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

Ortiz-Solà¹, J., Viñas¹*, I., Colás-Medà², P., Anguera², M., Abadias²*, M.

² Institut de Recerca i Tecnologia Agroalimentàries (IRTA). XaRTA-Postharvest. Edifici Fruitcentre, Parc Científic i Tecnològic Agroalimentari de Lleida, Parc de Gardeny, 25003 Lleida.

* Corresponding autor: M. Abadias (isabel.abadias@irta.cat) / I. Viñas (ivinas@tecal.udl.cat)
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 $< 1.70 \text{ (detection limit, dl)} - 5.89 \log_{10} \text{CFU/g (mean 3.78 log}_{10}\text{CFU/g)}$ for TAM; $2.10 - 5.86 \log_{10} \text{CFU/g (mean 3.80 log}_{10}\text{CFU/g)}$ for M&Y; and $<0.70 \text{ (dl)} - 4.91 \log_{10} \text{CFU/g (mean 2.15 log}_{10}\text{CFU/g)}$ for TC in fresh strawberries. In frozen strawberries, the counts were $<1.70$ (dl) – $3.66 \log_{10} \text{CFU/g (mean 2.30 log}_{10}\text{CFU/g)}$ for TAM; $<1.70$ (dl) – $2.76 \log_{10} \text{CFU/g (mean 1.82 log}_{10}\text{CFU/g)}$ for M&Y; and $<0.70$ (dl) – $1.74 \log_{10} \text{CFU/g (mean 0.77 log}_{10}\text{CFU/g)}$ 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).
According to the reported outbreaks connected to fresh produce, very little information can be found on the prevalence of *Salmonella* spp., Shiga toxin-producing *Escherichia coli* (STEC) and *Listeria monocytogenes* in strawberries, but everything seems to indicate that it was low (Bohaychuk et al., 2009; Yoon et al., 2010; Delbeke et al., 2015; Johannessen et al., 2015). An outbreak involving *E. coli* O157:H7 was reported in the United States in 2011 that caused 15 cases (2 deaths) and were related to the consumption of strawberries contaminated in the field by wildlife contact with deer faeces (Laidler et al., 2013). Moreover, in an investigation executed by the US Food and Drug Administration (FDA), *Salmonella* was noticed in 1 sample from a total of 143 imported strawberry samples (FDA, 2001). Furthermore, *L. monocytogenes* serogroup 4 was isolated by enrichment from 1 out of 173 strawberry samples obtained from Norwegian retail markets (Johannessen et al., 2002).

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

Currently, there is little information concerning the microbial quality and safety of strawberries grown in Spain. In addition, such a study has never been conducted in the country. Therefore, in this study we evaluated freshly harvested and retail strawberries (including frozen strawberries) from Spain for the presence of NoV genogroup I and II (GI and GII); human pathogenic bacteria, including *Salmonella* spp. and *Listeria monocytogenes*; and other
microbial parameters, including total aerobic mesophilic microorganisms, moulds and yeasts, total coliforms and *Escherichia coli*. 
2. Materials and Methods

2.1. Sampling and samples preparation

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

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

The 25 g samples in sterile blender bags with BPW were homogenized in Masticator Homogenizator (IUL S.A. Instruments, Barcelona). The homogenates were 1:10 diluted with Peptone solution (PS: 0.1% peptone, 0.85% NaCl). For total aerobic mesophilic microorganisms (TAM), 100 µL of dilutions were plated in duplicate PCA (Plate Count Agar, Biokar Diagnostics) plates and incubated for (72±3) h at 30 ±1 ° C as indicated in ISO 4833-2:2013. According to ISO 21527-1:2008, the same dilutions were plated in duplicate Dichloran Rose Bengal Chloramphenicol Agar (DRBC, Biokar Diagnostics) plates used for selective isolation...
of fungi –moulds and yeasts - of significance in food spoilage and incubated at 25 ± 1°C for 3 – 5 days.

To enumerate total coliform microorganisms (TC), 1 mL of dilution was transferred in duplicate to sterile Petri dishes and 12-15 mL of VRBL (Violet Red Bile Lactose Agar, Biokar Diagnostics) at 44 - 47 °C were poured into each Petri dish and carefully mixed with the inoculum. The mixture was allowed to solidify and afterwards, 4-6 mL of the VRBL medium were poured onto the surface of the inoculated medium and allowed to solidify before incubation at 37 ± 1°C for 24±2 h as defined in ISO 4832:2006. After 24h of incubation, characteristic violet colonies with a 0.5 mm diameter were counted (sometimes colonies were red covered).

For the quantification of *Escherichia coli*, 1 mL of the homogenate was plated in duplicate in the selective chromogenic medium TBX (Tryptone Bile Glucuronic Agar, Biokar Diagnostics) and plates were incubated at 44 ± 1°C for 18-24 h as indicated in ISO 16649-2:2001. After incubation period, blue-green colonies were counted. No confirmation test was done for TC and *E. coli*.

After microbial counts, the bag containing the homogenates of strawberries in BPW were incubated for 18 ± 2 h at 37 ± 1°C (non-selective pre-enrichment) for *Salmonella* spp. detection.

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

For *Salmonella* spp. detection, the procedures indicated in ISO 6579:2003 were followed. Briefly, 100 µL of non-selective pre-enrichment BPW homogenate commented above was transferred to 10 mL Rappaport-Vassiliadis-Soya Peptone Broth (RVS; Biokar Diagnostics) tubes and incubated at 41.5 °C for 24 ± 3 h for selective enrichment. In parallel, another 1 mL was added to tubes with 10 mL of Broth dehydrated base medium with novobiocin (MKTTn*; Biokar Diagnostics) (0,1% v/v) and incubated at 37 °C for 24 ± 3 h. After selective enrichment, the cultures obtained from RVS and MKTTn were streaked onto Xylose-Lysine-Desoxycholate Agar (XLD; Biokar Diagnostics) and Hektoen Enteric agar (HK; Biokar Diagnostics). The plates were incubated at 37 °C for 24 h and examined for typical colonies. The presence of *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.
For the virus extraction, 25 ± 0.3 g of sample were weighted in a 100 mL sterile bag with 40 ± 1 mL of Tris (hydroxymethyl) glycine beef extract (TGBE; Biokar Diagnostics), 0.5 mL of *Aspergillus* pectinase (Sigma P2611-50 mL, 3800 U/mL) and 10 ± 0.5 μl of Mengovirus Extraction Control kit (KMG, ceeramTools, Biomerieux, France) as control of virus extraction process. The contents were incubated at room temperature with constant rocking (60 oscillations/min) with an orbital shaker (Unimax 1010, Heidolph Instruments, Germany) for 20 min. The pH of the eluate was monitored at 10 min intervals using pH indicator strips (pH-Fix 6.0-10.0; VWR chemicals, United States) and, if the pH fell below 9.0, it was adjusted to 9.5 ± 0.5 using a 10 M NaOH solution. The period of incubation was extended by 10 min every time the pH was adjusted. The contents of the bag were decanted from the filtered compartment into a 50-mL sterile centrifuge tube (Corning) and centrifuged at 11,000 × g for 20 ± 2 min at 5 ± 3 °C. The resulting supernatant was transferred to a new centrifuge tube. The pH was adjusted to 7 ± 0.5 using a 5 M HCl solution. Then, 7.5 mL of the solution 5×PEG/NaCl (500 g/PEG 8000, 1.5 mol/l NaCl) (0.25% v/v TGBE) were also added, homogenized by shaking for 60 ± 5 s and incubated with constant rocking at 60 oscillations/min at 5 ± 3 °C for 60 ± 5 min. The samples were centrifuged at 11,000 × g for 30 min at 5 ± 3 °C. Supernatant was removed and the pellet was dried and suspended with 500 μl of Phosphate-buffered saline (PBS) at 56 °C. The two samples of 500 μl were combined (1000 μl) in a 2 mL Eppendorf tube. A volume of 1000 ± 10 μl of chloroform – butanol (VWR chemicals, United States) was added and homogenized for 15-30 s with vortex. Samples were incubated for 15±1 min at room temperature and centrifuged at 13,500 × g for 15 ± 5 min at 5 ± 3 °C. Three layers were formed; the upper aqueous phase was carefully transferred to a 2 mL Eppendorf tube to proceed with the subsequent extraction of the RNA. Samples from this step were either processed immediately, stored at 5 ± 3 °C for a maximum of 24 h or frozen at < -15 °C for up to 6 months.

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

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

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

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

2.8. Statistical Analysis
The data obtained were processed in Microsoft® Excel software and adjusted to models of logistic regression with program help JMP 13 software (SAS Institute Inc., Cary, USA). Results are expressed by mean ± standard deviation (SD) for all the samples present in this study. The detection limit (dl) was $1.70 \log_{10}$ CFU/g for TAM and M&Y and $0.70 \log_{10}$ CFU/g for total coliforms and *E.coli*. For samples with microbial counts below dl, an arbitrary value of $\frac{1}{2}$ detection limit was used for calculations. All data were checked for significant differences by analysis of variance test (ANOVA). The criterion for statistical significance was $p < 0.05$. JMP 13 software was used to develop principal component analysis (PCA) biplot. The PCA was performed to characterize the samples according to microbiological attributes by the presence in $\log_{10}$ CFU/g of TAM, M&Y and TC, and taking into account the kind of strawberries (fresh or 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 \( \leq 1.70 \) (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).
In fresh strawberries, fungi counts were similar to those obtained in TAM (Fig. 2A). The results reported fungal populations ranging between 2.10 and 5.86 log$_{10}$ CFU/g (mean 3.80 log$_{10}$ CFU/g). The range in which this microbial group was found in other studies with fresh-cut strawberry was 2.00 – 7.10 log$_{10}$ CFU/g (Abadias et al. 2008). Graça et al. (2017) found that strawberries, pineapples and mango presented the highest mean fungal counts (5.20, 5.10 and 4.70 log$_{10}$ CFU/g, respectively). Furthermore, Tournas et al. (2006) detected in fresh-cut strawberries 4.36 log$_{10}$ CFU/g counts of M&Y with Cladosporium spp. in 100 % occurrence of all samples. The low pH of berries decreases the viability of bacterial species, making it easier for moulds and yeasts to grow on and spoil fruits (Brackett, 1987). The mean average of the total yeast counts (3.04 log$_{10}$ CFU/g) were significantly lower than the mean average of the total mould counts (3.37 log$_{10}$ CFU/g) (p<0.05). The normal population of yeast on fresh and undamaged fruits is generally low (less than 3.00 log$_{10}$ CFU/g) (Tournas et al., 2005). It has been seen that the principal fungi present in fresh strawberries were the moulds Botrytis cinerea, Rhizopus spp., Penicillium spp., Alternaria spp., Cladosporium spp., Aureobasidium pullulans and the yeast Cryptococcus spp. (Tournas et al., 2005).

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

3.1.3. Total Coliform Counts and E. coli

The results showed large variations of total coliforms (TC) numbers depending of different featured samples (Fig. 2A). The mean of TC in fresh strawberries were 2.15 log$_{10}$ CFU/g with a range of <0.70 (dl) – 4.91 log$_{10}$ CFU/g. However, the TC population does not exceed 2 log$_{10}$ CFU/g in 74/152 fresh samples (48.7%). Roth et al. (2018) found lower TC levels for berries, with a mean of 0.52 log$_{10}$ CFU/g. Conversely, they found significantly higher levels of total
coli forms on spinach and leafy greens with similar values to this study (1.60 – 2.30 log_{10} CFU/g). Yoon et al. (2011) found that the interval of coliforms found in the leaves of the strawberries was 1.20–3.20 log_{10} CFU/leaf. It was established that specific types of produce, like berries, sprouts and leafy greens, are more at risk of infection and constituted an important source of pathogens in the documented outbreaks (Berger et al., 2010; Doyle & Erickson, 2008; EFSA, 2013; 2014).

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

*E. coli* was not detected in any of the fresh and frozen strawberry samples analyzed (n = 186). On microbiological criteria defined in EC Regulation 2073/2005 (EC Regulation, 2005), *E. coli* is controlled only in pre-cut fruit and vegetable or in fresh juices. If fresh and frozen strawberries were subject to the same legislative control as pre-cut fruits and vegetables and fruit juices, all strawberry samples from this study would meet the criteria defined by the regulation. Similarly, no *E. coli* was detected in strawberries from USA farmers’ fields market, including both organic and conventional farms (Mukherjee et al., 2004). In another study that surveyed more than 2,000 produce samples from 63 farms in USA, 1% of berries (2 out 194 samples) were positive for generic *E coli* (Mukherjee et al., 2006). In Europe, Delbeke et al. (2015) found generic *E. coli* on only 2 of 72 (2.8 %) of strawberry samples from primary production in Belgium, at concentrations 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) of strawberry field samples contaminated with *E. coli* in the Czech Republic, and 1.4% from marketplaces (1 of 70).

3.1.4. Principal Component analysis

A global overview of all data was done according to the level of processing (fresh or frozen), origin (field or supermarket), location, variety and harvest season. These results can be seen in Fig. 3 which depicts the scores and loadings according to principal components 1 (PC1) and 2.
These components accounted for 58.7 and 17.2% of the total variance, respectively. As is evident, a pattern with two different groups can be observed: fresh strawberries tend to be located at higher values of PC1 and PC2 while frozen strawberries tend to show lower scores on PC1 and PC2. Thus, as seen before, fresh strawberries were associated with higher values of all the microbial analyzed whereas the freezing conditions of frozen strawberries negatively affect the presence of microorganisms. No different groups were observed according to variety and harvest season.

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

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

### 3.2. Foodborne Pathogens
3.2.1. Foodborne Pathogenic Bacteria

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

3.2.2. Norovirus GI and GII

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

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

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

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

The authors declare no conflict of interest.
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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.

- Frozen Sample
  - 25 g
  - DEFROST
  - TGBE/Aspergillus pectinase/Mengovirus

- Virus extraction
  - BioMérieux NucliSENS®

- RNA extraction
  - BioMérieux ceeramTools® RT-qPCR Kit

- cDNA Real-Time RT-PCR
  - Process control detection
  - NoV GI/GII detection

- Results interpretation
Figure 2. Microbiological quality ($\log_{10}$ CFU/g) of fresh (A) and frozen (B) strawberries. TAM (■) = Total aerobic mesophilic count; M (□) = Moulds; Y (△) = Yeasts; M&Y (■) = Moulds and Yeasts; TC (□) = Total coliforms. The limit detection for TAM and M&Y was $1.70 \log_{10}$ UFC/g and for TC and *E. coli* was $0.70 \log_{10}$ UFC/g.
Figure 3. Biplot (scores and loadings) of PC1 vs. PC2 corresponding to a full-data PCA model for strawberries from Spain according to microbiological counts. TAM = Total aerobic mesophilic count; M = Moulds; Y = Yeasts; TC = Total Coliforms. Codes of variables are:

Fresh samples: Supermarket Samples (▼) and Field samples (△); Frozen samples (●).