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Microbial Quality of Raw and Ready-to-eat Mung Bean Sprouts produced in Italy

Running title: Microbial quality of Mung Bean Sprouts

Iacumin Lucilla and Comi Giuseppe*

Department of Agricultural, Food, Environmental and Animal Science, University of Udine, Via Sondrio 2/a, 33100 Udine, Italy.

***Corresponding author:** Giuseppe Comi, Department of Agricultural, Food, Environmental and Animal Science, University of Udine, Via Sondrio 2/a, 33100 Udine, Italy

Email: giuseppe.comi@uniud.it; tel. number +390432558129; fax: +390432558130.

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Abstract

The aim of the study was to determine the microbial quality of mung bean sprouts produced in Italy. In particular the presence of pathogenic microorganisms (Shiga Toxin *Escherichia coli* – STEC, *Salmonella* spp. and *Listeria monocytogenes*), total coliforms, total viable count (TVC) and *E. coli* were determined. The study covered five years of sprouts production. The results demonstrated that no pathogenic microorganisms were present and the microbial load was less than 6 log CFU/g. Consequently up to now the Mung Bean Sprouts produced in Italy seem to be acceptable for the consumption. Additionally aim was to value the fate of different strains of STEC, *L. monocytogenes* and *Salmonella* spp. intentionally inoculated in Mung Bean Seeds during sprouting and the use of chlorinated water to reduce their concentration in seeds and sprouts. Data demonstrated that they increased over 5-6 log CFU/g within 3 days from the inocula. The cholinated washing solution allowed to reduce about 3 and 7 log CFU/g of the investigated strains in seeds and sprouts, respectively. However it was not possible to completely eliminate the pathogens from either Mung Bean Seeds or Sprouts. Despite these encouraging results, the producer attention to the hygienic quality should never be reduced in order to obtain safe Mung Bean Sprouts.

33 1. Introduction

34 Recently the use of mung bean sprouts, originated in Far East areas, has been wide spread to the
35 western world. They are obtained germinating seeds, are considered fashionable and healthy
36 ingredients (Kuo et al., 2004). Yet, they are also recognised fundamental for human diet providing
37 health benefits because they are important source of proteins, vitamins, minerals and fibers (Martínez-
38 Villaluenga et al., 2008). The flow sheet of production includes that Mung Bean Sprouts must be
39 grown in environmentally controlled conditions, in warm (22-24 °C) and in high humidity (95%) for
40 sprouting for three-five days (Peles et al., 2012; Taormina et al., 1999a). Seeds are usually
41 contaminated by high microbial loads, including yeasts, Pseudomonads, Enterobacteria, Lactic acid
42 bacteria. Due to humid and nutritious sprouting conditions and being sprouts good media, the
43 microbial loads can increase at level of 10¹⁰ CFU/g (Zheng et al., 2015,2016; Randazzo et al., 2009;
44 Peñas et al., 2008; Ghandi & Matthews, 2003; Harris et al., 2003; Robertson et al., 2002; Prokopowich
45 & Blank, 1991). Pathogenic microorganisms can also contaminate them, either as primary
46 contamination (while growing in and during harvest) or secondary contamination, during washing,
47 slicing, soaking, packaging and preparation (Harris et al., 2003). The pathogen contamination of
48 sprouts comes from the seeds. Consequently it is largely recognized that the sprout-associated
49 outbreaks have been due to seeds contaminated with pathogenic microorganisms (NACMCF, 1999;
50 Peles et al., 2012) rather than post-production contamination (Xiao et al., 2014). Pathogenic
51 microorganisms load increase because of the high volumes of water used during sprout production
52 and sprouting temperature especially in systems where sprouts are exposed to a common “water bath”
53 and frequently or continuously mixed, such as in a rotary drum (Xiao et al., 2014). For these reasons,
54 sprouted seeds have been implicated in various and serious outbreaks caused by *Salmonella* and Shiga
55 toxin-producing *Escherichia coli* (STEC). Indeed, *Salmonella Bareilly* in United Kingdom (Cleary
56 et al., 2010), *S. Newport* in Germany and Netherlands (Bayer et al., 2014) and *S. Enteritidis* in 2014
57 in the USA (FDA, 2014) produced outbreak associated with bean sprouts. *Salmonella* and
58 *Escherichia coli* O157:H7 are the most frequently associated causative agents of outbreaks caused by

alfalfa and mung bean sprouts consumption in North America (CDC, 2012; Health Canada, 2012; Fett, 2005;) clearly indicating that mung bean sprouts constitute a significant food safety risk (Gómez-Aldapa et al., 2013). Furthermore also STEC produced various major outbreak. In particular in Northern Germany, together with other 14 EU countries, in USA and Canada, different outbreaks were associated with consumption of sprouted-fenugreek seeds (EFSA, 2012; Health Canada, 2012; Buchholz et al. 2011). Considering sprouts pose a very complex challenge to bacterial pathogen enrichment and detection, the aim of this work was to value the microbial quality of mung bean sprouts produced and sold in Italy. Additionally aims were to value the fate of different strains of STEC, *L. monocytogenes* and *Salmonella* spp., intentionally inoculated in Mung Bean Seeds during sprouting and the use of chlorinated water to reduce their concentration in seeds and in sprouts.

2. Material and methods

2.1 Media and sampling

Mung Bean Sprouts were collected from different Italian retailers. Three hundred and sixty Mung Bean Sprouts samples have been analysed during 5 years, from January/1st/2012 to December/31st/2016, of sampling; 6 samples for each months. They were sampled and analysed after 1 their production, considering their shelf-life was about 5 days at a temperature less than 7 °C. Also Mung Bean Seeds were analysed. They were collected in one out of the two facilities, that produce Mung Bean Sprouts in Italy. In particular it produces about the 90% of the Mung Bean Sprouts sold in Italy. Three hundred and sixty Mung Bean Seeds samples of 15 lots have been analysed during 5 years, from January/1st/2012 to December/31st/2016, of sampling; 6 samples for each months.

A sample of 10 g of the sprouts or seeds were serially diluted with saline-peptone water (8 g/l NaCl, 1 g/l bacteriological peptone; Oxoid, Italy, distilled water 1000 ml) in stomacher bags. An aliquot of 0.1 ml of each serial dilution was plated onto agar for counts of different groups of microorganisms: the Total Viable Count (TVC) was evaluated on Plate Count Agar (Oxoid, Italy) incubated at 30 °C for 48-72 h; total Coliforms (TC) and *Escherichia coli* was grown on Violet Red Bile Lactose Agar

85 (VRBLA) (Oxoid, Italy) incubated respectively at 37 °C at 44 °C for 24 h; *Salmonella* spp. was
86 evaluated by the ISO (6579-1 2002 Cor.1:2004 Microbiology of food and animal feeding stuffs –
87 Horizontal method for the detection of *Salmonella* spp.) method, *Listeria monocytogenes* by ISO
88 (11290-1,2:1996 Adm.1:2004. Microbiology of food and animal feeding stuffs – Horizontal method
89 for the detection of *Listeria monocytogenes*) method, and STEC was evaluated with CEN ISO TS
90 13136 - Commission Regulation EU n. 209/13 - 11/03/2013 amending regulation EC 2073/2005 as
91 regards microbiological criteria for sprouts and the sampling rules for poultry carcasses and fresh
92 poultry meat (Official J. European Union L 68/19, 12/01/2013) method.

94 2.2 Total Coliforms and presuntive *E. coli* isolation and identification

95 One thousand and eighty colonies were randomly collected from VRBLA agar plate used to count
96 total coliforms of Mung Bean Sprouts. Briefly, from one agar plate containing between 30 and 150
97 colonies of each samples, 3 colonies, presuntive total coliforms, were randomly isolated. The colonies
98 were streaked on Plate Count Agar and then stored at $-80_{\text{SEP}}^{\text{[11]}}^{\text{[11]}}\text{°C}$ in Brain Heart Infusion Broth (Oxoid,
99 Italy) supplemented with glycerol (30% final concentration, Sigma-Aldrich, Germany). The isolates
100 were subjected to Gram staining, Oxydase and catalase test and were then identified according to the
101 molecular method (PCR-DGGE and sequencing) reported by Iacumin et al. (2009). The PCR
102 products were run in DGGE, and the isolates were grouped according to the migration profile. Only
103 if the migration was again identical to the original band cut, the clone was sent for sequencing to a
104 commercial facility (MWG Biotech, Germany). The BLAST program was used for the analysis of
105 the sequences obtained (Altschul et al., 1997).

106

107

108

109 2.3 Fate of Total Viable Count and Total Coliforms during storing (4 ± 2 °C) for 5 days

110 Three different sequential lots of production were analysed at 0 and 5 days (End of Shelf-life). Each
111 lot included 20 samples: 10 analysed each time. The methods are the same above reported.

112

113 2.4 Bacterial strains and inoculum preparation

114 The bacteria strains included for *Listeria monocytogenes*: *Listeria monocytogenes* Scott A, *Listeria*
115 *monocytogenes* NCTC 10887 (serotype 1/2b), *Listeria monocytogenes* NCTC 10527 (serotype 4b);
116 for *Salmonella*: *Salmonella enteritidis*, *Salmonella typhimurium* and *Salmonella derby*; for
117 *Escherichia coli* (STEC): *E.coli* O157: H7 strains ATCC 43888; *E.coli* O103H2 ED-173 Istituto
118 Superiore Sanità, Rome; *E.coli* O26H11 E-D 56, Istituto Superiore della Sanità, Rome. Each strain
119 was grown in Brain Heart Infusion Agar (Oxoid, Italy). The inocula were obtained according to Xiao
120 et al., (2014) modified method. Briefly: Four milliliters of an overnight culture in Brain Heart
121 Infusion^[SEP] broth (Oxoid, Italy) of each strains were subjected to a centrifugation at 13,400 ^[SEP]rpm for
122 10 min at room temperature, and the pellets ^[SEP]were resuspended in saline-peptone water (8 g/l NaCl,
123 1 g/l bacteriological peptone; Oxoid, Italy, distilled water 1000 ml). ^[SEP]The concentration of each
124 suspension was about 10⁶ – 10⁷ CFU/ml. Equal volumes of cell suspensions of each species were
125 combined as a cocktail in order to obtain three different suspensions containing *L. monocytogenes*,
126 *Salmonella* spp. and STEC with desired cell concentrations for seed inoculation. Each suspensions
127 was diluted in peptoned water and 0.1 of the dilution was plated in Plate Count Agar (Oxoid, Italy)
128 incubated at 37 °C for 24-48 h in order to value their concentration. For inoculation, different portions
129 of 600 g seeds were immersed in 800 mL of each appropriate inoculum suspension with gentle
130 swirling for 5 min at room temperature. The final concentration of each inoculated microorganisms
131 in the seeds was about 10²~10³ CFU/g. The seeds were air-dried overnight under a laminar flow
132 biological safety hood at room temperature and stored at 4 °C for up to 48 h. Then part of the seeds
133 were subjected to sprouting and part directly treated with chlorine water in order to reduce the level
134 of the contamination.

135 2.5 Sprouting

136 The inoculated Mung Bean Seeds (20 g) were placed in polypropylene sprouting jars and soaked in
137 sterile distilled water for 24 h at room temperature (22 ± 1 °C). Then each jar was kept in the dark at
138 22 °C with a relative humidity of $75 \pm 5\%$ for 5 days and rinsed with distilled water each 5 hours
139 daily. Fifteen sprouting jars for each microbial strains were produced.

140

141 2.6 Mung bean sprout sampling

142 Three jars for each microbial strains were sampled at times: 1-Seed; 2-Seed after soaking, 3-One day
143 sprouting; 4-Three days sprouting and 5-Five days sprouting. *L. monocytogenes* was count by ISO
144 11290-2; *Salmonella* spp. in Brilliant Green Agar (Oxoid, Italy) according ISO 6579-1 (2002) and
145 STEC in Sorbitol MacConkey agar SMAC (March & Ratnam, 1986).

146

147 2.7 Reduction by Chlorinated water

148 Mung Bean Sprout – about 300 g of Mung Bean Seeds were additionally inoculated and sprouted,
149 obtaining 15 samples of 20 g for each investigated microorganisms. At 5 days sprouting, the samples
150 were subjected by 4 sequential washings in chlorinated water (100 ppm) for 5 min each. Among each
151 chlorine washing, the samples were floated for 5 min in aciduled water (10 ppm citric acids). Three
152 samples for each microorganism were analyzed after each washing. Chlorine solution was prepared
153 by adding 2.5% sodium hypochlorite to 0.05 M potassium phosphate buffer (pH 6.8, 21 °C) and then
154 diluted to reach the final concentration (100 ppm). The free chlorine in solution and the residual
155 chlorine in the sprouts after the 4 washing were determined with chlorine test kits. Free chlorine
156 concentration in treatment solutions was determined immediately before application to product.

157 Mung Bean Seeds – Part of the Seeds initially inoculated for sprouting was treated with chlorinated
158 water (100 ppm). In particular 10 seed samples (10 g) of each strains were analysed after the inoculum
159 (control) and 10 were subjected to chlorinated water treatment. Both treated and not treated samples
160 were analysed by the above method and treated samples also by enrichment methods: ISO 11290-1
161 for *Listeria monocytogenes*; ISO 6579-1 for *Salmonella* spp.; ISO TS 13136 for STEC.

162

163 2.8 Statistical analysis

164 The values of the various parameters were compared by one-way analysis of variance. The averages
165 of the microbial loads were compared with Tukey's honest significance test using the Statistical
166 Graphics software package ($p < 0.05$).

167

168 3. Results and Discussion

169 The mean and the standard deviation of TVC, Total coliforms and *E. coli* in mung bean seeds and
170 sprouts was investigated over a period of 5 years from 2012 to 2016. In seeds the TVC means were
171 less than 4 log UFC/g, and are quite similar to the mean of the total Coliforms, demonstrating that
172 the Coliforms are the major contaminants of Mung Bean Seeds (Table 1). In sprouts the TVC
173 concentration was equal or less than 6 log UFC/g, and the total Coliforms equal or less than 4 log
174 UFC/g (Table 2). *E. coli* concentration was always present less than the lower detection limit of
175 method (< 10 CFU/g) in both Mung Bean Seeds and Sprouts and that also confirmed the total absence
176 of STEC. In addition no *Salmonella* spp. and *L. monocytogenes* were detected either at seeds or
177 sprouts level. Both TVC and Total Coliforms loads have not significant differences among the 5
178 years of investigations ($p > 0.05$).

179 During the 5 days storing of Mung Bean Sprouts, the TVC and the TC increased up to 1 log CFU/g
180 (Table 3). Consequently it was observed a significant differences in the loads between 0 and 5 days
181 ($p < 0.05$).

182 The investigated Mung Bean Sprouts are grown in plastic washtubs, kept at 22 °C in relative humidity
183 (R.H.) about 90% and watered each 5 hours. So, the abundance of nutrient from sprouting seeds, the
184 temperature of growing and the R.H. of the cellars represent an ideal environment for microbial
185 growth (Peles et al., 2012; Taormina et al., 1999a). During their germination, different complex
186 compounds like lipids, carbohydrates, proteins are broken down into simple and digestible nutrients,

187 that with the vitamins naturally present, become a good food source for humans but also for spoiler
188 and pathogenic microorganisms (Peles et al., 2012).

189 Seeds contain a large microbial concentrations ranging between 10^3 to 10^7 CFU/g (Peles et al., 2012),
190 and these levels are subjected to increase during sprouting reaching up to 10^{11} CFU/g (Peñas et al.,
191 2008; Gabriel et al., 2007; Ghandi & Matthews, 2003; Lang et al. 2000; Prokopowich & Blank,
192 1991;). Various surveys have revealed that Total Aerobic Counts can reach levels of 10^3 to 10^6 CFU/g
193 on alfalfa seeds (Baker et al., 2016; Prokopowich & Blank, 1991; Andrews et al., 1982;1979), levels
194 up to 10^4 CFU/g on mung beans (Andrews et al., 1982), up to 10^5 CFU/g on onion seeds
195 (Prokopowich & Blank, 1991) and up to 10^7 CFU/g on rice seeds (Piernas & Guiraud, 1997). In our
196 investigation, the TVC seeds contamination was lower than the ones obtained by the above authors.
197 Indeed the difference is about 1 log CFU/g.

198 The Mung Bean Seeds microorganisms originated from the soil and from technological method of
199 plant production. The investigated seeds originated from Australia and either the tecnological
200 production or the environmental parameters of the producing area are unknown. Considering the low
201 level of contamination, it was concluded that probably they come from area where the Good
202 Production Practice are deeply applied.

203 Also the TVC load of the investigated sprouts produced and sold in Italy are lower than the ones
204 produced and sold in other parts of Europe or of the World. Several authors reported TVC between
205 10^8 and 10^{11} CFU/g in alfalfa, mung bean or onion sprouts (Peñas et al., 2008; Gabriel et al., 2007;
206 Ghandi & Matthews, 2003; Viswanathan et Kaur, 2001; Lang et al. 2000; Prokopowich & Blank,
207 1991; Patterson & Woodburn, 1980). The bacterial growth is favoured by the traditional sprouting
208 conditions determined by 2–7 days of sprouting, temperatures of 22-24 °C and physico-chemical
209 characteristics such as optimum nutrients availability, pH, water activity (Peles et al., 2012; Taormina
210 et al., 1999a;). In Italy sprouting of Mung Bean Seeds last 5 days. Considering the means of the initial
211 TVC and TC contaminations and the means of both microbial groups in the sprouts at the moment of
212 their harvesting, it seems that the increasing is about 1.8 and 0.4 log CFU/g, respectively. The TVC

213 levels of increasing were less than observed by other authors. In particular Peles et al., (2012)
214 observed that the TVC increasing was just evident after 12 h from the beginning of the sprouting
215 process. The TVC changed from 4.9 log CFU/g to 6.2 log CFU/g and continuously increased day after
216 day reaching 7.9 log CFU/g at the end of sprouting. The level of the increasing was 3 log CFU/g.
217 Different authors obtained same results. In particular during the sprouting they determined 2, 3 and
218 4 log cycles of increasing. The level seems to be due on the seeds such as kidney bean, rice, wheat,
219 lupin, fenugreek, alfalfa and mung bean (Weiss et al., 2007; Martínez-Villaluenga et al., 2006;
220 Kimanya et al., 2003; Splittstoesser et al., 1983; Andrews et al., 1982).

221 Also the concentration of TC of the investigated sprouts was less than the one observed by other
222 authors, which found level of growth up to 2-3 log CFU/g during sprouting. The authors demonstrated
223 that the level of growth depended of the kinds of seeds (Peles et al., 2012; Weiss et al., 2007;
224 Martínez-Villaluenga et al., 2006; Soylemez et al., 2001; Piernas et Guiraud, 1997; Prokopowich et
225 Blank, 1991). Peles et al., (2012), in particular, found 6.9 log total Coliforms/g in one-day sprouts
226 and 7.4 log CFU/g after three days sprouting, observing an increasing up to 3.5 log cycles CFU/g. So
227 it is possible that the low growth of TVC and TC in the investigated sprouts could depend on the
228 kinds of seeds, that in this case were Mung Bean Seeds. In addition the low concentration of TVC
229 and TC in sprouts was also due to the use of sanitized apparatus and of drinking water for watering.

230 In each case the final TVC concentration did not allow to prolong the shelf-life up to 5 days of the
231 investigated sprouts. However also in the case of TVC concentrations exceeding 7 log CFU/g, the
232 sprouts appearance is not negatively affecting (Taormina et al., 1999a).

233 Seeds may contain various microbial loads (Baker et al., 2016; Peles et al., 2012;), represented by
234 environmental microorganisms such as Enterobacteria, *Pseudomonas*, Lactic acid bacteria and yeast,
235 that can rapidly grow (Randazzo et al., 2009; Robertson et al., 2002; Prokopowich & Blank, 1991)
236 and reach high concentration levels ($>10^{10}$ CFU/g) (Peñas et al., 2008; Ghandi & Matthews, 2003;
237 Prokopowich & Blank, 1991; Patterson & Woodburn, 1980). In our study it was focused the isolation
238 and identification of Coliforms, which represent the main microorganisms contaminating either seeds

239 or sprouts. The Coliforms, despite they are easy to grow, required more detailed analyzes because
240 some colonies appeared with dubious colors and morphology, that could be confuse as *Escherichia*
241 *coli* or Corineforms (Galli et al., 1990). The coliforms, which fall within Enterobacteriaceae family,
242 include typically environmental microorganisms (e.g. total Coliforms) and fecal Coliforms or thermo-
243 tolerant Coliforms (44.5 °C) whose natural habitat is the human or animal intestine. In the
244 investigated samples, *Escherichia coli*, the only coliform that includes pathogenic biotypes and
245 pathotypes, was never found. The main isolated coliforms belong to the genera *Klebsiella*,
246 *Enterobacter* and *Citrobacter*. The isolated and identified strains are reported in Table 4. As shown
247 the main strains belonged to *Enterobacter cloacae* and *Klebsiella oxytoca*. Also *Klebsiella*
248 *pneumoniae*, *K. aerogenes* and *Citrobacter freundii* were isolated. For these isolates, it is not correct
249 using the denomination of "fecal coliforms" since they are present in waters of industrial effluents, in
250 the soil and in environments burdened by excessive organic load, consequently they are not always
251 of faecal derivation (Galli et al., 1990; Splittstoesser et al., 1983), but environmental contamination.
252 The packaging used (although there is an atmosphere consisting of air and a high relative humidity
253 85-100%), and the storage temperature do not seem to favor the development of coliforms and other
254 spoiling microorganisms. Despite Galli et al., (1990) have shown that in the fresh vegetable
255 products, the predominant microorganisms grow and produce spoilage and depreciate it, which loses
256 consistency and oxidizes, it was observed that in Mung Bean Sprout, the bud initially ivory-white,
257 become orange/rust at the end of the shelf-life, as a consequence of phenomenas of oxidation and
258 chemical and enzymatic browning (data not shown). So it could be concluded that in these products,
259 microorganisms seem to have only a marginal role in their decay.

260 In the last decade, the consumption of Mung Bean Sprouts has increased in many countries, and in
261 Italy in particular. However, their great consumption has led to an increasing in the number of sprout-
262 associated food-borne illness outbreaks, with at least 40 outbreaks reported in several countries
263 (Baker et al., 2016; Health Canada, 2012). Various outbreaks have been identified many country such
264 as North America (CDC, 2012; Health Canada, 2012; Fett, 2005), and the most frequently associated

265 causative agents are *Salmonella* and *Escherichia coli* O157:H7 (CDC, 2012; Fett, 2005). In particular,
266 Mung Bean Sprouts were responsible of over 600 reported cases for a salmonellosis outbreak in
267 different area of Canada, in 2005 (Health Canada, 2012). Similarly other vegetable sprouts,
268 contaminating by Enterohemorrhagic *E. coli* were responsible of various outbreaks in Europe (Baker et
269 al., 2016; Ding, 2016; Baranzoni et al., 2014). Consequently Mung Bean Sprouts constitute a
270 significant food safety risk (FDA, 2016; EFSA, 2011a,b,c, 2012), because they are eaten raw in many
271 countries, including Europe, USA and Mexico. The absence of any heat treatment increases the
272 potential infection risk associated with sprout consumption (Knödel et al., 2016; Sadler-Reeves et al.,
273 2015), considering that bacterial pathogens can contaminate fresh vegetables as primary
274 contamination (while growing and during harvest) or secondary contamination (during washing,
275 slicing, soaking, packaging and preparation) (Knödel et al., 2016; Harris et al., 2003). However,
276 sprout-associated outbreaks have largely been linked to seeds contaminated with pathogenic
277 microorganisms (Knödel et al., 2016; NACMCF, 1999) rather than post-production contamination.
278 EFSA (2012) reported over 50 outbreaks, due to *Salmonella* and STEC being the most frequent
279 responsible agents. The largest outbreak occurred in 2011 in Germany, due to Shiga toxin-producing
280 *E. coli* O104 H4 present in fenugreek sprouts grown in an organic farm and obtained from
281 contaminated seeds (Buchholz, 2011), imported from Egypt.

282 Usually pathogens contaminate seeds. Consequently seeds are generally recognized as the main
283 source of bacterial pathogens in most sprout-related outbreaks reported by the NACMCF (1999) and
284 various authors (Peles et al., 2012; Taormina et al. 1999b). In this case the control of each lot of seeds,
285 used to produce the investigated sprouts, never demonstrated the presence either of STEC or
286 *Salmonella* spp. and *L. monocytogenes*. According to Commission Regulation (EU) No. 209/2013,
287 Italian producers control the seeds and the sprouts before the sale for the presence of STEC. Up to
288 now, STEC, were never found in all the lots of Mung Bean Seeds and consequently also in the sprouts
289 made with those seeds. Again, at level of one of the two Italian producers, the major producer, the
290 absence of the above three strains of pathogens has been demonstrated since 1982, when this producer

291 started his activity. From 1982, sprouts of this producer are monthly sampled by Italian Official
292 Control Laboratories (ATS-Azienda Sanitaria Territoriale) in order to monitor the presence of
293 pathogenic microorganisms and up to now they never found STEC, *L. monocytogenes* and *Salmonella*
294 spp. However, considering the potential outbreak problems occurred in Europe and in many other
295 part of the world and despite the total absence of pathogenic microorganisms in the investigated
296 sprouts, the additional aim was to study the fate of pathogenic microorganisms during Mung Bean
297 Seeds sprouting and a method based on chlorinated water to decontaminate them before selling. So
298 seeds, artificially contaminated with STEC, *L. monocytogenes* and *Salmonella* spp., were sprouted
299 and analysed in different moments of sprouting in order to value their fates. Then chlorine solutions
300 were used in order to decontaminate the sprouts and seeds by these pathogenic microorganisms. As
301 expected all the investigated pathogenic strains grew on sprouts reaching levels of about 10⁹ CFU/g
302 within 5 days sprouting (Table 5). After one day sprouting the level of the inoculated strains were
303 similar to the initial inocula. At 3 days sprouting, they grew up to 7-8 log CFU/g ($p < 0.05$) and at 5
304 days the concentrations reached values up to 9 log CFU/g. In particular the *L. monocytogenes* and
305 STEC concentrations were significantly different also between 3 and 5 days sprouting, reaching 9.6
306 and 9.8 log CFU/g, respectively ($p < 0.05$). In contrast between the same sprouting period, *Salmonella*
307 spp. concentration did not change at significant level ($p > 0.05$). So the increasing levels for all the
308 inoculated strains were up to 6.5 log CFU/g.

309 The watering, the water and sprouting rooms temperatures permit their growth. The temperature
310 affects *E. coli* and other pathogenic microorganisms growth as demonstrated by Gómez-Aldapa et al.
311 (2013) in Mung Bean Sprout and Charkowski et al. (2002) in alfalfa seeds. Previous different authors
312 found same results. Gómez-Aldapa et al. (2013) demonstrated that all diarrheagenic *E. coli*
313 pathotypes (DEPs) strains grew during germination and sprouting of Mung Bean Seeds sprouted at
314 20 ± 2 or 30 ± 2 °C. In particular the DEPs populations increased from approximately 1.5 log up to
315 approximately 5 log and 7 log CFU/g at 20 ± 2 °C and 30 ± 2 °C, respectively. Their growth was
316 associated to total aerobic culturable bacteria, that increased from approximately 2.5 log to 8.4 and

317 8.9 log CFU/g at both the sprouting temperatures. Finally they concluded that DEPs slowly
318 decreased until day 10. In addition our data are in agreement with previous research of Warriner et
319 al. (2003) and Castro-Rosas and Escartín (2000) which observed up to 5 log CFU/g of *Salmonella*
320 *typhi* and *E. coli* O157:H7 growth during germination and sprouting of alfalfa seeds and Mung Bean
321 Seeds, respectively. Again, Xiao et al., (2014) observed during radish sprouting and microgreen high
322 level of survival and growth of *E. coli* O157:H7 and O104:H4, which was responsible of recent food
323 borne cases in Germany. They found at the end of sprouting levels of about 7.6 log CFU/g and 5.0 log
324 CFU/g for sprouts and microgreens, respectively. In addition, Knödler et al., (2016) investigated the
325 survival of *E. coli* O154H4, responsible of the recent outbreak in Germany, on fenugreek seeds
326 (*Trigonella foenum-graecum*) and formulated the hypothesis that it did not come from seeds
327 externally contaminated in Egypt, because of the large period from the seeds shipment from Egypt
328 (November 2009) and the first infections in Germany (2011) and consequently they suggested,
329 according to Radosavljevic et al., (2015) that should not exclude a contamination at later stages
330 through contact with infected individuals, eventually during the storage, transport, and repackaging
331 process at the distributor in Europe (Knödler et al., 2016).

332 Also *Salmonella* and *L. monocytogenes* represent a microbial risk for Mung Bean Sprouts and other
333 sprouted vegetables. The EFSA (2011a,b,c) reported that 34 out of 43 outbreaks were associated with
334 consumption of various sprouted seeds contaminating by *Salmonella*. Different serotypes were
335 implicated and in particular for Mung Bean Sprout, *Salmonella* enterica serovar Bareilly (*Salmonella*
336 *Bareilly*) was responsible of a large outbreaks in the UK in 2010 (Cleary et al. 2010), affecting 231
337 cases including one death. Consequently, 9 Official Control Laboratories in England and Northern
338 Ireland during January to March 2011 (Sadler-Reeves et al., 2015) analysed 554 samples of Bean
339 Sprouts or other sprouted seeds, collected at retail sale and submitted in order to value the presence
340 of *Salmonella* spp. In particular 23 % of samples were labelled as ready-to-eat, 61% as raw or ready-
341 to-cook, and the remaining had no indication on their use. *Salmonella* enterica serovar *Abetetuba*

342 (11:k:1.5) were detected only from four samples; 2 out 4 from products sold as ready-to-eat and 2 as
343 ready-to-cook.

344 *L. monocytogenes* can grow, as demonstrated in this work, during the germination of sprouts (Table
345 5). Piernas and Guiraud (1997) found that *L. monocytogenes*, inoculated in rice seeds, grew during
346 germination without any apparent antagonistic from the background microbiota. Usually *L.*
347 *monocytogenes* seems to be rarely present in seeds and sprouts (Symes et al., 2015; Palmai et al.,
348 2002). However it was implicated in some incidents (FDA, 2016; CDC, 2014).

349 Considering the risk for consumers, different treatments have been tested to reduce seeds
350 contamination by food pathogens (Nei et al., 2013; Studer et al., 2013; Fransisca et al., 2012; Kim et
351 al., 2010; Taormina and Beuchat, 1999a). Physical (e.g. dry heat, hot water, high hydrostatic pressure,
352 irradiation), biological (e.g. antagonistic microorganisms and their metabolites) and chemical
353 processes (i.e. chlorine, ozone and organic acids) strategies (Ding and Fu, 2016; Sikin et al., 2013)
354 have been explored to minimize the risk associated with bacterial pathogens on seeds and beans used
355 for sprouting (Trzaskowska et al., 2018). Seeds and sprouts disinfection remains the most important
356 crucial step in the safety of vegetable sprouts (Trzaskowska et al., 2018). The Canadian Food
357 Inspection Agency (CFIA, 2007) and FDA (2016,2014) recommend a minimum of 3 log of microbial
358 reduction in seeds by the application of a sanitation step.

359 In this work, it was investigated the use of chlorinated water in order to reduce the sprouts
360 contamination. As shown in table 6, it was suggested to decontaminate Mung Bean Sprouts by 4
361 sequential washings with chlorinated water. The initial contamination was up to 9 log CFU/g and
362 represents the concentration of the inoculated seeds after sprouting. The 4 sequential washing with
363 chlorinated water (100 ppm) decreased till less than 10 CFU/g, and this decreasing was higher than
364 the one suggested by both CFIA (2007) and FDA (2016,2014). However this treatment produces 3
365 problems. First, the use of a chlorine solution to wash sprouts is illegal in Italy; second, there is a
366 chlorine residual in the sprouts (10-15 ppm); third, the chlorine rapidly changes the color of the
367 sprouts becoming pale yellow or orange-rust. Usually after traditional washing with a solution of

368 citric acid, the color of the sprouts is white, and this color is wadely accepted by the consumers. In
369 contrast consumers consider that a pale yellow color is an oxidative index. In addition, after opening
370 the treated sprouts packagings, an intense chlorine smell occurred, despite additionally sequential
371 washings with drinkling water after the treatment. In each case it was not possible to reduce the
372 chlorine concentration to 20-30 ppm, because it is not sufficient to reduce the contamination of the
373 investigated pathogenic microorganisms up to 3 log CFU/g (data not shown). For this reason it could
374 be concluded that it needs to decontaminate directly the seeds before sprouting, as just suggested by
375 other authors (Trzaskowska et al., 2018; Knödler et al., 2016). Our data (Table 7) demonstrate that it
376 is possible to reduce of about 3 log the initial contamination, even though the treatment did not assure
377 0 tolerance for the inoculated pathogenic strains. It was impossible to complete eradicate the
378 pathogens. At the end of the seeds treatment they can be found at level of enrichment cultures (Table
379 7). So the use of chlorine at high or low concentration level does not permit to complete eliminate the
380 hazzard and the risk of pathogenic microorganisms in seeds and sprouts. Other authors used combined
381 treatments or hurdle to reduce the level of undesirable microorganisms on seeds and consider them
382 more effective than individual treatments (Ding et al., 2013; Nei et al., 2013). The latest authors
383 reduced up to 5 log CFU/g of both *S. enterica* and *E. coli* O157:H7 on mung bean washed with hot
384 water at 85 °C for 40 s followed by soaking in a 2000 ppm chlorine solution for 2 h. However the
385 authors did not report the value of chlorine residual and eventually the presence of chlorine smell in
386 the sprouts, as demonstrated in our work using a 100 ppm chlorine solution. In addition other methods
387 has been evalued in order to decontaminate vegetable sprouts including ozonated water (Sharma et
388 al., 2002), pulsed ultraviolet light (Sharma & Demirci, 2003), γ -radiation (Thayer et al., 2003), single
389 or combined treatment with hot water followed by exposure to H₂O₂ (Hong et Kang, 2016) or to
390 H₂O₂ and acetic acid (Trzaskowska et al., 2018; Studer et al., 2013) or only with acetic acid (Lang et
391 al., 2000), ultrasound followed by a washing solution of ClO₂ (3 ppm) (Millan-Sango 2017; Holliday
392 et al., 2001; Beuchat et al., 1997). Either single or combined treatment had good bactericidal effect
393 (up to 3-5 log CFU/g) and significative reduction impacts ($p < 0.05$), but they did not permit to

394 completely eliminate *E.coli* and *Salmonella* on alfalfa and mung bean sprouts. Consequently there is
395 so far no guarantee of a seed treatment, able to eradicate contamination with bacterial pathogens
396 before seed germination (Knödler et al., 2016). With respect to avoiding sprout-related STEC, *L.*
397 *monocytogenes* and *Salmonella* spp. outbreaks in the future, it was suggested a short treatment of the
398 seeds, such as chlorinated water for washing, that this work has shown to be effective against all
399 tested tested or using single or combined procedure (Knödler et al., 2016), that are cheap, already
400 part of the FDA guidelines for sprout production in the United States of America (Knödler et al.,
401 2016; HPA, 2009; NACMCF, 1999).

402

403 **4. Conclusion**

404 This study provides information on the hygienic quality and microbial status of Mung Bean Sprouts
405 produced in Italy. The microbial loads was less than the ones observed in seeds and sprouts produced
406 in other countries and in particular the pathogenic microorganisms, responsible of recently outbreaks
407 (Buchholz et al. 2011; Health Canada, 2012; EFSA, 2012), were never present. Despite these
408 encouraging results, the attention to the hygienic quality should never be reduced and it is of great
409 importance to minimize bacterial contamination. In particular, it was suggested to reduce and
410 eliminate pathogenic microorganisms directly from seeds and to minimize microbial contamination
411 of sprouts prior to consumption.

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Table 1: Microbial quality of Mung Bean seeds

Microbial group	Year of sampling				
	2012	2013	2014	2015	2016
Total viable count	3.5 ± 0.1a	4.1 ± 0.5a	3.3 ± 0.3a	3.2 ± 0.8a	3.0 ± 1.0a
Total coliforms	3.1 ± 0.2a	2.8 ± 0.4a	3.1 ± 0.1a	2.8 ± 0.4a	2.7 ± 0.4a
<i>Escherichia coli</i>**	< 10	< 10	< 10	< 10	< 10
*<i>Salmonella</i> spp.	Absence	Absence	Absence	Absence	Absence
*<i>L.monocytogenes</i>	Absence	Absence	Absence	Absence	Absence
*STEC	Absence	Absence	Absence	Absence	Absence

606 Legend: Data log CFU/g; ** CFU/g; STEC: Shiga Toxin *E. coli*; *: Absence in 25 g.

607 Mean with different letters within a row (following the lines) are significantly

608 different (p < 0.05).

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616 Table 2: Microbial quality of Mung Bean sprouts

Microbial group	Year of sampling				
	2012	2013	2014	2015	2016
Total viable count	5.5 ± 0.3a	5.2 ± 1.1a	5.3 ± 0.8a	5.1 ± 0.5a	5.1 ± 0.4a
Total coliforms	3.4 ± 0.8a	3.3 ± 1.1a	3.2 ± 0.5a	3.5 ± 0.4a	3.1 ± 0.9a
<i>Escherichia coli</i>**	< 1	< 1	< 1	< 1	< 1
*<i>Salmonella</i> spp.	Absence	Absence	Absence	Absence	Absence
*<i>L.monocytogenes</i>	Absence	Absence	Absence	Absence	Absence
*STEC	Absence	Absence	Absence	Absence	Absence

617 Legend: Data log CFU/g; ** CFU/g; STEC: Shiga Toxin *E. coli*; *: Absence in
618 25 g. Mean with different letters within a row (following the lines) are
619 significantly different (p < 0.05).

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623 Table 3: Coliform strains

Strains	%	Accession number
<i>Klebsiella pneumoniae</i>	14	NZ_CP006662.2
<i>Klebsiella oxytoca</i>	20	NZ_PCMV01000088.1
<i>Klebsiella (Enterobacter) aerogenes</i>	12	NZ_MTZP01001321.1
<i>Citrobacter freundii</i>	14	NZ_KQ464182.1
<i>Enterobacter cloacae</i>	40	NG_050406.1

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626 Table 4: Fate of Total Viable Count (TVC)
627 and Total Colifoms (TC) during the storage
628 of Mung bean Sprouts

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Days	Sprouts (CFU/g)	
	TVC	TC
0	5.2 ± 0.1a	3.2 ± 0.1a
5	6.8 ± 0.4b	4.5 ± 0.2b

630 Mean with different letters within a row
631 (following the colons) are significantly
632 different (p < 0.05).

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637 Table 5: Fate of different pathogenic microorganisms intentionally inoculated during Mung Beans
638 sprouting.

Microorganisms	Seed	Seed after soaking	One day sprouting	Three day sprouting	Five day sprouting
<i>Salmonella</i> spp.	2.5 ± 0.2a	2.7 ± 0.1a	2.7 ± 0.3a	8.1 ± 0.1b	9.1 ± 0.8b
<i>L. monocytogenes</i>	2.1 ± 0.2a	2.3 ± 0.2a	2.5 ± 0.3a	7.2 ± 0.1b	9.6 ± 0.3c
<i>E. coli</i> STEC	2.1 ± 0.3a	2.2 ± 0.1a	2.4 ± 0.2a	8.5 ± 0.4b	9.8 ± 0.1c

639 Legend: Data are log CFU/g mean ± standard deviation of triplicate experiments; Mean with
640 different letters within a row (following the lines) are significantly different (p < 0.05).

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Table 6: Reduction of the investigated pathogens after 4 sequential washings in chlorinated water solution (100 ppm)

Microorganisms	Before washing	After 1 st washing	After 2 nd washing	After 3 rd washing	After 4 th washing
<i>Salmonella</i> spp.	9.1 ± 0.8b	7.7 ± 0.3a	5.4 ± 0.3a	2.1 ± 0.1b	1.8 ± 0.2b
<i>L. monocytogenes</i>	9.6 ± 0.3c	7.3 ± 0.4a	5.5 ± 0.3a	2.2 ± 0.2b	1.9 ± 0.2b
<i>E. coli</i> STEC	9.8 ± 0.1c	7.2 ± 0.2a	5.4 ± 0.2a	1.9 ± 0.4b	1.8 ± 0.1b

Dara: Log CFU/g; Clorine residual after 4 washing 10-15 ppm.

Table 7: Reduction of the investigated pathogens

Microorganisms	Seeds Log CFU/g	After soaking CFU/g	After soaking Presence in 25 g
<i>Salmonella</i> spp.	3.1 ± 0.2	< 10	+
<i>L. monocytogenes</i>	3.6 ± 0.1	< 10	+
<i>E. coli</i> STEC	3.3 ± 0.2	< 10	+

Seeds treated in chlorinated water solution (100 ppm)

Time of soaking: 60 min