prepare gels containing insect protein, 1.44 g of insect protein extract was weighed and dispersed in 2 mL of water; then the suspension was sterilized in a bacteriological autoclave for 20 min at 121 °C. An amount (0.19 g) of gelatin (E-441) were hydrated with some drops of peptone water. The protein suspension (1.44 g in 2 mL of water) and the hydrated gelatin (0.19g) were then mixed and distributed in falcon tubes (2 mL of mixture); later 8 mL of peptone water was added to each tube containing the mixture. Then 1 mL of inoculum of *E. coli* or *S. Typhimurium* from the 2 mL cryovials previously defrozen were added and the whole mixture was homogenized to achieve even cell distribution and a concentration level of approximately 10⁶-10⁷ CFU/mL. After homogenization, the tubes were stored under refrigeration at 4°C for 2 hours to facilitate gelation. The gels thus formed were packed in vacuum bags and subjected to different High Hydrostatic Pressure treatments.

The pressures used varied depending on the microorganism under study. In the case of *E. coli*, 200, 250, and 350 MPa were used for times ranking from 1.5 to 7 min, while for *S. Typhimurium* the pressures were 300, 325, and 350 MPa for times ranking from 1.5 to 7 min.

The study of the natural flora contaminating the protein extract (Total Aerobic Mesophilic bacteria and Molds and Yeasts) was carried out as described above but in

this case the protein suspension was not sterilized, nor inoculated before the gel was formed. The pressures applied in this case were 200, 250 and 350 MPa for times ranking from 1.5 to 7 min.

High Hydrostatic Pressure (HHP) treatments were performed in a pilot-scale unit (High-Pressure Food Processor, EPSI NV, Belgium). All the treatments were applied in duplicate, (two repetitions), with at least two replicates per treatment and two bags per replicate.

HHP-treated samples were placed in a stomacher bag and disaggregated. Then, serial decimal dilutions of samples were performed in 0.1% sterile peptone water (Scharlab Chemie S. A., Barcelona, Spain). The enumeration of viable cells of *S. Typhimurium* and *E. coli* was carried out on Tryptic Soy Agar (TSA) (Scharlab Chemie S. A., Barcelona, Spain). Plate Count Agar (PCA) (Scharlab Chemie S. A., Barcelona, Spain) was used for Total Aerobic Mesophilic bacteria, and for Yeasts and Molds the medium used was Potato Dextrose Agar (PDA) supplemented with 10% of tartaric acid (Scharlab Chemie S. A., Barcelona, Spain). *S. Typhimurium* and *E. coli* plates were incubated at 37°C for 24 hours while Total Aerobic Mesophilic bacteria were incubated at 30 °C for 48 h and for Yeast and Mold the incubation conditions were 25 °C for 5–7 days.

2.4 Modelling

All data from survivor curves were fitted by a non-linear regression to the Weibull model using equation 1 (Mafart et al., 2002) in order to obtain the kinetic parameter δ and the shape parameter p .

$$\text{Log}_{10}(N) = \text{Log}_{10}(N(0)) - (t/\delta)^p \quad \text{Equation 1}$$

where $N(0)$ is the initial number of microorganisms, t is the treatment time, δ is the kinetic parameter and p is the shape parameter

2.5 Data analysis

Analyses of survivor's post-treatment data and modelling were performed using Statgraphics ® Centurion XV software (Statpoint Technologies, Inc., USA).

3. Results and Discussion

To develop suitable high hydrostatic pressure (HHP) processes for a new food based on gel particles containing insect protein, a kinetic study was carried out considering different groups of microorganisms at different treatment pressures. Those types of studies are essential from the food safety perspective.

The results for inactivation of total aerobic mesophilic (TAM) bacteria, and of yeasts and molds (Y&M) as natural contaminating microorganisms in protein gel particle samples treated using various pressures and times are shown in figures 1 and 2, respectively. As shown, in all cases the HHP treatments reduced the number of viable cells naturally contaminating the insect protein. The higher the pressure, the lower the survival rate for the same time interval. This means that a high-pressure process could be a suitable control measure to reduce the level of both groups of microorganisms naturally contaminating the protein included in gel particles. The logarithm of survivors for total aerobic mesophilic (TAM) bacteria showed a reduction of 3.02 log cycles obtained by applying a pressure of 350 MPa for 4 min, or 200 MPa for 7 minutes. The reduction achieved for yeast and mold (Y&M) counts was 3.03 log cycles under the same conditions as for TAM. Both results are similar and contrast with those obtained

by Kashiri et al (2018) and Marín et al (2019) where TAM appeared more resistant to high pressure than yeast and molds in *Hermetia illucens* larvae naturally contaminated with those groups of microorganisms, although the initial contamination in all cases was similar. The aforementioned studies considered the overall contamination level of larvae even though the larval feeding behavior would indicate that the main contributor to contamination is the insect surface rather than its interior. It is possible that gels contribute to the small differences observed in the effect of pressure between each group of microorganisms (TAM and Y&M) when distributed inside insect protein gels. Thus gels could reduce the differences in resistance to pressure between TAM and Y&M in contrast to the differences between those groups of microorganisms contaminating the surface of *Hermetia illucens* larvae. Rumpold et al (2014) also carried out studies to compare volumetric and surface decontamination techniques for innovative processing technologies applied to inactivation of microorganisms contaminating mealworm larvae (*Tenebrio molitor*). Authors reported that the application of HHP treatments inactivated overall natural microbial contamination. At least 600 MPa for 10 min was required to achieve an inactivation reduction of three log cycles. Those inactivation levels are very low considering the processing conditions applied (600 MPa for 10 min) compared with those obtained in the present paper at 350 MPa for 4 min, or 200 MPa for 7 minutes. The authors concluded that the low inactivation level could be due to the low water availability in the samples, which were vacuum-packed without added water. It is important to note that water is essential in pressure transmission.

Figure 3 shows the effect of pressure on intentionally inoculated *E. coli* O157:H7 inside the insect protein gel. The reduction achieved for this microorganism was 3.05 log cycles at 200 MPa for 7 minutes and 3.2 log cycles at 350 MPa for 4 minutes. The

inactivation reached was lower than the 5 log cycles of reduction recommended by FDA for non-thermal technologies. Comparing inactivation levels of *E. coli* O157:H7 in the insect-protein gel with those obtained in intentionally surface-inoculated *Hermetia illucens* larvae by Kashiri et al (2018), the latter reported a reduction below 1.5 log cycles after applying a pressure of 250 MPa for 5 min, while the present study achieved 2.0 log cycles under the same treatment conditions. Treatment at 350 MPa for 10 min produced a reduction of 3.93 log cycles for *E. coli* O157:H7 as compared with 3.2 log cycles obtained in the present work after a treatment of 350 MPa for 4 minutes. Again, as indicated above for natural contaminating microorganisms, the gel appears to influence the reduction level of the microorganism inoculated in the particle.

Inactivation curves for intentionally inoculated *S. Typhimurium* in gels with insect protein are shown in figure 4. In the case of *Salmonella*, 6.2 log cycles were achieved after 5 min at 350 MPa. A treatment of 300 MPa for 7 min was enough to reach 5 log cycles of reduction. Marín et al (2019) found 4.1 log cycles for *Samonella* inoculated on the surface of *Hermetia illucens* after a treatment of 350 MPa for 7 minutes. According to those results, *Salmonella* was more sensitive to pressure treatment in the gels with insect protein, as occurred with the natural contaminating microorganisms and intentionally inoculated *E coli*.

To establish a suitable preservation process, it is necessary to have information about kinetic parameters. In the present work, survivor curves did not follow a log linear kinetic pattern; consequently, a nonlinear model should be used to describe the survivor curves. In those cases, the Weibull model as indicated in equation 1 has been successfully used for different authors to describe non-linear curves (Fernandez et al. 1987; Peleg and Cole 1986).

Table 1 shows the kinetic parameter δ for each group of microorganisms and pressure used and table 2, shows the goodness of fit for the model. As can be seen in table 2 good fitting parameters were obtained, indicating that the Weibull model was useful in describing survivor curves obtained in this work. The shape parameter p of the Weibull model varied from 1.2 to 2.9 for *E. coli* O157:H7 indicating upward inactivation curves and from 0.69 to 2.9 for *Salmonella*, indicating that some inactivation curves were downward (p parameter less than 1) and other inactivation curves were upward (p parameter higher than 1)

Kinetic parameter δ represents the resistance of the microorganism to the stressing factor used, in this case HHP. As shown in table 1, the kinetic parameter δ decreased as the pressure increased indicating that the different groups of microorganisms were less resistant to high pressure levels. However, for *Salmonella* the kinetic parameter was similar at both 325 and 350 MPa.

Comparing the baro-resistance of the different groups of microorganisms, *Salmonella* was clearly the least resistant, followed by molds and yeast and *E. coli*. By contrast, TAM proved to be the most resistant group. This high resistance of the TAM group might be because the insect protein in the gel may possibly be contaminated with bacterial spores, such as natural contaminating microorganism, which are more resistant to HHP than vegetative cells. Rumpold et al. (2014) reported high baro-resistance for overall microbial counts (natural contaminating bacteria) and explained this result by considering that the treatment conditions chosen (up to 600 MPa for 15 min) were not sufficient to inactivate spore forming bacteria. The presence of bacterial spores on insects was also reported by Osimani et al. (2016 and 2018). Authors carried out a study focused on exploring the proximate composition and microbial diversity of edible insects marketed in the European Union. Among the different microorganisms

contaminating the insects, they found *Bacillus* and *Clostridium* bacterial spores. Fasolato et al. (2018) also identified bacterial spores with values ranging between 1.3-4.81 log₁₀ CFU/g. Other Gram positive microorganisms apart *Clostridium* or *Bacillus* and their spores that could be considered as contaminants of insects, for example *Staphylococcus aureus*. There are also other non-pathogenic microorganisms such as *Pseudomonas*, *Staphylococcus*, *Streptococcus*, *Micrococcus*, *Lactobacillus*, *Enterococcus* or *Acinetobacter* contaminating insects. They present some degree of baroresistance to HHP treatments, particularly on mild pasteurization HHP treatments. At this point, it is necessary to clarify that nor all TAM bacteria are baroresistant, but only some of the microorganism, particularly those Gram positive.

Regarding pathogenic microorganisms inoculated in the gel containing insect protein, *E. coli* O157:H7 showed higher baro-resistance than *Salmonella* according to the kinetic parameter δ of the Weibull model. Consequently, the target pathogenic microorganism to be used in developing HHP treatments for those insect-based products should be *E. coli* O157:H7. *Escherichia coli* spp is common in insects; it was characterized by Osimani et al. (2016) in edible insects by using PCR-DGGE and was also found in *Hermetia illucens* larvae by Marín et al. (2019).

As far as we know, there are few studies that kinetically characterize microbial inactivation in food matrices containing insects or insect protein. Marín et al (2019) published δ kinetic parameter for *Salmonella* Typhimurium, which was intentionally surface inoculated in *Hermetia illucens* larvae of 0.01 (min×mL/CFU) at 350 MPa. According to the results shown in table 1, a δ kinetic parameter of 0.17 min×mL/CFU was obtained for the same pressure level of 350 MPa. This difference appears to indicate a lower inactivation rate of *Salmonella* inoculated in the gel particle containing insect protein. Regarding *E. coli* O157:H7 in intentionally surface inoculated *Hermetia*

illucens larvae, Kashiri et al. (2018) reported a δ kinetic parameter of 0.11 min×mL/CFU at 350 MPa while, as indicated in table 1, a δ kinetic parameter of 1.58 was found in inoculated gels containing insect protein. Again, this difference could indicate a lower inactivation rate when the microorganism was inoculated in gel particles containing insect protein. Nevertheless, the goodness of fit to the Weibull model in Kashiri et al. (2018) and Marin et al. (2019) studies was not as accurate as the Biphasic model which was the best fit to the inactivation data.

4. Conclusions

High hydrostatic pressure can reduce both the natural and the inoculated load of pathogenic microorganisms in gels containing insect protein. The inactivation level depended on the microbiological group considered; the most resistant group was the total aerobic bacteria (TAB). With respect to pathogenic microorganisms, *E. coli* O157:H7 proved more resistant than *Salmonella* Typhimurium; therefore, *E. coli* should be considered as the target microorganism for developing HHP treatments to process such gels. None of the treatments applied achieved 5-log reductions of the *E. coli* O157:H7 inoculated load, thus it is necessary to increase the pressure over 350 MPa or the treatment time at that pressure. Both types of treatments are affordable for the food industry, and are thus good microbiological control measures of choice for insect protein derived products.

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423 **Figure captions**

424 Figure 1: Survivor curves for total aerobic mesophilic bacteria (TAM) at different
425 pressures

426 Figure 2: Survivor curves for yeast and molds at different pressures

427 Figure 3: Survivor curves for *E. coli* O157:H7 at different pressures

428 Figure 4: Survivor curves for *Salmonella* Typhimurium at different pressures

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431 Table 1: Kinetic parameter values for the different tested microorganisms

Pressure (MPa)	<i>S. Typhimurium</i>	<i>E. coli</i> O157:H7	Total Aerobic Mesophilic	Molds and Yeast
	$\delta \pm \sigma$ (minxml/CFU)	$\delta \pm \sigma$ (minxml/CFU)	$\delta \pm \sigma$ (minxml/CFU)	$\delta \pm \sigma$ (minxml/CFU)
200	ND	4.8±0.07	4.52±0.20	4.40±0.20
250	ND	2.38±0.08	2.47±0.30	2.10±0.20
300	2.08±0.40	ND	ND	ND
325	0.16±0.52	ND	ND	ND
350	0.17±0.54	1.58±0.02	2.21±0.21	1.27±0.11

432 ND= Non Determined

433

434 Table 2: Goodness of fit and RMSSE for the Weibull model used to describe the
435 experimental data

Pressure (MPa)	<i>S. Typhimurium</i>		<i>E. coli</i> O157:H7		Total Aerobic Mesophilic		Molds and Yeast	
	RMSSE	Adjusted R^2	RMSSE	Adjusted R^2	RMSSE	Adjusted R^2	RMSSE	Adjusted R^2
200	ND	ND	0.11	0.99	0.15	0.96	0.09	0.99
250	ND	ND	0.12	0.97	0.13	0.98	0.11	0.98
300	0.53	0.93	ND	ND	ND	ND	ND	ND
325	0.30	0.98	ND	ND	ND	ND	ND	ND
350	0.82	0.85	0.10	0.99	0.20	0.96	0.12	0.98

436 ND= Non Determined

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440 Figure 1: Survivor curves for Total Aerobic Mesophilic bacteria (TAM) at different
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