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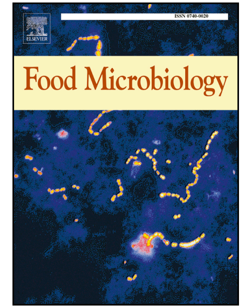

The microbial safety of seaweed as a feed component for black soldier fly (*Hermetia illucens*) larvae

Isobel Swinscoe, David M. Oliver, Robin Ørnsrud, Richard S. Quilliam

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

3

4 Isobel Swinscoe[1*], David M. Oliver[1], Robin Ørnsrud[2], Richard S. Quilliam[1]

5 [1]Biological and Environmental Sciences, Faculty of Natural Sciences, University of Stirling, Stirling, UK
6 FK9 4LA

7 [2]Institute of Marine Research, P.O. box 1870 Nordnes, NO-5817 Bergen; Norway .

8 [*]Corresponding author: Email: richard.quilliam@stir.ac.uk

9

10 **Key words:** BSF larvae; food safety; human pathogens; *Listeria*; macroalgae; sustainable food
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12

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14 **ABSTRACT**

15 Farmed insects can offer an environmentally sustainable aquafeed or livestock feed ingredient. The
16 value of black soldier fly (*Hermetia illucens*) (BSF) larvae however, could be improved by enrichment
17 in omega-3 through the dietary inclusion of seaweed. However, the industry practice of drying
18 seaweed at low temperatures to retain nutritional properties may benefit the survival of human
19 pathogenic bacteria, particularly if the seaweed has been harvested from contaminated water. Here
20 we have demonstrated that *E. coli* and *E. coli* O157:H7 died-off in seaweed dried at 50 °C, although
21 both were detected in the dried powder following 72 h storage. *V. parahaemolyticus* fell below the
22 level of detection in stored seaweed after drying at ≥ 50 °C, but *L. monocytogenes* remained
23 detectable, and continued to grow in seaweed dried at ≤60 °C. Therefore, drying seaweed at low
24 temperatures risks pathogen carry-over into insects destined for animal feed. BSF larvae reared on
25 an artificially contaminated seaweed-supplemented diet also became contaminated by all four
26 bacteria present in the supplement. Water quality at seaweed harvesting sites, seaweed desiccation,
27 and insect rearing practices, represent critical points where development of regulatory standards
28 could achieve targeted control of pathogenic hazards.

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29 **1. INTRODUCTION**

30 Seaweed meal is a recognised animal feed substrate in the EU (Reg (EC) 68/2013; EC, 2013a). It
31 can provide a supplementary source of energy, proteins, minerals, lipids, vitamins and antioxidants
32 (with-bioactive value) for livestock and aquaculture, and most recently, for the mass production of
33 insect larvae (Rajauria, 2015; Liland *et. al.,* 2017). The concept of insect protein as a sustainable
34 animal feed ingredient has gathered increasing acceptance across Europe and is now permitted in
35 aquafeed within the EU (Reg (EC) 893/2017; EC, 2017a). Recent innovative efforts to combine these
36 two ingredients into aquaculture feed for farmed carnivorous fish has seen advances in the mass
37 production of seaweed-fed black soldier fly larvae (BSFL), *Hermetia illucens* (L.) (Diptera:
38 Stratiomyidae) (Belghit *et. al.,* 2018; Swinscoe *et al*., 2019). The benefit of feeding insect larvae with
39 seaweeds includes utilizing a renewable feed resource that does not compete with sources of
40 human food or require land use, additional water or industrial fertilization. In Europe, seaweed for
41 animal feed is typically wild harvested from coastal marine waters (Makkar *et. al.,* 2016); however,
42 wild harvested seaweeds can also become colonised by human pathogenic bacteria e.g. species of
43 *Vibrio* and strains of *Escherichia coli* (Elbashir *et. al.,* 2018; Quilliam *et al.,* 2014; Mahmud *et. al.,*
44 2007; 2008). Molecular methods have detected *Salmonella enterica* ser. Typhimurium, *V.*
45 *parahaemolyticus* and *E. coli* O157:H7 on the farmed kelp *Saccharina latissima*, and potentially
46 toxin-producing, spore-forming *Bacillus licheniformis* and *Bacillus pumilus* have both been isolated
47 from the cultivated kelps *Alaria esculenta* and *S. latissima* (Barberi *et. al*., 2019; Blikra *et. al*., 2019).
48 In addition, Cladophora (a freshwater species of macroalgae) has been shown to harbour *E. coli*,
49 *Campylobacter*, *Shigella*, *Salmonella* and *C. botulinum* (Byappanahalli *et. al*., 2009; Ishii *et. al*., 2006).
50 Therefore, before seaweed supplements in BSFL diets can be advocated for mass-reared insect
51 production, critical control points (CCPs) during the production of seaweed-fed BSFL must be
52 identified (Swinscoe *et al*., 2019) in order to guarantee safety of this novel animal feed if it is to
53 enter the human food chain (Reg (EC) 183/2005; EC, 2005).

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54 Standardised processing methods in the feed and food industries are key to product quality and
55 safety, but such a system is currently lacking in the seaweed industry. There are also no
56 microbiological standards for seaweed meal in the EU, and those for insect processed animal
57 proteins (PAPs) in feed are limited to maximum levels of *Clostridium perfringens*, *Salmonella* spp and
58 Enterobacteriaceae (Reg (EC) 142/2011; EC, 2011). There is limited evidence for *Listeria* spp. being
59 present on freshly harvested seaweed (Banach *et. al*., 2020). However, its ubiquity in food and feed
60 processing environments (Carpentier and Cerf, 2011) and resulting opportunity for contamination of
61 feed materials warrants inclusion of *L. monocytogenes* in inactivation studies of processing effects
62 on seaweed-associated pathogens. Although processing-based interventions for controlling
63 microbial contamination of seaweeds have been explored, e.g. washing and drying (del Olmo et. al.,
64 2018; Hyun et. al., 2018), the full range of potential microbiological hazards associated with seaweed
65 entering the feed and food chain are not necessarily controlled by existing industrial practices, or
66 accounted for by current feed hygiene regulations.

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

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78 Insect farming to produce animal feed is still a nascent industry in the EU but it is widely
79 acknowledged that the microbiological safety of insects is fundamentally influenced by the hygienic
80 status of their feed (Van der Spiegel *et. al.,* 2013). Autochthonous bacteria and allochthonous
81 opportunistic bacteria (including human pathogens) colonise insects either parentally or horizontally
82 from their environment, and are harboured in the insect gastrointestinal tract (GIT), which together
83 with the mouthparts and body surface is the main niche for insect-associated bacteria (Schluter *et.*
84 *al*., 2017). Commensal, food spoilage and human pathogenic bacteria, including Enterobacteriaceae,
85 *Pseudomonas* spp. and *Clostridium* sp. have been isolated from BSFL (Jeon *et. al*., 2011; Wynants *et.*
86 *al*., 2019). Thus, good manufacturing and hygiene practices (GMP and GHP) specific to each insect
87 species, the feed substrate, the life stage at harvest, and the production environment need
88 considerable development as CCPs emerge at which pathogens may be introduced, persist or
89 replicate in the insect product (Van Raamsdonk *et. al.,* 2017). Therefore, the aims of this study were
90 to: (1) Determine colonisation dynamics of a range of human pathogenic bacteria on a combined
91 mixture of submerged brown, red and green seaweeds in an intertidal simulation of exposure to a
92 wastewater pollution event. (2) Evaluate the effect of typical industrial processing practices
93 (washing, drying and storage) on the survival of bacteria attached to seaweeds. (3) Assess the
94 survival dynamics of these bacterial contaminants when fed to BSFL as a powdered seaweed feed
95 supplement. (4) Identify CCPs where feed manufacturers can target control of bacterial hazards
96 during production of seaweed feed and its application as a feed supplement for the mass rearing of
97 BSFL.

98

99 **2. MATERIALS AND METHODS**

100 2.1 Bacteriological safety of processed seaweed (Experiment 1)

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101 A model system of postharvest industrial processing of seaweed was developed involving
102 sequential stages of washing, drying, milling and storage. Sampling for bacteriological quality was
103 conducted at key stages of the process.
104
105 2.2 Seaweed material
106 Living, attached intertidal seaweeds of the species *Laminaria digitata* (Hudson)
107 (Phaeophyceae), *Fucus serratus* (L.) (Phaeophyceae), *Palmaria palmata* (L.) (Rhodophyta) and *Ulva*
108 *lactuca* (L.) (Chlorophyta), together with seawater from the surf zone, were collected at low tide
109 from Elie, Fife, Scotland (56°11.191'N, 2°48.679'W). *Ascophyllum nodosum* (L.) (Phaeophyceae) was
110 gathered from Ganavan Bay, Oban, Scotland (56°26'05.1'N, 5°28'51.3'W) a day later. Seaweed was
111 rinsed in tap water for 3 min to remove sand and epiphytic flora and fauna. All seaweed and
112 seawater samples were stored at 4 °C and utilised within 24 h. To enumerate background *E. coli* and
113 total heterotrophic bacteria (THB) associated with the seaweed, 500 g of each species was
114 individually homogenised for 3 min using a hand blender (Bosch MSM6700GB). Four 10 g replicate
115 samples of the homogenate of each seaweed species was added to 10 ml of sterile seawater
116 (sterilised by autoclaving) and vortexed for 1 min. The supernatant was serially diluted using sterile
117 seawater and spread plated onto either Membrane Lactose Glucuronide Agar (MLGA) (CM1031,
118 Oxoid) or R2A agar (CM0906, Oxoid) to quantify *E. coli* and THB respectively. MLGA plates were
119 inverted and incubated at 37 °C for 24 h and R2A plates at 18 °C for 48 h. Seawater samples (n = 4)
120 were shaken and 100 ml vacuum-filtrated through a 0.45 µm cellulose nitrate membrane (Sartorius,
121 Goettingen, Germany). The membrane was transferred to MLGA or R2A plates and incubated as
122 described above. Bacterial concentrations were expressed as CFU (colony forming units) g^{-1} seaweed
123 (dry matter), or CFU 100 ml^{-1} seawater.
124
125 2.3 Inoculum preparation

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126 In addition to commensal *E. coli* isolated from a beach wastewater outlet, three bacterial
127 pathogens were used in this study: a non-toxigenic serotype of *E. coli* O157:H7 originally isolated
128 from a farm drain, *Listeria monocytogenes* from a mushroom production facility and an
129 environmental strain of *Vibrio parahaemolyticus.* To produce bacterial cells tolerant of seawater for
130 use in our experiments, each bacterial species was added to sterile seawater for 3 h at 10 °C. A 100
131 ml sample (*n* = 4) was vacuum filtered and the membrane transferred to the relevant selective agar
132 plate. The environmental *E. coli* was grown on MLGA, and *E. coli* O157:H7 on Sorbitol MacConkey
133 Agar (SMAC) (CM0813, Oxoid) supplemented with cefixime and potassium tellurite (CT) (SR0172,
134 Oxoid); *L. monocytogenes* was grown on Listeria Selective Agar (Oxford Formulation) (CM0856,
135 Oxoid) supplemented with Modified Listeria Selective Supplement (Oxford) (SR0206, Oxoid) and *V.*
136 *parahaemolyticus* grown on TCBS (Thiosulfate citrate bile salts sucrose agar; CM0333, Oxoid).
137 Following incubation at 37 °C for 24 h, single colonies of each species were picked off the plate and
138 *E. coli*, *E. coli* O157:H7 and *L. monocytogenes* individually cultured in Luria-Bertani (LB) broth
139 (CM1018, Oxoid), and *V. parahaemolyticus* in Alkaline Peptone Water (APW) (CM1028, Oxoid), at 37
140 °C for 18 h at 100 rev min^{-1}. Cells were centrifuged and washed three times in Phosphate Buffered
141 Saline (PBS), and re-suspended in PBS prior to use.
142

143 2.4 Simulated microbial contamination of pre-harvested seaweed

144 Fresh samples of *L. digitata*, *F. serratus*, *A. nodosum*, *P. palmata* and *U. lactuca* were combined
145 in equal quantities (40 g each) in 500 ml glass jars (*n* = 32). *L. digitata*, *F. serratus* and *A. nodosum*
146 were comprised of approximately 10 % stipe and 90 % frond, whereas *P. palmata* and *U. lactuca*
147 consisted of 100 % frond. The stipes and fronds of *L. digitata*, *F. serratus* and *A. nodosum* were cut
148 into 5 cm lengths to enable accurate weighing of each seaweed species into replicate batches. Eight
149 replicate jars were used for each temperature (room temperature (RT; approx. 20 °C), 40 °C, 50 °C

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150 and 60 °C) of which four replicate jars were inoculated with bacterial pathogens, and four non-
151 inoculated jars used to assess pH and the a_w of seaweed.

152 Seawater tolerant cells of *E. coli*, *E. coli* O157:H7, *L. monocytogenes* and *V. parahaemolyticus*
153 suspended in PBS were combined and added to 1600 ml of non-sterile seawater. The resulting
154 pathogen-seawater was mixed to ensure even distribution of cells, and the concentration of each
155 bacterial species determined by plating onto selective media as described above (*n* = 4 for each
156 bacterial species). The concentrations of each bacteria in the pathogen-seawater cocktail were as
157 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
158 10^9 CFU ml^{-1}; *V. parahaemolyticus* = 6.8 x 10^9 CFU ml^{-1}. Aliquots of 200 ml of the contaminated
159 seawater were poured into each of the glass jars (*n* = 16), which completely submerged the seaweed
160 mixture. Aliquots of 200 ml of non-inoculated non-sterile seawater were poured into each of the jars
161 (*n* = 16) used for pH and a_w measurements. Screw lids were used and all jars secured within a
162 temperature controlled rotating incubator at 100 revs min^{-1} for 24 h at 20.5 °C ± 3 °C.

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

171

172 2.5 Simulated post-harvest seaweed processing
173

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

186 The remaining seaweed in each of the eight jars was immediately washed and transferred to
187 individual foil trays measuring 20 (l) x 10 (w) x 5 (d) cm. The seaweed was spread out evenly to an
188 approximate depth of 4 cm and placed in a drying oven at either 40 °C, 50 °C or 60 °C, to simulate
189 the lower end of the range employed in hot air convection or oven drying by the seaweed industry
190 (Gupta *et. al.* 2011). A temperature logger was placed in the centre of the four non-inoculated
191 seaweed replicate trays. During drying, the actual temperatures achieved were 41.8 °C ± 0.03, 49.1
192 °C ± 0.14, and 64.2 °C ± 0.21. In addition, eight uncovered trays of seaweed were placed on the
193 bench top within the same laboratory to provide a room temperature (RT) treatment (22.7 °C ±
194 0.04).

195 Bacterial concentrations on the inoculated seaweed were enumerated during the drying
196 process at 24, 72, 120 and 168 h, using the methods above with the exception of seaweed dried at
197 50 °C and 60 °C from 72 h onwards, which was sufficiently desiccated to be ground to a fine powder
198 using a pestle and mortar. To determine bacterial concentrations in this seaweed powder, 2 g of

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

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

210

211 2.5 Bacteriological safety of seaweed as BSFL feed (Experiment 2)
212
213 A simulation of mass rearing of BSFL on feed supplemented with pathogen-contaminated seaweed
214 powder was undertaken. Larvae and the feed substrate were sampled throughout the rearing period
215 up to the point of pre-pupae harvest to assess both the microbial load of the feed and the hygienic
216 status of the larvae.2.7 Preparation of BSF colonies

217 Two colonies of BSF were established from larvae sourced online (livefoodsbypost.co.uk and
218 InternetReptile.com) in insect rearing tents measuring 75 (w) x 75 (d) x 115 (h) cm (BugDorm-2400,
219 bugdorm.com), in a controlled environment walk-in room (Reftech B.V., Netherlands) at 30 °C ± 2 °C,
220 a relative humidity of 70 % and a photoperiod of 12 h. One tent contained two 5 L plastic boxes
221 (Addis Ltd., UK) each containing approximately 1000 larvae, which were reared on a 15:3:1 mixture
222 of wheat bran (Harbro Ltd., Aberdeenshire), whey protein (Holland and Barrett International, UK)
223 and fruit and vegetable waste. Every 2 days, feed substrate was supplemented to a depth of
224 approximately 12 cm and 200 ml of water was added. Holes in the base of the containers enabled

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225 drainage of excess liquid to prevent waterlogging and anoxic conditions developing in the feed
226 substrate. Within the tent, cardboard boxes containing shredded newspaper provided dark
227 sheltered conditions for pupation. Once adult flies emerged, sliced fruit was placed on the surface of
228 the feed substrate and water (< 20 ml) was sprayed into the tent hourly during the day. Corrugated
229 cardboard strips were laid across the feed container above the level of the feed to provide dry
230 crevices in which the female flies laid their eggs. As soon as eggs were observed in a cardboard strip,
231 the strip was transferred to another insect tent and suspended above a tray containing feed
232 substrate comprised of the same ingredients as described above. After hatching, the larvae dropped
233 from the cardboard strip into the substrate, and were harvested for future experiments at
234 approximately 1 week old.

235 2.8 Preparation of seaweed powder and inoculation procedure
236
237 Seaweed and seawater was collected at the same time as above, and stored at 4 °C prior to
238 use. The seaweeds (stipes and fronds) were separated by species, washed clean of visible epiphytic
239 flora and fauna using tap water, and oven dried in single layers in foil trays (22 x 22 x 6 cm) at 50 °C
240 for 72 h. Each species of dried seaweed was then ground into a fine powder using a pestle and
241 mortar to pass through a 500 µm sieve. Composite 400 g mixtures (comprised of 80 g each of *L.*
242 *digitata, F. serratus, A. nodosum, P. palmata and U. lactuca*), were placed in three separate
243 stomacher bags. The seaweed powder in two bags was inoculated with 1.5 L of seawater containing
244 *E. coli*, *E. coli* O157:H7, *L. monocytogenes* and *V. parahaemolyticus* produced as described above,
245 and the contents of each bag agitated by hand for 5 min to ensure thorough mixing. The initial
246 concentration of each bacterial species in the contaminated seawater was: *E. coli* (6.7×10^9 CFU ml^{-}
247 ^{1}), *E. coli* O157:H7 (7.15×10^9 CFU ml^{-1}), *L. monocytogenes* (7.35×10^9 CFU ml^{-1}) and *V.*
248 *parahaemolyticus* (6.4×10^9 CFU ml^{-1}). The remaining 400 g of seaweed powder was mixed with 1.5 L
249 of non-inoculated seawater following the method described above.
250

251 2.9 Simulated mass-production of BSFL reared on seaweed supplement

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

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

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278 quantified at each time point by homogenising 10 g of material, and enumerating bacteria in the
279 supernatant. Bacterial concentrations in larvae were expressed as CFU larvae^{-1}, representative
280 substrate samples were dried at 80 °C for 24 h such that bacterial concentrations could be expressed
281 as CFU g^{-1} dry matter content.

282

283 2.10 Statistical analyses
284

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

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

309

310 **3. RESULTS**

311

312 3.1 Background bacteriological status of seaweed and seawater

313

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

319

320 3.2 Bacteriological safety of processed seaweed

321

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

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336 **Table 1**. The concentrations of *E. coli*, *E. coli* O157:H7, *L. monocytogenes* and *V. parahaemolyticus* in
337 the pathogen-seawater cocktail before the addition of seaweed, compared with the seawater and
338 seaweed after 24 h (note different units for seawater and seaweed). Within each row, data points
339 that do not share a letter are significantly different from each other (applicable to water treatments
340 only). 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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349 **Table 2**. Concentrations of *E. coli*, *E. coli* O157:H7, *L. monocytogenes* and *V. parahaemolyticus*
350 attached to seaweed before and after seaweed was washed. Within each row, data points that do
351 not share a letter are significantly different from each other. Data points are the mean of 16
352 replicates ± 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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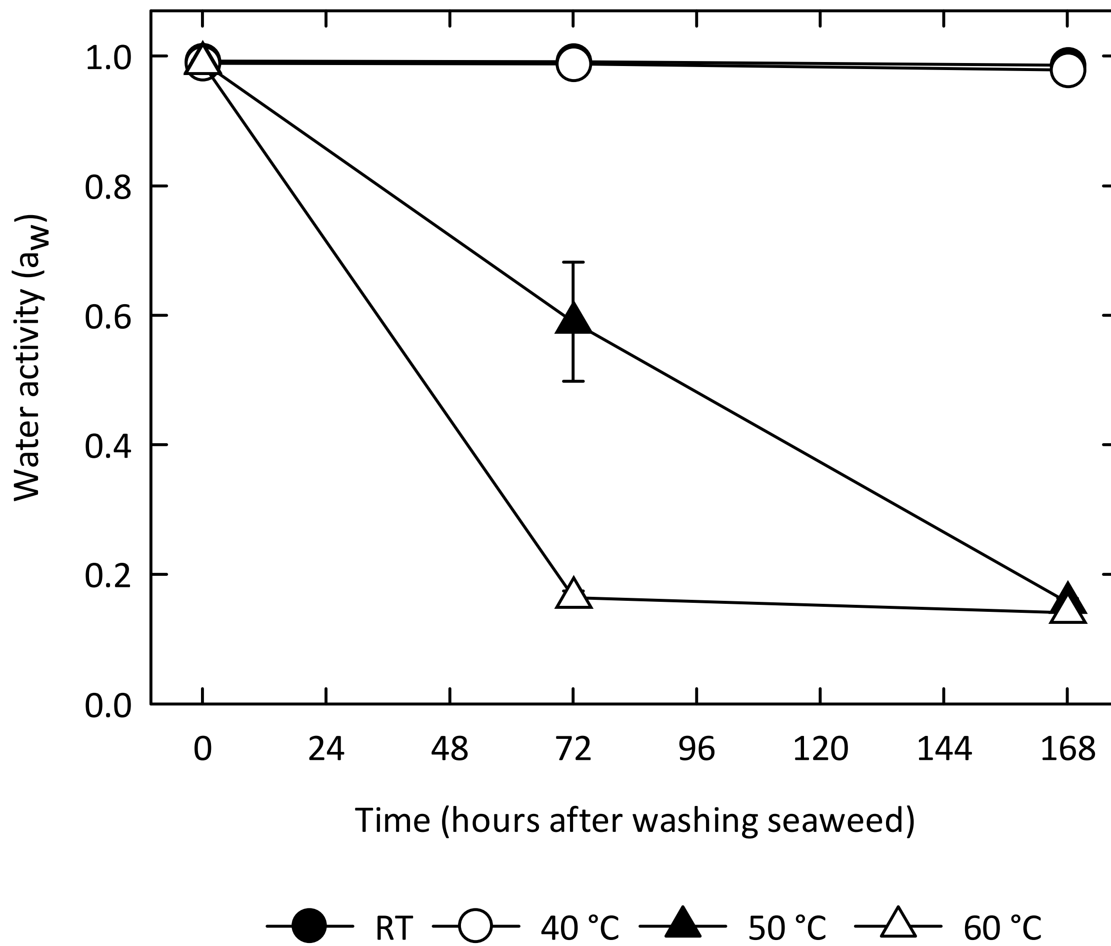

355

356 **Figure 1.** Water activity (a_w) in seaweed after washing and drying at RT (room temperature), 40 °C,
357 50 °C and 60 °C for 72 h and 168 h. Data points are the mean of four replicates ± SE.

358

359 The a_w of seaweed dried at room temperature or at 40 °C did not significantly change during
360 the drying process, whereas by 72 h and 168 h the a_w of seaweed dried at 50 °C and 60 °C had
361 significantly decreased ($P < 0.05$) (Fig. 1). At room temperature, the desiccation of seaweed had no
362 effect on the concentrations of *E. coli* or *E. coli* O157:H7 over the drying period (Fig. 2a and c),
363 whereas the concentration of both *L. monocytogenes* and *V. parahaemolyticus* significantly
364 increased ($P < 0.05$) during the drying process (Fig. 2e and g). Drying seaweed at 40 °C also had no
365 effect on concentrations of *E. coli* attached to the seaweed (Fig. 2a); however, drying at 50 °C or 60

366 °C resulted in significant *E. coli* die-off to undetectable levels by 168 h or within 24 h respectively ($P <$
367 0.05). Subsequent storage of the seaweed for 72 h did not affect *E. coli* levels, regardless of the
368 temperature at which the seaweed had previously been dried, and although *E. coli* grew during
369 storage from undetectable levels to ~2 log_{10} CFU in seaweed previously dried at 50 °C, this was not a
370 significant increase (Fig. 2b).

371 Drying seaweed at 40 °C and 50 °C led to significant *E. coli* O157:H7 die-off to undetectable
372 levels after 168 h ($P < 0.05$), whilst drying seaweed at 60 °C resulted in rapid die-off of the pathogen
373 by 24 h (Fig. 2c). Storage for 72 h had no effect on pathogen levels in seaweed dried at 40 °C or 60
374 °C, which remained undetectable in both cases, or in seaweed dried at room temperature (Fig. 2d).
375 Growth of *E. coli* O157:H7 was detected in stored seaweed, which had been dried at 50 °C, though
376 this was not a significant increase.

377 *L. monocytogenes* survival on seaweed was significantly reduced by ~5 log_{10} CFU between 72
378 h and 120 h by drying at 50 °C ($P < 0.05$) (Fig. 2e). *L. monocytogenes* survival on seaweed was
379 unaffected by drying at 40 °C or 60 °C (Fig. 2e) and persisted at ~3 log_{10} CFU after 168 h of drying at
380 60 °C (Fig. 2e). Storage for 72 h did not alter levels of *L. monocytogenes* attached to the seaweed,
381 regardless of the previous drying temperature (Fig. 2f). Drying at 40 °C significantly decreased the
382 concentration of *V. parahaemolyticus* from ~6 log_{10} CFU to ~1 log_{10} CFU after 168 h ($P < 0.05$) (Fig.
383 2g). *V. parahaemolyticus* was undetectable on seaweed following 168 h drying at 50 °C ($P < 0.05$),
384 and after the first 24 h at 60 °C ($P < 0.05$). Storage for 72 h did not affect *V. parahaemolyticus* levels,
385 regardless of the temperature at which the seaweed had previously been dried (Fig. 2h). During
386 drying, the pH of the seaweed dried at RT, 40 °C, 50 °C and 60 °C significantly increased from ~pH 6.5
387 to ~pH 7.5 ($P < 0.05$). However, after 72 h storage, seaweed dried at all temperatures with the
388 exclusion of the 40 °C treatment became more acidic ($P < 0.05$).

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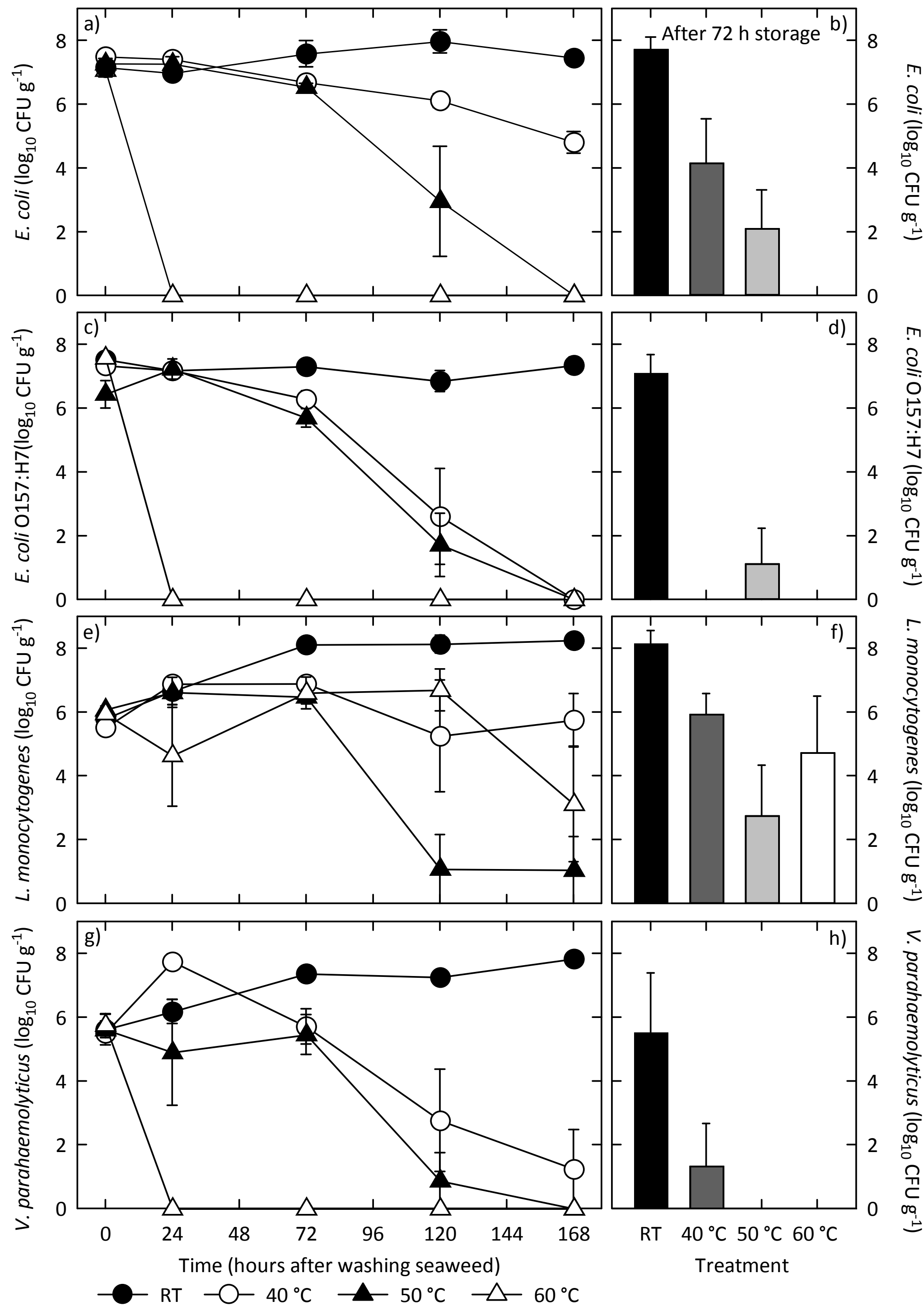

389

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

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395 3.3 Bacteriological safety of seaweed as BSFL feed
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397 The mean weight of individual larvae significantly increased from 0.12 ± 0.01 g on day 3 to
398 0.26 ± 0.005 g on day 5 ($P < 0.05$), although the onset of pre-pupation from day 6 led to an overall
399 decline in average weight. Water content in the inoculated substrate containing larvae was
400 significantly lower than in substrate with no larvae ($P < 0.05$). In general, the concentrations of *E.*
401 *coli*, *E. coli* O157:H7, *L. monocytogenes* and *V. parahaemolyticus* associated with BSF larvae were
402 significantly lower than in the substrate throughout the entire sampling period (Fig. 3a-d). However,
403 whilst the concentration of *E. coli* and *E. coli* O157:H7 associated with larvae significantly fell over
404 the 8 days ($P < 0.05$), the larval loads of *L. monocytogenes* and *V. parahaemolyticus* did not change
405 over the same period (Fig. 3a-d). In the absence of larvae, concentrations of *E. coli*, *E. coli* O157:H7,
406 *L. monocytogenes* and *V. parahaemolyticus* in the feed substrate decreased significantly on day 2 (P
407 < 0.05 in all cases); consequently, all four bacteria were 1 – 2 $\log_{10}$ CFU higher in substrate in which
408 larvae were present on days 3 and 4 ($P < 0.05$) (Fig.3a- d). The pH of non-inoculated feed in the
409 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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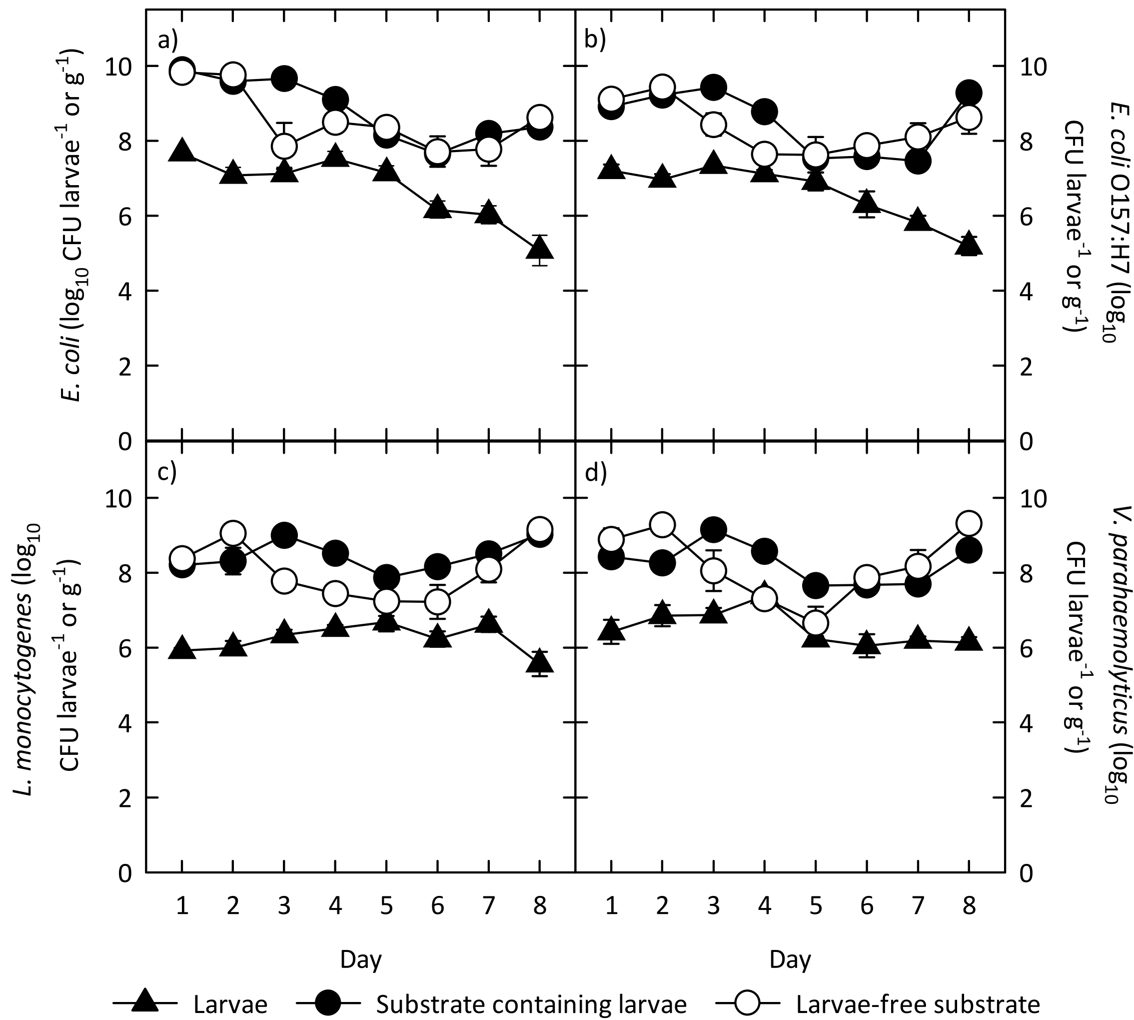

413

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

417

418 **4. DISCUSSION**
419

420 4.1 Bacteriological safety of processed seaweed
421

422 This study demonstrates that the current post-harvest processes of washing and drying
423 seaweed intended for animal feed can fail to eradicate (and can even encourage the survival of) *E.*
424 *coli* and selected human pathogenic bacteria if seaweed has a high level of contamination at the

425 point of seaweed harvest. The inadequacy of these manufacturing practices therefore, can result in a
426 dried seaweed product in which human pathogenic bacteria can persist during storage, although
427 survival will be variable depending on the stain or species of the pathogen. Our results have
428 highlighted that the industry objective of maximising the nutritional benefits of seaweed by
429 minimising the drying temperature comes at the cost of ensuring a microbiologically safe product.

430 *E. coli*, *E. coli* O157:H7, *L. monocytogenes* and *V. parahaemolyticus* all attached to
431 submerged senescing seaweed, which has not been previously shown for *L. monocytogenes* in the
432 natural environment. The persistence of these pathogens in seawater and their attachment to
433 seaweed reflect their biofilm-forming ability and subsequent increased tolerance to the osmotic
434 stress of seawater. The diversity of seaweed-associated bacterial biofilms can differ seasonally,
435 between seaweed species sharing the same habitat, and may diverge from the surrounding
436 planktonic bacterial communities (Bengtsson *et. al*., 2010; Singh and Reddy, 2014; Lachnit *et. al*.,
437 2009). Improved practices in the cleaning and processing of commercially targeted seaweed species
438 (del Olmo *et. al*., 2018), rather than sole reliance on water quality management at harvesting sites,
439 will therefore enable better control of seaweed-associated pathogen risks to consumers. Biofilm
440 formation on the seaweed by the four bacteria would also have contributed to the inefficiency of the
441 cleaning stage, and *V. parahaemolyticus* in particular can resist removal from seaweed by washing,
442 (Mahmud *et. al.,* 2007). Free chlorine is present in most tap water at concentrations typically within
443 the 0.2 - 1 mg/L range (WHO, 1996), which is capable of killing planktonic *E. coli* O157:H7 cells (Shen
444 *et. al.,* 2013). However, any such reduction of seaweed-associated bacterial concentrations is likely
445 to have been offset by neutralisation of free chlorine due to rapid reaction with seaweed exudates
446 (Shen *et. al.,* 2013). Mechanically cutting the seaweed would have released sugars, such as mannitol,
447 which may also have facilitated the growth of these pathogens in otherwise stressful conditions. For
448 example, *E. coli* replication in Cladophora leachate has been shown to positively correlate with
449 leachate concentration (Byappanahalli *et. al*., 2003). Furthermore, multi-species biofilms, as utilised
450 in this study, can resist chlorine disinfectant effects to a greater degree than can mono-species

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451 biofilms (Schwering *et. al*., 2013). Increasing the duration of the washing step therefore, is not likely
452 to have improved the effectiveness of pathogen removal.

453 During the drying of seaweed at ≥ 50 °C, the a_w of the feed material was reduced. Die-off of
454 *E. coli*, *E. coli* O157:H7 and *V. parahaemolyticus* due to 50 °C heat stress was delayed compared with
455 more rapid bacterial inactivation occurring at 60 °C, probably as a result of a_w levels that allowed
456 microbial survival within the first 72 h. *L. monocytogenes* exhibited poorer heat resistance at 50 °C
457 compared with the 60 °C treatment, most likely a result of the a_w being insufficiently low in the first
458 72 h to protect the bacterial cells from 50 °C heat damage. Drying seaweed at 60 °C had no effect on
459 *L. monocytogenes*, which is Gram-positive and therefore relatively desiccation tolerant, yet within 24
460 h led to log-linear inactivation of the more desiccation intolerant Gram-negative *E. coli*, *E. coli*
461 O157:H7 and *V. parahaemolyticus* populations to undetectable levels.

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

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475 effect on pathogens sufficiently rapidly to circumvent the property of thermal resistance of bacterial
476 cells by a low a_w and prevent re-emergence of the bacteria in stored feed.

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

491 Although not considered in this study, it is important to note that the microbiota of brown, red
492 and green seaweeds are often dominated by the bacterial genus Bacillus (del Olmo *et. al*., 2018).
493 Spore-forming bacteria are causative agents of foodborne disease, ubiquitous in the natural and
494 food production environments, and highly resistant to processing stresses (Wells-Bennik *et. al*.,
495 2016). It has for example been shown that application of 80 °C heat to the edible brown seaweeds
496 *Alaria esculenta* and *Saccharina latissima* for 30 minutes failed to inactivate toxin-producing, spore-
497 forming *B. lichenformis* and *B. pumilus* (Blikra *et. al*., 2019). Spore inactivation in food material
498 requires application of > 95 °C heat, far above the 40 °C drying temperature favoured by the
499 seaweed industry for nutrient retention (Gupta *et. al*., 2010).

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500 4.2 Microbial safety of seaweed as BSFL feed

501

502 This study has shown that BSFL can become rapidly contaminated (attachment to the
503 exoskeleton and via GIT recontamination) from their feed substrate, indicating that at the point of
504 harvest a decontamination step would be required. The high concentrations at which bacteria were
505 inoculated into the substrate (which is comparable to using seaweeds that had been dried at low
506 temperatures) prevented the BSFL from reducing their internal pathogen loads at the point of
507 harvest. However, BSFL exhibited a capacity to suppress larval-associated *E. coli* strains, indicating a
508 potential to clear these bacteria from the GIT if ingested at lower concentrations. In this study, the
509 concentration of bacteria associated with the larvae was less than the substrate throughout the
510 rearing period, which may indicate effective digestion, inactivation, or antimicrobial action in the
511 larval GIT (Wynants *et. al.,* 2018a). The expression of antimicrobial peptides (AMP) by BSFL is
512 particularly marked when fed protein-rich diets such as that provided in this study, and larvae can
513 adapt the diversity of their AMP in response to the microbiome of their environment, enabling them
514 to exploit diverse diets (Vogel *et. al.,* 2018). The decline in larvae-associated *E. coli* and *E. coli*
515 O157:H7 loads during larval development to pre-pupation could reflect selective inactivation of
516 ingested *E. coli* strains in the larval GIT via exposure to increasing levels of GIT antimicrobials
517 (Wynants *et. al.,* 2018a; Engel and Moran, 2013; De Smet *et. al.,* 2018). Importantly, neither *E. coli*
518 nor any of the pathogenic bacteria colonised or accumulated in the larval GIT during rearing.

519 The concentrations at which the four bacteria were introduced to the seaweed powder
520 supplement far exceeded the levels persisting in the stored seaweed powder following drying at 50
521 °C. The concentration of bacterial contaminants in insect feed may overwhelm GIT antibacterial
522 action or outcompete native GIT microbiota, and may thus enable colonisation (and subsequent
523 growth) of the pathogen in larval GITs (Wynants *et. al*., 2019). This may explain the persistence *L.*
524 *monocytogenes* and *V. parahaemolyticus* at high concentrations in the BSF larvae, particularly if
525 these bacterial species were not selectively inactivated in the BSFL GIT, although in this study GIT

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526 colonisation was not demonstrated. In this study, pathogenic bacteria may not be eliminated by
527 either gut voidance during metamorphosis into pre-pupae, or by 48 h starvation of larvae free of
528 contact with their faeces prior to harvesting (Wynants *et. al.,* 2017). Furthermore, the spore-forming
529 *B. cereus* is a typical member of BSFL GIT microbiota, and therefore if ingested in feed via a
530 contaminated seaweed supplement, this pathogen may not be inactivated, and may even replicate,
531 in the GIT (Jeon *et. al*., 2011). Therefore, sterilisation of the larvae meal and lipids during subsequent
532 processing steps is recommended.

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

541 A global increase in human *Vibrio* infections are associated with increased sea surface
542 temperatures (Vezzulli *et. al.,* 2015), and may be exacerbated by an increased risk of *Vibrio* spp
543 associated with seaweed entering human food chains via its use as feed and food. Bacteriological
544 criteria for pathogenic *Vibrio* spp. in seaweed-fed insects, particularly for species enriched in
545 seaweed-sourced omega-3 for direct human nutrition, should be established for products before
546 they leave the food-processing environment. *Vibrio* spp. are rare in most edible insect but have been
547 detected in edible giant water bugs and mealworm frass (Osimani *et. al*., 2018a and 2018b).
548 Seaweed growing in coastal waters and harvested for feed and food may also pose a public health
549 risk as reservoirs of terrestrial and aquatic sources of multi-antimicrobial resistant (AMR) bacteria.

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550 Antibiotic residues in feed may be one explanation for the occurrence of AMR genes in industrially
551 reared mealworms and crickets (Vandeweyer *et. al.,* 2019).

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

563 5. CONCLUSION

564
565 Ensuring production of safe novel animal feed ingredients requires understanding of both the
566 specific bacterial hazards associated with the novel ingredients, and the response of those bacteria
567 to abiotic and biotic processing stresses. Persistence in seawater, and rapid colonisation of brown,
568 red and green seaweeds, by some key human pathogens, indicates that water quality at seaweed
569 harvesting sites should be managed as part of Good Agricultural Practice (GAP) as the first line of
570 defence to reduce the overall contamination load at the start of the production chain. In the
571 seaweed feed sector, washing and drying seaweed are not intended or expected to remove bacterial
572 contaminants, but low temperature desiccation favoured by the industry encourages pathogen
573 persistence and growth during storage. This indicates a need for industry-wide adoption of a
574 minimal seaweed drying temperature-time-a_w treatment to guarantee product quality during its
575 shelf life. Seaweed feed, like all raw feed materials, represents a potential source of bacteriological

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576 hazards which must be managed through GMP and GHP in insect production. The bacteriological risk
577 profile of BSFL and other insects using seaweed as a feed supplement will reflect the unique
578 dynamics between insect species and bacterial species and strains. Development of robust HACCP
579 guidelines, and improved GAP, bacteriological standards and GMP, for each stage of the production
580 chain will encourage regulatory and commercial acceptability of seaweed-fed insects for both feed
581 and food.

582

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Drying seaweed at low temperatures retains its nutritional properties

Nutritional benefits of insect larvae can be improved by dietary inclusion of seaweed

Lower drying temperatures risks pathogen carry-over into insects destined for feed

Black soldier fly larvae fed seaweed can become contaminated with human pathogens

Industry-wide regulation needed before seaweed-fed insects promoted as feed and food

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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