

**The microbial safety of seaweed as a feed component for black soldier fly
(*Hermetia illucens*) larvae**

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ABSTRACT

Farming insects for use as an alternative aquafeed or livestock ingredient can deliver environmental and nutritive benefits. The larvae of the terrestrial black soldier fly (*Hermetia illucens*) (BSF) however, could be improved by the dietary inclusion of seaweed to incorporate valuable nutrients associated with the marine environment, e.g. omega-3. Standardised processing methods in the feed and food industries are key to product quality and safety; although currently such systems are limited for seaweed and insect processing. The industry practice of drying seaweed at low temperatures to retain nutritional properties may benefit the survival of human pathogenic bacteria, particularly if the seaweed has been harvested from contaminated water. Here we have determined the risk of bacterial (*E. coli*, *E. coli* O157:H7, *Listeria monocytogenes* and *Vibrio parahaemolyticus*), survival during seaweed drying and processing as feed for insect larvae and the subsequent risk of BSF larvae contamination. All four bacteria colonised seaweed and resisted removal by washing. *E. coli* and *E. coli* O157:H7 died-off in seaweed dried at 50 °C, although were detected in the dried powder following 72 h storage, indicating an increase in water activity (a_w) in the stored product. *V. parahaemolyticus* fell below the level of detection in stored seaweed after drying at ≥ 50 °C, but *L. monocytogenes* remained detectable, and continued to grow in seaweed dried at ≤ 60 °C. BSF larvae reared on an artificially contaminated seaweed-supplemented diet became colonised by all four bacteria present in the supplement. Therefore, drying seaweed at lower temperatures in order to preserve the maximum nutritive potential also risks pathogen carry-over (particularly if harvested from contaminated water) into BSF larvae destined for livestock feed. Seaweed processing and storage conditions, and the subsequent production of insect feed, represent critical control points where good manufacturing practices are needed to target the control of pathogenic hazards. Therefore, robust environmental management of seaweed harvesting waters is needed in order to provide the regulatory framework for including seaweeds, and the processing of insect larvae, into sustainable livestock and aquaculture feed chains.

1. INTRODUCTION

Seaweed meal is a recognised animal feed substrate in the EU (Reg (EC) 68/2013; EC, 2013a). It can provide a supplementary source of energy, proteins, minerals, lipids, vitamins and antioxidants (with-bioactive value) for livestock and aquaculture, and most recently, for the mass production of insect larvae (Rajauria, 2015; Liland *et al.*, 2017). The concept of insect protein as a sustainable animal feed ingredient has gathered increasing acceptance across Europe, and is now permitted in aquafeed within the EU (Reg (EC) 893/2017; EC, 2017a). Recent innovative efforts to combine these two ingredients into aquaculture feed for farmed carnivorous fish has seen advances in the mass production of seaweed-fed black soldier fly larvae (BSFL), *Hermetia illucens* (L.) (Diptera: Stratiomyidae) (Belghit *et al.*, 2018; Swinscoe *et al.*, 2019). The benefit of feeding insect larvae with seaweeds includes utilizing a renewable feed resource that does not compete with sources of human food or require land use, additional water or industrial fertilization. In Europe, seaweed for animal feed is typically wild harvested from coastal marine waters (Makkar *et al.*, 2016); however, wild harvested seaweeds can also become colonised by human pathogenic bacteria, e.g. species of *Vibrio* and strains of *Escherichia coli* (Elbashir *et al.*, 2018; Quilliam *et al.*, 2014; Mahmud *et al.*, 2007; 2008). Therefore, before seaweed supplements in BSFL diets can be advocated for mass-reared insect production, critical control points (CCPs) during the production of seaweed-fed BSFL must be identified (Swinscoe *et al.*, 2019) in order to guarantee safety of this novel animal feed if it is to enter the human food chain (Reg (EC) 183/2005; EC, 2005a).

Standardised processing methods in the feed and food industries are key to product quality and safety, but such a system is currently lacking in the seaweed industry. There are also no microbiological standards for seaweed meal in the EU, and those for insect processed animal proteins (PAPs) in feed are limited to maximum levels of *Clostridium perfringens*, *Salmonella* spp and Enterobacteriaceae (Reg (EC) 142/2011; EC, 2011), and in food to maximum levels of *Salmonella* spp and *L. monocytogenes* (Reg (EC) 2073/2005; EC, 2005b). Although processing-based interventions for controlling microbial

contamination of seaweeds have been explored, e.g. washing and drying (del Olmo et. al., 2018;; Hyun et. al., 2018), the full range of potential microbiological hazards associated with seaweed entering the feed and food chain are not necessarily controlled by existing industrial practices, or accounted for by current feed hygiene regulations.

Typical post-harvest processing of seaweed for animal feed involves (i) washing to remove visible epiphytic flora and fauna; (ii) reduction of bulk and water activity (a_w) by hot air drying, which inhibits microbial growth and biochemical degradation; (iii) milling, packaging and storage at room temperature for up to one year. Washing seaweeds however, fails to eradicate Enterobacteriaceae, coliforms or *V. parahaemolyticus*, and *E. coli* can replicate on seaweed during desiccation and storage (del Olmo et. al. 2018; Mahmud et. al. 2008)). Importantly, the higher the seaweed drying temperature, the greater the nutritional loss of the seaweed biomass. The industrial drying of seaweeds therefore needs to be balanced between using a temperature that can sufficiently desiccate the seaweed and destroy bacterial contaminants against potential nutritional losses. Nutritional loss occurs through the denaturation of proteins, oxidation of lipids and the loss of anti-oxidant activity in the seaweed product (Stevant et. al. 2018; Lage-Yusty et. al. 2014; Moreira et. al. 2016; Gupta et. al. 2011).

Insect farming to produce animal feed is still a nascent industry in the EU but it is widely acknowledged that the microbiological safety of insects is fundamentally influenced by the hygienic status of their feed (Van der Spiegel et. al., 2013). Thus, good manufacturing and hygiene practices (GMP and GHP) specific to each insect species, the feed substrate, the life stage at harvest, and the production environment need considerable development as CCPs emerge at which pathogens may be introduced, persist or replicate in the insect product (Van Raamsdonk et. al., 2017). Therefore, the aims of this study were to: (1) Determine colonisation dynamics of a range of human pathogenic bacteria on a combined mixture of submerged brown, red and green seaweeds in an intertidal simulation of exposure to a wastewater pollution event. (2) Evaluate the effect of typical industrial

processing practices (washing, drying and storage) on the survival of bacteria attached to seaweeds. (3) Assess the survival dynamics of these bacterial contaminants when fed to BSFL as a powdered seaweed feed supplement. (4) Identify CCPs, and recommend GMP and bacteriological standards to control bacterial hazards at CCPs, during production of seaweed feed and its application as a feed supplement for the mass rearing of BSFL.

2. MATERIALS AND METHODS

2.1 Bacteriological safety of processed seaweed (Experiment 1)

A model system of postharvest industrial processing of seaweed was developed involving sequential stages of washing, drying, milling and storage. Sampling for bacteriological quality was conducted at key stages of the process.

2.2 Seaweed material

Living, attached intertidal seaweeds of the species *Laminaria digitata* (Hudson) (Phaeophyceae), *Fucus serratus* (L.) (Phaeophyceae), *Palmaria palmata* (L.) (Rhodophyta) and *Ulva lactuca* (L.) (Chlorophyta), together with seawater from the surf zone, were collected at low tide from Elie, Fife, Scotland (56°11.191'N, 2°48.679'W). *Ascophyllum nodosum* (L.) (Phaeophyceae) was gathered from Ganavan Bay, Oban, Scotland (56°26'05.1'N, 5°28'51.3'W) a day later. Seaweed was rinsed in tap water for 3 min to remove sand and epiphytic flora and fauna. All seaweed and seawater samples were stored at 4 °C and utilised within 24 h. To enumerate background *E. coli* and total heterotrophic bacteria (THB) associated with the seaweed, 500 g of each species was individually homogenised for 3 min using a hand blender (Bosch MSM6700GB). Four 10 g replicate samples of the homogenate of each seaweed species was added to 10 ml of sterile seawater (sterilised by autoclaving) and vortexed for 1 min. The supernatant was serially diluted using sterile seawater and spread plated onto either Membrane Lactose Glucuronide Agar (MLGA) (CM1031, Oxoid) or R2A agar

(CM0906, Oxoid) to quantify *E. coli* and THB respectively. MLGA plates were inverted and incubated at 37 °C for 24 h and R2A plates at 18 °C for 48 h. Seawater samples ($n = 4$) were shaken and 100 ml vacuum-filtrated through a 0.45 µm cellulose nitrate membrane (Sartorius, Goettingen, Germany). The membrane was transferred to MLGA or R2A plates and incubated as described above. Bacterial concentrations were expressed as CFU (colony forming units) g⁻¹ seaweed (dry matter), or CFU 100 ml⁻¹ seawater.

2.3 Inoculum preparation

In addition, to an environmental isolate of *E. coli*, three bacterial pathogens were used in this study: a non-toxigenic serotype of *E. coli* O157:H7, *Listeria monocytogenes* and *Vibrio parahaemolyticus*. To produce bacterial cells tolerant of seawater for use in our experiments, each bacterial species was added to sterile seawater for 3 h at 10 °C. A 100 ml sample ($n = 4$) was vacuum filtered and the membrane transferred to the relevant selective agar plate. The environmental *E. coli* was grown on MLGA, and *E. coli* O157:H7 on Sorbitol MacConkey Agar (SMAC) (CM0813, Oxoid) supplemented with cefixime and potassium tellurite (CT) (SR0172, Oxoid); *L. monocytogenes* was grown on Listeria Selective Agar (Oxford Formulation) (CM0856, Oxoid) supplemented with Modified Listeria Selective Supplement (Oxford) (SR0206, Oxoid) and *V. parahaemolyticus* grown on TCBS (Thiosulfate citrate bile salts sucrose agar; CM0333, Oxoid). Following incubation at 37 °C for 24 h, single colonies of each species were picked off the plate and *E. coli*, *E. coli* O157:H7 and *L. monocytogenes* individually cultured in Luria-Bertani (LB) broth (CM1018, Oxoid), and *V. parahaemolyticus* in Alkaline Peptone Water (APW) (CM1028, Oxoid), at 37 °C for 18 h at 100 rev min⁻¹. Cells were washed three times in Phosphate Buffered Saline (PBS), and re-suspended in PBS prior to use.

2.4 Simulated microbial contamination of pre-harvested seaweed

Fresh samples of *L. digitata*, *F. serratus*, *A. nodosum*, *P. palmata* and *U. lactuca* were combined in equal quantities (40 g each) in 500 ml glass jars ($n = 32$). *L. digitata*, *F. serratus* and *A. nodosum* were

comprised of approximately 10 % stipe and 90 % frond, whereas *P. palmata* and *U. lactuca* consisted of 100 % frond. The stipes and fronds of *L. digitata*, *F. serratus* and *A. nodosum* were cut into 5 cm lengths to enable accurate weighing of each seaweed species into replicate batches. Eight replicate jars were used for each temperature (room temperature (RT; approx. 20 °C), 40 °C, 50 °C and 60 °C) of which four replicate jars were inoculated with bacterial pathogens, and four non-inoculated jars at each temperature were used to assess pH and the a_w of seaweed.

Seawater tolerant cells of *E. coli*, *E. coli* O157:H7, *L. monocytogenes* and *V. parahaemolyticus* suspended in PBS were combined and added to 1600 ml of non-sterile seawater. The resulting pathogen-seawater was mixed to ensure even distribution of cells, and the concentration of each bacterial species determined by plating onto selective media as described above ($n = 4$ for each bacterial species). The concentrations of each bacteria in the pathogen-seawater cocktail were as follows: *E. coli* = 6.32×10^9 CFU ml⁻¹; *E. coli* O157:H7 = 7.0×10^9 CFU ml⁻¹; *L. monocytogenes* = 5.9×10^9 CFU ml⁻¹; *V. parahaemolyticus* = 6.8×10^9 CFU ml⁻¹. Aliquots of 200 ml of the contaminated seawater were poured into each of the glass jars ($n = 16$), which completely submerged the seaweed mixture. Aliquots of 200 ml of non-inoculated non-sterile seawater were poured into each of the jars ($n = 16$) used for pH and a_w measurements. Screw lids were used and all jars secured within a temperature controlled rotating incubator at 100 revs min⁻¹ for 24 h at 20.5 °C \pm 3 °C.

The seawater was removed from each jar using a sieve, and concentrations of the bacteria remaining in the seawater were enumerated on selective media. Bacteria attached to the seaweed were quantified by removing a 10 g seaweed sample from each of the inoculated jars, homogenising the sample for 3 min with a hand blender, and vortexing the homogenate in 10 ml of PBS for 1 min. The concentration of all four bacteria suspended in the supernatant were quantified on selective media. In addition, 5 g of seaweed was removed from each of the non-inoculated jars ($n = 16$), and vortexed for 1 min in 5 ml distilled water to determine the pH using an HI 2550 Multiparameter bench meter (HANNA instruments, Bedfordshire, UK).

2.5 Simulated post-harvest seaweed processing

The first stage of industrial post-harvest processing of seaweed involves a washing step after harvesting in order to remove sand and debris. To simulate this, the seaweed from each jar was transferred to a sieve (mesh diameter 1 mm) and rinsed with cold tap water for 1 min. Each seaweed sample was stirred gently using a sterile metal spatula in order to maintain the flow of water through the sieve. The concentration of *E. coli*, *E. coli* O157:H7, *L. monocytogenes* and *V. parahaemolyticus* still attached to the seaweed post-washing were quantified by homogenisation of the seaweed and plating out onto selective media. The pH of post-washed batches of seaweed from the non-inoculated groups was also measured. Following the washing step, a 10 g sample of seaweed was taken from each replicate jar of the non-inoculated groups, finely chopped to approximately 5 mm² and the *a_w* measured using an AquaLab CX-2 (METER Group, Inc. USA), calibrated with a saturated solution of potassium sulphate, with the cooled mirror dew point technique (providing an accuracy of ± 0.005 according to the AquaLab Operator's Manual).

The remaining seaweed in each of the eight jars was immediately washed and transferred to individual foil trays measuring 20 (l) x 10 (w) x 5 (d) cm. The seaweed was spread out evenly to an approximate depth of 4 cm and placed in a drying oven at either 40 °C, 50 °C or 60 °C, to simulate the lower end of the range employed in hot air convection or oven drying by the seaweed industry (Gupta *et. al.* 2011). A temperature logger was placed in the centre of the four non-inoculated seaweed replicate trays. During drying, the actual temperatures achieved were 41.8 °C \pm 0.03, 49.1 °C \pm 0.14, and 64.2 °C \pm 0.21. In addition, eight uncovered trays of seaweed were placed on the bench top within the same laboratory to provide a room temperature (RT) treatment (22.7 °C \pm 0.04).

Bacterial concentrations on the inoculated seaweed were enumerated during the drying process at 24, 72, 120 and 168 h, using the methods above with the exception of seaweed dried at 50 °C and 60 °C from 72 h onwards, which was sufficiently desiccated to be ground to a fine powder using a pestle and mortar. To determine bacterial concentrations in this seaweed powder, 2 g of powder

was added to 20 ml of PBS ($n = 4$), the homogenate vortexed for 1 min, and bacteria enumerated as described above. After 72 h and 168 h drying, the a_w was measured in a 10 g sample of non-inoculated seaweed from each replicate tray, following the method described above, or carried out on the seaweed powder for samples dried at 50 °C and 60 °C.

After 168 h drying, seaweed from all trays was transferred to individual enclosed plastic boxes and stored at RT. Moisture loss from seaweed that had been dried at RT and 40 °C was insufficient to enable the seaweed to be ground to a powder prior to storage. Each seaweed mix from these groups was therefore individually homogenised with no added liquid for 3 min using a hand blender to approximately 5 mm² prior to storage. Seaweed dried at 50 °C and 60 °C was ground to a fine powder (approximately 0.5 – 1 mm²) using a pestle and mortar prior to storage. After 72 h storage, bacterial concentrations in all seaweed samples were quantified as described above.

2.5 Bacteriological safety of seaweed as BSFL feed (Experiment 2)

A simulation of mass rearing of BSFL on feed supplemented with pathogen-contaminated seaweed powder was undertaken. Larvae and the feed substrate were sampled throughout the rearing period up to the point of pre-pupae harvest to assess both the microbial load of the feed and the hygienic status of the larvae.

2.7 Preparation of BSF colonies

Two colonies of BSF were established from larvae sourced online (livefoodsbypost.co.uk and InternetReptile.com) in insect rearing tents measuring 75 (w) x 75 (d) x 115 (h) cm (BugDorm-2400, bugdorm.com), in a controlled environment walk-in room (Reftech B.V., Netherlands) at 30 °C ± 2 °C, a relative humidity of 70 % and a photoperiod of 12 h. One tent contained two 5 L plastic boxes (Addis Ltd., UK) each containing approximately 1000 larvae, which were reared on a 15:3:1 mixture of wheat bran (Harbro Ltd., Aberdeenshire), whey protein (Holland and Barrett International, UK) and fruit and

vegetable waste. Every 2 days, feed substrate was supplemented to a depth of approximately 12 cm and 200 ml of water was added. Holes in the base of the containers enabled drainage of excess liquid to prevent waterlogging and anoxic conditions developing in the feed substrate. Within the tent, cardboard boxes containing shredded newspaper provided dark sheltered conditions for pupation. Once adult flies emerged, sliced fruit was placed on the surface of the feed substrate and water (< 20 ml) was sprayed into the tent hourly during the day. Corrugated cardboard strips were laid across the feed container above the level of the feed to provide dry crevices in which the female flies laid their eggs. As soon as eggs were observed in a cardboard strip, the strip was transferred to another insect tent and suspended above a tray containing feed substrate comprised of the same ingredients as described above. After hatching, the larvae dropped from the cardboard strip into the substrate, and were harvested for future experiments at approximately 1 week old.

2.8 Preparation of seaweed powder and inoculation procedure

Seaweed and seawater was collected at the same time as above, and stored at 4 °C prior to use. The seaweeds (stipes and fronds) were separated by species, washed clean of visible epiphytic flora and fauna using tap water, and oven dried in single layers in foil trays (22 x 22 x 6 cm) at 50 °C for 72 h. Each species of dried seaweed was then ground into a fine powder using a pestle and mortar to pass through a 500 µm sieve. Composite 400 g mixtures (comprised of 80 g each of *L. digitata*, *F. serratus*, *A. nodosum*, *P. palmata* and *U. lactuca*), were placed in three separate stomacher bags. The seaweed powder in two bags was inoculated with 1.5 L of seawater containing *E. coli*, *E. coli* O157:H7, *L. monocytogenes* and *V. parahaemolyticus* produced as described above, and the contents of each bag agitated by hand for 5 min to ensure thorough mixing. The initial concentration of each bacterial species in the contaminated seawater was: *E. coli* (6.7×10^9 CFU ml⁻¹), *E. coli* O157:H7 (7.15×10^9 CFU ml⁻¹), *L. monocytogenes* (7.35×10^9 CFU ml⁻¹) and *V. parahaemolyticus* (6.4×10^9 CFU ml⁻¹). The remaining 400 g of seaweed powder was mixed with 1.5 L of non-inoculated seawater following the method described above.

2.9 Simulated mass-production of BSFL reared on seaweed supplement

Approximately one week old larvae ($n = \sim 700$; mean weight per larvae = $0.0807 \text{ g} \pm 0.004$) were removed from the rearing substrate and placed in two empty 5 L plastic boxes (with paper towelling secured over a hole (10 x 10 cm) in the lid to enable gas exchange) for 24 h to allow the larvae to purge their digestive tracts. Twelve 5 L plastic boxes were established each containing 900 g of feed substrate (765 g wheat bran and 135 g whey protein). The inoculated dried seaweed powder was added to eight replicate boxes of feed substrate (100 g per box), whilst the remaining four boxes of feed received 100 g of non-inoculated seaweed powder. Each box had 1.6 L of tap water added, and the feed mixture stirred for 5 min to ensure thorough mixing. Larvae were added to four of the feed boxes ($n = 80$ to each box) containing inoculated seaweed powder. No larvae were added to the remaining four boxes containing inoculated seaweed powder, which represented the control. Larvae ($n = 80$) were added to each of the four boxes containing non-inoculated seaweed powder. A temperature logger was placed in the centre of the feed within each box containing larvae and non-inoculated seaweed powder. Feed was not replenished during the experiment, although 300 ml of tap water was added to every box (inoculated and non-inoculated groups) on day 3 to maintain feed moisture levels.

Sampling of larvae and substrate began at 24 h, and continued daily for 8 days, when the majority of larvae had become pre-pupae. Larvae from the inoculated substrate were sampled by removing a scoop of substrate ($\sim 100 \text{ g}$) with a metal ladle from each box, removing the first three larvae observed in that material, and returning the substrate to the box. Sterile forceps were used to remove the larvae, which were then anaesthetised with 10 s exposure to CO_2 . Visibly attached feed and frass were removed from the larvae exoskeletons using forceps, and the combined weight of the three larvae was recorded. For each sample, three larvae were homogenised in 1 ml PBS in a 1.5 ml Eppendorf tube using a micro pestle (Anachem Ltd., Bedfordshire, UK), then transferred to a 15 ml tube (Sarstedt, Germany) and a further 1 ml PBS added. The homogenate was vortexed for 1 min, and

bacteria enumerated as described above. Bacterial concentrations in the substrate were also quantified at each time point by homogenising 10 g of material, and enumerating bacteria in the supernatant. Bacterial concentrations in larvae were expressed as CFU larvae⁻¹, representative substrate samples were dried at 80 °C for 24 h such that bacterial concentrations could be expressed as CFU g⁻¹ dry matter content.

2.10 Statistical analyses

Friedman's ANOVAs with pairwise comparisons or step-down follow-up analysis were used to compare water activity (a_w) within each treatment, and Kruskal-Wallis analysis examined differences in a_w between treatments at each sampling stage. One-way ANOVAs were used to determine the survival capacity of each bacteria in seawater, the attachment efficiency of each bacteria to submerged seaweed, and differences between bacterial levels in seawater and seaweed. Tukey post hoc testing was applied to *E. coli* and *V. parahaemolyticus* concentrations. However, Levene's tests indicated that *E. coli* O157:H7 and *L. monocytogenes* concentrations violated the assumption of homogeneity of variances, thus Games-Howell post-hoc testing was applied. Changes in concentrations of each bacteria between initial levels in the contaminated seawater and concentrations remaining in seawater and attached to seaweed combined after 24 h were examined using t-tests. The effect of washing seaweed on bacterial attachment of *E. coli* O157:H7 was tested using paired t-tests. The effect of duration of drying at a given temperature and of storage on bacterial concentrations were tested using Friedman's ANOVA as the data were not normally distributed despite log transformation, followed by pairwise comparisons with adjusted p -values or step-down follow-up analysis. Differences between temperature treatments in bacterial concentrations on seaweed at each sampling stage during drying were tested using Kruskal-Wallis analysis, with pairwise comparisons or step-down follow-up analysis. A Mauchly's test following a split-plot ANOVA to examine changes in seaweed pH between and within treatments indicated violation of the assumption of sphericity, therefore Greenhouse-Geisser tests were used.

Bacterial concentrations associated with larvae, their substrate and the larvae-free control substrate over time were analysed using split-plot ANOVAs, followed by Bonferroni post hoc tests. Changes in pH of the non-inoculated feed were tested with a repeated measures ANOVA with Bonferroni post hoc testing. All analyses were conducted using SPSS 21.0 software (SPSS Inc. Chicago, IL, USA).

3. RESULTS

3.1 Background bacteriological status of seaweed and seawater

E. coli was not detected on the freshly harvested seaweed used in both Experiments 1 and 2, and was present at a very low concentration ($< 10 \text{ CFU } 100 \text{ ml}^{-1}$) in the seawater from which the seaweed was harvested. Total heterotrophic bacteria were present in low abundance on all species of seaweed and in seawater, the highest concentrations being detected on *L. digitata* and in seawater (data not shown).

3.2 Bacteriological safety of processed seaweed

After 24 h in a rotating incubator at room temperature ($20.5 \text{ }^{\circ}\text{C} \pm 3 \text{ }^{\circ}\text{C}$), concentrations of *E. coli*, *E. coli* O157:H7, *L. monocytogenes* and *V. parahaemolyticus* in the seawater had fallen by $\sim 2 \log_{10}$ CFU, and *E. coli* and *E. coli* O157:H7. Cell concentrations in seawater and on seaweed are provided in Table 1. The concentration of all four bacteria associated with the seaweed significantly increased ($P < 0.05$ in all cases) after it had been washed under running tap water (Table 2). However, washing seaweed after 24 h submergence in seawater did not affect seaweed pH.

Table 1. The concentrations of *E. coli*, *E. coli* O157:H7, *L. monocytogenes* and *V. parahaemolyticus* in the pathogen-seawater cocktail before the addition of seaweed, compared with the seawater and seaweed after 24 h (note different units for seawater and seaweed). Within each row, data points that do not share a letter are significantly different from each other (applicable to water treatments only). Data points are the mean of 16 replicates \pm SE.

Bacteria	Artificially contaminated seawater (\log_{10} CFU ml^{-1})	After 24 h	
		Seawater (\log_{10} CFU ml^{-1})	Seaweed (\log_{10} CFU g^{-1})
<i>E. coli</i>	6.31 ± 0.1^a	4.62 ± 0.1^b	6.83 ± 0.05
<i>E. coli</i> O157:H7	7.0 ± 0.04^a	4.51 ± 0.1^b	6.8 ± 0.1
<i>L. monocytogenes</i>	5.88 ± 0.03^a	4.06 ± 0.07^b	5.01 ± 0.4
<i>V. parahaemolyticus</i>	6.8 ± 0.2^a	4.4 ± 0.1^b	5.3 ± 0.2

Table 2. Concentrations of *E. coli*, *E. coli* O157:H7, *L. monocytogenes* and *V. parahaemolyticus* attached to seaweed before and after seaweed was washed. Within each row, data points that do not share a letter are significantly different from each other. Data points are the mean of 16 replicates \pm SE.

Bacteria	Pre-wash (\log_{10} CFU g ⁻¹)	Post-wash (\log_{10} CFU g ⁻¹)
<i>E. coli</i>	6.84 \pm 0.05 ^a	7.24 \pm 0.08 ^b
<i>E. coli</i> O157:H7	6.8 \pm 0.10 ^a	7.21 \pm 0.20 ^b
<i>L. monocytogenes</i>	5.01 \pm 0.40 ^a	5.83 \pm 0.07 ^b
<i>V. parahaemolyticus</i>	5.3 \pm 0.20 ^a	5.62 \pm 0.20 ^b

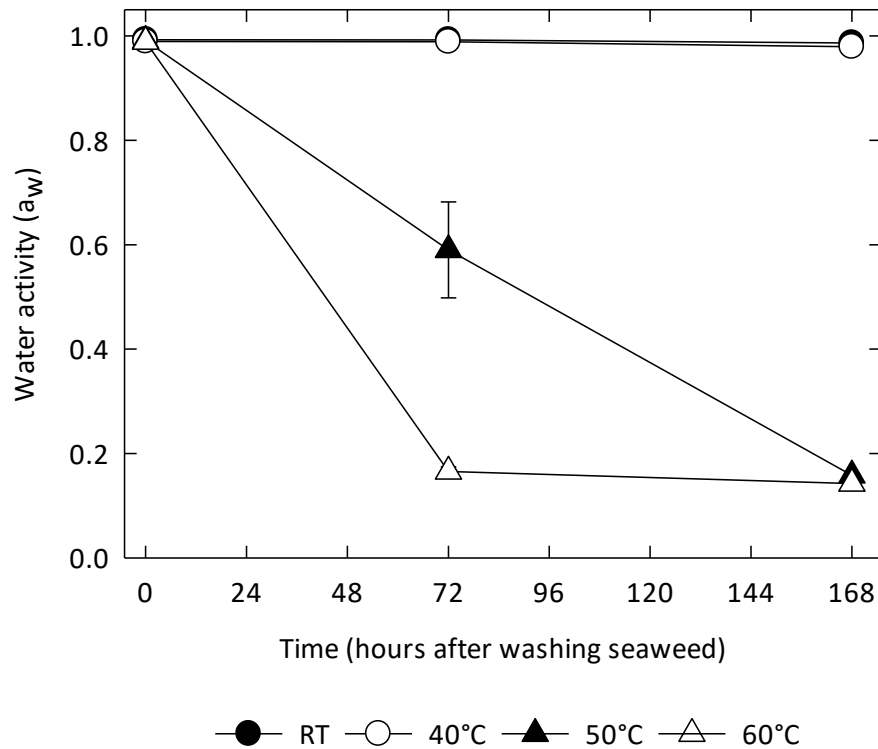


Figure 1. Water activity (a_w) in seaweed after washing and drying at RT (room temperature), 40 °C, 50 °C and 60 °C for 72 h and 168 h. Data points are the mean of four replicates \pm SE.

The a_w of seaweed dried at room temperature or at 40 °C did not significantly change during the drying process, whereas by 72 h and 168 h the a_w of seaweed dried at 50 °C and 60 °C had significantly decreased ($P < 0.05$) (Fig. 1). At room temperature, the desiccation of seaweed had no effect on the concentrations of *E. coli* or *E. coli* O157:H7 over the drying period (Fig. 2a and c), whereas the concentration of both *L. monocytogenes* and *V. parahaemolyticus* significantly increased ($P < 0.05$) during the drying process (Fig. 2e and g). Drying seaweed at 40 °C also had no effect on concentrations of *E. coli* attached to the seaweed (Fig. 2a); however, drying at 50 °C or 60 °C resulted in significant *E. coli* die-off to undetectable levels by 168 h or within 24 h respectively ($P < 0.05$). Subsequent storage of the seaweed for 72 h did not affect *E. coli* levels, regardless of the temperature at which the

seaweed had previously been dried, and although *E. coli* grew during storage from undetectable levels to $\sim 2 \log_{10}$ CFU in seaweed previously dried at 50 °C, this was not a significant increase (Fig. 2b).

Drying seaweed at 40 °C and 50 °C led to significant *E. coli* O157:H7 die-off to undetectable levels after 168 h ($P < 0.05$), whilst drying seaweed at 60 °C resulted in rapid die-off of the pathogen by 24 h (Fig. 2c). Storage for 72 h had no effect on pathogen levels in seaweed dried at 40 °C or 60 °C, which remained undetectable in both cases, or in seaweed dried at room temperature (Fig. 2d). Growth of *E. coli* O157:H7 was detected in stored seaweed, which had been dried at 50 °C, though this was not a significant increase.

L. monocytogenes survival on seaweed was significantly reduced by $\sim 5 \log_{10}$ CFU between 72 h and 120 h by drying at 50 °C ($P < 0.05$) (Fig. 2e). *L. monocytogenes* survival on seaweed was unaffected by drying at 40 °C or 60 °C (Fig. 2e), and persisted at $\sim 3 \log_{10}$ CFU after 168 h of drying at 60 °C (Fig. 2e). Storage for 72 h did not alter levels of *L. monocytogenes* attached to the seaweed, regardless of the previous drying temperature (Fig. 2f). Drying at 40 °C significantly decreased the concentration of *V. parahaemolyticus* from $\sim 6 \log_{10}$ CFU to $\sim 1 \log_{10}$ CFU after 168 h ($P < 0.05$) (Fig. 2g). *V. parahaemolyticus* was undetectable on seaweed following 168 h drying at 50 °C ($P < 0.05$), and after the first 24 h at 60 °C ($P < 0.05$). Storage for 72 h did not affect *V. parahaemolyticus* levels, regardless of the temperature at which the seaweed had previously been dried (Fig. 2h). During drying, the pH of the seaweed dried at RT, 40 °C, 50 °C and 60 °C significantly increased from \sim pH 6.5 to \sim pH 7.5 ($P < 0.05$). However, after 72 h storage, seaweed dried at all temperatures with the exclusion of the 40 °C treatment became more acidic ($P < 0.05$).

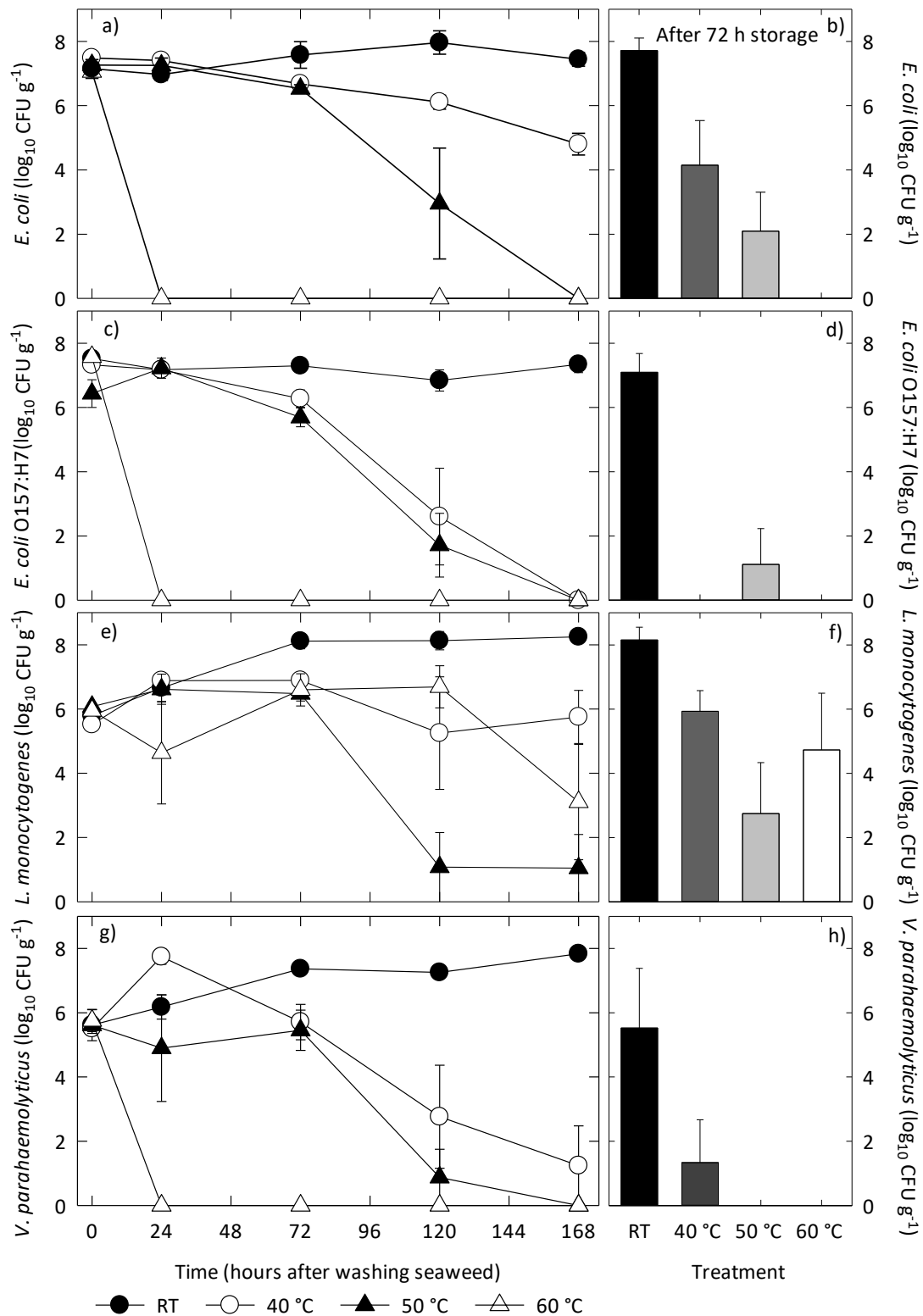


Figure 2. Survival of *E. coli* (a), *E. coli* O157:H7 (c), *L. monocytogenes* (e) and *V. parahaemolyticus* (g) on seaweed during drying at room temperature (filled circle), 40 °C (open circle), 50 °C (filled triangle) and 60 °C (open triangle). All seaweed samples had been washed just prior to the drying process beginning. Following the drying process all seaweed samples were stored for 72 h and pathogen survival enumerated again (b, d, f, h). Data points are the mean of four replicates ± SE.

3.3 Bacteriological safety of seaweed as BSFL feed

The mean weight of individual larvae significantly increased from 0.12 ± 0.01 g on day 3 to 0.26 ± 0.005 g on day 5 ($P < 0.05$), although the onset of pre-pupation from day 6 led to an overall decline in average weight. Water content in the inoculated substrate containing larvae was significantly lower than in substrate with no larvae ($P < 0.05$). In general, the concentrations of *E. coli*, *E. coli* O157:H7, *L. monocytogenes* and *V. parahaemolyticus* associated with BSF larvae were significantly lower than in the substrate throughout the entire sampling period (Fig. 3a-d). However, whilst the concentration of *E. coli* and *E. coli* O157:H7 associated with larvae significantly fell over the 8 days ($P < 0.05$), the larval loads of *L. monocytogenes* and *V. parahaemolyticus* did not change over the same period (Fig. 3a-d). In the absence of larvae, concentrations of *E. coli*, *E. coli* O157:H7, *L. monocytogenes* and *V. parahaemolyticus* in the feed substrate decreased significantly on day 2 ($P < 0.05$ in all cases); consequently, all four bacteria were $1 - 2 \log_{10}$ CFU higher in substrate in which larvae were present on days 3 and 4 ($P < 0.05$) (Fig.3a- d). The pH of non-inoculated feed in the presence of larvae significantly increased from 3.6 ± 0.11 on day 1 to 6.4 ± 0.13 by day 8 ($P < 0.05$).

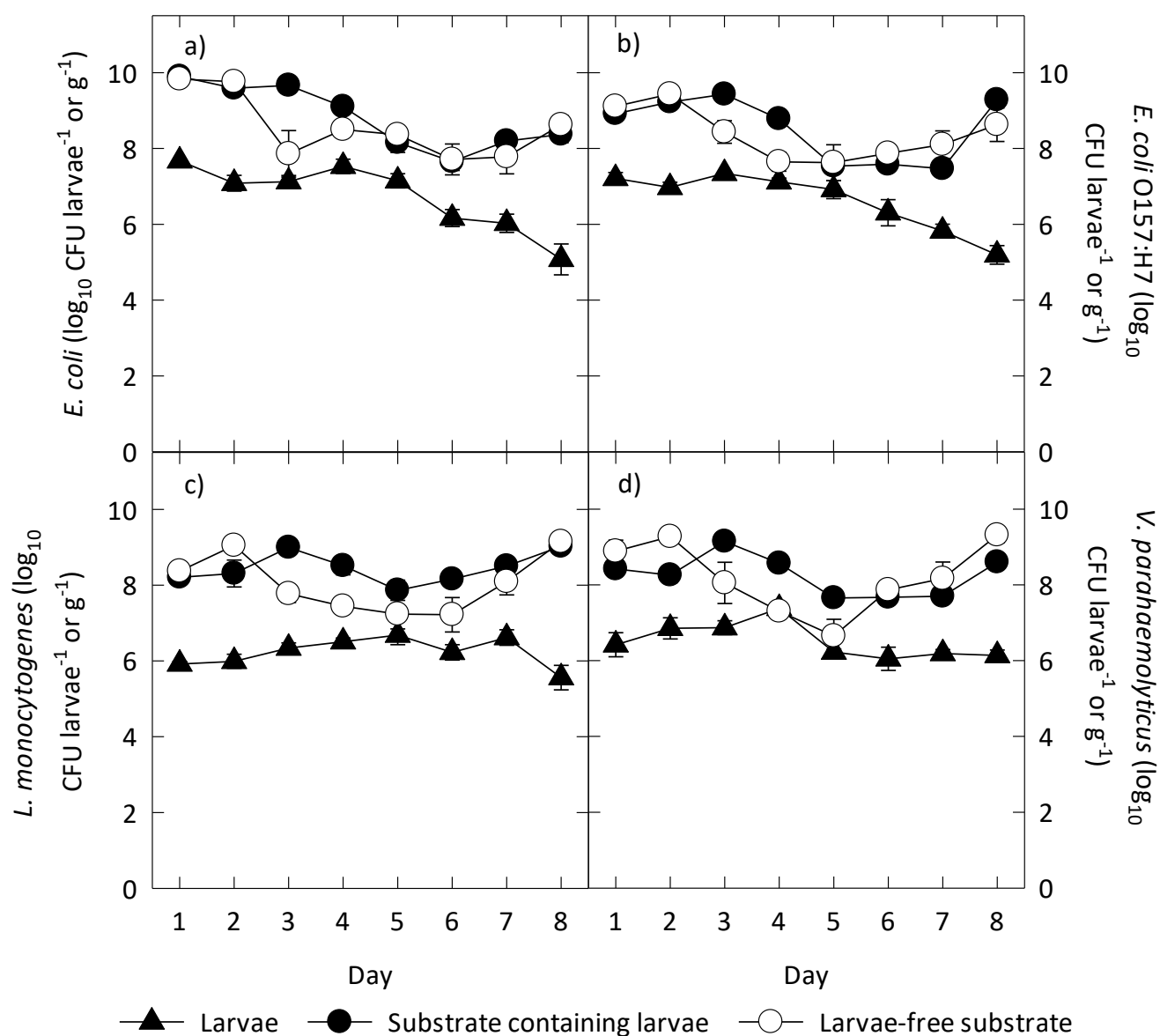


Figure 3. Concentration of *E. coli* (a), *E. coli* O157:H7 (b), *L. monocytogenes* (c) and *V. parahaemolyticus* (d) associated with BSFL (filled triangle), the substrate containing the BSFL (filled circle) and the larvae-free substrate (open circle). Data points are the mean of four replicates \pm SE.

4. DISCUSSION

4.1 Bacteriological safety of processed seaweed

This study demonstrates that the current post-harvest processes of washing and drying seaweed intended for animal feed can fail to eradicate (and can even encourage the survival of) *E. coli* and selected human pathogenic bacteria if seaweed has a high level of contamination at the point of seaweed harvest. The inadequacy of these manufacturing practices therefore, can result in a dried seaweed product in which human pathogenic bacteria can persist during storage, although survival will be variable depending on the strain or species of the pathogen. Our results have highlighted that the industry objective of maximising the nutritional benefits of seaweed by minimising the drying temperature comes at the cost of ensuring a microbiologically safe product.

E. coli, *E. coli* O157:H7, *L. monocytogenes* and *V. parahaemolyticus* all attached to submerged senescing seaweed, which has not been previously shown for *L. monocytogenes* in the natural environment. The persistence of these pathogens in seawater and their attachment to seaweed reflect their biofilm-forming ability and subsequent increased tolerance to the osmotic stress of seawater. Biofilm formation on the seaweed by the four bacteria would also have contributed to the inefficiency of the cleaning stage, and *V. parahaemolyticus* in particular can resist removal from seaweed by washing, (Mahmud *et. al.*, 2007). Free chlorine is present in most tap water at concentrations typically within the 0.2 - 1 mg/L range (WHO, 1996), which is capable of killing planktonic *E. coli* O157:H7 cells (Shen *et. al.*, 2013). However, any such reduction of seaweed-associated bacterial concentrations is likely to have been offset by neutralisation of free chlorine due to rapid reaction with seaweed exudates (Shen *et. al.*, 2013). Mechanically cutting the seaweed would have released sugars, such as mannitol, which may also have facilitated the growth of these pathogens in otherwise stressful conditions. Increasing the duration of the washing step therefore, is not likely to have improved the effectiveness of pathogen removal.

During the drying of seaweed at ≥ 50 °C, the a_w of the feed material was reduced. Die-off of *E. coli*, *E. coli* O157:H7 and *V. parahaemolyticus* due to 50 °C heat stress was delayed compared with more rapid bacterial inactivation occurring at 60 °C, probably as a result of a_w levels that allowed microbial survival within the first 72 h. *L. monocytogenes* exhibited poorer heat resistance at 50 °C compared with the 60 °C treatment, most likely a result of the a_w being insufficiently low in the first 72 h to protect the bacterial cells from 50 °C heat damage. Drying seaweed at 60 °C had no effect on Gram-positive *L. monocytogenes*, yet within 24 h led to log-linear inactivation of the more desiccation intolerant Gram-negative *E. coli*, *E. coli* O157:H7 and *V. parahaemolyticus* populations to undetectable levels.

Attaining a long shelf-life for seaweed meal by preventing microbial decay through desiccation is reliant on achieving a well-controlled and homogenous drying treatment; however, the temperature within convection ovens can vary significantly resulting in non-uniform heat dispersion throughout the product (Bonazzi and Dumoulin, 2011; Roos *et. al.*, 2018). In addition, the high salt content of seaweed may inhibit bacterial growth due to its disruptive effect on the osmotic balance of cells, whilst also contributing to the lowering of a_w and thus the thermal resistance of bacterial cells, particularly *L. monocytogenes* (Burgess *et. al.*, 2016). Despite these confounding factors, reduction of some key bacterial contaminants even from high initial concentrations in seaweed during the drying CCP is possible in order to attain control of bacterial growth during the storage CCP of the product. Although higher drying temperatures achieve shorter drying times (Chenlo *et. al.*, 2018), drying seaweed at a lower temperature retains a higher proportion of nutritional properties within the final seaweed product adding value to animal feed (Sappati *et. al.*, 2018). Our results have shown that with the exception of *L. monocytogenes*, drying temperatures of 60 °C exert a lethal effect on pathogens sufficiently rapidly to circumvent the property of thermal resistance of bacterial cells by a low a_w and prevent re-emergence of the bacteria in stored feed.

Growth from previously undetectable levels of *E. coli* and *E. coli* O157:H7 in seaweed powder stored at ambient temperature following 50 °C drying suggests that, in favourable conditions, bacteria were able to replicate, or that viable but non-culturable (VBNC) cells were able to recover culturability (Orruno *et. al.*, 2017). The storage bags were not airtight, which would have allowed the dried seaweed powder to absorb atmospheric moisture and thus increase the a_w (Hyun *et. al.*, 2018). If bacterial cells do enter a VBNC state during the processing of low moisture feed, there is the potential for prolonged survival and subsequent growth under favourable conditions further along the processing chain. This is of particular concern for pathogens with a low infective dose such as *E. coli* O157:H7, where a relatively small number of persistent cells can pose a significant public health risk (Esbelin *et. al.*, 2018). Application of 72 °C heat for 2 minutes is generally considered to assure sterilisation of food products contaminated with *Listeria* spp. (Smelt and Brul, 2014). Future assessments are needed to quantify the growth potential of pathogenic bacteria over an extended duration, e.g. the typical one year shelf life of dried seaweed powder, to fully appreciate the risk of pathogen persistence in seaweed feed.

4.2 Microbial safety of seaweed as BSFL feed

This study has shown that BSFL can become rapidly contaminated (attachment to the exoskeleton and via GIT recontamination) from their feed substrate, indicating that at the point of harvest a decontamination step would be required. The high concentrations at which bacteria were inoculated into the substrate (which is comparable to using seaweeds that had been dried at low temperatures) prevented the BSFL from reducing their internal pathogen loads at the point of harvest. However, BSFL exhibited a capacity to suppress larval-associated *E. coli* strains, indicating a potential to clear this bacteria from the GIT if ingested at lower concentrations. The concentration of bacteria associated with the larvae was less than the substrate throughout the rearing period, which may indicate effective digestion, inactivation, or antimicrobial action in the larval GIT (Wynants *et. al.*, 2018a). The

expression of antimicrobial peptides (AMP) by BSFL is particularly marked when fed protein-rich diets such as that provided in this study, and larvae can adapt the diversity of their AMP in response to the microbiome of their environment, enabling them to exploit diverse diets (Vogel *et. al.*, 2018). The decline in larvae-associated *E. coli* and *E. coli* O157:H7 loads during larval development to pre-pupation could reflect selective inactivation of ingested *E. coli* strains in the larval GIT via exposure to increasing levels of GIT antimicrobials (Wynants *et. al.*, 2018a; Engel and Moran, 2013; De Smet *et. al.*, 2018). Importantly, neither *E. coli* nor any of the pathogenic bacteria colonised or accumulated in the larval GIT during rearing. The concentrations at which the four bacteria were introduced to the seaweed powder supplement far exceeded the levels persisting in the stored seaweed powder following drying at 50 °C. In this study, pathogenic bacteria may not be eliminated by either gut voidance during metamorphosis into pre-pupae, or by 48 h starvation of larvae free of contact with their faeces prior to harvesting (Wynants *et. al.*, 2017). Therefore, sterilisation of the larvae meal and lipids during subsequent processing steps is recommended.

The hydration of the inoculated substrate with tap water potentially containing free chlorine is likely to have had a negligible effect on such high pathogen concentrations present in the feed and therefore in the larvae. Furthermore, in the acidic conditions of the substrate, chlorine may have been largely present in the hypochlorous acid form; this reacts rapidly with organic matter to form combined chlorine compounds, which exhibit limited antimicrobial activity (Delaquis *et. al.*, 2004). During this trial, the pH of the larval feed increased from acidic to near neutral, which is associated with the release of ammonia from BSFL excretion (Rehman *et. al.*, 2017). However, all three pathogens seemed to overcome the inhibitory effects of this change in pH environment.

A global increase in human *Vibrio* infections are associated with increased sea surface temperatures (Vezzulli *et. al.*, 2015), and may be exacerbated by an increased risk of *Vibrio* spp associated with seaweed entering human food chains via its use as feed and food. Bacteriological criteria for pathogenic *Vibrio* spp. in seaweed-fed insects, particularly for species enriched in seaweed-

sourced omega-3 for direct human nutrition, should be established for products before they leave the food-processing environment. Seaweed growing in coastal waters and harvested for feed and food may also pose a public health risk as reservoirs of terrestrial and aquatic sources of multi-antimicrobial resistant (AMR) bacteria. Antibiotic residues in feed may be one explanation for the occurrence of AMR genes in industrially reared mealworms and crickets (Vandeweyer *et. al.*, 2019).

Pathogen levels in feed are a function of the ability of the specific bacteria to tolerate and adapt to the intrinsic nature of the feed material, and the physio-chemical stresses incurred during processing of the product. The introduction of seaweed-fed insect larvae as a novel aquafeed ingredient will expand the feed resource base and contribute to future-proofing sustainability of the animal-based feed and food chain, but inadequate control of bacterial pathogens in the feed could ultimately pose health risks to the farmed animal and/or human consumers. Understanding opportunities for microbial contamination and growth at critical stages of the farm-to-fork continuum is key to microbiological risk reduction. As with traditional animal feed, quality control of pre-harvest seaweed as part of good agricultural practice should be seen as the principle means by which the feed industry can control the potential presence of seaweed-associated pathogenic bacteria in BSF pre-pupae.

5. CONCLUSION

Ensuring production of safe novel animal feed ingredients requires understanding of both the specific bacterial hazards associated with the novel ingredients, and the response of those bacteria to abiotic and biotic processing stresses. Persistence in seawater, and rapid colonisation of brown, red and green seaweeds, by some key human pathogens, indicates that water quality at seaweed harvesting sites should be considered a key CCP at the start of the production chain. In the seaweed feed sector, washing and drying seaweed are not intended or expected to remove bacterial contaminants, but low temperature desiccation favoured by the industry encourages pathogen persistence and growth during storage. This indicates a need for industry-wide adoption of a minimal

536 seaweed drying temperature-time- a_w treatment to guarantee product quality during its shelf life.
537 Seaweed feed, like all raw feed materials, will be a key CCP in the management of bacteriological
538 hazards in insect production. The bacteriological risk profile of BSFL and other insects using seaweed
539 as a feed supplement will reflect the unique dynamics between insect species and bacterial species
540 and strains. Development of robust HACCP guidelines, and improved good agricultural practice,
541 bacteriological standards and GMP, for each stage of the production chain will encourage regulatory
542 and commercial acceptability of seaweed-fed insects for both feed and food.

543

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