Microbial quality of raw and ready-to-eat mung bean sprouts produced in Italy

Published
DOI:10.1016/j.fm.2019.03.014

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.

Keywords: Mung Bean Sprout; Microbial Quality; STEC.

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.

1. Introduction

Recently the use of mung bean sprouts, originated in Far East areas, has been wide spread to the western world. They are obtained germinating seeds, are considered fashionable and healthy ingredients (Kuo et al., 2004). Yet, they are also recognised foundamental for human diet providing health benefits because they are important source of proteins, vitamins, minerals and fibers (Martínez-Villaluenga et al., 2008). The flow sheet of production includes that Mung Bean Sprouts must be grown in environmentally controlled conditions, in warm (22-24 °C) and in high humidity (95%) for sprouting for three-five days (Peles et al., 2012; Taormina et al., 1999a). Seeds are usually contaminated by high microbial loads, including yeasts, Pseudomonads, Enterobacteria, Lactic acid bacteria. Due to humid and nutritious sprouting conditions and being sprouts good media, the microbial loads can increase at level of 1010 CFU/g (Zheng et al., 2015,2016; Randazzo et al., 2009; Peńas et al., 2008; Ghandi & Matthews, 2003; Harris et al., 2003; Robertson et al., 2002; Prokopowich & Blank, 1991). Pathogenic microorganisms can also contaminate them, either as primary contamination (while growing in and during harvest) or secondary contamination, during washing, slicing, soaking, packaging and preparation (Harris et al., 2003). The pathogen contamination of sprouts comes from the seeds. Consequently it is largely recognized that the sprout-associated outbreaks have been due to seeds contaminated with pathogenic microorganisms (NACMCF, 1999; Peles et al., 2012) rather than post-production contamination (Xiao et al., 2014). Pathogenic microorganisms load increase because of the high volumes of water used during sprout production and sprouting temperature especially in systems where sprouts are exposed to a common “water bath” and frequently or continuously mixed, such as in a rotary drum (Xiao et al., 2014). For these reasons, sprouted seeds have been implicated in various and serious outbreaks caused by Salmonella and Shiga toxin-producing *Escherichia coli* (STEC). Indeed, *Salmonella Bareilly* in United Kingdom (Cleary et al., 2010), *S. Newport* in Germany and Netherlands (Bayer et al., 2014) and *S. Enteritidis* in 2014 in the USA (FDA, 2014) produced outbreak associated with bean sprouts. *Salmonella* and *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/1rt/2012 to December/31rt/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/1rt/2012 to December/31rt/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 ºC for 48-72 h; total Coliforms (TC) and *Escherichia coli* was grown on Violet Red Bile Lactose Agar

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

2.2 Total Coliforms and presuntive E. coli isolation and identification

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

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

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

2.4 Bacterial strains and inocolum preparation

The bacteria strains included for *Listeria monocytogenes*: *Listeria monocytogenes* Scott A, *Listeria monocytogenes* NCTC 10887 (serotype 1/2b), *Listeria monocytogenes* NCTC 10527 (serotype 4b); for *Salmonella*: *Salmonella enteritidis*, *Salmonella typhimurium* and *Salmonella derby*; for *Escherichia coli* (STEC): *E.coli* O157: H7 strains ATCC 43888; *E.coli* O103H2 ED-173 Istituto Superiore Sanità, Rome; *E.coli* O26H11 E-D 56, Istituto Superiore della Sanità, Rome. Each strain was grown in Brain Heart Infusion Agar (Oxoid, Italy). The inocula were obtained according to Xiao et al., (2014) modified method. Briefly: Four milliliters of an overnight culture in Brain Heart Infusion broth (Oxoid, Italy) of each strains were subjected to a centrifugation at 13,400 rpm for 10 min at room temperature, and the pellets were resuspended in saline-peptone water (8 g/l NaCl, 1 g/l bacteriological peptone; Oxoid, Italy, distilled water 1000 ml). The concentration of each suspension was about 10_6 – 10_7 CFU/ml. Equal volumes of cell suspensions of each species were combined as a cocktail in order to obtain three different suspensions containing *L. monocytogenes*, *Salmonella* spp. and STEC with desidered cell concentrations for seed inoculation. Each suspensions was diluted in peptoned water and 0.1 of the dilution was plated in Plate Count Agar (Oxoid, Italy) incubated at 37 °C for 24-48 h in order to value their concentration. For inoculation, different portions of 600 g seeds were immersed in 800 mL of each appropriate inoculum suspension with gentle swirling for 5 min at room temperature. The final concentration of each inoculated microorganisms in the seeds was about 10_2~10_3 CFU/g. The seeds were air-dried overnight under a laminar flow biological safety hood at room temperature and stored at 4 °C for up to 48 h. Then part of the seeds were subjected to sprouting and part directly treated with chlorine water in order to reduce the level of the contamination.

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 jair was kept in the dark at
138 22 °C with a relative humidity of 75 ± 5% for 5 days and rinsed with distilled water each 5 hours
139 daily. Fifteen sprouting jairs for each microbial strains were produced.
140
141 *2.6 Mung bean sprout sampling*
142 Three jairs 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 Brillian 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 analized 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 diluited 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 inocolum
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.

2.8 Statistical analysis

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

3. Results and Discussion

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

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

The investigated Mung Bean Sprouts are grown in plastic washtubs, kept at 22 °C in relative humidity (R.H.) about 90% and watered each 5 hours. So, the abundance of nutrient from sprouting seeds, the temperature of growing and the R.H. of the cellars represent and ideal environments for microbial growth (Peles et al., 2012; Taormina et al., 1999a). During their germination, different complex compounds like lipids, carbohydrates, proteins are broken down into simple and digerible 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 1011 CFU/g (Peńas 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 103 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 (Peńas 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 begining of the sprouting
215 process. The TVC changed from 4.9 log CFUg 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; Martìnez-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 Martìnez-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 apparathus 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 enviromental microorgansms 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) (Peńas 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 spoilaging 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

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

Usually pathogens contaminate seeds. Consequently seeds are generally recognized as the main source of bacterial pathogens in most sprout-related outbreaks reported by the NACMCF (1999) and various authors (Peles et al., 2012; Taormina et al. 1999b). In this case the control of each lot of seeds, used to produce the investigated sprouts, never demonstrated the presence either of STEC or *Salmonella* spp. and *L. monocytogenes*. According to Commission Regulation (EU) No. 209/2013, Italian producers control the seeds and the sprouts before the sale for the presence of STEC. Up to now, STEC, were never found in all the lots of Mung Bean Seeds and consequently also in the sprouts made with those seeds. Again, at level of one of the two Italian producers, the major producer, the 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 microrganisms 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 clorinate solutions
300 were used in order to decontaminate the sprouts and seeds by these pathogenic microorgasnisms. As
301 espected all the investigated pathogenic strains grew on sprouts reaching levels of about 10^9 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 significative 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 significative 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 temperaratures 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 slowely
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 responsable of recent food
323 borne cases in Germany. They foud 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 *Abaetetuba*

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 (Trząskowska et al., 2018). Seeds and sprouts disinfection remains the most important
356 crucial step in the safety of vegetable sprouts (Trząskowska 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 (Trząskowska 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 H_2O_2 (Hong et Kang, 2016) or to
390 H_2O_2 and acetic acid (Trząskowska et al., 2018; Studer et al., 2013) or only with acetic acid (Lang et
391 al., 2000), ultrasound followed by a washing solution of ClO_2 (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, responsable 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.
412
413

References

Robertson, L.J., Johannessen, G.S., Gjerde, B.K., Loncarevic, S., 2002, Microbiological analysis of seed sprouts in Norway. International Journal of Food Microbiology, 75, pp. 119-126

Altschul, S.F., Madden, T.L., Shaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402. Baker, C.A., Rubinelli, P.M., Hong Park, S., Carbonero, F., Ricke, S.C., 2016. Shiga toxin-producing *Escherichia coli* in food: Incidence, ecology, and detection strategies. Food Contr. 59, 407-419.

Andrews, W.H., Mislivec, P.B., Wilson, C.R., Bruce,V.R., Polema, P.L., Gibson, R., Trucksess, M.W., Young, K., 1982. Microbial hazards associated with bean sprouting. J. AOAC 65, 241-248.

Andrews, W.H., Wilson, C.R., Poelma, P.L., Romero, A., Mislivec, P.B., 1979. Bacteriological survey of sixty health foods. Appl. Environ. Microbiol. 37, 559-566.

Baranzoni, G.M., Fratamico, P.M., Rubio, F., Glaze, T., Bagi, L.K., Albonetti, S., 2014. Detection and isolation of Shiga toxin-producing *Escherichia coli* (STEC) O104 from sproouts. Int. J. Food Microbiol. 173, 99–104.

Bayer, C., Bernard, H., Prager, R., Rabsch, W., Hiller, P., Malorny, B., Pfefferkorn, B., Frank, C., de Jong, A., Friesema, I., Stark, K., Rosner, B.M., 2014. An outbreak of *Salmonella Newport* associated with mung bean sprouts in Germany and the Netherlands, October to November 2011. Euro Surveill. 19 (1) (pii = 20665. Available at: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20665).

Beuchat, L.R., 1997. Comparison of chemical treatments to kill *Salmonella* on alfalfa seeds destined for sprout production. Int. J. Food Microbiol. 34, 329-333.

Buchholz, U., 2011. German outbreak of *Escherichia coli* O104:H4 associated with sprouts. N. Engl. J. Med. 365, 1763–1770.

Castro-Rosas, J., Escartín, E.F., 2000. Survival and growth of *Vibrio cholerae* O1, *Salmonella typhi* and *Escherichia coli* O157:H7 in alfalfa sprouts. J. Food Sci. 65, 162–165.

CDC, Centers for Disease Control and Prevention, 2014. Wholesome Soy Products, Inc. Sprouts and Investigation of Human Listeriosis Cases accessed 6.16.16. http://www.cdc.gov/listeria/outbreaks/bean-sprouts-11-14/index.html.

CDC. 2012. *Escherichia coli*. Available: http,//www.cdc.gov/ecoli/general/ #who Accessed March 20, 2018.

CFIA, Canadian Food Inspection Agency, 2007. Code of Practice for the Hygienic Production of Sprouted Seeds accessed 9.6.16. http://www.inspection.gc.ca/ food/fresh-fruits-and-vegetables/food-safety/sprouted-seeds/eng/ 1413825271044/1413825272091#a8.

Charkowski, A.O., Barak, J.D., Sarreal, C.Z., Mandrell, R.E., 2002. Differences in growth of *Salmonella* enterica and *Escherichia coli* O157:H7 on Alfalfa Sprouts. Appl. Environ. Microbiol. 68, 3114–3120.

Cleary, P., Browning, L., Coia, J., Cowden, J., Fox, A., Kearney, J., Lane, C., Mather, H. et al. 2010. A foodborne outbreak of *Salmonella Bareilly* in the United Kingdom. Euro Surveill 15, 1–3.

Ding, H., Fu, T.J., 2016. Assessing the public health impact and effectiveness of interventions to prevent *Salmonella* contamination of sprouts. J. Food Prot. 79, 37-42.

Ding, H., Fu, T.J., Smith, M.A., 2013. Microbial contamination in sprouts: how effective is seed disinfection treatment? J. Food Sci. 78, R495-R501.

EFSA, 2011a. Scientific opinion on the risk posed by Shiga toxin-producing *Escherichia coli* (STEC) and other pathogenic bacteria in seeds and sprouted seeds. EFSA J. 9, 2424.

EFSA, 2011b. Tracing Seeds, in Particular Fenugreek (*Trigonella Foenum-graecum*) Seeds, in Relation to the Shiga Toxin-producing *E. coli* (STEC) O104:H4 2011 Outbreaks in Germany and France. 2011.

463 EFSA, 2011c. Urgent advice on the public health risk of Shiga-toxin producing *Escherichia coli* in
464 fresh vegetables. EFSA J. 9, 2274. Euro Surveill. 19 (1) (pii = 20665. Available at: http://www.
465 eurosurveillance.org/ViewArticle.aspx?ArticleId=20665).
466 EFSA, 2012. Scientific opinion on the risk posed by Shiga toxin-producing *Escherichia coli* (STEC)
467 and other pathogenic bacteria in seeds and sprouted seeds. EFSA J. 9, 1–101.
468 FDA, 2014a. FDA Investigates Multistate Outbreak of *Salmonella Enteritidis* Linked to Mung Bean
469 Sprouts Produced by Wonton Foods Inc. of New York. (Availabel at: http://www.
470 fda.gov/Food/RecallsOutbreaksEmergencies/Outbreaks/ucm424426.htm).
471 FDA, US Food and Drug Administration, 2016. Outbreaks - Outbreak Investigations accessed
472 6.16.16. http://www.fda.gov/Food/RecallsOutbreaksEmergencies/ Outbreaks/ucm272351.htm.
473 Fett, W.F., 2005. Interventions to ensure the microbiological safety of sprouts. In: Sapers, G.M.,
474 Gorny, J.R., Yousef, A.E. (Eds.), Microbiology of fruit and vegetables. CRC Press, Boca Raton, Fl,
475 pp. 187–210.
476 Fransisca, L., Park, H.K., Feng, H., 2012. *E. coli* O157:H7 population reduction from alfalfa seeds
477 with malic acid and thiamine dilauryl sulfate and quality evaluation of the resulting sprouts. J. Food
478 Sci. 77, M121-M126.
479 Gabriel, A.A., Berja, M.C., Estrada, A.P., Lopez, M.A., Nery, J.B., Villaflor, E.B., 2007.
480 Microbiology of retail mung bean sprouts vended in public markets of National Capital Region,
481 Philippines. Food Contr. 18, 1307-1313.
482 Galli A., Franzetti L., 1991. I batteri Gram negativi nelle insalate della IV gamma. Ann. Microbiol.
483 41, 41-51.
484 Ghandi, M., Matthews, K.R., 2003. Efficacy of chlorine and calcinated calcium treatment of alfalfa
485 seeds and sprouts to eliminate *Salmonella*. Int. J. Food Microbiol. 87, 301-306
486 Gómez-Aldapa, C.A., Rangel-Vargas, E., Bautista-De León, H., Vázquez-Barrios, M.E., Gordillo-
487 Martínez, A.J., Castro-Rosas, J., 2015. Behavior of enteroaggregative *Escherichia coli*, non-O157-

488 shiga toxin-producing *E. coli*, enteroinvasive *E. coli*, enteropathogenic *E. coli* and enterotoxigenic *E.*
489 *coli* strains on mung bean seeds and sprout. Int. J. Food Microbiol. 166, 364–368.
490 Harris, L.J., Farber, J.N., Beuchat, L.R., Parish, M.E., Suslow, T.V., Garrett, E.H., Busta, F.F., 2003.
491 Outbreaks associated with fresh produce: Incidence, growth, and survival of pathogens in fresh and
492 fresh cut produce. Compr. Rev. Food Sci. F. 2, 78–141.
493 Health Canada, 2012. Risk associated with sprouts. Available at: http://www.hc-sc.gc.ca/ hl-vs/iyh-
494 vsv/food-aliment/sprouts-germes-eng.php (Accessed July 31, 2013.).
495 Health Protection Agency (2009) Guidelines for Assessing the Microbiological Safety of Ready-to-
496 eat Foods Placed on the Market. London: HPA.
497 Holliday, S.L., Scouten, A.J., Beuchat, L.R., 2001. Efficacy of chemical treatments in eliminating
498 *Salmonella* and *Escherichia coli* O157:H7 on scarified and polished alfalfa seeds. J. Food Prot. 64,
499 1489-1495.
500 Hong, E.J., Kang, D.H., 2016. Effect of sequential dry heat and hydrogen peroxide treatment on
501 Reduction of *Salmonella Typhimurium* on alfalfa seeds and seeds germination. Food Microbiol. 53,
502 9-14. Part B.
503 Iacumin, L., Cecchini, F., Manzano, M., Osualdini, M., Boscolo, D., Orlic, S., Comi, G., 2009.
504 Description of the microflora of sourdoughts by culture-dependent and culture independent methods.
505 Food Microbiol. 26, 128-135.
506 Kim, H., Bang, J., Beuchat, L.R., Ryu, J.H., 2010. Synergistic effect of chlorine dioxide and drying
507 treatments for inactivating *Escherichia coli* O157:H7 on radish seeds. J. Food Prot. 73, 1225-1230.
508 Kimanya, M.E., Mamiro, P.R.S., Van Camp, J., Devlieghere, F., Opsomer, A., Kolsteren, P.,
509 Debevere, J., 2003. Growth of *Staphylococcus aureus* and *Bacillus cereus* during germination and
510 drying of finger millet and kidney beans. Int. J. Food Sci. Technol. 38, 119-125.
511 Knödler, M., Berger, M., Dobrindt, U., 2016. Long-term survival of the Shiga toxin-producing
512 *Escherichia coli* O104:H4 outbreak strain on fenugreek seeds. Food Microbiol. 59, 190-195.

513 Kuo, Y.H., Rozan, P., Lambein, F., Frías, J., Vidal-Valverde, C., 2004. Effects of different
514 germination conditions on the contents of free protein and non-protein amino acids of commercial
515 legumes. Food Chem. 86, 537-545.
516 Lang, M.M., Ingham, B.H., Ingham, S.C., 2000. Efficacy of novel organic acid and hypochlorite
517 treatments for eliminating *Escherichia coli* O157:H7 from alfalfa seeds prior to sprouting. Int. J. Food
518 Microbiol. 58, 73-82.
519 March, S. B., Ratnam, S., 1986. Sorbitol-MacConkey medium for detection of *Escherichia coli*
520 0157:H7 associated with hemorrhagic colitis. J. Clin. Microbiol. 23, 869-872.
521 Martínez-Villaluenga, C., Frías, J., Gulewicz, P., Gulewicz, K., Vidal-Valverde, C., 2008. Food
522 safety evaluation of broccoli and radish sprouts. Food Chem. Toxicol. 46, 1635-1644.
523 Martínez-Villaluenga, C., Gulewicz, P., Pérez, A., Frías, J., Vidal-Valverde, C., 2006, Influence of
524 lupin (*Lupinus luteus L.* cv. 4492 and *Lupinus angustifolius L.* var. Zapaton) and fenugreek
525 (*Trigonella foenum-graecum L.*) germination on microbial population and biogenic amines. J.
526 Agricult. Food Chem. 54, 7391-7398.
527 Millan-Sango, D., Sammut, E., Van Impe, J.F., Valdramidis, V.P., 2017. Decontamination of alfalfa
528 and mung bean sprouts by ultrasound and aqueous chlorine dioxide. LWT, Food Sci. Rechn., 78, 90-
529 96.
530 NACMCF (National Advisory Committee on Microbiological Criteria for Food), 1999.
531 Microbiological safety evaluations and recommendations on sprouted seeds. Int. J. Food Microbiol.
532 52, 123–153.
533 Nei, D., Bari, M.L., Enomoto, K., 2013. Validation of hot water and chlorine treatments to inactivate
534 pathogens inoculated on mung bean seeds: influence of the seed production area. Food Contr. 32,
535 186-189.
536 Palmai, M., Buchanan, 2002. Growth of *L. monocytogenes* during alfalfa sprouts. Food Microbiol.
537 19, 195-200.

538 Patterson, J.E., Woodburn, M.J., 1980. *Klebsiella* and other bacteria on alfalfa and bean sprouts at
539 the retail level. J. Food Sci. 45, 492-495.
540 Peles F., Győri, Z., Bácskai, T., Szabó, Zs., Murvai, M., Kovács, B. 2012. Microbiological quality of
541 organic wheat grains and sprout. Analele Universităţii din Oradea, Fascicula Protecţia Mediului Vol.
542 XVIII, 53-60.
543 Peńas, E., Gómez, R., Frías, J., Vidal-Valverde, C., 2008. Application of high pressure treatment on
544 alfalfa (*Medicago sativa*) and mung bean (*Vigna radiata*) seeds to enhance the microbial safety of
545 their sprouts. Food Control 19, 698- 705.
546 Piernas, V., Guiraud, J.P., 1997. Disinfection of rice seeds prior to sprouting. J. Food Sci. 62, 611-
547 615.
548 Prokopowich, D., Blank, G., 1991, Microbiological evaluation of vegetable sprouts and seeds. J. Food
549 Protect. 54, 560-562 .
550 Radosavljevic, V., Finke, E.J., Belojevic, G., 2015. *Escherichia coli* O104:H4 outbreak in Germany
551 clarification of the origin of the epidemic. Eur. J. Public Health 25, 125-129.
552 Randazzo, C.L., Scifò, G.O., Tomaselli, F., Caggia, C., 2009. Polyphasic characterization of bacterial
553 community in fresh cut salads. Int. J. Food Microbiol. 128, 484-490.
554 Sadler-Reeves, L., Jorgensen, F., Lane, C., Willis, C., McLauchlin, J., Aird, H., de Pinna, E., Elviss,
555 N., Fox, A., Kaye, M., 2015. The occurrence of *Salmonella* in raw and ready-to-eat bean sprouts and
556 sprouted seeds on retail sale in England and Northern Ireland. Lett. Appl. Microbiol. 62, 126—129.
557 Sharma, R.R., Demirci, A., 2003. Inactivation of *Escherichia coli* O157:H7 on inoculated Alfalfa
558 Seeds with Pulsed Ultraviolet Light and Response Surface Modeling. J. Food Sci. 68(4), 1448-1453.
559 Sharma, R.R., Demirci, A., Beuchat, L.R., Fett, W.F., 2002. Inactivation of *Escherichia coli* O157H7
560 on inoculated alfalfa seeds with ozonated water and heat treatment. J. Food Protect. 65 (3), 447-451.
561 Sikin, A.M.D., Zoellner, C., Rizvi, S.S.H., 2013. Current intervention strategies for the microbial
562 safety of sprouts. J. Food Prot. 76, 2099-2099.

563 Soylemez, G., Brashears, M.M., Smith, D.A., Cuppett, S.L., 2001. Microbial quality of alfalfa seeds
564 and sprouts after a chlorine treatment and packaging modifications. J. Food Science 66, 153-157.
565 Splittstoesser, D.F., Queale, D.T., Andaloro, B.W., 1983. The microbiology of vegetable sprouts
566 during commercial production. J. Food Safety 5, 79-86.
567 Studer, P., Heller, W.E., Hummerjohann, J., Drissner, D., 2013. Evaluation of aerated steam treatment
568 of alfalfa and mung bean seeds to eliminate high levels of *Escherichia coli* O157:H7 and O178:H12,
569 *Salmonella enterica*, and *Listeria monocytogenes*. Appl. Environ. Microbiol. 79, 4613-4619.
570 Symes, S., Goldsmith, P., Haines, H., 2015. Microbiological safety and food handling practices of
571 seed sprout products in the Australian State of Victoria. J. Food Prot. 78, 1387-1391.
572 Taormina, P.J., Beuchat, L.R., 1999a. Comparison of chemical treatments to eliminate
573 enterohemorrhagic *Escherichia coli* O157:H7 on alfalfa seeds. J. Food Prot. 62, 318-324.
574 Taormina, P.J., Beuchat, L.R., Slutsker, L., 1999b. Infections associated with eating seed sprouts: an
575 international concern. Em. Infect. Dis. 5, 626-634.
576 Thayer, D.W., Boyd, G., Fett, W.F. 2003. γ-Radiation Decontamination of Alfalfa Seeds Naturally
577 Contaminated with *Salmonella Mbandaka.* J. Food Sci. 68 (5) 1777-1781.
578 Trząskowska, M., Dai, Y., Delaquis, P., Wang, S. 2018. Pathogen reduction on mung bean reduction
579 of *Escherichia coli* O157:H7, Salmonella enterica and Listeria monocytogenes on mung bean using
580 combined thermal and chemical treatments with acetic acid and hydrogen peroxide. Food Microbiol.
581 76, 62-68.
582 Viswanathan, P., Kaur, R., 2001, Prevalence and growth of pathogens on salad vegetables, fruits and
583 sprouts. Int. J. Hyg. Environ. Health 203, 205-213.
584 Warriner, K., Spaniolas, S., Dickinson, M., Wright, C., Waites, W.M., 2003. Internalization of
585 bioluminescent *Escherichia coli* and *Salmonella Montevideo* in growing bean sprouts. J. Appl.
586 Microbiol. 95, 719–727.

587 Weiss, A., Hertel, C., Grothe, S., Ha, D., Hammes, W.P., 2007. Characterization of the cultivable
588 microbiota of sprouts and their potential for application as protective cultures. Syst. Appl. Microbiol.
589 30, 483-493.

590 Xiao, Z, Nou, X., Luo, Y., Wang, Q., 2014. Comparison of the growth of *Escherichia coli* O157: H7
591 and O104: H4 during sprouting and microgreen production from contaminated radish seeds. Food
592 Microbiol. 44, 60-63.

593 Zheng, Q., Mikšs-Krajnik, M., D'Souza, C., Yang, Y., Heo, D.J., Kim, S.K., Lee, S.C., Yuk, H.G.,
594 2016. Evaluation of real-time PCR coupled with immunomagnetic separation or centrifugation for
595 the detectionof healthy and sanitizer-injured Salmonella spp. on mung bean sprouts. Int. J. Food
596 Microbiol. 222, 48-55.

597 Zheng, Q., Mikšs-Krajnik, M., D'Souza, C., Yang, Y., Heo, D.J., Kim, S.K., Lee, S.C., Yuk, H.G.,
598 2015. Growth of healthy and sanitizer-injured Salmonella cells on mung bean sprouts in different
599 commercial enrichment broths. Food Microbiol. 52, 159–168.

600

601
602
603
604
605 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
Escherichia coli**	< 10	< 10	< 10	< 10	< 10
****Salmonella* spp.**	Absence	Absence	Absence	Absence	Absence
****L.monocytogenes***	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 ($p < 0.05$).

609
610
611
612
613
614
615

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
Escherichia coli**	< 1	< 1	< 1	< 1	< 1
****Salmonella* spp.**	Absence	Absence	Absence	Absence	Absence
****L.monocytogenes***	Absence	Absence	Absence	Absence	Absence
***STEC**	Absence	Absence	Absence	Absence	Absence

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

Table 3: Coliform strains

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

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

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

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

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

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

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

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

Microorganisms	Before washing	After 1st washing	After 2nd washing	After 3rd washing	After 4th washing
***Salmonella* spp.**	9.1 ± 0.8b	7.7 ± 0.3a	5.4 ± 0.3a	2.1 ± 0.1b	1.8 ± 0.2b
L. monocytogenes	9.6 ± 0.3c	7.3 ± 0.4a	5.5 ± 0.3a	2.2 ± 0.2b	1.9 ± 0.2b
E. coli 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
***Salmonella* spp.**	3.1 ± 0.2	< 10	+
L. monocytogenes	3.6 ± 0.1	< 10	+
E. coli STEC	3.3 ± 0.2	< 10	+

Seeds treated in chlorinated water solution (100 ppm)
Time of soaking: 60 min