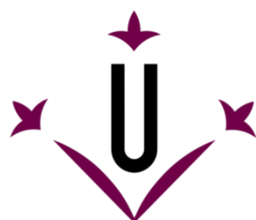

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Occurrence of selected viral and bacterial pathogens and microbiological quality of fresh and frozen strawberries sold in Spain

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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).

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

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

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

91 microbial parameters, including total aerobic mesophilic microorganisms, moulds and yeasts,

92 total coliforms and *Escherichia coli*.

93 ## 2. Materials and Methods

94 ### 2.1. *Sampling and samples preparation*

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

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

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

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

137 **2.3.** ***Detection of*** **Salmonella** ***spp.***

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

Diagnostics) followed by biochemical confirmation using API 20E® (BioMérieux SA, France). One typical colony was chosen per selective plate. If the first colony was negative, another four colonies were selected and examined. A positive control of *Salmonella* spp. was done using the strain BAA 709 (*Salmonella enterica* subsp. *enterica* (Smith) Weldin serotype Michigan).

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

The presence of *L. monocytogenes* was examined according to the procedures described in ISO 11290-2:1998. Firstly, 100 µL of selective culture enrichment (25 g sample plus 225 mL Half Fraser medium) was transferred to tubes with 10 mL of Fraser broth with Fraser supplement (1:10 v/v) (Biokar Diagnostics) and incubated for 18 ± 2 h at 30 ± 1 ° C. In parallel, 100 µL of the homogenate was spread on Palcam Agar (Biokar Diagnostics) and Compass *Listeria* Agar (Biokar Diagnostics). Both the tubes with Fraser broth and the plates were incubated for 48 h at 37 °C. Once this time had elapsed, 100 µL culture taken from the Fraser tubes was spread on Palcam and Compass, repeating the same procedure as previously mentioned. Plates were counted, and the possible suspected colonies of *L. monocytogenes* were isolated on TSAYE plates and incubated at 37 °C for 24 h with the same procedure criteria explained above for detection of *Salmonella*. The experiment was monitored with a positive control of *L. monocytogenes* strain CECT 4031.

For the confirmation of *L. monocytogenes*, one typical colony per plate and positive (CECT4031) and negative control were subjected to biochemical tests: Gram staining and tested for the production of catalase, beta-hemolysis, and fermentation of carbohydrates (xylose, mannitol, and rhamnose). Finally, API LISTERIA® (BioMérieux SA, France) was performed.

2.5. *Detection of Norovirus GI and GII*

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

174 For the virus extraction, 25 ± 0.3 g of sample were weighted in a 100 mL sterile bag with 40 ± 1
175 mL of Tris (hydroxymethyl) glycine beef extract (TGBE; Biokar Diagnostics), 0.5 mL of
176 *Aspergillus* pectinase (Sigma P2611-50 mL, 3800 U/ mL) and 10 ± 0.5 µl of
177 Mengovirus Extraction Control kit (KMG, ceeramTools, Biomerieux, France) as control of
178 virus extraction process. The contents were incubated at room temperature with constant
179 rocking (60 oscillations/min) with an orbital shaker (Unimax 1010, Heidolph Instruments,
180 Germany) for 20 min. The pH of the eluate was monitored at 10 min intervals using pH
181 indicator strips (pH-Fix 6.0-10.0; VWR chemicals, United States) and, if the pH fell below 9.0
182 it was adjusted to 9.5 ± 0.5 using a 10 M NaOH solution. The period of incubation was
183 extended by 10 min every time the pH was adjusted. The contents of the bag were decanted
184 from the filtered compartment into a 50-mL sterile centrifuge tube (Corning) and centrifuged at
185 11,000 × *g* for 20 ± 2 min at 5 ± 3 °C. The resulting supernatant was transferred to a new
186 centrifuge tube. The pH was adjusted to 7 ± 0.5 using a 5 M HCl solution. Then, 7.5 mL of the
187 solution 5×PEG/NaCl (500 g/PEG 8000, 1.5 mol/l NaCl) (0.25% v/v TGBE) were also added,
188 homogenized by shaking for 60 ± 5 s and incubated with constant rocking at 60 oscillations/min
189 at 5 ± 3 °C for 60 ± 5 min. The samples were centrifuged at 11,000 × *g* for 30 min at 5 ± 3 °C.
190 Supernatant was removed and the pellet was dried and suspended with 500 µl of Phosphate-
191 buffered saline (PBS) at 56 ° C. The two samples of 500 µl were combined (1000 µl) in a 2 mL
192 Eppendorf tube. A volume of 1000 ± 10 µl of chloroform – butanol (VWR chemicals, United
193 States) was added and homogenized for 15-30 s with vortex. Samples were incubated for 15±1
194 min at room temperature and centrifuged at 13,500 × *g* for 15 ± 5 min at 5 ± 3 °C. Three layers
195 were formed; the upper aqueous phase was carefully transferred to a 2 mL Eppendorf tube to
196 proceed with the subsequent extraction of the RNA. Samples from this step were either
197 processed immediately, stored at 5 ± 3 °C for a maximum of 24 h or frozen at < -15 ºC for up to
198 6 months.

199 **2.6.** ***Commercial RNA extraction of BioMérieux NucliSENS®***

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

207 **2.7.** ***One-Step Commercial RT-qPCR Kit of CeeramTools***

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

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

225 **2.8.** ***Statistical Analysis***

226 The data obtained were processed in Microsoft® Excel software and adjusted to models of
227 logistic regression with program help JMP 13 software (SAS Institute Inc., Cary, USA). Results
228 are expressed by mean ± standard deviation (SD) for all the samples present in this study. The
229 detection limit (dl) was 1.70 log_{10} CFU/g for TAM and M&Y and 0.70 log_{10} CFU/g for total
230 coliforms and *E.coli*. For samples with microbial counts below dl, an arbitrary value of ½
231 detection limit was used for calculations. All data were checked for significant differences by
232 analysis of variance test (ANOVA). The criterion for statistical significance was $p < 0.05$. JMP
233 13 software was used to develop principal component analysis (PCA) biplot. The PCA was
234 performed to characterize the samples according to microbiological attributes by the presence in
235 log_{10} CFU/g of TAM, M&Y and TC, and taking into account the kind of strawberries (fresh or
236 frozen, their origin (supermarket or field), location and season.

237 # 3. Results and Discussion

238 ## 3.1. *Microbiological quality of strawberries*

239 ### *3.1.1. Total aerobic mesophilic (TAM) counts.*

240 The initial TAM counts of fresh untreated strawberries (Fig. 2A) ranged from ≤ 1.70 (detection
241 limit, dl) – 5.89 $\log_{10}$ CFU/g (mean 3.78 $\log_{10}$ CFU/g). In our study, 88.2% (134/152) of the
242 samples analyzed had a TAM count < 5 $\log_{10}$ CFU/g and only 19.7% (30/152) of samples were
243 < 2 $\log_{10}$ CFU/g. Abadias et al. (2008) found that 90.4% of their fresh-cut fruit samples had
244 TAM counts inferior to 5 $\log_{10}$ CFU/g. The mean initial TAM count of our investigation was
245 almost in the same range as that reported by Hassenberg et al. (2010) in fresh strawberries (4.27
246 $\log_{10}$ CFU/g). In other study conducted in 61 samples of fresh berries, TAM count ranged
247 between 1.7 and 6.9 $\log_{10}$ CFU/g, with a mean value of 2.77 $\log_{10}$ CFU/g and 86 % of
248 prevalence (65/75) (Macori et al., 2018). On the food chain production, fresh strawberries
249 receive minimal processing to avoid being damaged and the consequent increased risk of
250 spoilage. Currently, the EU regulation on microbial criteria for foodstuffs (EC 2073/2005 and
251 subsequent modifications) does not include maximum levels of TAM in fresh and
252 pre-cut fruit.

253 For frozen strawberries, TAM counts (Fig. 2B) ranged from <1.70 (dl) - 3.66 $\log_{10}$ CFU/g
254 (mean 2.30 $\log_{10}$ CFU/g). TAM population was above the detection limit in 26 of 31 frozen
255 samples (83.9 % prevalence). To our knowledge, there are no similar studies concerning the
256 microbial quality of frozen strawberries, except those concerning incidence of viruses.
257 According to Rivas-Pala et al. (1984), counts of mesophilic aerobes should not exceed 5.70
258 $\log_{10}$ CFU/g (5×10^5 CFU/g) of mesophilic aerobes and 2.48 $\log_{10}$ CFU/g (3×10^2 CFU/g) of
259 total coliforms in frozen fruits and vegetables. Therefore, all samples analyzed are acceptable as
260 they fulfil the recommended specifications for frozen foods and vegetables.

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

264 ### *3.1.2. Moulds and yeasts (M&Y).*

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

280 In frozen strawberries, M&Y counts were between 1.70 (dl) – 2.76 $\log_{10}$ CFU/g (mean 1.82
281 $\log_{10}$ CFU/g), and 83.9 % of samples (26/31) had M&Y counts below detection limit (Fig. 2B).
282 Microbial populations on frozen strawberries decreased due to the cell damage that occurred
283 during freezing, probably due to the formation of intracellular ice. Slow freezing involves the
284 apparition of large ice crystals and is beneficial from a microbiological standpoint killing more
285 microorganisms (Jeremiah, 1996).

286 3.1.3. ***Total Coliform Counts and* E. coli**

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

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

298 In frozen strawberries, the population of TC was below the detection limit in 14 of 31 samples
299 (45.2 %) with a range between <0.70(dl) – 1.74 log_{10} CFU/g (mean 0.77 log_{10} CFU/g).
300 According to Rivas-Pala et al. (1896), the interval of TC to consider frozen fruit or vegetable
301 safe was between 2.00 - 2.48 log_{10}.

302 *E. coli* was not detected in any of the fresh and frozen strawberry samples analyzed (n = 186).
303 On microbiological criteria defined in EC Regulation 2073/2005 (EC Regulation, 2005), *E. coli*
304 is controlled only in pre-cut fruit and vegetable or in fresh juices. If fresh and frozen
305 strawberries were subject to the same legislative control as pre-cut fruits and vegetables and
306 fruit juices, all strawberry samples from this study would meet the criteria defined by the
307 regulation. Similarly, no *E. coli* was detected in strawberries from USA farmers' fields market,
308 including both organic and conventional farms (Mukherjee et al., 2004). In another study that
309 surveyed more than 2,000 produce samples from 63 farms in USA, 1% of berries (2 out 194
310 samples) were positive for generic *E coli* (Mukherjee et al., 2006). In Europe, Delbeke et al.
311 (2015) found generic *E. coli* on only 2 of 72 (2.8 %) of strawberry samples from primary
312 production in Belgium, at concentrations of 1.0 log_{10} CFU/g and 3.0 Log_{10} CFU/g and
313 Dziedzinska et al. (2018) found 9.0 % (14 of 156) of strawberry field samples contaminated
314 with *E. coli* in the Czech Republic, and 1.4% from marketplaces (1 of 70).

315 ***3.1.4. Principal Component analysis***

316 A global overview of all data was done according to the level of processing (fresh or frozen),
317 origin (field or supermarket), location, variety and harvest season. These results can be seen in
318 Fig. 3 which depicts the scores and loadings according to principal components 1 (PC1) and 2

319 (PC2). These components accounted for 58.7 and 17.2% of the total variance, respectively. As
320 is evident, a pattern with two different groups can be observed: fresh strawberries tend to be
321 located at higher values of PC1 and PC2 while frozen strawberries tend to show lower scores on
322 PC1 and PC2. Thus, as seen before, fresh strawberries were associated with higher values of all
323 the microbial analyzed whereas the freezing conditions of frozen strawberries negatively affect
324 the presence of microorganisms. No different groups were observed according to variety and
325 harvest season.

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

339 On the other hand, it was seen that the prevalence of total coliforms was statistically higher ($P <$
340 0.05) in field samples than those from a supermarket. Results reported by Roth et al. (2018)
341 showed that total coliforms were more prevalent and at higher levels on farmers' market-
342 collected produce (50.8 %) than supermarket-collected samples (34%). Delbeke et al. (2015)
343 concluded that the field being a potential vehicle that connects the contaminated irrigation water
344 with the fruit.

345 **3.2.** ***Foodborne Pathogens***

346 ***3.2.1. Foodborne Pathogenic Bacteria***

347 Regarding the food safety microorganisms determined, neither *Salmonella* spp. nor
348 *L. monocytogenes* were detected in any of the tested samples. This was consistent with other
349 published studies on fresh and fresh-cut produce (Johnston et al., 2006; Abadias et al., 2008;
350 Santos et al., 2012; Johannessen et al., 2015; Denis et al., 2016; Macori et al., 2018). In other
351 studies, pathogens were not found on strawberries grown in fields or produced in supermarkets
352 of the United States (Mukherjee et al., 2006) and Europe (Delbeke et al., 2015; Graça et al.,
353 2017). Similarly, Macori et al. (2018) did not find *L. monocytogenes* in 75 berry batches from
354 50 different producers. On the contrary, Dziedzinska et al. (2018) found one sample (0.6 %) of
355 fresh strawberry from the Czech Republic contaminated by *L. monocytogenes*, the producer's
356 field having a contamination level lower than 100 CFU/g. Other investigations corroborated the
357 small incidence of the pathogens: Hadjilouka et al. (2014) reported presence of *L.*
358 *monocytogenes* in 3.8% and Ceuppens et al. (2015) found a prevalence of 2.9% in strawberry
359 fruit.

360 ***3.2.2. Norovirus GI and GII***

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

364 For fresh strawberries, the results of our study were comparable with data reported in previous
365 investigations. In Italy, Terio et al. (2017) showed no presence of NoV in 911 fresh strawberry
366 samples studied. Moreover, Li et al. (2018) reported that the low incidence of NoV was 0.24 %
367 for 2,015 fresh berry samples (including strawberries) collected between 2009 and 2016 from
368 different countries including Germany, Bulgaria, France, Poland, Switzerland, Czech Republic,
369 USA, Spain, Russia, and Turkey. Dziedzinska et al. 2018 analyzed the presence of NoV in
370 strawberries and only found two contaminated strawberry field samples (1.3%) and one
371 contaminated sample of NoV in a fresh strawberry purchased from a supermarket in the Czech
372 Republic (1.4%). In case of other fresh berries, Maunula et al. (2013) analyzed 60 samples of

373 fresh raspberries at point of sale in four European countries and no NoV-positive samples were
374 identified, as in this study.
375 Recently, Chatziprodromidou et al. (2018) has reviewed the viral outbreaks linked to fresh and
376 frozen produce and reported that most NoV outbreaks (87.2 %) were produced by frozen food
377 stuffs, and the greatest common produce suspected for viral outbreaks were frozen raspberries
378 (23.7%) and frozen berries (19.1%). Mäde et al. (2013) analyzed 11 samples of frozen
379 strawberries implicated in a Germany outbreak and found 7 positive samples of NoV (63.6%).
380 The incidence of NoV in strawberry matrix can be explained because viral contamination can
381 occur in all parts of food chain (Dziedzinska et al., 2018). Since viruses are not able to replicate
382 extracellularly, their presence can only be due to contaminated irrigation water during pre-
383 harvest and the handling of a contaminated person during (post)harvest. The measures applied
384 by industries used to prevent growth or eliminate bacteria, are not necessarily useful for
385 foodborne viruses. Some measures taken to control bacteria preserve viral particles, as is the
386 case for refrigeration/freezing (Stals et al., 2011). For this reason, in the last 5 years (2015-
387 2018) there have been 39 notifications (contamination incidents) concerning foodborne
388 pathogens in berries in the RASFF (*Rapid Alert System for Food and Feed*) portal, 10 of them
389 related to strawberries (9 frozen and 1 fresh samples), 8 of them concerning norovirus and 2
390 hepatitis A virus. One contamination incident was related to NoV contaminated strawberries
391 from Spain.

392 ## 4. Conclusions

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

405

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Conflict of interest

The authors declare no conflict of interest.

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

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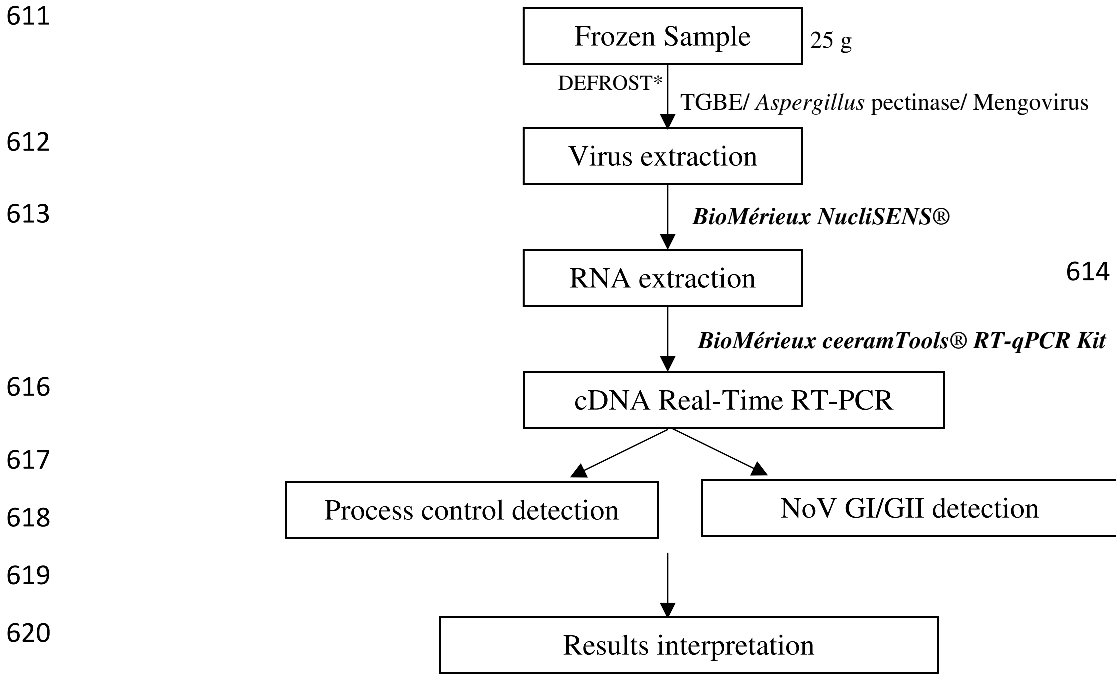

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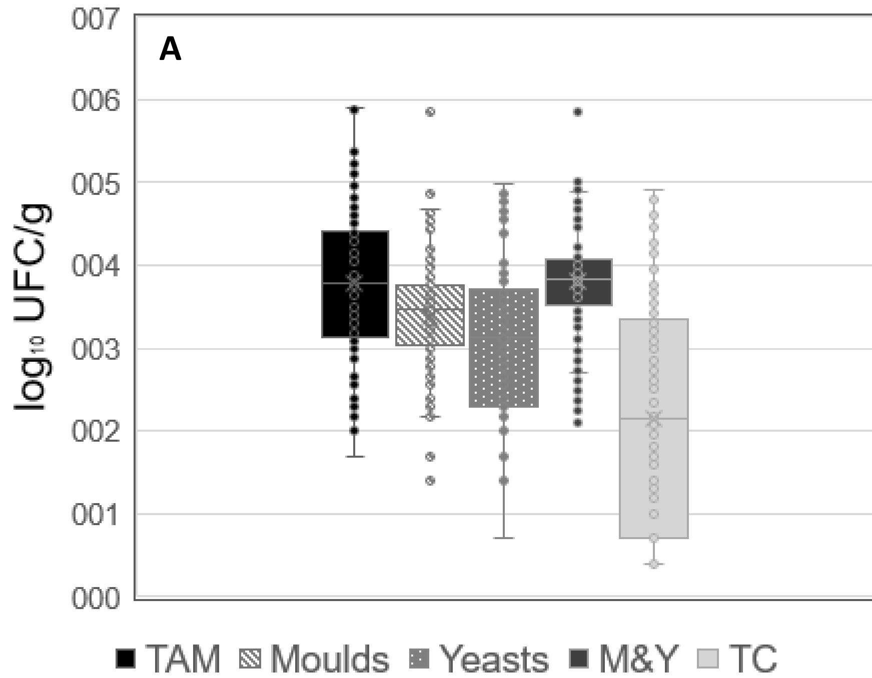
626 **Figure 2**. Microbiological quality (log_{10} CFU/g) of fresh (A) and frozen (B) strawberries. TAM

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

628 Moulds and Yeasts; TC (□) = Total coliforms. The limit detection for TAM and M&Y was

629 1.70 log_{10} UFC/g and for TC and *E. coli* was 0.70 log_{10} UFC/g.



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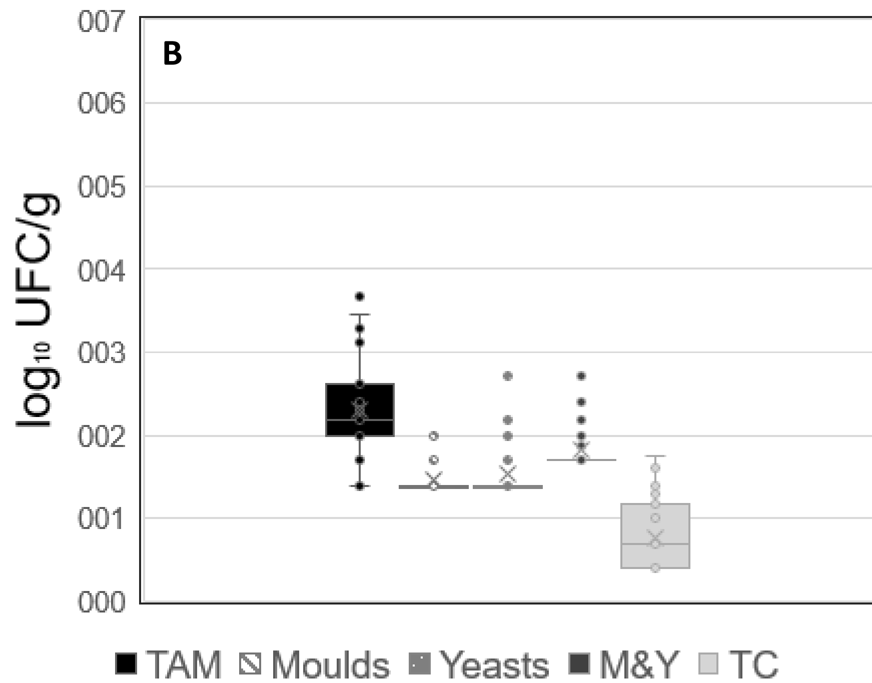

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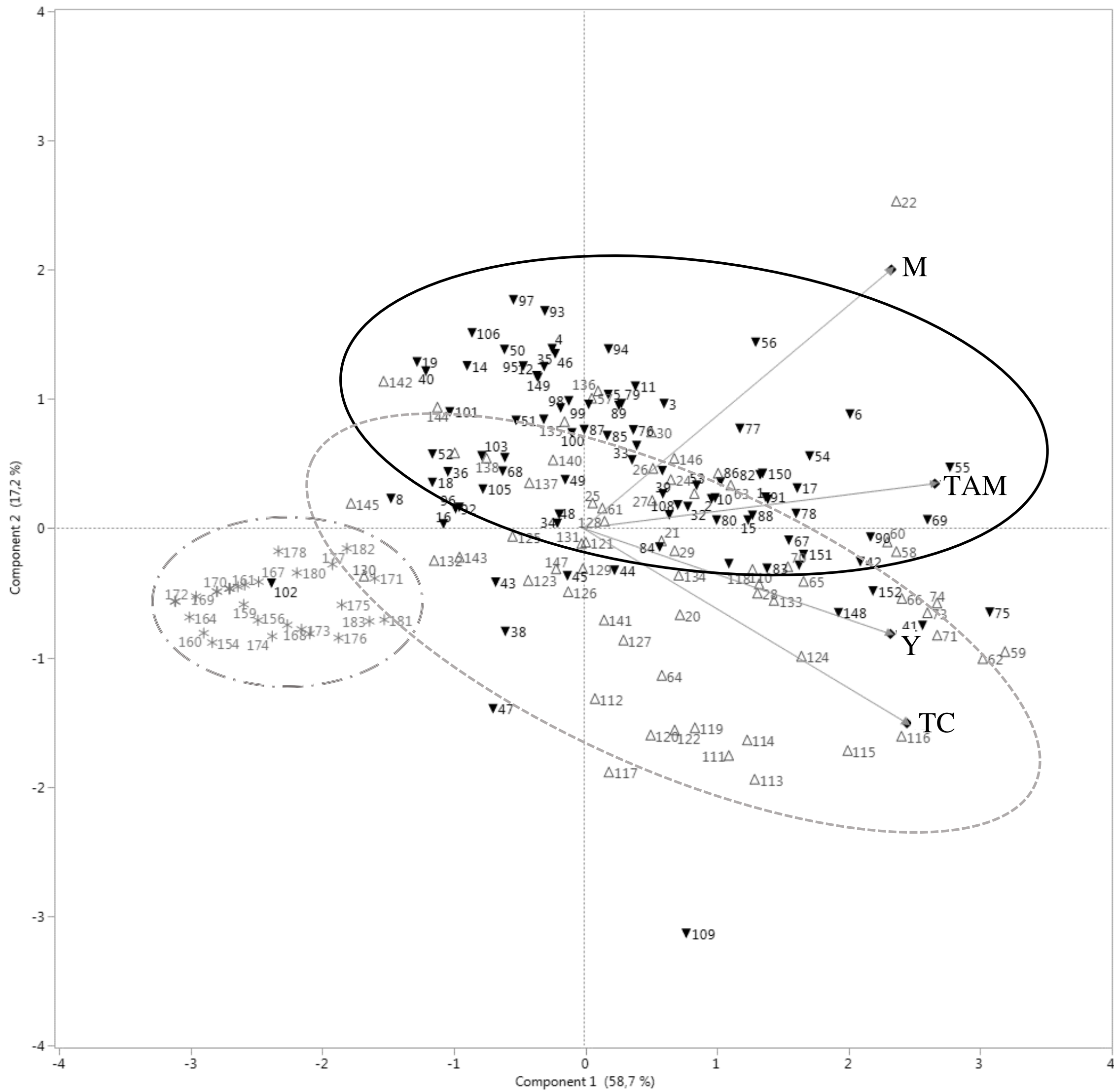
633 **Figure 3**. Biplot (scores and loadings) of PC1 vs. PC2 corresponding to a full-data PCA model
634 for strawberries from Spain according to microbiological counts. TAM = Total aerobic
635 mesophilic count; M = Moulds; Y = Yeasts; TC = Total Coliforms. Codes of variables are:
636 Fresh samples: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



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