

Research Note

Microbiological Quality of Saffron from the Main Producer Countries

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ABSTRACT

A microbiological study of saffron spice was undertaken in the context of a European research project (Methodologies for Implementing International Standards for Saffron Purity and Quality, the acronym for which is SAFFIC), analyzing 79 samples obtained from the main producer countries, namely Greece, Iran, Italy, Morocco, and Spain. Current microbiological quality criteria are the same as for other spices, but saffron is added in minute quantities during the cooking process, so the health risk associated with microbial contamination might be lower. We did not detect *Salmonella* either by culture or by PCR methods in any sample, and *Escherichia coli* was only found in five samples. *Enterobacteriaceae* were frequently found (70.9% of the samples), but most of them belonged to species of probable environmental origin. Aerobic sporulated bacteria were also common, but only three samples contained *Bacillus cereus* at low levels (<200 CFU g⁻¹). *Clostridium perfringens* counts were also very low, with only one sample reaching >100 CFU g⁻¹, an acceptable value. Overall, microbial contamination in saffron was markedly lower than it was in other spices.

Spices have been used to prepare foods for centuries worldwide, mainly because of their flavoring properties. In ancient times, spices were so valuable that they were used as a form of currency. Currently, this is still done in some regions in the case of saffron, which remains probably the most expensive spice. Saffron consists of the dried stigmas of *Crocus sativus* L., either as filaments or in powder form. The saffron flower has one bright-red stigma divided into three filaments remaining united through a small portion of orangey stigma. This spice is valued for its abilities of coloring and flavoring, and for its aromatic strength.

As with many other agricultural products, spices are exposed to a wide range of environmental microbial contamination during collection, processing, and in the retail markets by dust, wastewater, and animal and even human excreta (5, 8, 10). Contaminated spices may cause a microbiological problem, depending on the end use. Saffron is added during cooking, so this risk is limited by the thermal processing of the food; however, some preparations involve cold infusion in water and oil extraction.

The goal of this work was to measure the microbial contamination of saffron, of either natural origin or resulting from collection and handling, in order to evaluate the possible health risks associated with this contamination. In the context of a European research project (Methodologies for Implementing International Standards for Saffron Purity

and Quality, the acronym for which is SAFFIC), a large number of samples were obtained globally from the main saffron producers. An objective of this project was to set new criteria for the microbiological quality for saffron. The presence of microorganisms (bacteria and fungi) was evaluated by classic plate count, but PCR was also used to detect possible nonviable *Salmonella* and *Escherichia coli*.

A comprehensive study on the microbial safety of spices has recently been published (12), but it only included two saffron samples. To our knowledge, this is the first microbiological study of saffron that includes a large number of samples of diverse origin.

MATERIALS AND METHODS

Saffron samples. Seventy-nine saffron samples were obtained directly from producers in sealed polyethylene bags. The origins of the samples were Iran (33 samples), Italy (15 samples), Greece (15 samples), Spain (14 samples), and Morocco (2 samples). Twenty-five grams of each sample was aseptically transferred to a homogenizer bag, and 225 ml of buffered peptone water (Pronadisa, CONDA, Madrid, Spain) and 0.1% Tween 80 (vol/vol) were added. (Tween 80 was included, given the presence of olive oil residues in the Italian samples, because in Sardinia the stigmas are wetted with virgin olive oil before drying.) After 10 min of hydration at room temperature, the sample was homogenized for 1 min in a homogenizer (Stomacher Lab Blender 400, Seward, Worthing, UK) and kept for 50 min at room temperature. Aliquots of this 10⁻¹ dilution were used for every microbial count, and the remaining volume was incubated for 24 h

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at 37°C for preenrichment purposes, and then used for detection of *Salmonella* and *Staphylococcus aureus*.

Microbiological analysis. Aerobic, unsporulated bacteria were evaluated by plate count in standard methods agar (Pronadisa) after a 48-h incubation at 30°C. For fungal counts, 1-ml-aliquot dilutions of 10^{-1} , 10^{-2} , and 10^{-3} were filtered through membranes (pore size of 0.45 µm; Millipore, Billerica, MA), which were then laid over Sabouraud-dextrose-chloramphenicol agar (Pronadisa) and then incubated for 2 to 4 days at 24°C.

Enterobacteriaceae, coliforms, and *E. coli* were enumerated by plate count in violet red bile glucose agar, violet red bile agar with lactose (Pronadisa), and Coli-ID agar (bioMérieux, Inc., Hazelwood, MO) after incubation at 30, 37, and 45°C, respectively. Suspected *Enterobacteriaceae* colonies were identified by the API 20E system (bioMérieux, Inc.).

To investigate the presence of sporulated bacteria, an aliquot of the 10^{-1} dilution was heated for 5 min at 80°C, and 0.1-ml aliquots of serial dilutions were spread either onto Mossel agar (Mannitol polymyxin-egg yolk; Pronadisa) and incubated for 48 h at 30°C for selective enumeration of *Bacillus cereus*, or onto sulfite-polymyxin-sulfadiazine agar (Pronadisa) plates to enumerate clostridia. In the latter case, an overlay of the same medium was used to cover the inoculum, and plates were then incubated for 72 h in an anaerobic atmosphere, either at 37°C for enumeration of sulfite-reducing sporulated bacteria, or at 45°C for *Clostridium perfringens*.

Plating was always done in duplicate, and the mean of countable colonies was calculated.

For detection of *Salmonella*, 1-ml aliquots taken from the preenrichment culture were inoculated in duplicate in selenite and Rappaport-Vassiliadis enrichment broth (Pronadisa) and incubated for 24 h at 37 and 45°C, respectively. Samples from the selenite medium were used to inoculate plates of selective differential media (*Salmonella-Shigella* and xylose-lysine-deoxycholate agar [Pronadisa]), whereas Hektoen agar and *Salmonella* chromogenic agar (Pronadisa) were inoculated from the Rappaport-Vassiliadis tube. All of these plates were incubated for 48 h at 37°C and then examined for the presence of characteristic colonies.

Detection of *S. aureus* was carried out by inoculation of 0.1-ml aliquots of the preenrichment culture in Baird-Parker agar plates (Pronadisa) and 48 h of incubation at 37°C.

PCR detection. PCR was used to detect *Salmonella* and *E. coli* in saffron samples after the preenrichment culture process described above. To eliminate sample debris, 500 µl of the preenrichment supernatant was filtered through a VectaSpin Micro system (Whatman, Maidstone, UK) by centrifugation for 3 min at $15,000 \times g$. Then, the filter and the supernatant were discarded, and 100 µl of PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, Carlsbad, CA) was added to the pellet and homogenized in a vortex mixer. The samples were heated for 10 min at 100°C, cooled for 2 min at room temperature, and centrifuged for 3 min at $15,000 \times g$. Fifty microliters of the supernatant was collected for DNA purification. In order to avoid the possible *Taq* polymerase inhibitors present in saffron, three different approaches were tested by using samples spiked with *Salmonella* serovar Typhimurium LT2 at a final concentration of 10^6 CFU ml $^{-1}$: dilution of the extracted DNA to 1/10 and 1/32 with distilled water, ethanol precipitation, or purification with GENECLEAN Turbo for PCR columns used according to the manufacturer's recommendations (Q·BIOgene, Inc., Montreal,

Quebec, Canada). The latter purification protocol always gave consistent results, and it was used thereafter.

PCR amplifications were carried out with TaqMan polymerase (Biotoools, B & M Labs, Madrid, Spain) by using the following primers: 5'-CGGTGGTTTTAAGCGTACTCTT-3' and 5'-CGAA-TATGCTCCACAAGGTTA-3' for amplification of the *invA* gene of *Salmonella* (7), and 5'-AAAACGGCAAGAAAAAGCAG-3' and 5'-ACGCGTGGTTACAGTCTTGCG-3' for amplification of the *uidA* gene of *E. coli* (4). PCR was performed in a Mastercycler gradient thermocycler (Eppendorf, Hamburg, Germany), with 25 cycles of amplification and annealing temperatures of 55°C for *Salmonella* and 52°C for *E. coli*. Products of amplification were analyzed by agarose gel electrophoresis. *Salmonella* Typhimurium LT2 and *E. coli* ATCC 29213 were used as controls, and amplification products compatible with the expected sizes of 796 and 1,476 bp, respectively, were obtained.

RESULTS AND DISCUSSION

The Spanish specifications for spices set maximum limits of 10^3 CFU g $^{-1}$ of sulfite-reducing sporulated anaerobic bacteria and 10 CFU g $^{-1}$ of *E. coli*, the absence of *Salmonella* in 25 g of sample and, in general, the absence of microbial pathogens (2). The International Commission on Microbiological Specifications for Foods (ICMSF) allows maximum limits of 10^6 CFU of total aerobic mesophilic bacteria (TAMB); 10^4 CFU of yeasts, molds, and coliforms; and 10^3 CFU of *E. coli* and *C. perfringens* per g of spice (8, 9). Finally, the Commission of the European Union (EU) recommends the enumeration of *B. cereus* and *C. perfringens* as well as to verify the absence of *Salmonella* in 25 g of sample (6). Therefore, we performed all of these microbiological determinations in the saffron samples, and the results are summarized in Table 1, whereas the distribution per countries of the samples with the highest microbial counts is outlined in Table 2.

Detection of *S. aureus* in spices is not specified by any normative, but we determined its presence or absence in 1 g, because the presence of this bacterium may be related to handling practices during harvesting or storage of saffron; only two samples (from Spain and Greece) gave a positive result.

All of the analyzed samples fulfilled the EU and Spanish criteria for absence of *Salmonella*. In order to confirm these results and to investigate whether nonviable *Salmonella* could be present, we tested 0.5-ml aliquots of the preenrichment cultures from 65 randomly chosen samples by PCR, plus seven samples that gave positive results either for *S. aureus* or *E. coli* (see below). All of the samples were negative for *Salmonella*.

As expected for vegetables, all samples contained TAMB (Table 1), but only 6 (7.6%) of them reached or slightly surpassed the limit of 10^6 CFU g $^{-1}$ set by the ICMSF (8) (Table 2). Aerobic sporulated bacteria were also found in all 62 samples that were analyzed for these bacteria, with counts between 10^2 and 10^5 CFU g $^{-1}$. We searched for *B. cereus* in all samples, bearing in mind the risk of food poisoning. This bacterium was frequently reported in Indian spices (not including saffron), with counts $>10^4$ CFU g $^{-1}$ in many of the analyzed samples (3), but in our study, it was only found in three samples, reaching 10^2

TABLE 1. Percentages of saffron samples containing microorganisms^a

Range (log CFU g ⁻¹)	TAMB	Aerobic sporulated bacteria ^b	<i>Enterobacteriaceae</i>	Coliforms	SRSB	<i>Clostridium perfringens</i>	Yeast	Molds
Not detected	0	0	29.1	30.4	35.4	51.9	49.4	22.8
<1	— ^c	1.6	—	—	16.5	16.5	—	—
1–2	0	3.2	5.1	7.6	44.3	30.4	6.3	40.5
2–3	2.5	25.4	12.7	13.9	3.8	1.3	16.5	30.4
3–4	44.3	65.1	20.3	20.3	0	0	25.2	6.3
4–5	25.3	4.8	20.3	19.0	0	0	1.3	0
5–6	21.5	0	11.4	8.9	0	0	1.3	0
6–7	6.3	0	1.3	0	0	0	0	0

^a TAMB, total aerobic mesophilic bacteria; SRSB, sulfite-reducing sporulated anaerobic bacteria.

^b Only 62 samples were analyzed for aerobic sporulated bacteria.

^c —, coincident with the detection limit of the method.

CFU g⁻¹ in two cases (Table 2). This level is considered satisfactory according to EU recommendations (<10³ CFU g⁻¹); indeed, it is too low to be considered as a risk of food poisoning unless significant bacterial growth occurs in the food.

Fifty-one (64.6%) samples contained sulfite-reducing, sporulated, anaerobic bacteria, but at very low counts. As may be expected from this result, levels of *C. perfringens* were also very low: 37 (46.8%) samples were positive, but only one reached 10² CFU g⁻¹ (Table 2). This value is acceptable according to EU recommendations, and it is much lower than the level that is potentially capable of causing food poisoning, which is estimated at 10⁵ CFU g⁻¹ (13), so active proliferation in the food would be necessary to present a health risk. The incidence of *C. perfringens* was similar to that reported in different Mexican and Indian

spices (3, 11), but higher than the incidences reported in spices from Argentina (12.2%) (1); none of these studies included saffron.

Enterobacteriaceae surpassing >10⁴ CFU g⁻¹ were found in 56 (70.9%) and 26 (32.9%) samples (Table 2). As these bacteria may represent either fecal or environmental contamination, we identified a number (45) of the isolated colonies. We found 82.8% of identified strains of probable environmental origin (26 *Pantoea* spp., 2 *Buttiauxella agrestis*, and 1 *Serratia plymuthica*) and 17.1% of strains of possible fecal origin (two *Enterobacter aerogenes*, two *Enterobacter cloacae*, and two *Klebsiella pneumoniae*). The prevalence of coliforms closely followed that of *Enterobacteriaceae*: 22 (27.9%) samples contained >10⁴ CFU g⁻¹ (Table 2), and most of them coincided with those containing a high number of *Enterobacteriaceae*.

TABLE 2. Distribution by country of the samples with the highest counts of TAMB, SRSB, *Enterobacteriaceae*, coliforms, *E. coli*, *B. cereus*, *C. perfringens*, and yeast

Microbial count	No. (%) of positive samples from each country ^a			
	Greece (n=15)	Iran (n=33)	Italy (n=15)	Spain (n=14)
TAMB ≥ 10 ⁶ CFU ^b	3 (20.0)	1 (3.0)	1 (6.7)	0
SRSB ≥ 10 ² CFU	2 (13.3)	0	0	1 (7.1)
<i>Enterobacteriaceae</i> ≥ 10 ⁴ CFU	5 (33.3)	21 (63.6)	0	0
Coliforms ≥ 10 ⁴ CFU ^b	5 (33.3)	16 (48.5)	1 (6.7)	0
<i>Escherichia coli</i> > 10 ¹ CFU ^c	2 (13.3)	2 (6.1)	0	0
<i>E. coli</i> > 10 ² < 10 ³ CFU	1 (6.7)	0	0	0
<i>Bacillus cereus</i> > 10 ² CFU ^d	0	0	2 (13.3)	0
<i>Clostridium perfringens</i> ≥ 10 ¹ CFU	6 (40.0)	11 (33.3)	5 (33.3)	3 (21.4)
<i>C. perfringens</i> ≥ 10 ² CFU ^e	1 (6.7)	0	0	0
Molds ≥ 10 ³ CFU	0	2 (6.1)	1 (6.7)	2 (14.3)
Yeast ≥ 10 ⁴ CFU ^b	0	2 (6.1)	0	0

^a Only two samples from Morocco were analyzed and both were positive for *C. perfringens* (>10¹ CFU); the remaining microbial counts were lower than the values reported here.

^b Surpassing the ICMSF criteria.

^c Surpassing the Spanish limits.

^d Considered as satisfactory by the EU recommendations.

^e Considered as acceptable (but not satisfactory) by the EU recommendations. The remaining microbial counts are satisfactory for any of the indicated criteria.

E. coli was only present in five (6.3%) samples, four of them surpassing the Spanish limits (10 CFU g⁻¹) but considered acceptable according to the ICMSF criteria (<10³ CFU g⁻¹) (Table 2). These five samples were also checked by PCR, and all of them presented positive amplification with *E. coli*-specific primers (data not shown). Rechecking by culture at the time PCR was performed (after 6 months of storage at room temperature) gave a negative result for all samples, indicating the poor long-term viability of this bacterium in saffron.

Molds were found in 77.2% of the samples, but always at low counts; only four samples reached or slightly surpassed 10³ CFU g⁻¹. A major concern would be the presence of *Aspergillus* spp., because some species can produce aflatoxins. Therefore, any suspicious colony was presumptively identified, and none of them was compatible with that genus. Most of the isolated fungi belonged putatively to the genus *Rhizopus*, based on morphological identification. These findings, together with the low counts detected, permitted us to discard a toxicity risk. Yeasts were also frequently isolated (50.6%), particularly in samples from Iran (87.9%). Two samples contained >10⁴ CFU g⁻¹, which would be considered unacceptable according to the ICMSF recommendations (8).

In summary, only 3 (3.8%) of 79 samples analyzed were unacceptable according to both Spanish and ICMSF specifications for *E. coli*, coliforms, and TAMB. Four (5.1%) samples were unacceptable according to the Spanish specifications for *E. coli*. Five (6.3%) surpassed the ICMSF limits for TAMB, and two (2.5%) other samples exceeded the limit for yeast contamination. Overall, we detected the highest microbial load in saffron samples from Iran (Table 2). This may be due to the warmer climate, but poor harvesting and sanitary practices during storage cannot be ruled out.

Remarkably, potential pathogens were either undetectable (*Salmonella*), incidental (*S. aureus*), or very low both in number and prevalence (*C. perfringens* and *B. cereus*) and always within safety regulations. As saffron is added to food *only* before cooking and not used in raw food, the presence of these bacteria cannot be considered a health risk. To our knowledge, there have been no reports on the presence of *Salmonella* in saffron. This fact is of special interest, because the EU recommendations and Spanish specifications require 25 g of sample to discard the presence of this bacterium, making the analysis of saffron very expensive. The very small amount of saffron used for cooking (about 1.5 × 10⁻² g per person) and the low counts of potential pathogens found here (Table 2) suggest that the amount used for microbiological analysis may be reduced. We propose the use of 5 g, obtained from a 25-g sample homogenized in a balls mill and used for all the chemical and microbiological analyses, to be diluted in 45 ml of preenrichment broth. Aliquots of this suspension

are then used for the enumeration of *E. coli*, *B. cereus*, and *C. perfringens*, and after preenrichment, the absence of *Salmonella* is checked. We recommend using the more stringent Spanish limits for *E. coli* (2) and the EU criteria (6) for the remaining bacteria.

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