Effect of processing on microbial safety of wild pepper (*Piper borbonense*) from Reunion Island

Mathieu Weil[a,b,*], Fabienne Remize[c], Noel Durand[d,e], Pascaline Alter[d,e], Mathilde Hoarau[a,b], Jean Christophe Meile[a,b]

[a] CIRAD, UMR Qualisud, F-97410 Saint-Pierre, Réunion, France.

[b] Qualisud, Université de Montpellier, CIRAD, Montpellier SupAgro, Université d'Avignon, Université de La Réunion, Montpellier, France.

[c] UMR QualiSud, Université de La Réunion, CIRAD, Université Montpellier, Montpellier SupAgro, Université d'Avignon, Sainte Clotilde, France.

[d] CIRAD, UMR Qualisud, F-34398 Montpellier, France.

[e] Qualisud, Université de Montpellier, CIRAD, Montpellier SupAgro, Université d'Avignon, Université de La Réunion, Montpellier, France.

***Corresponding author**: Mathieu Weil, CIRAD – UMR Qualisud, F-97410 Saint Pierre – BP 180, Réunion, France - Tel: +262 2 62 49 28 62; E–mail address: mathieu.weil@cirad.fr*

Declarations of interest: none

Abstract

The management of microbial contamination is an important issue in spice trade. For common domesticated black pepper (*Piper* spp.), the control relies mainly on post-process decontamination. The aim of the present study was to examine microbial contamination of wild pepper (*Piper borbonense)* from Reunion Island and investigate the effects of different processing paths on microbiological quality and fungal ecology. The fresh pepper microbial counts ranged from 4.6 to 6.8 log $CFUg^{-1}$. Blanching had a positive significant impact on the microbiological quality of pepper whereas sweating led to microbial growth up to 5 log $CFUg^{-1}$ and, therefore, should be avoided. Microbial counts for dried pepper were 1.33 log $CFUg^{-1}$; 3.37 log $CFUg^{-1}$; 1.67 log $CFUg^{-1}$ and 1.3 log $CFUg^{-1}$ for coliforms, TAMB, *Staphylococcus*, yeast and moulds, respectively. Potential mycotoxin producers were identified from pepper samples but aflatoxins and ochratoxin A levels detected were far below the regulation limits. The initial diversity of fungal contamination is prominent for the final quality of pepper in contrast to the impact of processes. The revisited wet process (blanching then drying), which positively affected all microbial loads, could be a good option for pepper transformation.

Keywords

Blanching; drying; sweating; microbiota; moulds; mycotoxins.

1. Introduction

Spices and herbs are used worldwide to enhance food flavour. Whereas herbs are obtained from the leaves, spices cover roots, stems, bulbs, barks, berries or seeds. Among those, black pepper (*Piper nigrum*) is one of the most extensively used spice in the world. In 2017, 690 000 tons of pepper were produced; nearly one-third of which was produced by Vietnam, the leading producer, ahead of Indonesia and India (FAO Statistics Division, 2019).

The highest demand of pepper comes from Europe and North America, where pepper is used for food industry, institutional catering and home-consumption. Therefore, the different geographical locations for production and consumption leads to high trade volumes. Dried black pepper trade accounted for twenty per cent of all spice imports in 2002 (Mokshapathy & Yogesh, 2013). Wild peppers represent an extremely low amount of trade, but their high sensory characteristics put them in a prominent place regarding their commercial value. For instance, black pepper is sold for around 30 euros per kg, when *Tsiprifery*, a wild Malagasy pepper, reaches 200 euros/kg in French delicatessens (Razafimandimby et al., 2017). *Piper borbonense*, the wild pepper from Reunion Island, though offering worthwhile potential regarding its original chemical composition (Weil, Shum, Meot, Boulanger, & Bohuon, 2017) is not cultivated, neither processed, nor commercialised yet.

Legal requirements for the entrance of pepper in the European market (CBI, 2019) are for the product to comply with maximum levels of mycotoxins, in particular aflatoxin and ochratoxin, and microbial contamination levels. For instance, in 2018, a total of 17 batches of black pepper received a border control notification, among those 9 were subjected to withdrawal, due to the positive detection of *Salmonella* (RASFF, 2019). Thus, many

studies have focused on the ability of *Salmonella* to grow or survive on black pepper over an extended storage time (Keller, VanDoren, Grasso, & Halik, 2013). However microbial contamination levels of black pepper is not restricted to pathogens, with the detection of total mesophilic bacteria in the range of 2 to 8 log $CFUg^{-1}$ (Chitrakar, Zhang, & Adhikari, 2018; Fogele, Granta, Valcina, & Berzins, 2018; Garbowska, Berthold-Pluta, & Stasiak-Rozanska, 2015; Klimešová et al., 2015; Mckee, 1995; Plany et al., 2018). *Bacillus* spp. or *Clostridium perfringens* or other spore-forming bacteria are often reported, as well as *Staphylococci* and *Enterobacteria*. Both *Salmonella* and Gram-positive bacteria counts did not decrease significantly over storage (Thanh et al., 2018). Assessment of mycological quality and mycotoxin contamination of black pepper showed that *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus niger* and *Penicillium* spp. were the main potentially mycotoxinogenous fungi associated to pepper (El Mahgubi et al., 2013; Fogele et al., 2018; Yogendrarajah, Deschuyffeleer, et al., 2014). As a consequence, aflatoxins, ochratoxin and other mycotoxins were occasionally detected at low concentrations, therefore, the risk associated to black pepper consumption was considered low (Yogendrarajah, Deschuyffeleer, et al., 2014; Yogendrarajah et al., 2015; Yogendrarajah, Jacxsens, et al., 2014; Yogendrarajah et al., 2016). In contrast to black pepper, there is no data concerning microbial contamination of wild pepper, such as *P. borbonense.*

The management of microbial contamination in spices relies mainly on good practices, but inactivation of bacteria may be performed. To this end, non-thermal methods are of high interest as their impact on aromatic compounds is low. Superheated steam, vacuum-steam pasteurization, radio-frequency heating, microwave- or gamma-irradiation have shown to be efficient to decrease *Salmonella* or *Escherichia coli* populations (Ban et al., 2018; Jeevitha, Sowbhagya, & Hebbar, 2016; Jeong & Kang, 2014; Shah, Asa, Sherwood,

93 Graber, & Bergholz, 2017; Song et al., 2014). Another approach is to investigate
94 processing practices in the light of microbiological contaminants. In Madagascar, wild
95 peppers are processed according to ‘‘dry” and ‘‘wet” processes (Weil et al., 2014). The
96 ‘‘dry” process only consists of single drying, whereas the ‘‘wet” process includes blanching
97 and sweating prior to drying. Blanching consists of dipping pepper berries in hot or boiling
98 water for a few minutes. According to Dhas and Korikanthimath (2003), it is used not only
99 to remove impurities and increase the speed of drying but also to decrease the microbial
100 load. Traditionally, sweating, i.e. keeping the hot blanched pepper in a blanket for 24 h is
101 used in Madagascar to brown pepper and is hypothesized to develop its aroma (as for
102 vanilla). Drying is performed to reduce water content and stabilize pepper for future
103 conservation by limiting microbial growth. Previous studies (Weil et al., 2017) showed,
104 chemical-sensorial criteria such as piperine and essential oil contents were quite resistant
105 to the process. Therefore, the choice of the process could be monitored according to
106 sanitary considerations. There is no literature investigating the impact of these processing
107 practices on microbial contaminants. The aim of this study was to examine the microbial
108 contamination of wild pepper (*P. borbonense*) from Reunion Island and investigate the
109 effects of different processing paths on the microbiological quality.
110
111 **2. Materials and methods**
112 **2.1. Pepper sampling**
113 Pepper (*P. borbonense*) berries on spikes were sampled from wild creepers in a place
114 called “Rivière Langevin” in a limited area of approximately 5 000 m^2 located in the
115 southernmost part of Reunion Island (- 21° 2' 04.49 S; 55° 38' 33.07 E). Six different
116 batches were harvested at ripened stage at five different dates: Batch A – 2nd of December

2014; Batch B – 6th of November 2013; Batch C 12th of December 2013; Batch D – 4th of December 2013; Batch E and Batch F – 13th of December 2013.

The batches were directly frozen at -80 °C until use (Freezer Froilabo – Bio Memory 690 liters, Paris, France). Before processing, around 800 g of each batch were manually destemmed then thawed on a disinfected inox plate during two hours at room temperature. Defrosted and unprocessed pepper is called "fresh pepper" (stage 1, **Figure 1**).

2.2. Pepper processing

Three transformation processes - two "wet" and one "dry" - were tested (**Figure 1).**

2.2.1. Wet processes

Two types of wet processes were tested. The first one (called "wet process") consisted in the succession of three unit operations: blanching, sweating and drying. The second one ("revisited wet process") consisted of two unit operations: blanching then drying. A quantity of 550 g of fresh pepper was blanched in a water bath (Memmert Gmbh type WB 22 Schwabach, Germany) at 75 °C, over 3 min at a ratio of 1:36 (m/m) pepper berries to water. After blanching either the pepper was: directly dried or sweated prior to drying. Sweating consisted of placing the pepper berries arranged in a compact 1 cm layer in aluminium trays (300 cm^2) in a climatic chamber (BIA Climatic – Type CL 125, Conflans Sainte Honorine, France) at 35 °C and 99 % RH for 24 h. Drying was performed by placing the same aluminium trays containing 225g of pepper berries arranged in a compact 1 cm layer for 24 h at 60 °C ± 1 °C, RH 20 % ± 2 % in the same climatic chamber. Hot air (60 ± 1 °C, RH 20 ± 2 %) was circulated over the surface of the layer. Wet process was applied to batches A, B, C, D (n=4); revisited wet process was applied to batches A, B, C, D, E, F (n=6).

2.2.2. Dry process

141 The dry process consisted in a single drying unit operation. Sample of 225 g of fresh
142 pepper arranged in a compact 1 cm layer was placed in an aluminium tray for 24 h at 60
143 °C ± 1 °C, RH 20 % ± 2 % in the climatic chamber. Hot air (60 ± 1 °C, RH 20 ± 2 %) was
144 circulated over the surface of the layer. Dry process was applied to batches D, E, F (n=3).

145 **2.3. Microbial enumeration**

146 Samples of 25 g of berries corresponding to different pepper stages were withdrawn after
147 each processing step (samples 1, 2, 3, 4, 6 for wet processes and 1, 5 for dry process).
148 Samples were homogenized with 25 mL of buffered peptone water (Biolife ref 4012782,
149 Milan, Italy) with a stomacher over 1min at maximal speed. Serial decimal (up to 10^{-8})
150 dilutions were prepared in the same buffer. Homogenates were frozen at -80 °C for further
151 molecular analyses as described in section 2.5.
152 For enumeration, 0.1 mL of dilution was plated onto agar plates. Total aerobic mesophilic
153 bacteria (TAMB) counts were determined on Plate Count Agar (Biokar ref BK144HA,
154 Allonne, France) supplemented with 4 µg.mL^{-1} cycloheximide and incubated for 72 h at
155 30°C. *Staphylococcus* spp. were enumerated on Baird Parker medium
156 (Acumedia/Neogen ref 7112, Laning, USA) after incubation for 48 h at 37 °C. Yeasts and
157 fungi were enumerated on Glucose Chloramphenicol broth supplemented with agar (GCA,
158 CONDA, ref 1258, Madrid, Spain) after incubation for 5 days at 25 °C. Coliforms were
159 enumerated by plating 1 mL of sample onto Petri dish supplemented with 12 mL of VRBL
160 (Violet Red Bile Lactose) agar (Biokar ref BK152HA, Allonne, France) after incubation for
161 48 h at 37 °C. Counts were expressed as decimal logarithm (log) of colony forming units
162 (CFU) per gram of sample.

163 **2.4. Fungal isolation and identification**

After incubation for 5 days at 25 °C, fungal isolates were selected from GCA plates based on their colony morphology. 26 Isolates that exhibited morphological colony differences were selected. Selected isolates were re-suspended on the same medium and grown in the same conditions. Thereafter, each isolate was stored in glycerol 20 % at -80 °C until further analyses.

2.4.1 Fungal DNA isolation

A CTAB-based method was used to extract DNA from fungal isolates (adapted from (Umesha, Manukumar, & Raghava, 2016). Fungal mycelia and spores were collected from plates by pipetting 50 µL of Triton-X100 then added to 500 µL of CTAB buffer [Autoclaved CTAB solution (0.1 M Tris pH 8, 1.4 M NaCl, 20 mM EDTA, 2 % CTAB (cetyltrimethyl ammonium bromide) to which 4 % polyvinylpyrrolidone and 0.5 % β-mercaptoethanol were further added] and one scoop of glass beads (Sigma G9772, Germany) in empty 1.5 mL tubes. After vortex mixing (Vortex Genius 3, IKA, Staufen, Germany) for 2 min, the tubes were incubated at 65 °C for 15 min. A mixture of 500 µL of chloroform:isoamyl alcohol (24:1) was added and mixed well by hand shaking then centrifuged for 5 min at maximum speed (14.000 x g, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). The upper (aqueous) phase was transferred into new 1.5 mL tube and 0.1 volume of 7.5 M ammonium acetate and 1 mL of absolute ethanol were added. After gentle mixing, the tubes were centrifuged for 30 min at maximum speed at 4 °C. The liquid was discarded and 500 µL of cold 70 % ethanol were added to wash the pellet. Tubes were centrifuged for 10 min at 4 °C at maximum speed. The liquid was carefully discarded; the DNA pellet was dried overnight and resuspended with 20 µL of molecular grade water.

2.4.2 Amplification and sequencing of fungal DNA

PCR amplification reactions were performed by mixing 5x Green Go*Taq* Buffer (Promega), 2 mM $MgCl_2$ (Qiagen), 200 µM of dNTPs (Promega), 1.25 U of Go*Taq* DNA polymerase (Promega), 0.2 mM of ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS4 (TCCTCCGCTTATTGATATGC) DNA primers (Sigma), 5 µL of fungal DNA extract, and Ultrapure Water (Eppendorf) qs 50 µL. PCR amplifications were performed in Veriti Thermal Cycler (Applied Biosystems,ThermoFisher Scientific, Waltham, MA, USA) with an initial denaturation step at 95 °C for 2 min followed by 35 cycles of 3 steps (denaturation at 95 °C for 15 sec, hybridization at 52 °C for 30 sec and elongation step at 72 °C for 30 s) and a final elongation step at 72 °C for 5 min. 5 µL of PCR reactions were deposited on 2 % agarose gel in TAE 1x buffer for a control electrophoresis. PCR amplicons of expected size (between 500 and 700 bp) were sent for sequencing to Macrogen (South Korea). The raw sequences data were edited using Bioedit Software and compared to the GenBank database using the BLAST program (Blast, 2018). Sequences having a percentage of identity of 97 % or more were considered to belong to the same species (Koljalg et al., 2013).

2.5. Fungal community analysis by PCR-DGGE

2.5.1 DNA extraction

A CTAB-based method was used (adapted from Hamdouche et al., 2015). Frozen pepper filtrates were thawed and 2 mL were sampled and centrifuged for 2 min at 500 *x g* to clear the lysate. The lysate volume was split into two microtubes containing 300 µL of glass beads (Sigma). The mix was vortexed at maximum speed for 5 min at room temperature then centrifuged for 15 min at 12000 *x g*. The supernatant was removed, and the pellet resuspended with 300 µL of lysis buffer [Triton X-100 2 %, SDS 1 %, NaCl 100 mM, Tris pH8 10 mM, EDTA pH8 1 mM] and the mix was incubated at room temperature for 5 min.

212 A volume of 100 µL of TE buffer [Tris pH8 10 mM, EDTA pH8 1 mM] was added and the
213 mix incubated for 5 min at room temperature. Then, 100 µL of Lysozyme (25 $mg.mL^{-1}$)
214 were added. After 5 min of incubation at room temperature, 100 µL of proteinase K (20
215 $mg.mL^{-1}$) were added and the mix was incubated for 20 min at 42 °C. Then, 50 µL of SDS
216 (20 %) were added and the mix incubated for 5 min at room temperature followed by 10
217 min at 4 2°C in water bath (Memmert Gmbh type WB 22 Schwabach, Germany). A volume
218 of 400 µL of CTAB [2 % in NaCl 3 M] was added and the mix incubated for 10 min at 65
219 °C.
220 A phenol/chloroform extraction was performed by addition of 700 µL of phenol solution
221 (Sigma ref. 77617). Tubes were agitated manually then centrifuged at 12000 *x g* for 15
222 min at room temperature. The upper (soluble) phase was transferred to a new microtube.
223 A second phenol/chloroform extraction was performed as described above. Then, 600 µL
224 of chloroform–isoamyl alcohol mixture (Sigma ref. 25666) were added, manually mixed
225 and centrifuged at 12000 *x g* for 10 min at room temperature. The upper phase was
226 transferred to a new microtube.
227 DNA was precipitated by adding 0.1 volume of sodium acetate (3 M, pH5), followed by 1
228 volume of 100 % isopropanol, manually mixed and left for 1 h at -20 °C. After centrifugation
229 at 12000 *x g* for 30 min and supernatant removal, the DNA pellet was washed by addition
230 of 500 µL 70 % ethanol. After centrifugation at 12000 *x g* for 5 min and supernatant
231 removal, the DNA pellet was air-dried and resuspended in 100 µL of water. DNA was
232 quantified by UV Spectrophotometry (Nanodrop 8000, Thermo Fisher Scientific Waltham,
233 MA, USA).

234 **2.5.2 PCR amplifications**

PCR amplification reactions were performed using 50 ng of DNA as described above except that GC-ITS1F (CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGCTTGGTCATTTAGAGGAAGTAA) and ITS2 (GCTGCGTTCTTCATCGATGC) DNA primers were used. PCR amplifications were performed in Veriti Thermal Cycler (Applied Biosystems ThermoFisher Scientific, Waltham, MA, USA) with an initial denaturation step at 95 °C for 2 min followed by 35 cycles of 3 steps, as mentioned previously. The agarose gels were run at 100 V for 30 min and stained for 15 min in ethidium bromide (0.5 µg.mL^{-1}) followed by 10 min in water, then observed and photographed on a UV transilluminator coupled with a CCD camera (Syngene, G:Box, Cambridge, United Kingdom).

2.5.3 Denaturing gradient gel electrophoresis (DGGE) analysis

PCR products were separated by DGGE, using an OmniPAGE VS20WAVE-DGGE system (Cleaver Scientific, Warwickshire, United Kingdom) according to the procedure of the manufacturer. From 10 to 30 µL (estimated from PCR amplification control electrophoresis in order to get homogeneous signals) of each PCR product were loaded onto 8 % (w/v) polyacrylamide gels (acrylamide : bisacrylamide, 37.5:1, Promega) in 1x TAE. Electrophoresis experiments were performed at 60 °C using a denaturing gradient ranging from 40 % to 70 % (100 % corresponded to 7 M urea and 40 % (v/v) formamide, Promega). The migration was carried out at 20 V for 10 min and then at 80 V for 16h. The gel was stained for 30 min with ethidium bromide solution (40 µg.mL^{-1}) then 30 minutes in water and photographed as described above.

2.6. Mycotoxin analysis: aflatoxins (AFs) and ochratoxin A (OTA) quantification

Each sample (5 grams of pepper) was homogenized with 25 mL methanol and agitated during 15 minutes with an orbital agitator (Ratek RSM7DC, Victoria, Australia). The extract was centrifuged at 6000 *x g* for 10 min. A volume of 5 mL of the filtrate was diluted with 15 mL of PBS buffer. The diluted extract was passed through an immuno affinity column (IAC) (Aflaochraprep, R-Biopharm). The IAC was washed twice with 10 mL of PBS before being eluted with 1 mL methanol and 1 mL of water. The obtained elute was collected into a glass bottle, identified by High Performance Liquid Chromatography (HPLC) and quantified by spectrofluorescence (Shimadzu RF 20A, Kyoto, Japan) after post column derivatization with electrochemical system (Kobra Cell™ R. Biopharm Rhône Ltd, Glasgow, UK). Fluorescence detection for AFs was set at 365 nm for excitation and 435 nm for emission and OTA was set at 333 nm for excitation and 460 nm for emission. The mobile phase A was water: methanol (55:45; v/v), 119 $mg.L^{-1}$ of potassium bromide and 350 $\mu L.L^{-1}$ of nitric acid (4 M) and the mobile phase B was water: methanol (20:80; v/v), 119 mg L^{-1} of potassium bromide and 350 µL of nitric acid 54 M). AFs and OTA standards were used for the construction of a five-point calibration curve of peak areas versus concentration ($ng.mL^{-1}$). The injection volume was 100 µL for both standard solutions and sample extracts. The operating conditions were as follow: injection volume of 100 µL of sample; C18 reverse-phase HPLC column, Uptisphere type, ODS, 5 µm particle size, 5 ODB, 250 x 4.6 mm, with identical pre-column, thermostatically controlled at 40 °C; isocratic flow rate of 0.8 $mL.min^{-1}$. Mobile phase gradient: mobile phase A: 100 % (0-26 min); 35 % (26-45 min); 100 % (45-50 min); The repeatability and detection limits on AFs were respectively 94.8 % and 0.05 $\mu g.kg^{-1}$. The repeatability and detection on OTA were respectively 96.5 % and 0.05 $\mu g.kg^{-1}$. The contents were calculated from a calibration

curve established with aflatoxins (TSL-108, Biopharm Rhône Ltd, Glasgow, UK) and ochratoxins standards (TSL-504, Biopharm Rhône Ltd, Glasgow, UK).

2.7. Statistical analyses

Differences in the mean values of the microbial loads were tested by an analysis of variance (ANOVA); the significance of differences between enumerations was determined using the Tukey test. Two mean values were considered significantly different when P value was below 0.05.

The PCR-DGGE fingerprinting was scored by presence and absence of co-migrating bands, regardless of their intensity. Pair wise community similarities were quantified using Dice similarity coefficient (S_D): $S_D = 2\ N_c / N_a + N_b$ where N_a represented the number of bands detected in sample A, N_b represented the number of bands in sample B, and N_c represented the number of bands common to both samples. The similarity index was expressed within a range of 0 (completely dissimilar) to 1 (perfect similarity).

Principal Component Analysis (PCA) was used to group samples according by the presence/absence of the 18 discriminant fungal markers detected by PCR-DGGE using XL STAT software (Spearman correlation). Markers IV, X and XI, detected in all samples, were not included in the PCA.

3. Results and Discussion

3.1. Microbiological quality

In this study, for each type of microbial counts, a decrease was considered as a positive impact and an increase as a negative impact on the microbiological quality of pepper.

3.1.1. Initial contamination of fresh pepper

304 The six fresh pepper batches used in this study exhibited a wide range in microbial counts.
305 This was observed for the four types of microbial groups studied. *Staphylococcus* ranged
306 from 3.6 to 6.5 log $CFUg^{-1}$, with a mean value of 4.6 ±1.2; coliforms ranged from 2.8 to 7
307 log $CFUg^{-1}$, with a mean value of 5.2 ± 1.5; TAMB ranged from 6.1 to 8.2 log $CFUg^{-1}$ with
308 a mean value of 6.8 ± 0.9; yeasts and moulds ranged from 5 to 6.9 log $CFUg^{-1}$ with a
309 mean value of 5.9 ± 0.6. Fresh pepper from batch A which exhibited the highest counts in
310 *Staphylococcus* also had the highest loads in coliforms and in TAMB.
311 The microbial counts variations observed between the different batches of fresh pepper
312 could be due to climate conditions linked to the harvest period. A rain shower for instance
313 can wash the product. Other environmental and intrinsic factors (such as chemical
314 composition of the pepper) could influence the contamination level.
315 As pepper is hardly ever consumed fresh (except in brine), and very few data on the
316 contamination of fresh pepper is available in the scientific literature. The microbial loads
317 found by Omafuvbe (2004) in fresh black pepper (*Piper guineense*) originated from Nigeria
318 were very similar to those measured in this study: coliforms ranged from 6.2 to 6.8 log
319 $CFUg^{-1}$; TAMB ranged from 6.8 to 7.0 log $CFUg^{-1}$ and yeasts and moulds ranged from 6.7
320 to 7.3 log $CFUg^{-1}$.

321 **3.1.2. Impact of blanching, sweating and drying on the microbial**
322 **counts**

323 Blanching reduced the microbial loads of all four types of microorganisms up to 4 log
324 $CFUg^{-1}$ and had, thus, a positive significant impact on the microbiological quality of pepper
325 (**Figure 2**). This is consistent with the work of Omafuvbe (2004) who applied a more
326 drastic blanching treatment to pepper (15 min in boiling water vs 3 min at 75 °C in this

study) and obtained a greater positive significant impact of blanching, reducing TAMB, coliform, yeast and mould contamination by approximately 6 log $CFUg^{-1}$.

On the opposite, sweating increased the microbial counts of all groups of microorganisms, up to 5 log $CFUg^{-1}$, resulting in a negative significant impact on pepper sanitary quality. Therefore, sweating, should not be used in pepper processing for safety reasons.

Drying, which is essential to stabilize pepper for future storage and thus increase shelf life, had no significant impact on the abundance of the microbiota **(Figure 2).**

A water-cleaning step consisting in mixing by hand during 3 min the fresh pepper in water at room temperature (20 °C) (data not shown) was also tested. This operation did not exhibit a significant impact on microbial counts, validating that blanching effect observed on the microbiota was thermal and not mechanical.

3.1.3. Impact of the different processes on microbial population

Pepper processing consisted of combinations of different unit operations. Three main pepper processing were performed in this study and their impact on microbial population was measured.

Wet processing is defined as the sequence of blanching, sweating and drying steps. As blanching had a positive significant impact, sweating a significant negative impact, and drying had no significant impact on the microbiota. Therefore, in all the wet processing appeared to have no significant impact on the counts of any of the four microbial groups studied **(Figure 2)**.

Dry processing of pepper is defined as the direct drying of fresh pepper berries. Drying had no significant impact on microbial counts. The impact of the dry process was therefore not significant for any of the four floras studied **(Figure 2).**

A revisited wet process was experimented by removing the sweating step of the wet process. This process resulted from the combination of blanching and drying. Due to blanching, the impact of the revisited wet process was significantly positive for all types of microbiota studied. Indeed, a reduction by 3 log $CFUg^{-1}$ for *Staphylococcus* counts, 2.5 log $CFUg^{-1}$ for coliform counts, 3 log $CFU.g^{-1}$ for TAMB counts and 4 log $CFUg^{-1}$ for yeast and mould counts was observed **(Figure 2).**

The blanching conditions applied in this study were 75 °C for 3 min. Pepper colour could be preserved by inactivating oxidative enzymes (polyphenol oxidase and peroxidase) responsible for browning. As determined in another study subsequent to this one (Weil, unpublished), enzyme inactivation was complete by applying 100 °C for 30 s. One can hypothesize that these more drastic blanching conditions would result in an increased inactivation of vegetative bacteria. With a z value of 10 °C, a decrease by 5 log can be expected from the increased time and temperature of treatment.

3.1.4. Dried pepper microbial counts

The microbial population levels of dried pepper were highly dependent on the type of process applied. For all four groups of microbiota, both wet and dry processes had no significant impact independently of the initial microbial load. For instance, TAMB counts were on average 7.8 and 7.2 log $CFUg^{-1}$ for the wet and the dry process respectively **(Figure 2)**. The most positive effect (i.e. lowest counts) was observed for the revisited wet process. The counts for the dried pepper were 1.33 ± 0.5 log $CFUg^{-1}$; 3.37 ± 0.5 log $CFUg^{-1}$; 1.67 ± 0.5 log $CFUg^{-1}$ and 1.50 ± 0.3 log $CFUg^{-1}$ for coliforms, TAMB, *Staphylococcus* and yeast and moulds, respectively **(Figure 2)**.

Previous studies reported microbiological quality of dried black pepper. In their reviews on microbial contamination of spices, Mckee (1995) and Chitrakar et al. (2018) reported

374 great variations in total bacterial counts measured in dried black peppers, ranging from a
375 few hundreds to more than 8 log $CFUg^{-1}$. The population of *Bacillus cereus* was up to 2.49
376 log $CFUg^{-1}$ (Fogele et al., 2018), whereas total counts of mesophilic aerobes ranged from
377 3 to 5 log $CFUg^{-1}$ (Garbowska et al., 2015; Klimešová et al., 2015).
378 Piperine is known to have antifungal and antibacterial activities. *P. borbonense* contains
379 low levels of piperine (around 0.2 %) when compared to *P. nigrum* (around 4 %). However,
380 the microbial loads observed for *P. borbonense* were in accordance with the
381 contamination rates reported in literature for *P. nigrum*.
382 Food operators shall ensure that foods satisfies safety requirements at all steps of
383 production, processing and distribution (Commission of the European Communities,
384 2002). The regulation concerning microbial contamination on pepper is scarce. The IPC
385 (International Pepper Community, 2015) defines limits for *P. nigrum* for two bacteria:
386 *E. coli* (under 3 $MPNg^{-1}$) and *Salmonella* (absence in 25 g).
387 Mckee (1995) reported the identification of *E. coli*, *Escherichia freundii*, *Serrati* sp.,
388 *Klebsiella* sp., *Bacillus* sp., *Staphylococcus* sp. and *Streptococcus* sp.. In the present
389 study the high counts of coliforms and *Staphylococcus* could include those bacterial
390 species.
391 The observed counts of yeasts and moulds ranged from 1.3 to 5.5 log $CFUg^{-1}$ for the
392 revisited and the other processes respectively and are consistent with values of 3.3-4.2
393 log $CFUg^{-1}$ previously reported on black pepper (El Mahgubi et al., 2013; Yogendrarajah,
394 Deschuyffeleer, et al., 2014). Drying had no significant impact on the abundance of the
395 microbiota but is essential to stabilize the product (Jin, Mujumdar, Zhang, & Shi, 2018).
396 The determination of the main fungal species and their ability to produce mycotoxins is of
397 importance. However, at this stage it could not be determined whether the observed

impacts were only quantitative, or also qualitative in terms of microbial diversity. The study of the fungal ecology was designed to answer this question.

3.2. Fungal ecology

3.2.1. Variations in fungal communities during pepper post-harvest treatments

Pepper samples were analysed by PCR-DGGE to study the pepper-associated fungal communities and their dynamics during both wet and dry processes. In this way, fungal fingerprints (profiles) were generated for each sample. Fingerprints are composed of bands each of which corresponds to a unique DNA sequence. In the present study, each DNA band was considered a fungal marker **(Figure 3 and Table 1_supp**).

3.2.1.1. Inter-batch variations in fungal communities

A total of 21 different fungal DNA markers were detected from all pepper samples analysed by PCR-DGGE (**figure 3**). A general feature is that each batch (A, B and C) showed a specific fungal signature (profile) underlying significant variations in fungal communities between batches **(Figures 3 and Table 1_supp**). A1 and B1 harbored a similarity Dice index of 0.50 as they shared 50 % of their fungal markers (6 on 12). B1 and C1 samples shared 8 markers (on 12 and 11 respectively) with a similarity index of about 0.70. A1 and C1 samples shared 7 markers (on 12 and 11 respectively) with an index of about 0.61 (**Figure 4**). Six fungal DNA markers were common to all batches, three being detected in all samples (i.e. in all stages, DNA markers IV, X and XI) and another three being detected at least in one sample/stage of each batch (I, II and XIV, **Figure 3 and Table 1_supp**).

Eight batch-specific fungal markers were defined including, respectively, one, four and three markers only detected in batch A (IX), B (XII, XV, XVII and XX) and C (III, XIII and

422 XXI). This suggests that there are significant variations in fungal diversity on unprocessed
423 fresh pepper samples. Samples A, B and C were collected at different periods. Therefore
424 the observed inter-batch fungal diversity could result from seasonal or environmental
425 variations. Statistical comparisons of fungal DNA profiles displayed using PCA confirmed
426 this observation (**Figure 4**).

427 **3.2.1.2. Intra-batch variations in fungal communities**

428 Batch A samples exhibited a total of 12 different fungal markers and two types of profiles:
429 a 12-marker profile observed in the three first stages “fresh” (A1), “blanched” (A2) and
430 “blanched-sweated” (A3) samples, while a 10-marker profile (with all markers common to
431 the 12-marker profile) could be detected in "blanched-sweated-dried" (A4) and “dried” (A5)
432 samples. The drying step appeared to lower the fungal diversity either in the wet or dry
433 processes. On the other hand, the blanching and sweating steps did not show any
434 measurable effect on fungal diversity **(Figure 3)**.

435 A total of 12 different fungal markers were detected and three types of profiles could be
436 observed in batch B samples. All 12 markers were detected in the profiles of fresh (B1)
437 and blanched (B2) samples. A second 9-marker profile was observed for the blanched-
438 sweated (B3) samples while a third 8-marker profile was observed for dry pepper samples
439 from both wet and dry methods (B4 and B5 samples). The sweating step decreased fungal
440 diversity (3 markers I, II and XX no more detected) for the wet method process. The drying
441 step also had a measurable impact on reducing detectable fungal diversity in both wet
442 and dry processed pepper samples. For the dry process, one third of the fungal markers
443 (4 on 12) were no more detected in the dry pepper sample (compare B1 and B5 in **Figure**
444 **3 and Table 1_supp**).

For both batches A and B, fungal diversity tended to decrease along the processes to reach fungal profiles that appeared indistinguishable between wet and dry processes (compare samples A4 and A5; B4 and B5 in figures 3 and 4).

However, batch C samples showed a different trend. A total of 15 different markers and four different profiles could be observed. A 11-marker profile for fresh (C1) and blanched (C2) samples, then a 14-marker profile for blanched-sweated sample (C3), including the occurrence of four markers (V, VII, VIII and XVI, all detected in batch A samples) and the disappearance of one marker (XVIII). Blanched-sweated-dried (C4) samples exhibited a 13-marker profile, composed of an additional marker (III) and the loss of two markers (I and II). Dried pepper (C5) obtained from the dry method is distinguishable from C4 sample with a 12-marker profile (XIV marker non-detectable). In batch C, blanching did not show any significant effect while sweating increased fungal diversity (**Figure 3 and Table 1_supp**). Drying step showed opposite trends in the wet and dry processes. While the number of detectable fungal markers was reduced in the wet process (14 to 13 markers) the drying step increased the number of fungal markers detectable in the dry process (12 to 13). While three markers were no longer detected in the wet method samples after drying step (I and II, between A1-A4, B1-B4 and C1-C4; XVIII between C1-C4), four markers were no longer detected in the dry method samples (I, II, between A1-A5, B1-B5 and C1-C5; XIV and XVIII between C1-C5). On the opposite, one marker appeared in C4 sample (III) and five markers in C5 sample (III, V, VII, VIII, XVI), compared to C1. In all, fungal variations observed within batches (pepper sampled after different steps of the processes) were less marked than inter-batch differences (Figures 3 and 4).

3.2.1.3. Identification of fungal isolates

From all batches, 26 fungal isolates were collected, according to morphologic and microscopic criteria, thus representing the fungal diversity of all pepper samples. They were identified by ITS region sequencing and were found to belong to 15 different taxa **(Table 1)**. Among those, seven (27 %) isolates belonged to the *Colletotrichum* genus, which is known to be ubiquist. Among the other fungal groups, *Fusarium solani*, *A. niger* and *Penicillium sp.* were identified. According to Sweeney and Dobson (1998) some of these isolates are potentially mycotoxigenic: *F. solani* can produce fusaric acid, naphtoquinones and trichothecenes; *Penicillium* species can produce Ochratoxin *A (OTA).* Mckee (1995) reported the presence of *Aspergillus* spp. and *Penicillium* spp. and revealed the presence of aflatoxin B1 in various samples of black pepper. The detection of different species of *Aspergillus* in black pepper, as well as *Fusarium* sp. and *Penicillium* sp., was confirmed by El Mahgubi et al. (2013). Similarly, Yogendrarajah, Deschuyffeleer, et al. (2014) showed the presence of *A. flavus*, A*. parasiticus*, *A. niger* and *Penicillium* spp. and detected the mycotoxins aflatoxins G2, G1, B2 and B1, fumonisin, sterigmatocystin ochratoxin and citrinin in black pepper from Sri Lanka. Omafuvbe (2004) isolated *Aspergillus, Fusarium, Itersonilia, Botrydiplodia, Mucor* and *Penicillium* species from pepper sampled after different steps of the process.

3.2.1.4. Relationship between quantitative and qualitative analyses of fungal populations

Profile analysis performed by PCR-DGGE highlighted the diversity of moulds. *Colletotrichum* genus occurrence was dominant when some potentially mycotoxigenic moulds (*Fusarium, Aspergillus, Penicillium)* were present. The results obtained show higher inter-batch than intra-batch variations, illustrating the weak influence of the operation units on the fungal diversity of pepper. This result should be confirmed taking

492 into consideration that detectable fungal DNA cannot be directly related to the living or
493 active status of the corresponding fungi.
494 Blanching had a significant positive impact on fungal abundance but no measurable effect
495 on fungal diversity. This could be explained by the possible detection of DNA from
496 inactivated moulds after the blanching step. The significant negative impact of sweating
497 on fungal abundance (up to 5 log increase) is correlated with changes in fungal diversity
498 for batches B and C. The drying step had no significant impact on fungal abundance.
499 However, it showed the most significant impact on reducing fungal diversity for batches A
500 and B. Fungal markers I and II were detected in fresh and blanched samples but were no
501 longer detectable in any (wet and dry processes) dried samples of all three batches.
502 The wet and dry processes did not show any obvious selection on fungal species.
503 However, Omafuvbe (2004) revealed the impact of drying for the inactivation of *A. flavus,*
504 *F. solani* and *Botrydiplodia theobromae.* It would have been interesting to test if variations
505 in fungal communities could be observed for the revisited wet process, as this process
506 had a significant positive impact on reducing all microbial loads in the present study.

507 **3.3. Contamination by mycotoxins**

508 The results obtained for aflatoxins and ochratoxin A on 12 samples of this study
509 corresponding to three stages: fresh (stage 1), blanched-sweated-dried (stage 4) and
510 blanched-dried (stage 6) peppers - corresponding to six different batches were all found
511 to be under the detection limit (0,05 ppb). This value is at least one hundred fold below
512 the regulation limit of 5 ppb for aflatoxins B1 and 15 ppb for ochratoxin A (Commission of
513 the European Communities, 2006). These observations are in accordance with the fact
514 that the processes tested did not include storage; this step is known to be critical for the
515 production of these two mycotoxins.

516 The identification of fungal isolates revealed potential mycotoxin producers. *A. niger* that
517 was also identified as the fungal marker II (**Figure 3**) is known to be a low OTA producer
518 (Abarca, Accensi, Bragulat, Castella, & Cabanes, 2003). In addition, fungal isolates and
519 markers were identified as belonging to the *Penicillium* genus. Several species are known
520 to be ochratoxin A producers (Larsen, Svendsen, & Smedsgaard, 2001). *F. solani,* that
521 was also identified among fungal isolates, is known to produce trichothecene mycotoxins
522 (Ueno et al., 1973). There is no regulation limit for this type of contaminants in pepper.
523 Even if the tested samples came from six different batches collected over two years, it
524 would be worthwhile to confirm that fresh *P. borbonense* is free from mycotoxin
525 contaminations by testing potential contaminations of pepper collected at other periods of
526 the year including rainy periods. The processes tested did not include storage although
527 storage is a critical step regarding mould proliferation and mycotoxins synthesis
528 (Yogendrarajah, Deschuyffeleer, et al., 2014). In the present case, the absence of storage
529 step could explain the absence of OTA detection, despite the identification of one
530 mycotoxigenic mould (*A. niger*). Therefore, it would be interesting to test the impact of
531 different storage conditions (temperature, duration, humidity/aw) on mycotoxin content of
532 dried pepper.
533

534 ## 4. Conclusion

535 The level of contamination of fresh *P. borbonense* as well as fungal diversity greatly vary
536 from one batch to another, as a result of the extrinsic and intrinsic factors influencing
537 microbial colonization and growth. Initial diversity of fungal contamination is prominent for
538 the final quality of pepper in contrast to the impact of unit operations (blanching, sweating,
539 drying) and their combination. However, the processing steps, especially blanching and

sweating exhibit a strong effect on microbial loads, respectively by decreasing and increasing. These observations show clearly that the sweating step should be eliminated in pepper processing for safety reasons.

The revisited wet process (blanching + drying) which positively affected all microbial loads could be a good option for pepper transformation. In such a revisited process, more drastic blanching conditions (100 °C for 30 sec instead of 75 °C for 3 min) approved to preserve essential oil and piperine rates and limit colour degradation, would be interesting as to reduce microbial contaminations. Concerning mould proliferation and mycotoxins synthesis, the post-processing storage conditions would also be of great importance.

Acknowledgments

This work was carried out as part of the CIRAD DPP COSAQ (period: 2015–2018) funded by a grant from European Union (ERDF) and the Conseil Régional de La Réunion

References

Abarca, M. L., Accensi, F., Bragulat, M. R., Castella, G., & Cabanes, F. J. (2003). Aspergillus carbonarius as the main source of ochratoxin A contamination in dried vine fruits from the Spanish market. *Journal of Food Protection, 66*(3), 504-506. doi: 10.4315/0362-028x-66.3.504

Ban, C., Lee, D. H., Jo, Y., Bae, H., Seong, H., Kim, S. O., Choi, Y. J. (2018). Use of superheated steam to inactivate Salmonella enterica serovars Typhimurium and Enteritidis contamination on black peppercorns, pecans, and almonds. *Journal of Food Engineering, 222*, 284-291. doi: 10.1016/j.jfoodeng.2017.11.036

Chitrakar, B., Zhang, M., & Adhikari, B. (2018). Dehydrated Foods: Are they Microbiologically Safe? *Critical Reviews in Food Science and Nutrition*, 1-12. doi: 10.1080/10408398.2018.1466265

Commission of the European Communities. (2002). General principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety.

Commission of the European Communities. (2006). *Regulation 1881/2006 - Maximum levels for certain contaminants in foodstuffs*.

Dhas, P., & Korikanthimath, V. (2003). Processing and quality of black pepper - a review. *Journal of spices and aromatical crops, 12*((1)), 1-13.

El Mahgubi, A., Puel, O., Bailly, S., Tadrist, S., Querin, A., Ouadia, A., . . . Bailly, J. D. (2013). Distribution and toxigenicity of Aspergillus section Flavi in spices marketed in Morocco. *Food Control, 32*(1), 143-148. doi: 10.1016/j.foodcont.2012.11.013

Fogele, B., Granta, R., Valcina, O., & Berzins, A. (2018). Occurrence and diversity of Bacillus cereus and moulds in spices and herbs. *Food Control, 83*, 69-74. doi: 10.1016/j.foodcont.2017.05.038

Garbowska, M., Berthold-Pluta, A., & Stasiak-Rozanska, L. (2015). Microbiological quality of selected spices and herbs including the presence of Cronobacter spp. *Food Microbiology, 49*, 1-5. doi: 10.1016/j.fm.2015.01.004

Hamdouche, Y., Tagro, G., Noel, D., Kedjebo, K., Montet, & Meile, J.-C. (2015). Dynamics of microbial ecology during cocoa fermentation and drying: Towards the identification of molecular markers. *Food Control, 48*, 117-122. doi: 10.1016/j.foodcont.2014.05.031

International Pepper Community. (2015). IPC Standard Specifications for Black/ White Pepper (Whole and Ground) and Whole Dehydrated Green Pepper.

Jeevitha, G. C., Sowbhagya, H. B., & Hebbar, H. U. (2016). Application of microwaves for microbial load reduction in black pepper (Piper nigrum L.). *Journal of the science of food and agriculture, 96*(12), 4243-4249. doi: 10.1002/jsfa.7630

Jeong, S. G., & Kang, D. H. (2014). Influence of moisture content on inactivation of Escherichia coli O157:H7 and Salmonella enterica serovar Typhimurium in powdered red and black pepper spices by radio-frequency heating. *International Journal of Food Microbiology, 176*, 15-22. doi: 10.1016/j.ijfoodmicro.2014.01.011

Jin, W., Mujumdar, A. S., Zhang, M., & Shi, W. F. (2018). Novel Drying Techniques for Spices and Herbs: a Review. [Review]. *Food Engineering Reviews, 10*(1), 34-45. doi: 10.1007/s12393-017-9165-7

Keller, S. E., VanDoren, J. M., Grasso, E. M., & Halik, L. A. (2013). Growth and survival of Salmonella in ground black pepper (Piper nigrum). *Food Microbiology, 34*(1), 182-188. doi: 10.1016/j.fm.2012.12.002

Klimešová, M., Horáček, J., Ondřej, M., Manga I., Koláčková, I., Nejeschlebová, L., & Ponížil, A. (2015). Microbial contaminations of spices used in production of meat products. *Potravinarstvo® Scientific Journal for Food Industry, 9*, 154-159. doi: 10.5219/440

Koljalg, U., Nilsson, R. H., Abarenkov, K., Tedersoo, L., Taylor, A. F. S., Bahram, M., . . . Larsson, K. H. (2013). Towards a unified paradigm for sequence-based identification of fungi. *Molecular Ecology, 22*(21), 5271-5277. doi: 10.1111/mec.12481

Larsen, T. O., Svendsen, A., & Smedsgaard, J. (2001). Biochemical characterization of ochratoxin A-producing strains of the genus Penicillium. *Applied and Environmental Microbiology, 67*(8), 3630-3635. doi: 10.1128/aem.67.8.3630-3635.2001

Mckee, L. H. (1995). Microbial-Contamination of Spices and Herbs - a Review. *Food Science and Technology-Lebensmittel-Wissenschaft & Technologie, 28*(1), 1-11.

Mokshapathy, S., & Yogesh, M. S. (2013). Production and Export Performance of Black Pepper. *International Journal of Humanities and Social Science Invention, 2*(4), 36-44.

Omafuvbe. (2004). Quality Assurance of Stored Pepper (Piper guineense) using contrlled processing methods. *Pakistan Journal of Nutrition*.

Plany, M., Soltys, K., Budis, J., Mader, A., Szemes, T., Siekel, P., & Kuchta, T. (2018). Potential of high-throughput sequencing for broad-range detection of pathogenic bacteria in spices and herbs. *Food Control, 83*, 118-122. doi: 10.1016/j.foodcont.2016.12.026

Razafimandimby, H., Benard, A. G., Andrianoelisoa, H., Tsy, J., Touati, G., Levesque, A., . . . Danthu, P. (2017). Tsiperifery, the wild pepper from Madagascar, emerging on the international spice market whose exploitation is unchecked: current knowledge and future prospects. *Fruits, 72*(6), 331-340. doi: 10.17660/th2017/72.6.1

Shah, M. K., Asa, G., Sherwood, J., Graber, K., & Bergholz, T. M. (2017). Efficacy of vacuum steam pasteurization for inactivation of Salmonella PT 30, Escherichia coli O157:H7 and Enterococcus faecium on low moisture foods. *International Journal of Food Microbiology, 244*, 111-118. doi: 10.1016/j.ijfoodmicro.2017.01.003

Song, W. J., Sung, H. J., Kim, S. Y., Kim, K. P., Ryu, S., & Kang, D. H. (2014). Inactivation of Escherichia coli O157:H7 and Salmonella Typhimurium in black pepper and red pepper by gamma irradiation. *International Journal of Food Microbiology, 172*, 125-129. doi: 10.1016/j.ijfoodmicro.2013.11.017

Sweeney, M. J., & Dobson, A. D. W. (1998). Mycotoxin production by Aspergillus, Fusarium and Penicillium species. *International Journal of Food Microbiology, 43*(3), 141-158. doi: 10.1016/s0168-1605(98)00112-3

Thanh, M. D., Frentzel, H., Fetsch, A., Krause, G., Appel, B., & Mader, A. (2018). Tenacity of Bacillus cereus and Staphylococcus aureus in dried spices and herbs. *Food Control, 83*, 75-84. doi: 10.1016/j.foodcont.2016.12.027

Ueno, Y., Sato, N., Ishii, K., Sakai, K., Tsunodia, H., & Enomoto, M. (1973). Biological and Chemical Detection of Trichothecene Mycotoxins of Fusarium Species. *Applied microbiology, 25*(4), 6.

Umesha, S., Manukumar, H. M., & Raghava, S. (2016). A rapid method for isolation of genomic DNA from food-borne fungal pathogens. [journal article]. *3 Biotech, 6*(2), 123. doi: 10.1007/s13205-016-0436-4

Weil, M., Descroix, F., Shum, A. C. S., Boulanger, R., Hoarau, M., Levesque, A., & Bohuon, P. (2014). Postharvest treatments of wild pepper (*Piper* spp.) in Madagascar. *Fruits, 69*(5), 371-380. doi: 10.1051/fruits/2014025

Weil, M., Shum, A. C. S., Meot, J. M., Boulanger, R., & Bohuon, P. (2017). Impact of blanching, sweating and drying operations on pungency, aroma and color of Piper borbonense. *Food Chemistry, 219*, 274-281. doi: 10.1016/j.foodchem.2016.09.144

Yogendrarajah, P., Deschuyffeleer, N., Jacxsens, L., Sneyers, P. J., Maene, P., De Saeger, S., . . . De Meulenaer, B. (2014). Mycological quality and mycotoxin contamination of Sri Lankan peppers (Piper nigrum L.) and subsequent exposure assessment. *Food Control, 41*, 219-230. doi: 10.1016/j.foodcont.2014.01.025

Yogendrarajah, P., Devlieghere, F., Ediage, E. N., Jacxsens, L., De Meulenaer, B., & De Saeger, S. (2015). Toxigenic potentiality of Aspergillus flavus and Aspergillus parasiticus strains isolated from black pepper assessed by an LC-MS/MS based multi-mycotoxin method. *Food Microbiology, 52*, 185-196. doi: 10.1016/j.fm.2015.07.016

Yogendrarajah, P., Jacxsens, L., Lachat, C., Walpita, C. N., Kolsteren, P., De Saeger, S., & De Meulenaer, B. (2014). Public health risk associated with the co-occurrence of mycotoxins in spices consumed in Sri Lanka. *Food and Chemical Toxicology, 74*, 240-248. doi: 10.1016/j.fct.2014.10.007

Yogendrarajah, P., Vermeulen, A., Jacxsens, L., Mavromichali, E., De Saeger, S., De Meulenaer, B., & Devlieghere, F. (2016). Mycotoxin production and predictive modelling kinetics on the growth of Aspergillus flavus and Aspergillus parasiticus isolates in whole black peppercorns (Piper nigrum L). *International Journal of Food Microbiology, 228*, 44-57. doi: 10.1016/j.ijfoodmicro.2016.03.015

Web references

Blast. (November 2018). Web blast. Basic Local Alignment Search Tool. Retrieved from https://blast.ncbi.nlm.nih.gov/Blast.cgi

CBI. (May 2019). *CBI Market Information. Exporting pepper to Europe*. Retrieved from https://www.cbi.eu/market-information/spices-herbs/pepper

RASFF. (January 2019). *European Commission. Rapid Alert System for Food and Feed*. Retrieved from https://webgate.ec.europa.eu/rasff-window/portal/?event=searchResultList

FAOSTAT. (June 2019). Food and Agriculture Organization of the United Nations. Retrieved from http://www.fao.org/faostat/en/#data/QC

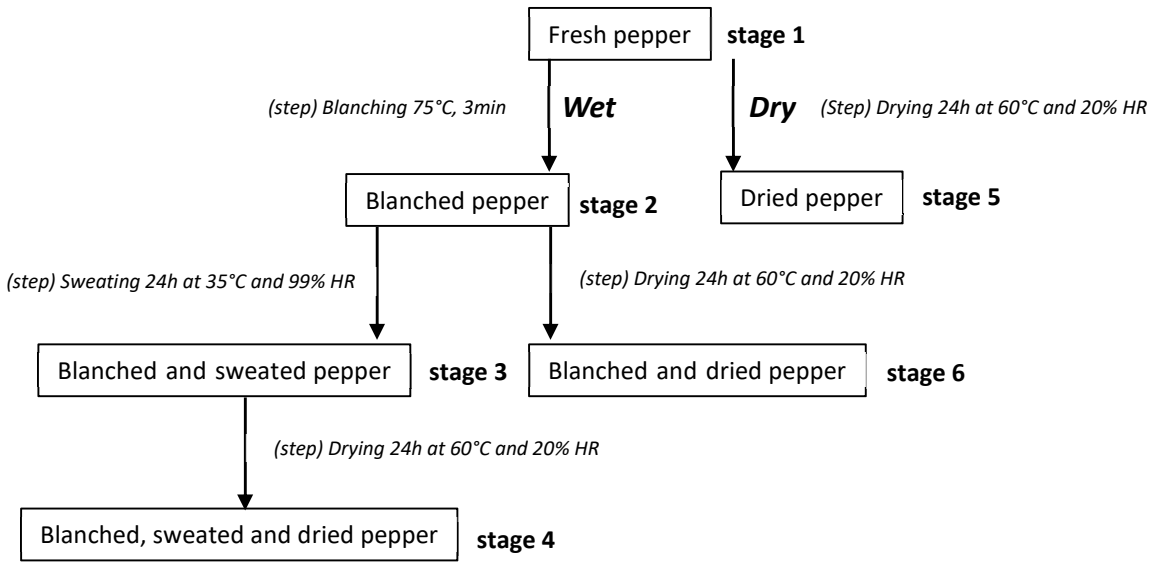
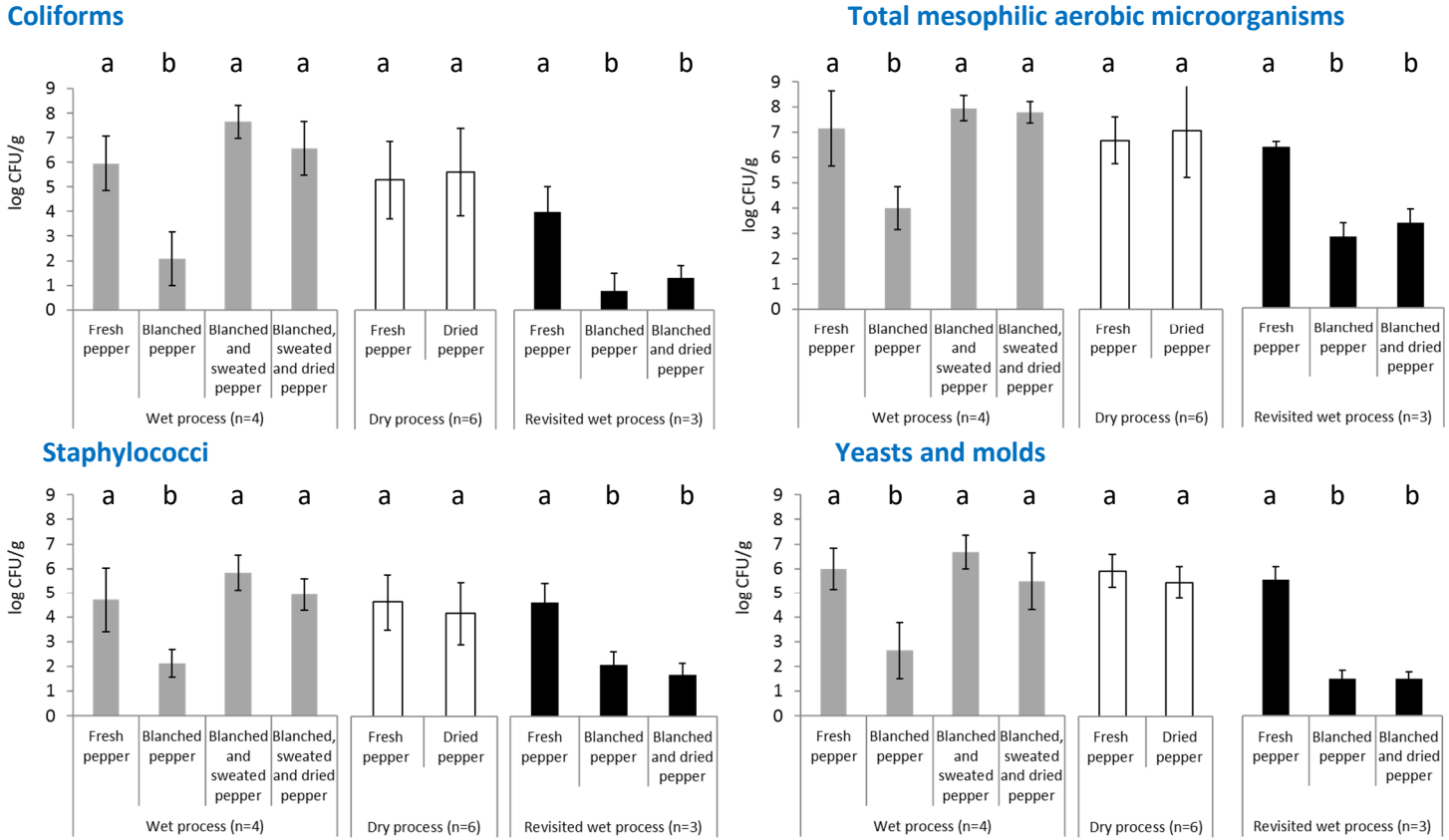

Fresh pepper
stage 1
(step) Blanching 75°C, 3min
Wet
Dry
(Step) Drying 24h at 60°C and 20% HR
Blanched pepper
stage 2
Dried pepper
stage 5
(step) Sweating 24h at 35°C and 99% HR
(step) Drying 24h at 60°C and 20% HR
Blanched and sweated pepper
stage 3
Blanched and dried pepper
stage 6
(step) Drying 24h at 60°C and 20% HR
Blanched, sweated and dried pepper
stage 4

Figure 1.

Figure 2.


Coliforms
Total mesophilic aerobic microorganisms
Staphylococci
Yeasts and molds
log CFU/g
a b a a a a a b b
Fresh pepper
Blanched pepper
Blanched and sweated pepper
Blanched, sweated and dried pepper
Dried pepper
Blanched and dried pepper
Wet process (n=4)
Dry process (n=6)
Revisited wet process (n=3)

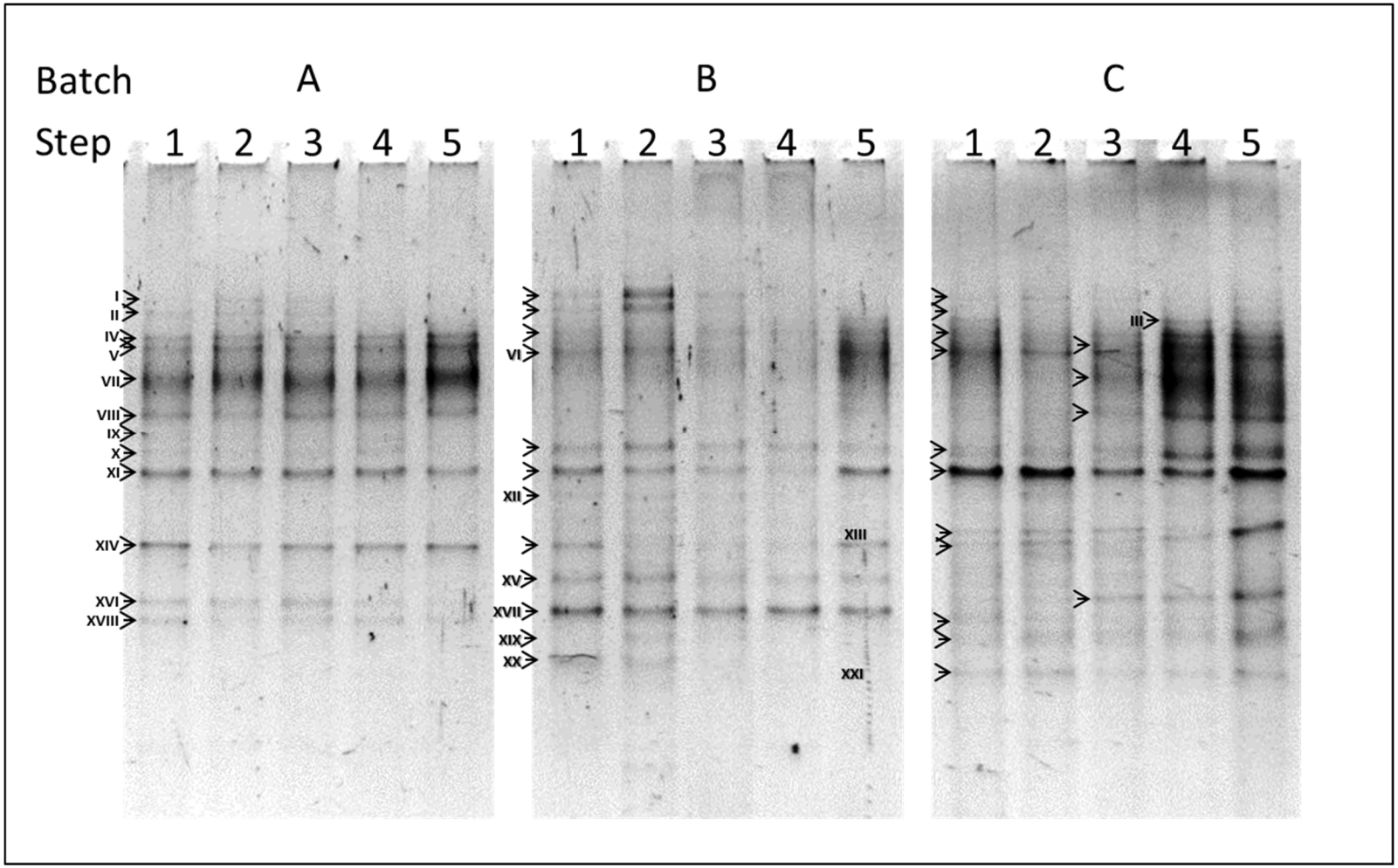

Batch
A
B
C
Step
1
2
3
4
5
I
II
IV
V
VII
VIII
IX
X
XI
XIV
XVI
XVIII
VI
XII
XIII
XV
XVII
XIX
XX
XXI
III

Figure 3.

Figure 4.

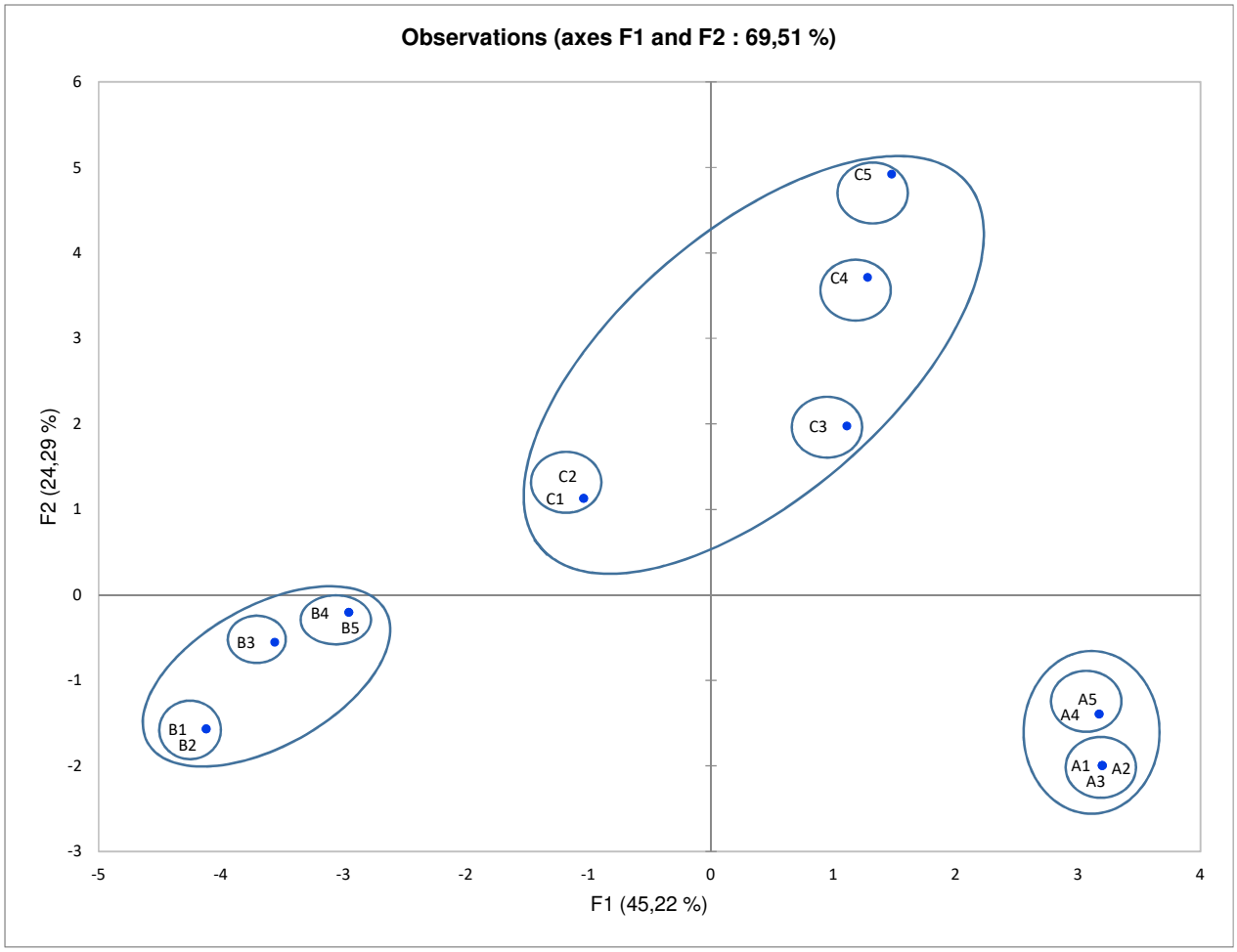

Observations (axes F1 and F2 : 69,51 %)
F2 (24,29 %)
F1 (45,22 %)
6
5
4
3
2
1
0
-1
-2
-3
-5
-4
-3
-2
-1
0
1
2
3
4
C5
C4
C3
C2
C1
B4
B5
B3
B1
B2
A5
A4
A1
A2
A3

Table 1. List of fungal species isolated from pepper

Most probable match	% identiy	% cover	E-value
Anthracocystis chrysopogonis	98	100	0.0
Aspergillus niger	100	100	0.0
Candida hawaiiana	99	91	1,00E-144
Cladiosporium sp.	100	100	0.0
Clonostachys rosea	100	100	0.0
Colletotrichum sp.	98	96	0.0
Cryptococcus sp.	99	99	0.0
Fusarium solani	100	100	4,00E-45
Hannaella oryzae	97	99	0.0
Meira geulakonigii	99	99	0.0
Penicillium sp.	100	100	0.0
Phomopsis sp.	99	100	5,00E-173
Pleosporineae (Phomea, Ampelimyces, Epicoccum)	100	100	0.0
Rhodosporidiobulus lusitaniae	99	100	0.0
Stagonosporopsis cucurbitacearum	98	99	5,00E-178

List of fungal species isolated from pepper samples at different steps of the process.
Most probable matches as well as sequence alignments parameters (percentage of identity, percentage of cover and e-values) are shown (from left to right columns).