This document is a postprint version of an article published in International Journal of Food Microbioloby © Elsevier after peer review. To access the final edited and published work see https://doi.org/10.1016/j.ijfoodmicro.2019.108392

Document downloaded from:

1 **Occurrence of selected viral and bacterial pathogens and microbiological quality**

2 **of fresh and frozen strawberries sold in Spain**

3

4 Ortiz-Solà[1], J., Viñas[1*], I., Colás-Medà[2], P., Anguera[2], M., Abadias[2*], M.

5

6 1 Universitat de Lleida. Departamento de Ciencia y Tecnología de Alimentos. XaRTA-Postharvest. Centro

7 Agrotecnio. Rovira Roure 191, 25198 Lleida.

8 2 Institut de Recerca i Tecnologia Agroalimentàries (IRTA). XaRTA-Postharvest. Edifici Fruitcentre, Parc

9 Científic i Tecnològic Agroalimentari de Lleida, Parc de Gardeny, 25003 Lleida.

10

11 * Corresponding autor: M. Abadias (isabel.abadias@irta.cat) / I. Viñas (ivinas@tecal.udl.cat)

12

Abstract

Strawberry production and exports have been increasing in Spain in the last decades. However, little information is available about their microbiological quality. Due to the growing concern of these fruits over their microbial safety, the objective of this investigation was to study the microbiological quality and the prevalence of the main foodborne pathogens on strawberries sold in Spain. Fresh (n=152) and frozen (n=31) samples were obtained from marketplaces and fields during 2017 and 2018. The samples were assayed for total aerobic mesophilic microorganisms (TAM), moulds and yeasts (M&Y), total coliforms (TC), *Escherichia coli*, *Salmonella* spp., *Listeria monocytogenes* as well as Norovirus (NoV) GI and GII. The microbiological counts ranged from <1.70 (detection limit, dl) – 5.89 $\log_{10}$ CFU/g (mean 3.78 $\log_{10}$ CFU/g) for TAM; 2.10 – 5.86 $\log_{10}$ CFU/g (mean 3.80 log10 CFU/g) for M&Y; and <0.70 (dl) – 4.91 $\log_{10}$ CFU/g (mean 2.15 log10 CFU/g) for TC in fresh strawberries. In frozen strawberries, the counts were <1.70 (dl) – 3.66 $\log_{10}$ CFU/g (mean 2.30 $\log_{10}$ CFU/g) for TAM; <1.70 (dl) – 2.76 $\log_{10}$ CFU/g (mean 1.82 $\log_{10}$ CFU/g) for M&Y; and <0.70(dl) – 1.74 $\log_{10}$ CFU/g (mean 0.77 $\log_{10}$ CFU/g) for TC. All the samples tested in this study were negative for *Salmonella* spp., *L. monocytogenes. E. coli* and NoV GI and GII genome. A global overview of all data was executed using *Principal Component Analysis* (PCA), and the results showed that the scores and loadings according to principal components 1 (PC1) and 2 (PC2) accounted for 75.9 % of the total variance, allowing a distinction between fresh and frozen samples. The presence of moulds was significantly higher in the supermarket samples whereas the presence of total coliforms was significantly higher in the field samples ($p<0.05$). Although pathogenic microorganisms were not found, preventive measures and prerequisites in the strawberries production chain must be considered in order to avoid possible foodborne diseases related to the microbiological quality of the fruit.

Keywords: Incidence, Salmonella, L. monocytogenes, Norovirus, Real Time RT-PCR.

1. INTRODUCTION

Worldwide production of strawberries has practically doubled during the last 15 years, with a production of 9,223,815 tn in 2017. Spain is in the 6th place of the top-10 producers in the world, with 278,664 tn in 2017 (FAOSTAT, 2019). Exportation constitutes 83% of this production, mainly as fresh berries during the period February to May and the main market is in northern Europe, principally Germany, France and UK (Simpson, 2018). Fresh market strawberries are account for approximately 80% of total production, while the rest are intended for industrial processing purposes, such as the production of yogurts, jams, jellies dessert toppings, etc. (Šamec et al., 2016). In contrast, in other countries such as Morocco or Egypt, 76 % of the production is for frozen fruit processing (Dira, 2016). Despite this, Spain was also in the top-10 list of frozen strawberries exports in 2017 (5.2 % of total exported frozen strawberries, €43.5 million) (CBI, Ministry of Foreign Affairs, 2019).

Therefore, strawberries represent a significant weight in terms of production value in Spain; however, little information is available about their natural microbiological criteria. Berries of any kind are generally considered to be low-risk foods because of their naturally low pH (Knudsen et al., 2001). Nevertheless, Jensen et al. (2013) found that the microbiota present on healthy strawberries was complex including potential plant pathogens, opportunistic human pathogens, plant disease biocontrol agents and mycotoxin producing moulds. Additionally, the EFSA (EFSA Panel on Biological Hazards, 2013), ranked the combination of strawberries and other berries with Norovirus (NoV) as the sixth most common risk linked to foodborne cases in humans originating from food of non-animal origin (FoNAO) in the EU. The combination of raspberries with *Salmonella* spp. and NoV were also considered as a problem as it was ranked as the fourth most common risk associated to foodborne human cases originating from FoNAO. Consequently, the Panel on Biological Hazards of EFSA (BIOHAZ) was asked to write an opinion about the risk of *Salmonella* spp. and NoV in red fruits and determine if it is necessary to establish a food safety criterion for these products, which are not included in the current legislation (Regulation 2073/2005 and subsequent modifications).

64 According to the reported outbreaks connected to fresh produce, very little information can be
65 found on the prevalence of *Salmonella* spp., Shiga toxin-producing *Escherichia coli* (STEC) and
66 *Listeria monocytogenes* in strawberries, but everything seems to indicate that it was low
67 (Bohaychuk et al., 2009; Yoon et al., 2010; Delbeke et al., 2015; Johannessen et al., 2015). An
68 outbreak involving *E. coli* O157:H7 was reported in the United States in 2011 that caused 15
69 cases (2 deaths) and were related to the consumption of strawberries contaminated in the field by
70 wildlife contact with deer faeces (Laidler et al., 2013). Moreover, in an investigation executed by
71 the US Food and Drug Administration (FDA), *Salmonella* was noticed in 1 sample from a total
72 of 143 imported strawberry samples (FDA, 2001). Furthermore, *L. monocytogenes* serogroup 4
73 was isolated by enrichment from 1 out of 173 strawberry samples obtained from Norwegian retail
74 markets (Johannessen et al., 2002).

75 On the other hand, foodborne viruses, especially calciviruses (noroviruses), are increasingly
76 reported as the cause of foodborne outbreaks in soft red fruits (Baert et al., 2011). In 2012, there
77 was a large multistate outbreak of norovirus (NoV) gastroenteritis in Germany, which affected
78 nearly 11,000 people and was linked to frozen strawberries from China (Bernard et al., 2014).
79 Moreover, 2 outbreaks of Hepatitis A virus (HAV) have been linked to the consumption of frozen
80 strawberries and mixed berries in Europe during 2012-2014 and 2 outbreaks of NoV in frozen
81 raspberries (Tavoschi et al., 2015). In the US, there was also an outbreak of HAV linked to the
82 consumption of frozen strawberries in 2016 (FDA, 2016). Concerning the prevalence of
83 norovirus, there are some reports of their occurrence in frozen strawberries (Mäde et al., 2013)
84 and other berries (fresh and frozen) (Maunula et al., 2013; Cook et al., 2018).

85 Currently, there is little information concerning the microbial quality and safety of strawberries
86 grown in Spain. In addition, such a study has never been conducted in the country. Therefore, in
87 this study we evaluated freshly harvested and retail strawberries (including frozen strawberries)
88 from Spain for the presence of NoV genogroup I and II (GI and GII); human pathogenic bacteria,
89 including *Salmonella* spp. and *Listeria monocytogenes.*; and other microbial parameters,

90 including total aerobic mesophilic microorganisms, moulds and yeasts, total coliforms and

91 *Escherichia coli*.

92 ## 2. Materials and Methods

93 ### 2.1. *Sampling and samples preparation*

94 Different fresh samples of strawberry (n = 152) were bought in 18 largest national supermarkets
95 (n=88) and collected from fields (n=64) around Spain in two consecutive seasons (2017 and
96 2018). Most of the samples (89 %) came from Huelva province, corresponding to the highest
97 production area in Spain. A limited number of frozen strawberries (n=31, 17 % of total) were also
98 included in the study. Fresh strawberry samples were transferred to the laboratory in a controlled
99 temperature box, stored at 4 °C, and analysed within 24 h. Frozen strawberries were stored in
100 their original packaging at −20 ± 2 °C until further usage. Sample data contained in the label
101 (origin, weight, brand, variety and batch number) and reception date were documented. Variety
102 was not available from all samples. Strawberries were cut and four subsamples of 25 g were used:
103 25 g were placed into a sterile filtered blender bag of 400 mL (BagPage®, Interscience) with 225
104 g of peptone buffered water (PBW, Biokar Diagnostics, France) for the microbiological quality
105 and *Salmonella* spp. detection, another 25 g of sample was placed in other sterile blender bag with
106 225 g of Half Fraser broth (Biokar Diagnostics) for detection of *Listeria monocytogenes* and the
107 other two subsamples were stored at -20°C ± 2 °C for the NoV detection. To determine the
108 microbiological parameters of frozen strawberries, samples were defrosted at room temperature
109 for approximately 30 min and analysed as indicated above.

110 ### 2.2. *Quantification of total aerobic mesophilic microorganisms, moulds and yeasts,*
111 *coliform bacteria and* Escherichia coli.

112 The 25 g samples in sterile blender bags with BPW were homogenized in Masticator
113 Homogenizator (IUL S.A. Instruments, Barcelona). The homogenates were 1:10 diluted with
114 Peptone solution (PS: 0.1% peptone, 0.85% NaCl). For total aerobic mesophilic microorganisms
115 (TAM), 100 µL of dilutions were plated in duplicate PCA (Plate Count Agar, Biokar Diagnostics)
116 plates and incubated for (72±3) h at 30 ±1 ° C as indicated in ISO 4833-2:2013.
117 According to ISO 21527-1:2008, the same dilutions were plated in duplicate Dichloran Rose
118 Bengal Chloramphenicol Agar (DRBC, Biokar Diagnostics) plates used for selective isolation of

119 fungi –moulds and yeasts - of significance in food spoilage and incubated at 25 ± 1°C for 3 – 5
120 days.
121 To enumerate total coliform microorganisms (TC), 1 mL of dilution was transferred in duplicate
122 to sterile Petri dishes and 12-15 mL of VRBL (Violet Red Bile Lactose Agar, Biokar Diagnostics)
123 at 44 - 47 ºC were poured into each Petri dish and carefully mixed with the inoculum. The mixture
124 was allowed to solidify and afterwards, 4-6 mL of the VRBL medium were poured onto the
125 surface of the inoculated medium and allowed to solidify before incubation at 37 ± 1ºC for 24±2
126 h as defined in ISO 4832:2006. After 24h of incubation, characteristic violet colonies with a 0.5
127 mm diameter were counted (sometimes colonies were red covered).
128 For the quantification of *Escherichia coli*, 1 mL of the homogenate was plated in duplicate in the
129 selective chromogenic medium TBX (Tryptone Bile Glucuronic Agar, Biokar Diagnostics) and
130 plates were incubated at 44 ± 1 ºC for 18-24 h as indicated in ISO 16649-2:2001. After incubation
131 period, blue-green colonies were counted. No confirmation test was done for TC and *E. coli*.
132 After microbial counts, the bag containing the homogenates of strawberries in BPW were
133 incubated for 18 ± 2 h at 37 ± 1ºC (non-selective pre-enrichment) for *Salmonella* spp. detection.

134 **2.3.** ***Detection of*** **Salmonella** ***spp.***

135 For *Salmonella* spp. detection, the procedures indicated in ISO 6579:2003 were followed. Briefly,
136 100 µL of non-selective pre-enrichment BPW homogenate commented above was transferred to
137 10 mL Rappaport-Vassiliadis-Soya Peptone Broth (RVS; Biokar Diagnostics) tubes and
138 incubated at 41.5 °C for 24 ± 3 h for selective enrichment. In parallel, another 1 mL was added to
139 tubes with 10 mL of Broth dehydrated base medium with novobiocin (MKTTn*; Biokar
140 Diagnostics) (0,1% v/v) and incubated at 37 ºC for 24 ± 3 h. After selective enrichment, the
141 cultures obtained from RVS and MKTTn were streaked onto Xylose-Lysine-Desoxycholate Agar
142 (XLD; Biokar Diagnostics) and Hektoen Enteric agar (HK; Biokar Diagnostics). The plates were
143 incubated at 37 °C for 24 h and examined for typical colonies. The presence of *Salmonella* spp.
144 was confirmed by streaking typical colonies on Nutrient Agar 2% (NA; Biokar Diagnostics)
145 followed by biochemical confirmation using API 20E® (BioMérieux SA, France). One typical
146 colony was chosen per selective plate. If the first colony was negative, another four colonies were

147 selected and examined. A positive control of *Salmonella* spp. was done using the strain BAA 709
148 (*Salmonella enterica* subsp. *enterica* (Smith) Weldin serotype Michigan).

149 **2.4.** ***Detection of*** **Listeria monocytogenes**

150 The presence of *L. monocytogenes* was examined according to the procedures described in ISO
151 11290-2:1998. Firstly, 100 µL of selective culture enrichment (25 g sample plus 225 mL Half
152 Fraser medium) was transferred to tubes with 10 mL of Fraser broth with Fraser supplement (1:10
153 v/v) (Biokar Diagnostics) and incubated for 18 ± 2 h at 30 ± 1 ° C. In parallel, 100 µL of the
154 homogenate was spread on Palcam Agar (Biokar Diagnostics) and Compass *Listeria* Agar (Biokar
155 Diagnostics). Both the tubes with Fraser broth and the plates were incubated for 48 h at 37 °C.
156 Once this time had elapsed, 100 µL culture taken from the Fraser tubes was spread on Palcam and
157 Compass, repeating the same procedure as previously mentioned. Plates were counted, and the
158 possible suspected colonies of *L. monocytogenes* were isolated on TSAYE plates and incubated
159 at 37 °C for 24 h with the same procedure criteria explained above for detection of *Salmonella*.
160 The experiment was monitored with a positive control of *L. monocytogenes* strain CECT 4031.
161 For the confirmation of *L. monocytogenes*, one typical colony per plate and positive (CECT4031)
162 and negative control were subjected to biochemical tests: Gram staining and tested for the
163 production of catalase, beta-hemolysis, and fermentation of carbohydrates (xylose, mannitol, and
164 rhamnose). Finally, API LISTERIA® (BioMérieux SA, France) was performed.

165 **2.5.** ***Detection of Norovirus GI and GII***

166 NoV were analysed according to the procedure indicated in ISO 15216-2:2013 (Fig. 1), which
167 consisted of three main steps: virus extraction, RNA extraction and Real-Time RT-PCR. Fresh
168 samples from 2017 season (n=76) and frozen samples from 2017 and 2018 seasons (n=31) were
169 evaluated. All frozen samples were defrosted at room temperature for 30 min, approximately.

170 For the virus extraction, 25 ± 0.3 g of sample were weighted in a 100 mL sterile bag with 40 ± 1
171 mL of Tris (hydroxymethyl) glycine beef extract (TGBE; Biokar Diagnostics), 0.5 mL of
172 *Aspergillus* pectinase (Sigma P2611-50 mL, 3800 U/ mL) and 10 ± 0.5 µl of
173 Mengovirus Extraction Control kit (KMG, ceeramTools, Biomerieux, France) as control of virus

174 extraction process. The contents were incubated at room temperature with constant rocking (60
175 oscillations/min) with an orbital shaker (Unimax 1010, Heidolph Instruments, Germany) for 20
176 min. The pH of the eluate was monitored at 10 min intervals using pH indicator strips (pH-Fix
177 6.0-10.0; VWR chemicals, United States) and, if the pH fell below 9.0 it was adjusted to 9.5 ±
178 0.5 using a 10 M NaOH solution. The period of incubation was extended by 10 min every time
179 the pH was adjusted. The contents of the bag were decanted from the filtered compartment into a
180 50-mL sterile centrifuge tube (Corning) and centrifuged at 11,000 × *g* for 20 ± 2 min at 5 ± 3 °C.
181 The resulting supernatant was transferred to a new centrifuge tube. The pH was adjusted to 7 ±
182 0.5 using a 5 M HCl solution. Then, 7.5 mL of the solution 5×PEG/NaCl (500 g/PEG 8000, 1.5
183 mol/l NaCl) (0.25% v/v TGBE) were also added, homogenized by shaking for 60 ± 5 s and
184 incubated with constant rocking at 60 oscillations/min at 5 ± 3 °C for 60 ± 5 min. The samples
185 were centrifuged at 11,000 × *g* for 30 min at 5 ± 3 °C. Supernatant was removed and the pellet
186 was dried and suspended with 500 µl of Phosphate-buffered saline (PBS) at 56 ° C. The two
187 samples of 500 µl were combined (1000 µl) in a 2 mL Eppendorf tube. A volume of 1000 ± 10 µl
188 of chloroform – butanol (VWR chemicals, United States) was added and homogenized for 15-30
189 s with vortex. Samples were incubated for 15±1 min at room temperature and centrifuged at
190 13,500 × *g* for 15 ± 5 min at 5 ± 3 °C. Three layers were formed; the upper aqueous phase was
191 carefully transferred to a 2 mL Eppendorf tube to proceed with the subsequent extraction of the
192 RNA. Samples from this step were either processed immediately, stored at 5 ± 3 °C for a
193 maximum of 24 h or frozen at < -15 ºC for up to 6 months.

194 **2.6. *Commercial RNA extraction of BioMérieux NucliSENS®***

195 RNA extraction was performed using the NucliSENS® Kit (NucliSENS Lysis Buffer 2 mL and
196 NucliSENS Magnetic-Extraction Reagents) and NucliSENS® MiniMag® Nucleic Acid
197 Purification System (bioMérieux SA, France), which complies with ISO/TS 15216-1&2. Provider
198 instructions were followed. The assay contain internal control RNA for each batch of samples
199 from the beginning of the procedure. For the extraction of RNA, work was carried out in a Class
200 II flow cabinet. After RNA extraction, samples were stored at -80 °C until RT-qPCR was done.

201 **2.7.** ***One-Step Commercial RT-qPCR Kit of CeeramTools***

202 Commercial RT-qPCR kit for detection of Norovirus GI and GII (KNVGI and KNVGII,
203 ceeramTools, Biomerieux, France) were used. They assembled RT-PCR reactions with included
204 master mix, enzyme mix, internal, positive, and negative controls. Manufacturer instructions were
205 followed. Primers for qPCR present in this reaction mixture were in accordance with those defined
206 in the standard ISO, and the RT and qPCR step occurred in the same reaction well. Internal
207 amplification control, positive and negative controls, were comprised in the experimental
208 procedure according to the manufacturer's instructions with the objective of validate the whole
209 process. To determine the proper extraction and quantification, all samples were determined by
210 the presence of Mengovirus from the "Mengo@ceeramTools™ Kit" (CEERAM S.A.S, La
211 Chapelle Sur Erdre, France) with a concentration of 1.61×10^5 viral particles/µL, including
212 process control and the minor curve. The process was considered validated if extraction recovery
213 was higher than 1 %.

214 RT-qPCR was performed on a 7500 Real Time PCR System (Applied Biosystems), and
215 amplification data were collected and analysed using the SDS 7500 instruments software. For the
216 generation of standard curves, control plasmids containing primer–probe binding sites were used
217 in the case of GI and GII NoV detection. Interpretation and expression of results were done
218 according to the above-mentioned ISO.

219 **2.8.** ***Statistical Analysis***

220 The data obtained were processed in Microsoft® Excel software and adjusted to models of logistic
221 regression with program help JMP 13 software (SAS Institute Inc., Cary, USA). Results are
222 expressed by mean ± standard deviation (SD) for all the samples present in this study. The
223 detection limit (dl) was 1.70 $\log_{10}$ CFU/g for TAM and M&Y and 0.70 $\log_{10}$ CFU/g for total
224 coliforms and *E.coli*. For samples with microbial counts below dl, an arbitrary value of ½
225 detection limit was used for calculations. All data were checked for significant differences by
226 analysis of variance test (ANOVA). The criterion for statistical significance was $p < 0.05$. JMP
227 13 software was used to develop principal component analysis (PCA) biplot. The PCA was
228 performed to characterize the samples according to microbiological attributes by the presence in

229 $\log_{10}$ CFU/g of TAM, M&Y and TC, and taking into account the kind of strawberries (fresh or

230 frozen, their origin (supermarket or field), location and season.

3. Results and Discussion

3.1. *Microbiological quality of strawberries*

3.1.1. Total aerobic mesophilic (TAM) counts.

The initial TAM counts of fresh untreated strawberries (Fig. 2A) ranged from ≤ 1.70 (detection limit, dl) – 5.89 log_{10} CFU/g (mean 3.78 log_{10} CFU/g). In our study, 88.2% (134/152) of the samples analyzed had a TAM count < 5 log_{10} CFU/g and only 19.7% (30/152) of samples were < 2 log_{10} CFU/g. Abadias et al. (2008) found that 90.4% of their fresh-cut fruit samples had TAM counts inferior to 5 log_{10} CFU/g. The mean initial TAM count of our investigation was almost in the same range as that reported by Hassenberg et al. (2010) in fresh strawberries (4.27 log_{10} CFU/g). In other study conducted in 61 samples of fresh berries, TAM count ranged between 1.7 and 6.9 log_{10} CFU/g, with a mean value of 2.77 log_{10} CFU/g and 86 % of prevalence (65/75) (Macori et al., 2018). On the food chain production, fresh strawberries receive minimal processing to avoid being damaged and the consequent increased risk of spoilage. Currently, the EU regulation on microbial criteria for foodstuffs (EC 2073/2005 and subsequent modifications) does not include maximum levels of TAM in fresh and pre-cut fruit.

For frozen strawberries, TAM counts (Fig. 2B) ranged from <1.70 (dl) - 3.66 log_{10} CFU/g (mean 2.30 log_{10} CFU/g). TAM population was above the detection limit in 26 of 31 frozen samples (83.9 % prevalence). To our knowledge, there are no similar studies concerning the microbial quality of frozen strawberries, except those concerning incidence of viruses. According to Rivas-Pala et al. (1984), counts of mesophilic aerobes should not exceed 5.70 log_{10} CFU/g (5×10^5 CFU/g) of mesophilic aerobes and 2.48 log_{10} CFU/g (3×10^2 CFU/g) of total coliforms in frozen fruits and vegetables. Therefore, all samples analyzed are acceptable as they fulfil the recommended specifications for frozen foods and vegetables.

Jensen et al. (2013) characterized the microbiota of strawberries from organic and conventional farming in Denmark and found that bacteria made up the largest proportion of the total microbiota, followed by yeasts.

3.1.2. Moulds and yeasts (M&Y).

258 In fresh strawberries, fungi counts were similar to those obtained in TAM (Fig. 2A). The results
259 reported fungal populations ranging between 2.10 and 5.86 log_{10} CFU/g (mean 3.80 log_{10} CFU/g).
260 The range in which this microbial group was found in other studies with fresh-cut strawberry was
261 2.00 – 7.10 log_{10} CFU/g (Abadias et al. 2008). Graça et al. (2017) found that strawberries,
262 pineapples and mango presented the highest mean fungal counts (5.20, 5.10 and 4.70 log_{10} CFU/g,
263 respectively). Furthermore, Tournas et al. (2006) detected in fresh-cut strawberries 4.36 log_{10}
264 CFU/g counts of M&Y with *Cladosporium spp*. in 100 % occurrence of all samples. The low pH
265 of berries decreases the viability of bacterial species, making it easier for moulds and yeasts to
266 grow on and spoil fruits (Brackett, 1987). The mean average of the total yeast counts (3.04 log_{10}
267 CFU/g) were significantly lower than the mean average of the total mould counts (3.37 log_{10}
268 CFU/g) ($p<0.05$). The normal population of yeast on fresh and undamaged fruits is generally low
269 (less than 3.00 log_{10} CFU/g) (Tournas et al., 2005). It has been seen that the principal fungi present
270 in fresh strawberries were the moulds *Botrytis cinerea*, *Rhizopus* spp., *Penicillium* spp.,
271 *Alternaria* spp., *Cladosporium* spp., *Aureobasidium pullulans* and the yeast *Cryptococcus* spp.
272 (Tournas et al., 2005).

273 In frozen strawberries, M&Y counts were between 1.70 (dl) – 2.76 log_{10} CFU/g (mean 1.82 log_{10}
274 CFU/g), and 83.9 % of samples (26/31) had M&Y counts below detection limit (Fig. 2B).
275 Microbial populations on frozen strawberries decreased due to the cell damage that occurred
276 during freezing, probably due to the formation of intracellular ice. Slow freezing involves the
277 apparition of large ice crystals and is beneficial from a microbiological standpoint killing more
278 microorganisms (Jeremiah, 1996).

279 3.1.3. ***Total Coliform Counts and* E. coli**

280 The results showed large variations of total coliforms (TC) numbers depending of different
281 featured samples (Fig. 2A). The mean of TC in fresh strawberries were 2.15 log_{10} CFU/g with a
282 range of <0.70 (dl) – 4.91 log_{10} CFU/g. However, the TC population does not exceed 2 log_{10}
283 CFU/g in 74/152 fresh samples (48.7%). Roth et al. (2018) found lower TC levels for berries,
284 with a mean of 0.52 log_{10} CFU/g. Conversely, they found significantly higher levels of total

285 coliforms on spinach and leafy greens with similar values to this study (1.60 – 2.30 log_{10} CFU/g).
286 Yoon et al. (2011) found that the interval of coliforms found in the leaves of the strawberries was
287 1.20–3.20 log_{10} CFU/leaf. It was established that specific types of produce, like berries, sprouts
288 and leafy greens, are more at risk of infection and constituted an important source of pathogens
289 in the documented outbreaks (Berger et al., 2010; Doyle & Erickson, 2008; EFSA, 2013; 2014).

290 In frozen strawberries, the population of TC was below the detection limit in 14 of 31 samples
291 (45.2 %) with a range between <0.70(dl) – 1.74 log_{10} CFU/g (mean 0.77 log_{10} CFU/g). According
292 to Rivas-Pala et al. (1896), the interval of TC to consider frozen fruit or vegetable safe was
293 between 2.00 - 2.48 log_{10}.

294 *E. coli* was not detected in any of the fresh and frozen strawberry samples analyzed (n = 186). On
295 microbiological criteria defined in EC Regulation 2073/2005 (EC Regulation, 2005), *E. coli* is
296 controlled only in pre-cut fruit and vegetable or in fresh juices. If fresh and frozen strawberries
297 were subject to the same legislative control as pre-cut fruits and vegetables and fruit juices, all
298 strawberry samples from this study would meet the criteria defined by the regulation. Similarly,
299 no *E. coli* was detected in strawberries from USA farmers' fields market, including both organic
300 and conventional farms (Mukherjee et al., 2004). In another study that surveyed more than 2,000
301 produce samples from 63 farms in USA, 1% of berries (2 out 194 samples) were positive for
302 generic *E coli* (Mukherjee et al., 2006). In Europe, Delbeke et al. (2015) found generic *E. coli* on
303 only 2 of 72 (2.8 %) of strawberry samples from primary production in Belgium, at concentrations
304 of 1.0 log_{10} CFU/g and 3.0 Log_{10} CFU/g and Dziedzinska et al. (2018) found 9.0 % (14 of 156)
305 of strawberry field samples contaminated with *E. coli* in the Czech Republic, and 1.4% from
306 marketplaces (1 of 70).

307 ***3.1.4. Principal Component analysis***

308 A global overview of all data was done according to the level of processing (fresh or frozen),
309 origin (field or supermarket), location, variety and harvest season. These results can be seen in
310 Fig. 3 which depicts the scores and loadings according to principal components 1 (PC1) and 2
311 (PC2). These components accounted for 58.7 and 17.2% of the total variance, respectively. As is

312 evident, a pattern with two different groups can be observed: fresh strawberries tend to be located
313 at higher values of PC1 and PC2 while frozen strawberries tend to show lower scores on PC1 and
314 PC2. Thus, as seen before, fresh strawberries were associated with higher values of all the
315 microbial analyzed whereas the freezing conditions of frozen strawberries negatively affect the
316 presence of microorganisms. No different groups were observed according to variety and harvest
317 season.

318 Even though there were some mixed samples, another pattern with two different groups can be
319 observed between samples obtained from primary production (field) and those from retail (Fig.
320 3). In general, the tendency of TAM and the mould counts in supermarket samples were higher
321 than in field samples, but only the mould population showed significant differences ($p < 0.05$)
322 respect to the origin of the strawberries. The packaging and storage are also relevant steps and it
323 is probable that they can provide conditions for contamination and growth of microorganisms in
324 fruits and vegetables (FDA, 2008). Lehto et al. (2011) detected high values of total aerobic
325 microorganisms (including moulds and yeasts) in the atmosphere of the storage areas, processing
326 and packaging of fruit and vegetable processing plants. In fact, the packaging material surfaces,
327 scales and floor cleaning equipment presented the highest mould counts. In Spain, it is a common
328 practice to pick up strawberries directly into the commercial packaging plastic container or
329 wooden box, in order to prevent too much handling of fruits, which increases damage and
330 spoilage.

331 On the other hand, it was seen that the prevalence of total coliforms was statistically higher ($P <$
332 0.05) in field samples than those from a supermarket. Results reported by Roth et al. (2018)
333 showed that total coliforms were more prevalent and at higher levels on farmers' market-collected
334 produce (50.8 %) than supermarket-collected samples (34%). Delbeke et al. (2015) concluded
335 that the field being a potential vehicle that connects the contaminated irrigation water with the
336 fruit.

337 **3.2.** ***Foodborne Pathogens***

338 ***3.2.1. Foodborne Pathogenic Bacteria***

339 Regarding the food safety microorganisms determined, neither *Salmonella* spp. nor
340 *L. monocytogenes* were detected in any of the tested samples. This was consistent with other
341 published studies on fresh and fresh-cut produce (Johnston et al., 2006; Abadias et al., 2008;
342 Santos et al., 2012; Johannessen et al., 2015; Denis et al., 2016; Macori et al., 2018). In other
343 studies, pathogens were not found on strawberries grown in fields or produced in supermarkets
344 of the United States (Mukherjee et al., 2006) and Europe (Delbeke et al., 2015; Graça et al., 2017).
345 Similarly, Macori et al. (2018) did not find *L. monocytogenes* in 75 berry batches from 50 different
346 producers. On the contrary, Dziedzinska et al. (2018) found one sample (0.6 %) of fresh
347 strawberry from the Czech Republic contaminated by *L. monocytogenes*, the producer's field
348 having a contamination level lower than 100 CFU/g. Other investigations corroborated the small
349 incidence of the pathogens: Hadjilouka et al. (2014) reported presence of *L. monocytogenes* in
350 3.8% and Ceuppens et al. (2015) found a prevalence of 2.9% in strawberry fruit.

351 ***3.2.2. Norovirus GI and GII***

352 In our study, NoV GI and GII genomes were not detected in both fresh and frozen samples
353 (n=108). The positive controls made in each phase of multistep virus and RNA extraction were
354 detected satisfactorily.

355 For fresh strawberries, the results of our study were comparable with data reported in previous
356 investigations. In Italy, Terio et al. (2017) showed no presence of NoV in 911 fresh strawberry
357 samples studied. Moreover, Li et al. (2018) reported that the low incidence of NoV was 0.24 %
358 for 2,015 fresh berry samples (including strawberries) collected between 2009 and 2016 from
359 different countries including Germany, Bulgaria, France, Poland, Switzerland, Czech Republic,
360 USA, Spain, Russia, and Turkey. Dziedzinska et al. 2018 analyzed the presence of NoV in
361 strawberries and only found two contaminated strawberry field samples (1.3%) and one
362 contaminated sample of NoV in a fresh strawberry purchased from a supermarket in the Czech
363 Republic (1.4%). In case of other fresh berries, Maunula et al. (2013) analyzed 60 samples of
364 fresh raspberries at point of sale in four European countries and no NoV-positive samples were
365 identified, as in this study.

366 Recently, Chatziprodromidou et al. (2018) has reviewed the viral outbreaks linked to fresh and
367 frozen produce and reported that most NoV outbreaks (87.2 %) were produced by frozen food
368 stuffs, and the greatest common produce suspected for viral outbreaks were frozen raspberries
369 (23.7%) and frozen berries (19.1%). Mäde et al. (2013) analyzed 11 samples of frozen
370 strawberries implicated in a Germany outbreak and found 7 positive samples of NoV (63.6%).
371 The incidence of NoV in strawberry matrix can be explained because viral contamination can
372 occur in all parts of food chain (Dziedzinska et al., 2018). Since viruses are not able to replicate
373 extracellularly, their presence can only be due to contaminated irrigation water during pre-harvest
374 and the handling of a contaminated person during (post)harvest. The measures applied by
375 industries used to prevent growth or eliminate bacteria, are not necessarily useful for foodborne
376 viruses. Some measures taken to control bacteria preserve viral particles, as is the case for
377 refrigeration/freezing (Stals et al., 2011). For this reason, in the last 5 years (2015-2018) there
378 have been 39 notifications (contamination incidents) concerning foodborne pathogens in berries
379 in the RASFF (*Rapid Alert System for Food and Feed*) portal, 10 of them related to strawberries
380 (9 frozen and 1 fresh samples), 8 of them concerning norovirus and 2 hepatitis A virus. One
381 contamination incident was related to NoV contaminated strawberries from Spain.

382 ## 4. Conclusions

383 This is the first study conducted in the country about microbial quality and safety of fresh and
384 frozen strawberries sold in Spain. All the samples tested were negative for the RNA of targeted
385 NoV GI and GII and foodborne pathogens such as *Salmonella* spp. and *Listeria monocytogenes*
386 resulted negative too, indicating that the strawberries sampled in the current study was
387 microbiologically safe. Even though studies reveal low incidence of pathogenic microorganisms,
388 several incidents related to this kind of products have occurred in the last few years. Nonetheless,
389 not only pathogenic bacteria are a concern in fresh and frozen strawberries. Other
390 microorganisms, including mesophylls, moulds and yeasts, and total coliforms, are also key for
391 the quality of these products. Their presence and growth may cause a decrease in the shelf-life,
392 leading to huge economic losses in fruit industry. Accordingly, more consideration should be
393 given to microbial quality of strawberries, which should be studied and assessed properly.

394

395 **Acknowledgements**

396 The authors are grateful to the Spanish Government (Ministerio de Economía y Competitividad,
397 research project AGL2016-78086-R) and the CERCA Programme of 'Generalitat de Catalunya'
398 for its financial support. J. Ortiz-Solà thanks the University of Lleida for its PhD grant (UdL 2018)
399 and Dr. Valero (Department of Bromatology and Food Technology, University of Cordoba) for
400 hosting at his laboratory for field samplings. The authors also appreciate the unconditional help
401 of Dr. A. Bosch and Dr. S. Guix (Molecular Biology of Enteric Viruses, Microbiology
402 Department, Universitat de Barcelona) for training us in the detection of norovirus and Dr. G.
403 Echeverria for her collaboration in the principal component analyses. Finally, we thank the
404 producers and industries for providing the samples.

405 **Conflict of interest**

406 The authors declare no conflict of interest.

407 ## References

408 Abadias, M., Usall, J., Anguera, M., Solsona, C., & Viñas, I. 2008. Microbiological quality of
409 fresh, minimally-processed fruit and vegetables, and sprouts from retail establishments.
410 *International Journal of Food Microbiology*, *123*(1–2), 121–129.
411 https://doi.org/10.1016/j.ijfoodmicro.2007.12.013

412 Baert, L., Mattison, K., Loisy-Hamon, F., Harlow, J., Martyres, A., Lebeau, B., & Uyttendaele,
413 M. 2011. Review: Norovirus prevalence in Belgian, Canadian and French fresh produce: A
414 threat to human health? *International Journal of Food Microbiology*, *151*(3), 261–269.
415 https://doi.org/10.1016/j.ijfoodmicro.2011.09.013

416 Berger, C. N., Sodha, S. V., Shaw, R. K., Griffin, P. M., Pink, D., Hand, P., & Frankel, G. 2010.
417 Fresh fruit and vegetables as vehicles for the transmission of human pathogens.
418 *Environmental Microbiology*, *12*(9), 2385–2397. https://doi.org/10.1111/j.1462-
419 2920.2010.02297.x

420 Bernard, H., Faber, M., Wilking, H., Haller, S., Höhle, M., Schielke, A., Ducomble, T., Siffczyk,
421 C., Merbecks, S., Fricke, G., Hamouda, O., Stark, K., & Werber, D. 2014. Large multistate
422 outbreak of norovirus gastroenteritis associated with frozen strawberries, Germany, 2012.
423 *Eurosurveillance*, *19*(8). https://doi.org/10.2807/1560-7917.ES2014.19.8.20719

424 Bohaychuk, V. M., Bradbury, R. W., Dimock, R., Fehr, M., Gensler, G. E., King, R. K., Rieve,
425 R., & Barrios, P. R. 2009. A microbiological survey of selected Alberta-grown fresh produce
426 from farmers' markets in Alberta, Canada. *Journal of Food Protection*, *72*(2), 415–420.
427 https://doi.org/10.4315/0362-028X-72.2.415

428 Brackett, R.E. 1987. Microbiological consequences of minimally processed fruits and vegetables.
429 J Food Quality 10, 195–206. https://doi.org/10.1111/j.1745-4557.1987.tb00858.x

430 CBI, Ministry of Foreign Affairs. 2019. Available at: https://www.cbi.eu/node/2764/pdf/.
431 Accession date 13/03/2019.

432 Ceuppens, S., Johannessen, G.S., Allende, A., Tondo, E.C., El-Tahan, F., Sampers, I., Jacxsens,
433 L., & Uyttendaele, M. 2015. Risk factors for *Salmonella*, shiga toxin-producing *Escherichia*
434 *coli* and *Campylobacter* occurrence in primary production of leafy greens and strawberries.
435 Int. J. Environ. Res. Public Health 12, 9809–9831. https://doi.org/10.3390/ijerph120809809

436 Chatziprodromidou, I. P., Bellou, M., Vantarakis, G., & Vantarakis, A. 2018. Viral outbreaks
437 linked to fresh produce consumption: a systematic review. *Journal of Applied Microbiology*,
438 *124*(4), 932–942. https://doi.org/10.1111/jam.13747

439 Cook, N., Williams, L., & D'Agostino, M. 2018. Prevalence of norovirus in produce sold at retail

in the United Kingdom. *Food Microbiology*, *79*, 85–89. https://doi.org/10.1016/j.fm.2018.12.003.

Delbeke, S., Ceuppens, S., Hessel, C. T., Castro, I., Jacxsens, L., De Zutter, L., & Uyttendaele, M. 2015. Microbial safety and sanitary quality of strawberry primary production in Belgium: Risk factors for *Salmonella* and shiga toxin-producing *Escherichia coli* contamination. *Applied and Environmental Microbiology*, *81*(7), 2562–2570. https://doi.org/10.1128/AEM.03930-14.

Denis, N., Zhang, H., Leroux, A., Trudel, R., & Bietlot, H. 2016. Prevalence and trends of bacterial contamination in fresh fruits and vegetables sold at retail in Canada. *Food Control*, *67*, 225–234. https://doi.org/10.1016/j.foodcont.2016.02.047.

Dira. 2019. Frozen strawberries from Morocco: Historic data and outlook 2016. Available at: https://www.dirafrost.com/en/news-2/detail/frozen-strawberries-from-morocco-historic-data-outlook-2016. Accession date: 13/03/2019.

Doyle, M. P., & Erickson, M. C. 2008. Summer meeting 2007 - The problems with fresh produce: An overview. *Journal of Applied Microbiology*, *105*(2), 317–330. https://doi.org/10.1111/j.1365-2672.2008.03746.x.

Dziedzinska, R., Vasickova, P., Hrdy, J., Slany, M., Babak, V., & Moravkova, M. 2018. Foodborne bacterial, viral, and protozoan pathogens in field and market strawberries and environment of strawberry farms. *Journal of Food Science*, *83*(12), 3069–3075. https://doi.org/10.1111/1750-3841.14401.

EFSA, Panel on Biological Hazards, 2013. Scientific Opinion on the risk posed by pathogens in food of non-animal origin. Part 1 (outbreak data analysis and risk ranking of food/pathogen combinations). https://doi.org/10.2903/j.efsa.2013.3025.

EFSA, Panel on Biological Hazards, 2014. Scientific Opinion on the risk posed by pathogens in food of non-animal origin. Part 2 (*Salmonella* and Norovirus in berries). EFSA J. 12, 3706. https://doi.org/10.2903/j.efsa.2014.3706.

European Commission, Regulation [EC] 2073/2005: microbiological criteria for foodstuffs, november 2005. Available at: https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32005R2073&from=ES. Accession date 10/03/2019.

Food And Agriculture Organitzation of the United Nations [FAOSTAT]. 2019. http://www.fao.org/faostat/en/#data/QC. Accession date: 06/03/2019.

Food and Drug Administration [FDA]. 2001. Survey of imported fresh produce FY 1999 field assignment. https://wayback.archive-

473 it.org/7993/20170404223238/https://www.fda.gov/Food/GuidanceRegulation/GuidanceDo
474 cumentsRegulatoryInformation/ProducePlantProducts/ucm118891.htm. Accession date
475 11/03/2019.

476 Food and Drug Administration [FDA]. 2008. Guidance for industry: Guide to minimize microbial
477 food safety hazards of fresh-cut fruits and vegetables. Electronic publication.
478 http://www.fda.gov/food/ guidancecomplianceregulatoryinformation/guidancedocuments/
479 produceandplanproducts/ucm064458.htm. Accession date 15/07/2019.

480 Food and Drug Administration [FDA]. 2016. Multistate outbreak of hepatitis A linked to frozen
481 strawberries (Final Update). https://www.cdc.gov/hepatitis/outbreaks/2016/hav-
482 strawberries.htm. Accession date 11/03/2019.

483 Graça, A., Esteves, E., Nunes, C., Abadias, M., & Quintas, C. 2017. Microbiological quality and
484 safety of minimally processed fruits in the marketplace of southern Portugal. *Food Control*,
485 *73*, 775–783. https://doi.org/10.1016/j.foodcont.2016.09.046.

486 Hadjilouka, A., Paramithiotis, & S., Drosinos, E.H. 2014. Prevalence of *Listeria monocytogenes*
487 and occurrence of listeriosis from ready-to-eat fresh fruits and vegetables. *Listeria*
488 *monocytogenes*: Food Sources, prevalence and management strategies. Innova. Science
489 Publishers, Inc., pp. 283–296.

490 Hassenberg, K., Geyer, M., & Herppich, W., 2010. Effect of acetic acid vapour on the natural
491 microflora and *Botrytis cinerea* of strawberries. Eur. J. Hortic. Sci. 75, 141–146.

492 Jensen, B., Knudsen, I. M. B., Andersen, B., Nielsen, K. F., Thrane, U., Jensen, D. F., & Larsen,
493 J. 2013. Characterization of microbial communities and fungal metabolites on field grown
494 strawberries from organic and conventional production. *International Journal of Food*
495 *Microbiology*, *160*(3), 313–322. https://doi.org/10.1016/j.ijfoodmicro.2012.11.005.

496 Jeremiah, L. E. 1996. Freezing effects on food quality. CRC Press, published february 6, 1996.
497 432 Pag. ISBN 9780824793500.

498 Johannessen, G. S., Loncarevic, S., & Kruse, H. 2002. Bacteriological analysis of fresh produce
499 in Norway. *International Journal of Food Microbiology*, *77*(3), 199–204.
500 https://doi.org/10.1016/S0168-1605(02)00051-X.

501 Johannessen, G. S., Eckner, K. F., Heiberg, N., Monshaugen, M., Begum, M., Økland, M., &
502 Høgåsen, H. R. 2015. Occurrence of *Escherichia coli, Campylobcter, Salmonella* and shiga-
503 toxin producing *E. coli* in norwegian primary strawberry production. *International Journal*
504 *of Environmental Research and Public Health*, *12*(6), 6919–6932.
505 https://doi.org/10.3390/ijerph120606919.

506 Johnston, L. M., Jaykus, L. A., Moll, D., Anciso, J., Mora, B., & Moe, C. L. 2006. A field study
507 of the microbiological quality of fresh produce of domestic and Mexican origin.
508 *International Journal of Food Microbiology*, *112*(2), 83–95.
509 https://doi.org/10.1016/j.ijfoodmicro.2006.05.002.

510 Jongen, W. 2005. Improving the safety of fresh fruit and vegetables. *Woodhead Publishing,* 656.
511 ISBN: 9781845690243. https://doi.org/10.1533/9781845690243.

512 Knudsen, D. M., Yamamoto, S. A., & Harris, L. J. 2001. Survival of *Salmonella* spp. and
513 *Escherichia coli* O157:H7 on fresh and frozen strawberries. *Journal of Food Protection*,
514 *64*(10), 1483–1488. https://doi.org/10.4315/0362-028X-64.10.1483.

515 Krimm, U., Abanda-Nkpwatt, D., Schwab, W., & Schreiber, L. 2005. Epiphytic microorganisms
516 on strawberry plants *(Fragaria ananassa* cv. Elsanta): Identification of bacterial isolates and
517 analysis of their interaction with leaf surfaces. *FEMS Microbiology Ecology*, *53*(3), 483–
518 492. https://doi.org/10.1016/j.femsec.2005.02.004.

519 Tavoschi, L., Severi, L., Niskanen, T., Boelaert, F., Rizzi, V., Liebana, E., Gomes Dias, J.,
520 Nichols, G., Takkinen, G., & Coulombier, D. 2015. Food-borne diseases associated with
521 frozen berries consumption: a historical perspective, European Union, 1983 to 2013.
522 *Eurosurveillance,* 20 (29), 1-11. https://doi.org/10.2807/1560-7917.ES2015.20.29.21193.

523 Lehto, M., Kuisma, R., Määttä, J., Kymäläinen, H. R., & Mäki, M. 2011. Hygienic level and
524 surface contamination in fresh-cut vegetable production plants. *Food Control*, *22*(3–4),
525 469–475. https://doi.org/10.1016/j.foodcont.2010.09.029.

526 Laidler, M. R., Tourdjman, M., Buser., G. L., Hostetler, T., Repp, K., Leman, R., Samadpour, M.,
527 & Keene, W. E. 2013. *Escherichia coli* O157:H7 infections associated with consumption of
528 locally grown strawberries contaminated by deer. *Clinical Infectious Diseases*, *57*(8), 1129–
529 1134. https://doi.org/10.1093/cid/cit468.

530 Li, D., Butot, S., Zuber, S., & Uyttendaele, M. 2018. Monitoring of foodborne viruses in berries
531 and considerations on the use of RT-PCR methods in surveillance. *Food Control*, *89*, 235–
532 240. https://doi.org/10.1016/j.foodcont.2018.02.024.

533 Macori, G., Gilardi, G., Bellio, A., Bianchi, D., Gallina, S., Vitale, N., & Decastelli, L. 2018.
534 Microbiological parameters in the primary production of berries: a pilot study. *Foods*, *7*(7),
535 105. https://doi.org/10.3390/foods7070105.

536 Mäde, D., Trübner, K., Neubert, E., Höhne, M., & Johne, R. 2013. Detection and typing of
537 norovirus from frozen strawberries involved in a large-scale gastroenteritis outbreak in
538 Germany. *Food and Environmental Virology*, *5*(3), 162–168.

539 https://doi.org/10.1007/s12560-013-9118-0.

540 Maunula, L., Kaupke, A., Vasickova, P., Söderberg, K., Kozyra, I., Lazic, S., & Cook, N. 2013.
541 Tracing enteric viruses in the European berry fruit supply chain. *International Journal of*
542 *Food Microbiology*, *167*(2), 177–185. https://doi.org/10.1016/j.ijfoodmicro.2013.09.003.

543 Mukherjee, A. 2006. Longitudinal microbiological survey of fresh produce grown by farmers in
544 the upper midwest. *Journal of Food Protection*, *69*(8), 1928. https://doi.org/10.4315/0362-
545 028X-69.8.1928.

546 Mukherjee, A., Speh, D., & Dyck, E. 2004. Preharvest evaluation of coliforms, *Escherichia coli*,
547 *Salmonella*, and *Escherichia coli* O157 : H7 in organic food. *Journal of Food Protection*,
548 *67*(5), 894–900.

549 Pan, F., Li, X., Carabez, J., Ragosta, G., Fernandez, K. L., Wang, E., & Atwill, E. R. 2015. Cross-
550 sectional survey of indicator and pathogenic bacteria on vegetables sold from Asian vendors
551 at farmers' markets in Northern California. J*ournal of Food Protection*, 78(3), 602–608.
552 https://doi.org/10.4315/0362-028X.JFP-14-095.

553 Rivas-Pala, T., Yangüela, J., Garcia, M.J., Ucar., A. & Herrera, A., 1984. Microbiological quality
554 of frozen beans (*Vicia faba*) and peas (*Pisum sativum*) obtained of supermarket.
555 *Alimentaria: Revista de Tecnología e Higiene de los Alimentos, 149*, 39-50.

556 Roth, L., Simonne, A., House, L., & Ahn, S. 2018. Microbiological analysis of fresh produce sold
557 at Florida farmers' markets. *Food Control*, *92*, 444–449.
558 https://doi.org/10.1016/j.foodcont.2018.05.030.

559 Šamec, D., Maretić, M., Lugarić, I., Mešić, A., Salopek-Sondi, B., & Duralija, B. 2016.
560 Assessment of the differences in the physical, chemical and phytochemical properties of
561 four strawberry cultivars using principal component analysis. *Food Chemistry*, *194*, 828–
562 834. https://doi.org/10.1016/j.foodchem.2015.08.095.

563 Santos, M. I., Cavaco, A., Gouveia, J., Novais, M. R., Nogueira, P. J., Pedroso, L., & Ferreira, M.
564 A. S. S. 2012. Evaluation of minimally processed salads commercialized in Portugal. *Food*
565 *Control*, *23*(1), 275–281. https://doi.org/10.1016/j.foodcont.2011.06.022.

566 Sarvikivi, E., Roivainen, M., Maunula, L., Niskanen, T., Korhonen, T., Lappalainen, M., & Kuusi,
567 M. 2012. Multiple norovirus outbreaks linked to imported frozen raspberries. *Epidemiology*
568 *and Infection*, *140*(2), 260–267. https://doi.org/10.1017/S0950268811000379.

569 Simpson, D. 2018. The Genomes of Rosaceous berries and their wild relatives. The economic
570 importance of strawberry crops. Springer, 1-7. https://doi.org/10.1007/978-3-319-76020-9

571 Stals, A., Baert, L., Van Coillie, E., & Uyttendaele, M. 2011. Evaluation of a norovirus detection
572 methodology for soft red fruits. *Food Microbiology*, *28*(1), 52–58.
573 https://doi.org/10.1016/j.fm.2010.08.004

574 Terio, V., Bottaro, M., Pavoni, E., Losio, M. N., Serraino, A., Giacometti, F., & Tantillo, G. 2017.
575 Occurrence of hepatitis A and E and norovirus GI and GII in ready-to-eat vegetables in Italy.
576 *International Journal of Food Microbiology*, *249*, 61–65.
577 https://doi.org/10.1016/j.ijfoodmicro.2017.03.008.

578 Tournas, V. H. & Katsoudas, E. 2005. Mould and yeast flora in fresh berries, grapes and citrus
579 fruits. *International Journal of Food Microbiology, 105*, 11-17.
580 https://doi.org/10.1016/j.ijfoodmicro.2005.05.002

581 Tournas, V. H., Heeres, J., & Burgess, L. 2006. Moulds and yeasts in fruit salads and fruit juices.
582 *Food Microbiology*, *23*(7), 684–688. https://doi.org/10.1016/j.fm.2006.01.003.

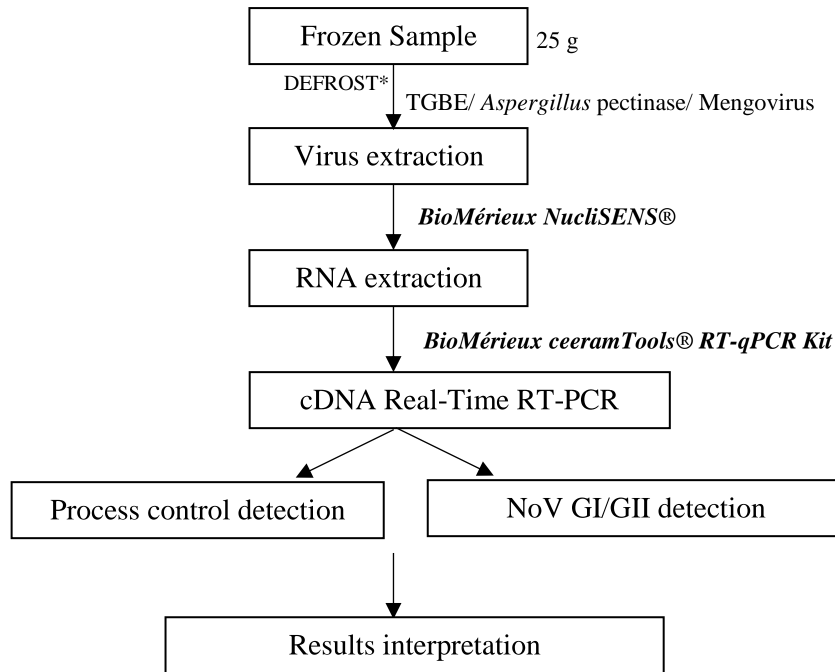
583 Yoon, Y., Geornaras, I., Scanga, J. A., Belk, K. E., Smith, G. C., Kendall, P. A., & Sofos, J. N.
584 2011. Probabilistic models for the prediction of target growth interfaces of *Listeria*
585 *monocytogenes* on ham and turkey breast products. *Journal of Food Science*, *76*(6), 450–
586 455. https://doi.org/10.1111/j.1750-3841.2011.02273.x.

587 Yoon, Y., Kim, K., Nam, M., Shim, W.-B., Ryu, J.-G., Kim, D.-H., & Chung, D.H. 2010.
588 Microbiological assessment in strawberry production and recommendations to establish a
589 good agricultural practice system. *Foodborne Pathogens and Disease*, *7*(12), 1511–1519.
590 https://doi.org/10.1089/fpd.2010.0611.

591

592

Figure 1. Schematic procedure for NoV detection according to ISO 15216-2:2013. Reverse transcription (RT) control consisting of MNV-1 RNA for process and control and NoV GI/GII detection.

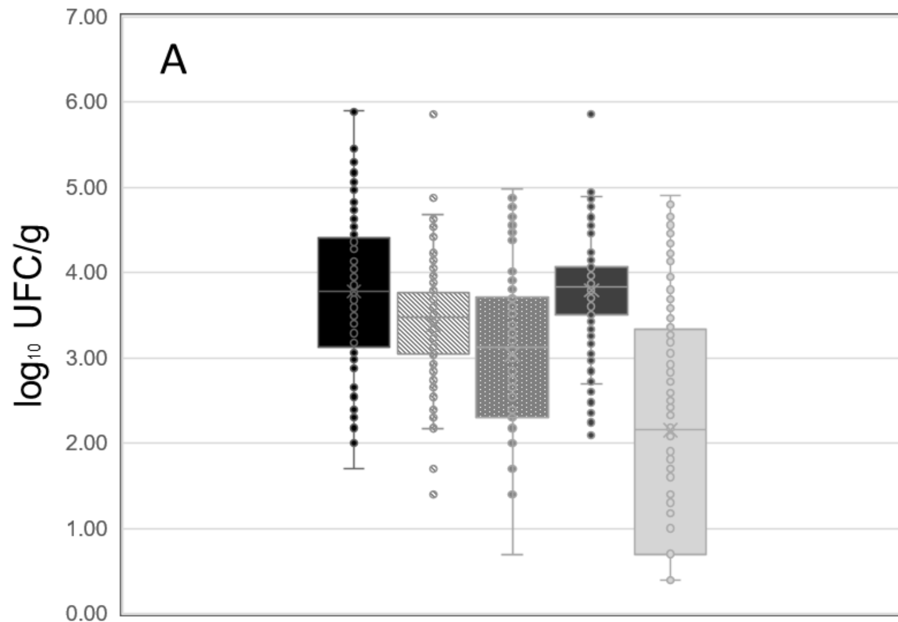


608 **Figure 2**. Microbiological quality (log_{10} CFU/g) of fresh (A) and frozen (B) strawberries. TAM

609 (■) = Total aerobic mesophilic count; M (▧) = Moulds; Y (▩) = Yeasts; M&Y (■) = Moulds

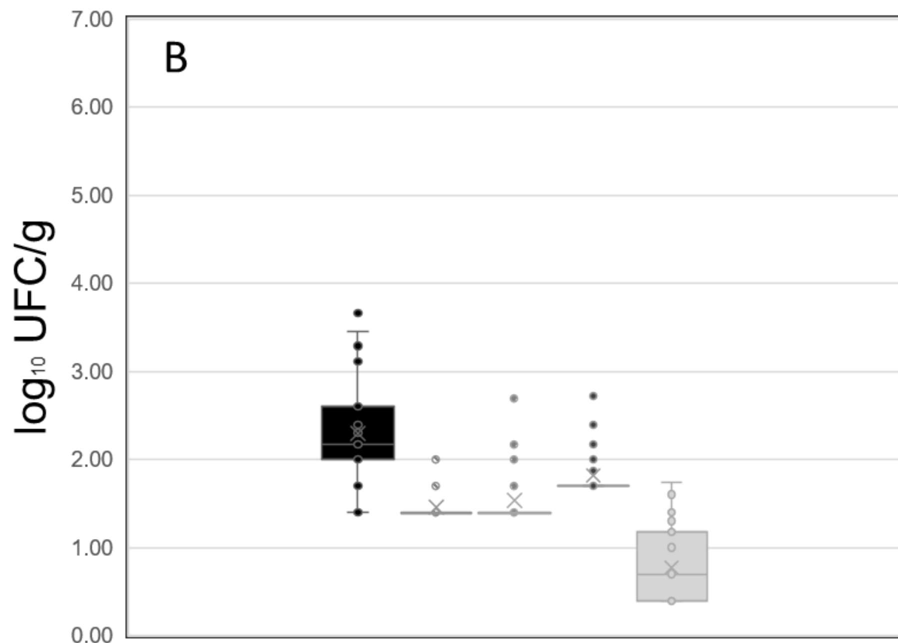
610 and Yeasts; TC (□) = Total coliforms. The limit detection for TAM and M&Y was 1.70 log_{10}

611 UFC/g and for TC and *E. coli* was 0.70 log_{10} UFC/g.



612

613

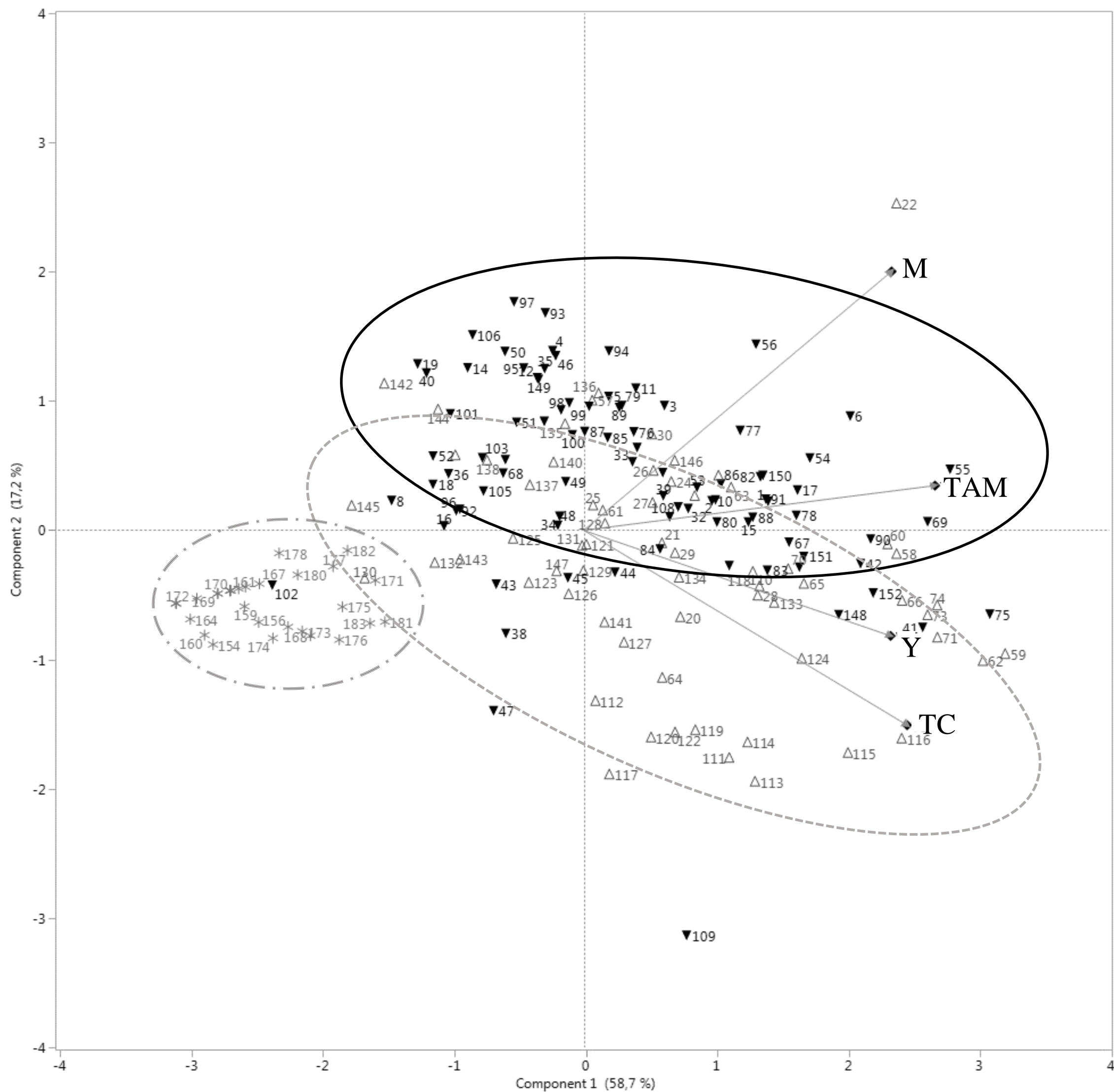


614

615

616

617 **Figure 3**. Biplot (scores and loadings) of PC1 vs. PC2 corresponding to a full-data PCA model
618 for strawberries from Spain according to microbiological counts. TAM = Total aerobic
619 mesophilic count; M = Moulds; Y = Yeasts; TC = Total Coliforms. Codes of variables are: Fresh
620 samples: Supermarket Samples (▼) and Field samples (△); Frozen samples (✳).



621