1 **The microbial safety of seaweed as a feed component for black soldier fly**

2 **(*Hermetia illucens*) larvae**

3

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14 **ABSTRACT**

15 Farming insects for use as an alternative aquafeed or livestock ingredient can deliver environmental
16 and nutritive benefits. The larvae of the terrestrial black soldier fly (*Hermetia illucens*) (BSF) however,
17 could be improved by the dietary inclusion of seaweed to incorporate valuable nutrients associated
18 with the marine environment, e.g. omega-3. Standardised processing methods in the feed and food
19 industries are key to product quality and safety; although currently such systems are limited for
20 seaweed and insect processing. The industry practice of drying seaweed at low temperatures to retain
21 nutritional properties may benefit the survival of human pathogenic bacteria, particularly if the
22 seaweed has been harvested from contaminated water. Here we have determined the risk of bacterial
23 (*E. coli*, *E. coli* O157:H7, *Listeria monocytogenes* and *Vibrio parahaemolyticus*), survival during
24 seaweed drying and processing as feed for insect larvae and the subsequent risk of BSF larvae
25 contamination. All four bacteria colonised seaweed and resisted removal by washing. *E. coli* and *E.*
26 *coli* O157:H7 died-off in seaweed dried at 50 °C, although were detected in the dried powder following
27 72 h storage, indicating an increase in water activity (a_w) in the stored product. *V. parahaemolyticus*
28 fell below the level of detection in stored seaweed after drying at ≥ 50 °C, but *L. monocytogenes*
29 remained detectable, and continued to grow in seaweed dried at ≤60 °C. BSF larvae reared on an
30 artificially contaminated seaweed-supplemented diet became colonised by all four bacteria present
31 in the supplement. Therefore, drying seaweed at lower temperatures in order to preserve the
32 maximum nutritive potential also risks pathogen carry-over (particularly if harvested from
33 contaminated water) into BSF larvae destined for livestock feed. Seaweed processing and storage
34 conditions, and the subsequent production of insect feed, represent critical control points where good
35 manufacturing practices are needed to target the control of pathogenic hazards. Therefore, robust
36 environmental management of seaweed harvesting waters is needed in order to provide the
37 regulatory framework for including seaweeds, and the processing of insect larvae, into sustainable
38 livestock and aquaculture feed chains.

39 **1. INTRODUCTION**

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

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

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

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

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

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

94

95 **2. MATERIALS AND METHODS**

96 2.1 Bacteriological safety of processed seaweed (Experiment 1)

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

100

101 2.2 Seaweed material

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

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

120

121 2.3 Inoculum preparation

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

137 2.4 Simulated microbial contamination of pre-harvested seaweed

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

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

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

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

165 2.5 Simulated post-harvest seaweed processing
166

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

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

187 Bacterial concentrations on the inoculated seaweed were enumerated during the drying
188 process at 24, 72, 120 and 168 h, using the methods above with the exception of seaweed dried at 50
189 °C and 60 °C from 72 h onwards, which was sufficiently desiccated to be ground to a fine powder using
190 a pestle and mortar. To determine bacterial concentrations in this seaweed powder, 2 g of powder

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

195 After 168 h drying, seaweed from all trays was transferred to individual enclosed plastic boxes
196 and stored at RT. Moisture loss from seaweed that had been dried at RT and 40 °C was insufficient to
197 enable the seaweed to be ground to a powder prior to storage. Each seaweed mix from these groups
198 was therefore individually homogenised with no added liquid for 3 min using a hand blender to
199 approximately 5 mm^2 prior to storage. Seaweed dried at 50 °C and 60 °C was ground to a fine powder
200 (approximately 0.5 – 1 mm^2) using a pestle and mortar prior to storage. After 72 h storage, bacterial
201 concentrations in all seaweed samples were quantified as described above.

202

203 2.5 Bacteriological safety of seaweed as BSFL feed (Experiment 2)
204
205 A simulation of mass rearing of BSFL on feed supplemented with pathogen-contaminated
206 seaweed powder was undertaken. Larvae and the feed substrate were sampled throughout the
207 rearing period up to the point of pre-pupae harvest to assess both the microbial load of the feed and
208 the hygienic status of the larvae.

209

210 2.7 Preparation of BSF colonies

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

217 vegetable waste. Every 2 days, feed substrate was supplemented to a depth of approximately 12 cm
218 and 200 ml of water was added. Holes in the base of the containers enabled drainage of excess liquid
219 to prevent waterlogging and anoxic conditions developing in the feed substrate. Within the tent,
220 cardboard boxes containing shredded newspaper provided dark sheltered conditions for pupation.
221 Once adult flies emerged, sliced fruit was placed on the surface of the feed substrate and water (< 20
222 ml) was sprayed into the tent hourly during the day. Corrugated cardboard strips were laid across the
223 feed container above the level of the feed to provide dry crevices in which the female flies laid their
224 eggs. As soon as eggs were observed in a cardboard strip, the strip was transferred to another insect
225 tent and suspended above a tray containing feed substrate comprised of the same ingredients as
226 described above. After hatching, the larvae dropped from the cardboard strip into the substrate, and
227 were harvested for future experiments at approximately 1 week old.
228

229 2.8 Preparation of seaweed powder and inoculation procedure
230

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

244

245 2.9 Simulated mass-production of BSFL reared on seaweed supplement

246

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

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

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

276 2.10 Statistical analyses
277

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

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

302

303 **3. RESULTS**

304
305 3.1 Background bacteriological status of seaweed and seawater

306
307 *E. coli* was not detected on the freshly harvested seaweed used in both Experiments 1 and 2,
308 and was present at a very low concentration (< 10 CFU 100 ml^{-1}) in the seawater from which the
309 seaweed was harvested. Total heterotrophic bacteria were present in low abundance on all species of
310 seaweed and in seawater, the highest concentrations being detected on *L. digitata* and in seawater
311 (data not shown).

312

313 3.2 Bacteriological safety of processed seaweed

314
315 After 24 h in a rotating incubator at room temperature (20.5 °C ± 3 °C), concentrations of *E.*
316 *coli*, *E. coli* O157:H7, *L. monocytogenes* and *V. parahaemolyticus* in the seawater had fallen by ~2 log_{10}
317 CFU, and *E. coli* and *E. coli* O157:H7. Cell concentrations in seawater and on seaweed are provided in
318 Table 1. The concentration of all four bacteria associated with the seaweed significantly increased (P
319 < 0.05 in all cases) after it had been washed under running tap water (Table 2). However, washing
320 seaweed after 24 h submergence in seawater did not affect seaweed pH.

321

322

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

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

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

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

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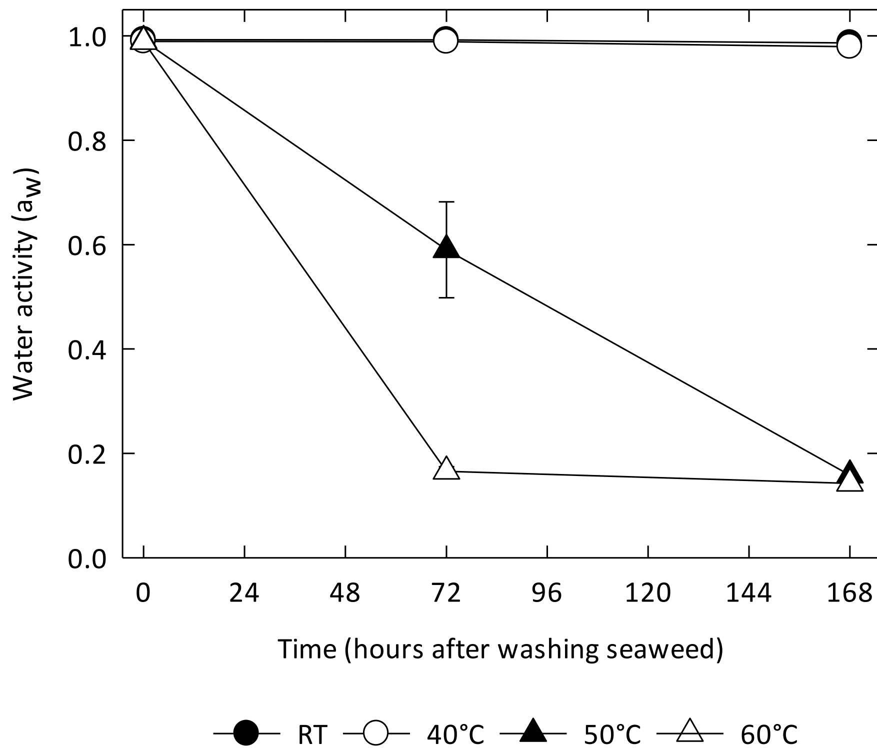

343

344 **Figure 1.** Water activity (a_w) in seaweed after washing and drying at RT (room temperature), 40 °C, 50
345 °C and 60 °C for 72 h and 168 h. Data points are the mean of four replicates ± SE.

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

357 seaweed had previously been dried, and although *E. coli* grew during storage from undetectable levels

358 to ~2 log_{10} CFU in seaweed previously dried at 50 °C, this was not a significant increase (Fig. 2b).

359 Drying seaweed at 40 °C and 50 °C led to significant *E. coli* O157:H7 die-off to undetectable

360 levels after 168 h ($P < 0.05$), whilst drying seaweed at 60 °C resulted in rapid die-off of the pathogen

361 by 24 h (Fig. 2c). Storage for 72 h had no effect on pathogen levels in seaweed dried at 40 °C or 60 °C,

362 which remained undetectable in both cases, or in seaweed dried at room temperature (Fig. 2d).

363 Growth of *E. coli* O157:H7 was detected in stored seaweed, which had been dried at 50 °C, though this

364 was not a significant increase.

365 *L. monocytogenes* survival on seaweed was significantly reduced by ~5 log_{10} CFU between 72

366 h and 120 h by drying at 50 °C ($P < 0.05$) (Fig. 2e). *L. monocytogenes* survival on seaweed was

367 unaffected by drying at 40 °C or 60 °C (Fig. 2e), and persisted at ~3 log_{10} CFU after 168 h of drying at

368 60 °C (Fig. 2e). Storage for 72 h did not alter levels of *L. monocytogenes* attached to the seaweed,

369 regardless of the previous drying temperature (Fig. 2f). Drying at 40 °C significantly decreased the

370 concentration of *V. parahaemolyticus* from ~6 log_{10} CFU to ~1 log_{10} CFU after 168 h ($P < 0.05$)(Fig. 2g).

371 *V. parahaemolyticus* was undetectable on seaweed following 168 h drying at 50 °C ($P < 0.05$), and after

372 the first 24 h at 60 °C ($P < 0.05$). Storage for 72 h did not affect *V. parahaemolyticus* levels, regardless

373 of the temperature at which the seaweed had previously been dried (Fig. 2h). During drying, the pH

374 of the seaweed dried at RT, 40 °C, 50 °C and 60 °C significantly increased from ~pH 6.5 to ~pH 7.5 ($P <$

375 0.05). However, after 72 h storage, seaweed dried at all temperatures with the exclusion of the 40 °C

376 treatment became more acidic ($P < 0.05$).

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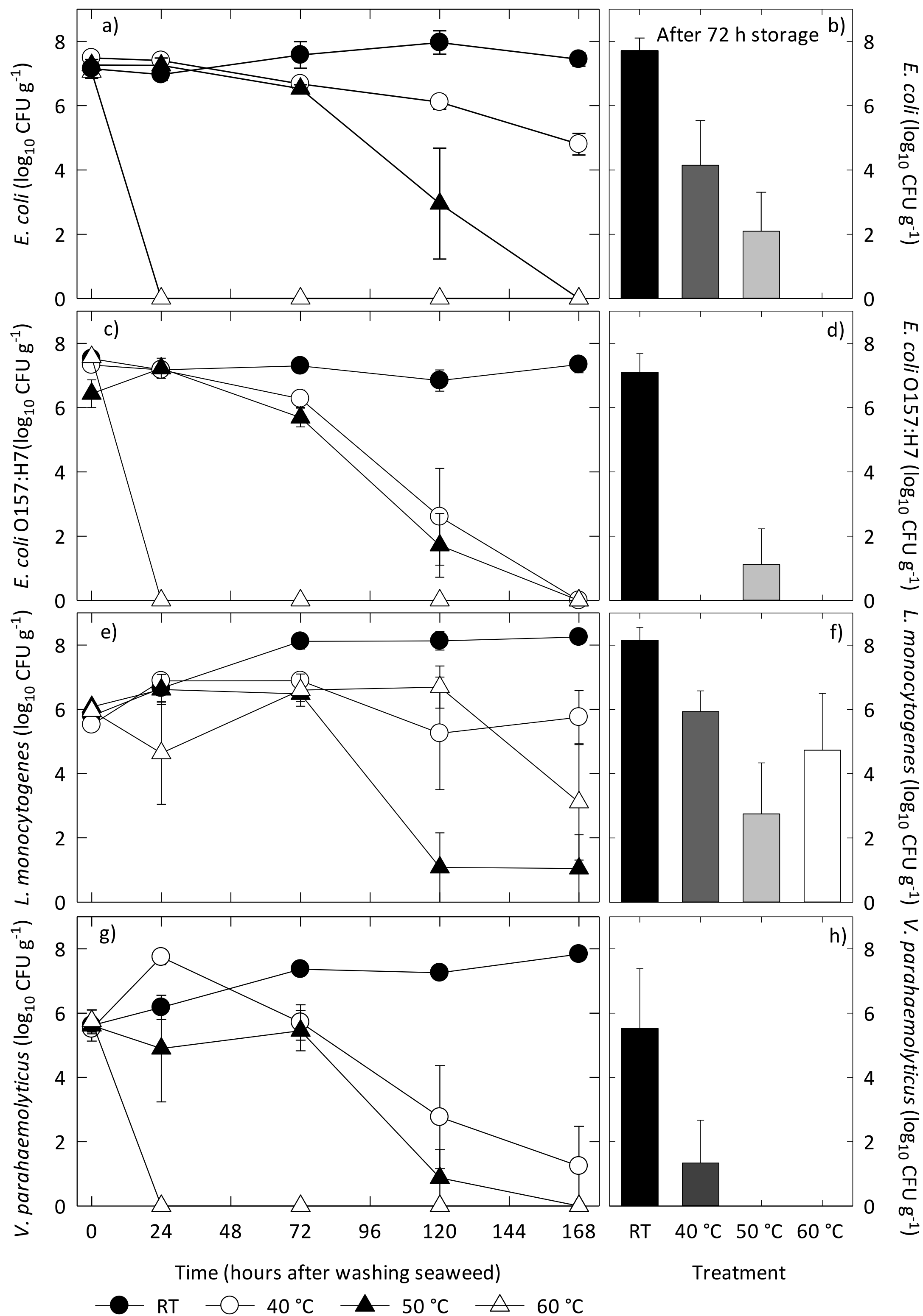

379 RT 40 °C 50 °C 60 °C

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

385 3.3 Bacteriological safety of seaweed as BSFL feed

386

387 The mean weight of individual larvae significantly increased from 0.12 ± 0.01 g on day 3 to 388 0.26 ± 0.005 g on day 5 (P < 0.05), although the onset of pre-pupation from day 6 led to an overall 389 decline in average weight. Water content in the inoculated substrate containing larvae was 390 significantly lower than in substrate with no larvae (P < 0.05). In general, the concentrations of *E. coli*, 391 *E. coli* O157:H7, *L. monocytogenes* and *V. parahaemolyticus* associated with BSF larvae were 392 significantly lower than in the substrate throughout the entire sampling period (Fig. 3a-d). However, 393 whilst the concentration of *E. coli* and *E. coli* O157:H7 associated with larvae significantly fell over the 394 8 days (P < 0.05), the larval loads of *L. monocytogenes* and *V. parahaemolyticus* did not change over 395 the same period (Fig. 3a-d). In the absence of larvae, concentrations of *E. coli*, *E. coli* O157:H7, *L.* 396 *monocytogenes* and *V. parahaemolyticus* in the feed substrate decreased significantly on day 2 (P < 397 0.05 in all cases); consequently, all four bacteria were 1 – 2 $\log_{10}$ CFU higher in substrate in which 398 larvae were present on days 3 and 4 (P < 0.05) (Fig.3a- d). The pH of non-inoculated feed in the 399 presence of larvae significantly increased from 3.6 ± 0.11 on day 1 to 6.4 ± 0.13 by day 8 (P <0.05).

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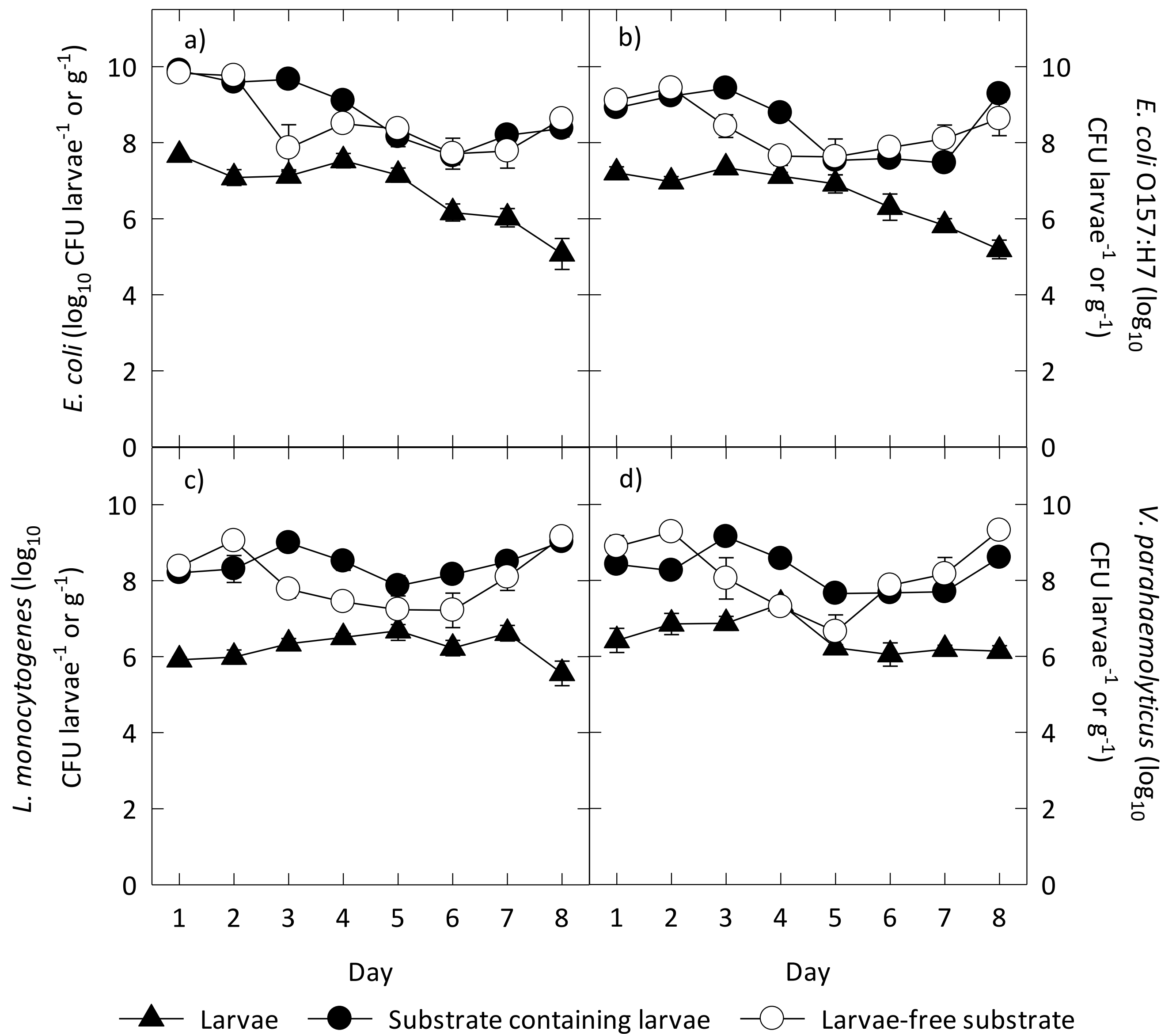

401

402 **Figure 3**. Concentration of *E. coli* (a), *E. coli* O157:H7 (b), *L. monocytogenes* (c) and *V. parahaemolyticus*

403 (d) associated with BSFL (filled triangle), the substrate containing the BSFL (filled circle) and the larvae-

404 free substrate (open circle). Data points are the mean of four replicates ± SE.

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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 stain 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.

435 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.

444 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.

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

473

474 4.2 Microbial safety of seaweed as BSFL feed

475

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

485 expression of antimicrobial peptides (AMP) by BSFL is particularly marked when fed protein-rich diets
486 such as that provided in this study, and larvae can adapt the diversity of their AMP in response to the
487 microbiome of their environment, enabling them to exploit diverse diets (Vogel *et. al.,* 2018). The
488 decline in larvae-associated *E. coli* and *E. coli* O157:H7 loads during larval development to pre-
489 pupation could reflect selective inactivation of ingested *E. coli* strains in the larval GIT via exposure to
490 increasing levels of GIT antimicrobials (Wynants *et. al.,* 2018a; Engel and Moran, 2013; De Smet *et. al.,*
491 2018). Importantly, neither *E. coli* nor any of the pathogenic bacteria colonised or accumulated in the
492 larval GIT during rearing. The concentrations at which the four bacteria were introduced to the
493 seaweed powder supplement far exceeded the levels persisting in the stored seaweed powder
494 following drying at 50 °C. In this study, pathogenic bacteria may not be eliminated by either gut
495 voidance during metamorphosis into pre-pupae, or by 48 h starvation of larvae free of contact with
496 their faeces prior to harvesting (Wynants *et. al.,* 2017). Therefore, sterilisation of the larvae meal and
497 lipids during subsequent processing steps is recommended.

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

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

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

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

526 5. CONCLUSION

527
528 Ensuring production of safe novel animal feed ingredients requires understanding of both the
529 specific bacterial hazards associated with the novel ingredients, and the response of those bacteria to
530 abiotic and biotic processing stresses. Persistence in seawater, and rapid colonisation of brown, red
531 and green seaweeds, by some key human pathogens, indicates that water quality at seaweed
532 harvesting sites should be considered a key CCP at the start of the production chain. In the seaweed
533 feed sector, washing and drying seaweed are not intended or expected to remove bacterial
534 contaminants, but low temperature desiccation favoured by the industry encourages pathogen
535 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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548 Austin (CEFAS) for providing the strain of *V. parahaemolyticus*.

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551 **REFERENCES**

552 Belghit, I., Liland, N., Waagbo, R., Biancarosa, I., Pelusio, N., Li, Y., Krogdahl, A. and Lock, E-J. (2018)
553 Potential of insect-based diets for Atlantic salmon (*Salmo salar*). Aquaculture 491: 72-81.

554 Burgess, C., Gianotti, A., Gruzdev, N., Holah, J., Knochel, S., Lehner, A., Margas, E., Schmitz Esser, S.,
555 Sela, S. and Tresse, O. (2016) The response of foodborne pathogens to osmotic and desiccation
556 stresses in the food chain. International Journal of Food Microbiology 221: 37-53.

557 De Smet, J., Wynants, E., Cos, P. and Van Campenhout, L. (2018) Microbial community dynamics during
558 rearing of black soldier fly larvae (*Hermetia illucens*) and its impact on exploitation potential. Applied
559 and Environmental Microbiology 84: e02722-17

560 Del Olmo, A., Picon, A. and Nunez, M. (2018) The microbiota of eight species of dehydrated edible
561 seaweeds from North West Spain. Food Microbiology 70: 224-231.

562 Delaquis, P., Fukumoto, L. Toivonen, P. and Cliff, M. (2004) Implications of wash water chlorination
563 and temperature for the microbiological and sensory properties of fresh-cut iceberg lettuce.
564 Postharvest Biology and Technology 31: 81-91.

565 Esbelin, J., Santos, T. and Hebraud, M. (2018) Desiccation: an environmental and food industry stress
566 that bacteria commonly face. Food Microbiology 69: 82-88.

567 European Commission (EC) (2005a) Council Regulation (EC) 183/2005/EC of 12 January 2005 laying
568 down requirements for feed hygiene. Official Journal of the European Union L35: 1-22.

569 European Commission (EC) (2005b) Commission Regulation (EC) 2073/2005 of 15 November 2005 on
570 microbiological criteria for foodstuffs. Official Journal of the European Union L338: 1-26.

571 European Commission (EC) (2011) Commission Regulation (EC) 142/2011 of 25 February 2011
572 implementing Regulation (EC) No 1069/2009 of the European Parliament and of the Council laying
573 down health rules as regards animal by-products and derived products not intended for human
574 consumption and implementing Council Dircective 97/78/EC as regards certain samples and items
575 exempt from veterinary checks at the border under that Directive. Official Journal of the European
576 Union L54: 1-254.

577 European Commission (EC) (2013) Commission Regulation (EU) 68/2013/EC of 16 January 2013 on the
578 Catalogue of feed materials. Official Journal of the European Union L29: 1-64.

579 European Commission (EC) (2017) Council Regulation (EC) 893/2017/EC of 24 May 2017 amending
580 Annexes I and IV to Regulation (EC) No 999/2001 of the European Parliament and of the Council and
581 Annexes X, XIV and XV to Commission Regulation (EU) No 142/2011 as regards the provisions on
582 processed animal protein. Official Journal of the European Union L138: 92-116.

583 Gupta, S., Cox, S. and Abu-Ghannam, N. (2011) Effect of different drying temperatures on the moisture
584 and phytochemical constituents of edible Irish brown seaweed. LWT- Food Science and Technology
585 44: 1266-1272.

586 Hyun, J., Kim, J., Choi, Y., Kim, E., Kim, J. and Lee, S. (2018) Evaluation of microbial quality of dried
587 foods stored at different relative humidity and temperature, and effect of packaging methods. Journal
588 of Food Safety: 38: e12433.

589 Lage-Yusty, M., Alvarado, G., Ferraces-Casais, P. and Lopez-Hernandez, J. (2014) Modification of
590 bioactive compounds in dried edible seaweeds. International Journal of Food Science and Technology
591 49: 298-304.

592 Liland, N., Biancarosa, I., Araujo, P., Biemans, D., Bruckner, C., Waagbo, R., Torstensen, B. and Lock, E-
593 J. (2017) Modulation of nutrient composition of black soldier fly (*Hermetia illucens*) larvae by feeding
594 seaweed-enriched media. PLoS ONE 12: e0183188

595 Mahmud, Z., Neogi, S., Kasu, A., Wada, T., Islam, M., Nair, G. and Ota, F. (2007) Seaweeds as a reservoir
596 for diverse *Vibrio parahaemolyticus* populations in Japan. International Journal of Food Microbiology
597 118: 92-96.

598 Mahmud, Z., Neogi, S., Kasu, A., Huong, B., Jahid, I., Islam, M. and Ota, F. (2008) Occurrence,
599 seasonality and genetic diversity of *Vibrio vulnificus* in coastal seaweeds and water along the Kii
600 Channel, Japan. FEMS Microbiological Ecology 64: 209-218.

601 Makkar, H., Tran, G., Heuze, V., Giger-Reverdin, S., Lessire, M., Lebas, F. and Ankers, P. (2016)
602 Seaweeds for livestock diets: A review. Animal Feed Science and Technology 212: 1-17.

603 Moreira, R., Chenlo, F., Sineiro, J., Arufe, S. and Sexto, S. (2016) Drying temperature effect on powder
604 physical properties and aqueous extract characteristics of *Fucus vesiculosus*. Journal of Applied
605 Phycology 28: 2485-2494.

606 Orruno, M., Kaberdin, V. and Arana, I. (2017) Survival strategies of *Escherichia coli* and *Vibrio* spp.:
607 contribution of the viable but nonculturable phenotype to their stress-resistance and persistence in
608 adverse environments. World Journal of Microbiology and Biotechnology: 33: 45.

609 Quilliam, R.S., Jamieson, J. and Oliver, D. (2014) Seaweeds and plastic debris can influence the survival
610 of faecal indicator organisms in beach environments. Marine Pollution Bulletin 84: 201-207.

611 Rajauria, G. (2015) Seaweeds: a sustainable feed source for livestock and aquaculture. In Tiwari, B.
612 and Try, D (Eds.) Seaweed sustainability. Food and non-food applications. Elsevier Academic Press. pp.
613 389-420.

614 Rehman, K., Rehman, A., Cai, M., Zheng, L., Xiao, X., Somroo, A., Wang, H., Li, W., Yu, Z. and Zhang, J.
615 (2017) Conversion of mixtures of dairy manure and soybean curd residue by black soldier fly larvae
616 (*Hermetia illucens* L.). Journal of Cleaner Production 154: 366-373.

617 Sappati, P., Nayak, B., Van Walsum, G. and Mulrey, O. (2018) Combined effects of seasonal variation
618 and drying methods on the physicochemical properties and antioxidant activity of sugar kelp
619 (*Saccharina latissima*). Journal of Applied Phycology 31: 1311-1332

620 Shen, C., Luo, Y., Nou, X., Wang, Q. and Millner, P. (2013) Dynamic effects of free chlorine
621 concentration, organic load, and exposure time on the inactivation of *Salmonella*, *Escherichia coli*
622 O157:H7, and non-O157 shiga toxin-producing *E. coli*. Journal of Food Protection 76: 386-393.

623 Smelt, J. and Brul, S. (2014) Thermal inactivation of microorganisms. Critical Reviews in Food Science
624 and Nutrition 54: 1371-1385.

625 Stevant, P., Indergard, E., Olafsdottir, A., Marfaing, H., Larssen, W., Fleurence, J., Roleda, M., Rustad,
626 T., Slizyte, R. and Nordtvedt, T. (2018) Effects of drying on the nutrient content and physico-chemical
627 and sensory characteristics of the edible kelp *Saccharina latissimi*. Journal of Applied Phycology 30:
628 2587-2599.

629 Swinscoe, I., Oliver, D.M., Gilburn, A.S., Lunestad, B., Lock, E.J., Ørnsrud, R. and Quilliam, R.S. (2019).
630 Seaweed-fed black soldier fly (Hermetia illucens) larvae as feed for salmon aquaculture: assessing the
631 risks of pathogen transfer. Journal of Insects as Food and Feed 5: 15-27.

632 Van der Spiegel, M., Noordam, M. and Van der Fels-Klerx, H.J. (2013) Safety of novel protein sources
633 (insects, microalgae, seaweed, duckweed, and rapeseed) and legislative aspects for their application
634 in food and feed production. Comprehensive Reviews in Food Science and Food Safety 12: 662-678.

635 Van Raamsdonk, L., van der Fels-Klerx, H. and Jong, J. (2017) New feed ingredients: the insect
636 opportunity. Food Additives and Contaminants: Part A. 34: 1384-1397.

637 Vandeweyer, D., Milanovic, V., Garofalo, C., Osimani, A., Clementi, F., Van Campenhout, L. and
638 Aquilanti, L. (2019) Real-time PCR detection and quantification of selected transferable antibiotic
639 resistance genes in fresh edible insects from Belgium and the Netherlands. International Journal of
640 Food Microbiology 290: 288-295.

641 Vezzulli, L., Pezzati, E., Brettar, I., Hofle, M. and Pruzzo, C. (2015) Effects of global warming on *Vibrio*
642 ecology. Microbiology Spectrum 3: 3.

643 Vogel, H., Muller, A., Heckel, D., Gutzeit, H. and Vilcinskas, A. (2018) Nutritional immunology:
644 diversification and diet-dependent expression of antimicrobial peptides in the black soldier fly
645 *Hermetia illucens*. Developmental and Comparative Immunology 78: 141-148.

646 World Health Organisation (WHO) (1996) Guidelines for drinking-water quality. Volume 2. Health
647 criteria and other supporting information. Second Edition. WHO, Geneva. Available at:
648 https://apps.who.int/iris/handle/10665/38551

649 Wynants, E., Frooninckx, L., van Miert, S., Geeraerd, A., Claes, J. and van Campenhout, L. (2019) Risks
650 related to the presence of *Salmonella* sp. during rearing of mealworms (Tenebrio molitor) for food or
651 feed: survival in the substrate and transmission to the larvae. Food Control 100: 227-234.

652