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1 **A new cause of spoilage in goose sausages**

2

3 Lucilla Iacumin<sup>1</sup>, Marisa Manzano<sup>1</sup>, Sara Panseri<sup>2</sup>, Luca Chiesa<sup>2</sup> and Giuseppe Comi<sup>1\*</sup>

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5 <sup>1</sup>Department of Food Science, Università degli Studi di Udine, Via Sondrio 2/a, 33100 Udine,

6 Italy.

7 <sup>2</sup>Department of Veterinary Science and Public Health, Università degli Studi di Milano, Via

8 Celoria 10, 20121 Milano, Italy.

9

10 \*Corresponding author:

11 Giuseppe Comi

12 Department of Food Science, University of Udine

13 Via Sondrio 2/A

14 33100 Udine, Italy

15 phone: +39 0432 558129; mobile: +39 338 9918561; fax: +39 0432 558130;

16 e-mail: [giuseppe.comi@uniud.it](mailto:giuseppe.comi@uniud.it)

17

18 **Abstract**

19 The aim of this work was to determine the microorganisms present and to investigate their

20 metabolites that cause spoilage of many goose sausages produced in Friuli, a northeast region

21 of Italy. The defect was observed by sensorial analysis using the “needle probing” technique;

22 the spoiled sausages were unsafe and not marketable. Despite the addition of starter, the

23 microorganisms, particularly enterococci and Enterobacteriaceae, grew during ripening and

24 produced a large amount of biogenic amines; therefore, these sausages represented a risk to

25 consumers. The production of those compounds was confirmed *in vitro*. Furthermore, a

26 second cause of spoilage was attributed to moulds that grew during ripening; the fungi grew

27 between the meat and casing, producing a large amount of total volatile nitrogen, and  
28 consequently an ammonia smell was present either in the ripening area or in the sausages.  
29 This is the first description of this type of defect in goose sausages.

30

## 31 **1. Introduction**

32 In Friuli, a large number of traditional sausages are produced using raw meat of different  
33 animals: pork, beef, wild game (deer) and poultry. In particular, goose meat mixed with pork  
34 fat is used to produce sausages that are characterized by a slight sour taste and a semi-rigid  
35 consistency, which is elastic but not rubbery. These goose sausages are prepared with fresh  
36 or frozen goose meat, pork lard, NaCl (2.8% maximum) and additives (nitrates, nitrites,  
37 spices). Sugars (mostly sucrose and dextrose) and microbial starters, consisting of  
38 coagulase-negative, catalase-positive cocci (CNCPC) and lactic acid bacteria (*Lactobacillus*  
39 *sakei*), are also used in both industrial and craft manufacturing. In fact, goose sausages are  
40 essentially produced by shops and other small producers (farms, frasche, typical taverns of  
41 Friuli) or by artisanal facilities, and consequently the quality is not standardized. Indeed,  
42 considering that appropriate drying and ripening chambers or systems with the complete  
43 control of relative humidity (R.H.) and temperature do not exist in such small facilities, each  
44 lot can have its own history and be completely different from other lots. However,  
45 production of an edible product requires evaluation of the choice of raw material, the natural  
46 microclimate of the drying/ripening rooms and the aptitude of the producers.  
47 Similar to traditional sausages made with pork meat and fat, goose sausage ripening is based  
48 on microbial and tissue enzymes (Comi et al., 2005, 2000; Coppola et al., 1998). CNCPC  
49 and lactic acid bacteria (LAB) are the main microorganisms responsible for ripening (Talon,  
50 2007; Iacumin et al., 2006; Metaxopoulos et al., 2001; Garcia-Verona et al., 2000). Although  
51 these bacteria are normally present in salt and both pork and goose meat, they are often  
52 intentionally added to fat and meat mixtures for sausages as microbial starters to ensure a

53 consistent aroma and flavor, to improve quality and to reduce the length of the curing period  
54 (Iacumin et al., 2006; Comi et al., 2005, 2000; Tjener et al., 2003; Luongo et al., 2001). To  
55 meet the increasing needs of new products requested by consumers, goose sausages  
56 represent an effort to generate alternatives. Goose and chicken meat and their products are  
57 preferred and largely consumed by the public; although chicken meat is often mixed with  
58 the meat of other animals, the combination of goose meat with other meat is quite rare  
59 (Gulbaz and Kamber, 2008). Recently, the Italian population has rediscovered products  
60 based on regional recipes, and goose sausages, which are common in villages throughout  
61 Italy, constitute an important resource. Accordingly, these sausages are widely produced and  
62 appreciated by consumers, who are weary of eating traditional sausages made with pork  
63 meat. The quality of goose sausages is variable and often distinct. However, defects can  
64 occur during goose breeding and slaughtering, and sausage manufacturing, making the  
65 sausages unfit for consumption. The quality of the raw material, bacterial metabolism, as  
66 well as temperature and R.H. values during production and storage can cause these defects.  
67 In addition, inadequate ripening may also lead to unpleasant odors or tastes.  
68 A small-scale facility produced two lots (a and b) of goose sausages. During their ripening,  
69 lot b presented a defect consisting in an ammonia smell, which was confirmed by a sensorial  
70 analysis, made by non professional assessors.  
71 Therefore, the aim of this work was to study the microorganisms and the metabolites  
72 responsible for the defects and spoilages of these goose sausages.

73

## 74 **2. Material and Methods**

### 75 2.1 Evaluation and identification of the defect

76 In January, a small-scale facility located in the Friuli area produced two lots of sausages (30  
77 each) with two different batches of goose meat (Lots a and b). Five days before the end of

78 ripening, the sausages of Lot b presented an ammonia smell, which was also widespread in  
79 the ripening room area. No ammonia smell was perceived in the area of the Lot a sausages.

80

#### 81 2.1.1. *Sensorial analysis*

82 In this facility, the workers are used to tasting each lot before selling in order to value its  
83 sensorial quality. Consequently also in this case, all the sausages of both lots were evaluated  
84 by the “needle probing” technique, by ten assessors of a non-professional panel (workers at  
85 the facility). The technique involves the rapid insertion of a thin horse bone into the  
86 sausages, resulting in the perception of odors (Barbuti et al., 2003).

87 Then 5 sausages of Lot b were sliced and tasted by the panelists in order to identify the  
88 flavor and to determine the defect.

89

#### 90 2.2. *Sampling*

91 Twenty unspoiled (Lot a) and 20 spoiled goose (Lot b) sausages were analyzed. The samples  
92 were collected at the end of the ripening period (45 days). Defects of the spoiled goose  
93 sausages were found late during ripening (5 days before the end) due to an ammonia smell  
94 that was widespread in the ripening rooms. Both lots of sausages, produced the same day  
95 with two different batches of goose meat, had the following composition: goose meat 70%,  
96 lard 30%, NaCl 2.8%, KNO<sub>3</sub> 0,02%, dextrose 0.1 %, black pepper 0.002%, nutmeg 0.002%.

97 Before adding each ingredient of the recipe, a starter composed of *Staphylococcus xylosus*  
98 and *L. sakei* (1/1 ratio) was added at a final concentration of 6 log CFU/g. A starter of  
99 *Penicillium nalgiovense* was spread by aerosol (approximately 3 log/cm<sup>2</sup>) onto the casings.

100 Natural casing was used.

101 Before analysis, the spoiled and unspoiled products were washed to eliminate moulds on the  
102 casings, which were then aseptically removed. Then each sausage was sterile sliced. Four  
103 slices of each sausage were used for color determination. The remaining slices were

104 homogenized in stomacher and the homogenate was used for microbial and physico-  
105 chemical analysis and for biogenic amines and volatile compounds determination.

106

### 107 2.3. Microbiological analysis

108 Ten g of the meat homogenate was serially diluted with saline-peptone water (8 g/l NaCl, 1  
109 g/l bacteriological peptone; Oxoid, Italy, distilled water 1000 ml) in stomacher bags. An  
110 aliquot of 1 or 0.1 ml of each serial dilution was plated onto agar for counts of different  
111 groups of microorganisms: the Total Viable Count (TVC) was evaluated on Plate Count  
112 Agar (PCA, Oxoid, Italy) incubated at 30 °C for 48-72 h; LAB were grown on De Man  
113 Rogosa Sharpe agar (MRS, Oxoid, Italy) incubated at 42 °C for 48 h; yeasts and moulds  
114 were grown on Malt Agar (MA, Oxoid, Italy) incubated at 25 °C for 72-96 h and  
115 distinguished by macroscopical and microscopical examination (Samson et al., 2004);  
116 *Escherichia coli* was grown on Violet Red Bile Lactose Agar (VRBLA, Oxoid, Italy)  
117 incubated at 44 °C for 24 h; Enterobacteriaceae were grown on Violet Red Bile Glucose  
118 Agar (VRBGA, Oxoid, Italy) incubated at 37 °C for 24 h; coagulase-positive, catalase-  
119 positive cocci (CPCPC) were grown on Baird-Parker agar medium (BP, Oxoid, Italy)  
120 supplemented with egg yolk tellurite emulsion (Oxoid, Italy) incubated at 35 °C for 24-48 h  
121 and confirmed by a coagulase test; coagulase-negative, catalase-positive cocci (CNCPC)  
122 were grown on Mannitol Salt Agar (MSA, Oxoid, Italy) incubated at 30 °C for 48 h;  
123 enterococci were grown on Kanamycin Aesculin Azide Agar (KAA, Oxoid, Italy) incubated  
124 at 37 °C for 48 h; sulfite-reducing Clostridia were quantified on Differential  
125 Reinforced Clostridia Medium (DRCM, VWR, USA) incubated at 37 °C for 24-48 h in an  
126 anaerobic jar with an anaerobic kit (gas pack anaerobic system, BBL, Becton Dickinson,  
127 USA). *Salmonella* spp. were evaluated by the ISO (6579-1 2002 Cor.1:2004 Microbiology  
128 of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp.)  
129 method and *Listeria monocytogenes* by another ISO (11290-1,2:1996 Adm.1:2004.

130 Microbiology of food and animal feeding stuffs – Horizontal method for the detection of  
131 *Listeria monocytogenes*) method.

132

### 133 2.3.1. Isolation and identification of enterococci

134 One hundred colonies were randomly isolated from KAA agar plates of the spoiled sausages  
135 containing between 30 and 50 colonies and purified on PCA (Oxoid, Italy), which was  
136 incubated at 37 °C for 48 h. After purification, the colonies were subjected to Gram staining  
137 and to a catalase test. Gram-positive streptococci and catalase-negative colonies were  
138 identified by API 20 Strep, according to the manufacturer's method (BioMerieux, France).

139

### 140 2.3.2. Isolation and identification of moulds

141 One hundred mould colonies grown on MA were isolated from the spoiled goose sausages,  
142 purified and transferred onto three different agar media: Czapek Dox Agar (Oxoid, Italy), MA  
143 and Salt-Malt Agar (5% malt extract, 5% NaCl, distilled water 1,000 ml, pH 6.2; Oxoid,  
144 Italy). The moulds were identified by morphological characters by macroscopical and  
145 microscopical examination (hyphas, spores and reproduction, colour of colony and type of  
146 mycelium) according to Samson et al. (2004). The identification was confirmed by PCR-  
147 DGGE and sequencing according to method reported in Iacumin et al. (2009) briefly: The  
148 DNA of each colony was amplified by Nested PCR (2 step amplification). Each amplicon was  
149 run in acrilamide gel (DGGE), then it was excised by gel cutting tips and subjected to a re-  
150 amplification with the same primers without GC clamp. The product was cloned into pGEM-  
151 T easy vector (Promega, Milan, Italy), following the instructions of the manufacturer. The  
152 insert of the appropriate clone was sequenced by a commercial facility (Eurofins MWG  
153 GmbH, Martinsried, Germany). Sequence comparisons were performed using the Blast  
154 program (Altschul et al., 1997).

155

156

157 *2.4. In vitro reproduction of the defect by moulds*

158 One hundred g of the unspoiled meat homogenates of goose sausages was boiled in water  
159 (200 ml) for 1 h. After boiling, the mixture was filtered through cotton wool and sterilized at  
160 115 °C for 15 minutes. The sterilized mixture was adjusted to 300 ml with distilled sterile  
161 water and distributed among 10 Petri plates (30 ml each). A loop of each isolated mould  
162 species was inoculated in the plates (one strain per plate), which were incubated for 7 days  
163 at 25 °C. Three replicates of each strain were performed. At the end of the incubation period,  
164 each mixture was filtered and analyzed for the presence of TVB-N (Total Volatile Basic  
165 Nitrogen), biogenic amines and acetic acid.

166

167 *2.5. Total Volatile Basic Nitrogen ( TVB-N), pH, acetic acid and color determination*

168 TVB-N was evaluated by the Pearson (1976) method; briefly: “The TVB-N is released by  
169 boiling the sample directly with magnesium oxide, which also prevents volatile acids from  
170 distilling over into the boric acid. The distillate is titrated with standard acid”. The pH of the  
171 product was measured directly by inserting a pH meter probe (Radiometer, Denmark) into  
172 the sample. The water activity ( $A_w$ ) was determined using a Hygromer AWVC (Rotronic,  
173 Italia). Acetic acid was detected using an Acetic acid kit (R-Biopharm, Italy) according to  
174 the manufacturer’s instructions. The color was measured using a Minolta Chromameter CR-  
175 200 (Singapore) and the CIE Lab system. After calibration with standard white tiles, the  
176 chromameter was positioned perpendicular to the patty surface, and 10 different positions  
177 were evaluated for each sample immediately after slicing. The evaluated parameters were  
178  $L^*$ ,  $a^*$  and  $b^*$ .  $L^*$  describes the white intensity or brightness, with values ranging from 0  
179 (black) to 100 (white). The  $a^*$  value describes the redness ( $a^* > 0$ ), and  $b^*$  describes the  
180 yellowness ( $b^* > 0$ ). The final value was expressed as the respective average of ten  
181 measurements.



182

183 *2.6. Biogenic amines in vitro and in spoiled and unspoiled sausages*

184 All the identified strains were tested for biogenic amine production on agar media,  
185 according to the Bover-Cid and Holzapfel (1999) method. Ten out 20 of spoiled and  
186 unspoiled meat homogenates were randomly sampled in order to detect the biogenic amines  
187 using the method proposed by Eerola et al., (1993) briefly: “Amines were separated using  
188 HPLC (HPLC Jasco 2089 quaternary pump, AS 2057 autosampler; Jasco, Ishikawa-cho,  
189 Japan). The separation was carried out by gradient elution with 0.1 mol L<sup>-1</sup> ammonium  
190 acetate/acetonitrile on a reverse-phase column (Spherisorb ODS-2; 5 µm, 125 × 4 mm;  
191 Waters Corporation, Milford, MA, USA) at a flow rate of 1 mL min<sup>-1</sup> using UV/VIS 2075  
192 detector operating at 254 nm for biogenic amine (Jasco, Ishikawa-cho, Japan).

193

194

195 *2.7. Volatile compound determination*

196 Ten out 20 of spoiled and 10 out 20 of unspoiled meat homogenates were randomly  
197 collected and analyzed for the presence of volatile compounds using SPME-GC-MS and a  
198 Finnigan Trace DSQ (Thermo Scientific Corporation, USA) with a Rtx-Wax capillary  
199 column (length 30 m x 0.25 mm id.; film thickness 0.25 µm; Restek Corporation, USA),  
200 according to the method reported by Chiesa et al. (2006). The volatile compounds were then  
201 identified by comparing the spectra obtained experimentally with spectra available in the  
202 Commercial Wiley library and an in-lab library. The results represent the average of all 10  
203 samples.

204

205 2.8. *Statistical analysis*

206 The values of the various parameters were compared using a one-way analysis of variance.  
207 Averages were compared with Tukey's honest significant test using the StatGraphics  
208 software package from Statistical Graphics (Rockville, Maryland).

209

210 **3.0 Results**

211 The ten assessors of the non-professional panel (workers at the facility) confirmed the  
212 ammonia smell in lot b by "needle probing" technique. Moreover, after tasting lot b, some of  
213 the panel members suffered from headaches (3 of 10), facial flushing (7 of 10) and bright  
214 red rash (7 of 10), symptoms that are typical in consumers who eat foods rich in biogenic  
215 amines. Consequently the sausages were also analysed for biogenic amine presence. Indeed  
216 all the panelists perceived also a vinegar odor.

217 The microbial and physico-chemical characteristics of the unspoiled and spoiled goose  
218 sausages are shown in Tables 1 and 2, respectively. The PCA and MRS counts are typical of  
219 sausages, and there was no significant difference ( $p > 0.05$ ) between the spoiled and  
220 unspoiled sausages. In particular, the PCA counts of both the sausages were approximately 6  
221 log CFU/g, and the MRS counts were approximately 8.6 log CFU/g. Yeasts and CNCPC  
222 concentrations also did not differ significantly ( $p > 0.05$ ) between the spoiled and unspoiled  
223 sausages. The yeast counts were less than 3 log CFU/g, and the CNCPC concentration was  
224 approximately 6 log CFU/g; these concentrations are also typical of traditional Italian  
225 sausages made with pork meat. The moulds, the enterococci and the Enterobacteriaceae  
226 concentrations differed significantly between the spoiled and unspoiled samples ( $p < 0.05$ ).  
227 The values of enterococci and moulds in the spoiled sausages were 3 logs higher than in the  
228 unspoiled samples. The high level of moulds in the spoiled sausages was not due to  
229 contamination during sampling because the casings of both groups were first brushed and  
230 washed. In the spoiled goose sausages, the moulds probably grew in the space between the

231 meat and casing. Indeed in some part of the spoiled sausages, between the casing and the  
232 meat, a white mycelium was observed, consequently this can prove the higher level of  
233 moulds in spoiled sausages. In the spoiled goose sausages, Enterobacteriaceae reached log  
234  $4.0 \pm 0.4$  CFU/g and were log  $2.0 \pm 0.1$  CFU/g in the unspoiled samples. CCPPC, sulfite-  
235 reducing Clostridia and *E. coli* were present at concentrations below the detection limit of  
236 the method (LOD < 10 CFU/g). *Listeria monocytogenes* was present at less than 100 CFU/g,  
237 and *Salmonella* was absent in a 25-g sample, according to REG. EEC 2073/05 (15/11/2005,  
238 L 338/1). The physico-chemical parameters demonstrated significant differences for the  
239 levels of TVB-N and histamine ( $p < 0.05$ ). Indeed, the spoiled products had a high  
240 concentration of histamine ( $415.25 \pm 115.01$  mg/kg), a level higher than the limit proposed  
241 for fish and fish products (REG. EEC 2073/05) and considered unsafe for consumers. In  
242 contrast, the values of histamine in the unspoiled sausages were low, approximately 80 times  
243 less than in the spoiled sausages. The cadaverine concentration of the spoiled sausages  
244 reached  $339.3 \pm 31.40$  mg/kg, 10 times more than the concentration found in the unspoiled  
245 sausages. Putrescine, spermine and spermidine concentrations were below the limit of  
246 detection (< 1 mg/kg). Moreover, the TVB-N value of the spoiled sausages was  
247 approximately twice that of the unspoiled sausages. The pH and  $A_w$  values of both sausage  
248 lots were similar at a level of 6.3 and 0.92, respectively. The brightness of the spoiled  
249 sausages, as expressed by the evaluation of  $L^*$ , was not significantly different ( $p > 0.05$ )  
250 from that of the unspoiled sausages (Table 2). Moreover, parameters  $a^*$  and  $b^*$  showed no  
251 changing, and the observed differences were not significant ( $p > 0.05$ ). It is clear that natural  
252 oxidative phenomena induced by microorganisms are involved with spoilage, and it is well  
253 known that heterofermenting LAB release small amounts of hydrogen peroxide and  
254 hydrogen sulfide, which produce discoloration and greening (Comi and Iacumin, 2012).  
255 However, it was not observed any greening or discoloration in the spoiled samples.

256 Table 3 shows the ability to produce biogenic amines of 100 enterococci isolates issued  
257 from spoiled sausages *in vitro*. Isolates were identified as *Enterococcus faecium* (N=70) and  
258 *Enterococcus faecalis* (N=30), two species which are typical sugar fermenting but able to  
259 decarboxylate amino acids and produce amines. As observed *in vitro*, all the isolated strains  
260 produced histamine, and 60 out of 70 *E. faecium* and 25 out 30 *E. faecalis* strains produced  
261 cadaverine. Conversely, only 10 out of 70 (both species combined) produced tyramine; 6 out  
262 of 70 *E. faecium* and 4 out of 30 *E. faecalis* were able to produce putrescine, spermine and  
263 spermidine. These data justify the low concentration of these biogenic amines found in the  
264 sausages (below the detection limit).

265 The moulds isolated belonged to 3 different species: *Penicillium nalgiovense* (85 isolates),  
266 which predominated, being inoculated as a starter; *P. chrysogenum* (8 isolates) and *P.*  
267 *viridicatum* (7 isolates) were also present. The compounds found *in vivo* (TVB-N, acetic  
268 acid) were produced by all the mould strains *in vitro* (Table 4), though no biogenic amines  
269 were produced by the moulds *in vitro* (Table 4). Either TVB-N or acetic acid contributed to  
270 the off-odor and off-flavor of the spoiled goose sausages, as perceived by the panelists.

271 The volatile compounds and their concentrations are shown in Table 5, which also shows the  
272 retention times, individual compounds and means of concentrations expressed in µg/kg  
273 product from ten analytical runs. The data demonstrate that the same substances were  
274 present in both sausage groups tested. The concentrations of these substances were  
275 determined by their amount relative to the internal standard (ethylpropionate).

276 For a better interpretation of the results obtained from the headspace, the 37 observed  
277 substances were divided into 6 classes: aldehydes (2), ketones (6), esters (1), hydrocarbons  
278 (7), alcohols (7), carboxylic acids (10), and others (4). Some classes and compounds are  
279 typical of well-ripened dry sausages. The concentrations of only 13 out of 37 compounds  
280 exhibited a significant difference between the spoiled and unspoiled goose sausages ( $p <$   
281 0.05). In fact, the amounts of 2-butanone, 2-pentanone, formic acid pentylester, 2,4-

282 dimethylexane, 3-ethylexane, ethylbenzene, 2-butanol, 1-propanol, 2-pentanol, acetic acid,  
283 2-methylpentanoic, butanoic acid and 3-methylpentanoic acid in the spoiled sausages were  
284 significantly different from the unspoiled ones. In addition, the differences in the  
285 concentrations of volatile organic compounds between the spoiled and unspoiled samples  
286 only partially influenced the off-odor. In fact, it appears that only the concentrations of  
287 TVB-N and acetic acid were important to the production of the off-odor and off-flavor of the  
288 spoiled product.

289

#### 290 **4.0. Discussion**

291 The spoilage of the goose sausages examined was due to the large growth of enterococci and  
292 moulds. As shown, the values of moulds and enterococci in the spoiled sausages were 3 logs  
293 higher than the values of these microbial groups in the unspoiled samples. The compounds  
294 responsible for the spoilage mainly included biogenic amines and TVB-N. The former are  
295 common in fermented meats and other foods (Roig-Sagués et al., 1999; Gardini et al., 2008),  
296 and the most important are histamine, putrescine, cadaverine, tyramine, tryptamine,  
297 spermine, and spermidine. These compounds originate from the decarboxylation of amino  
298 acids, and consequently foods rich in proteins are a potential risk (Hernández-Jover et al.,  
299 1997). The effects observed in some of the panelists during the tasting and the sensorial  
300 analysis are justified by the presence of histamine and cadaverine, over 300 mg/kg. Biogenic  
301 amines are often present in Spanish and Italian sausages. (Hernandez-Jover et al., 1996,  
302 1997; Roig-Sagués et al., 1999; Bover-Cid et al., 2000). However, their data do not agree  
303 with ours because in the present work, only cadaverine, and histamine were found, whereas  
304 a large amount of tyramine (600 mg/kg), putrescine (up to 450 mg) were found in those  
305 earlier studies. Tyramine production, as well as histamine, depends on lactobacilli and  
306 enterococci (Suzzi and Gardini 2003; Buňková et al., 2009; Pircher et al., 2007; Ladero et  
307 al., 2012; Marcobal et al., 2012; Gardini et al., 2008), but in this work, only a few of the

308 isolated enterococci were able to produce tyramine. Tyramine was not found consequently it  
309 must be excluded that lactobacilli could have contributed to the production of biogenic  
310 amines, considering that only lactobacilli possess the tyrosine decarboxylase enzyme (Suzzi  
311 and Gardini, 2003; Buňková et al., 2009; Pircher et al., 2007).

312 LAB, CNCPC and CCPPC lack histidine decarboxylation capability (Landete et al., 2007,  
313 2008), whereas LAB and staphylococci cannot produce cadaverine and putrescine (Pircher  
314 et al., 2007). Consequently, it appears that only Enterobacteriaceae and enterococci could be  
315 responsible for the production of biogenic amines, particularly histamine, in fermented foods  
316 (Suzzi and Gardini, 2003; Gardini et al., 2008), considering that Enterobacteriaceae  
317 decarboxylase activity can continue after the cell autolysis (Rossi et al., 2011; Kanki et al.,  
318 2007). In the tested spoiled goose sausages, the Enterobacteriaceae could have had a limited  
319 activity, being present at level of 4 log CFU/g. Conversely, the number of enterococci in the  
320 spoiled goose sausages was higher, up to 6 log CFU/g; therefore, it is possible to speculate  
321 their main role in biogenic amine production. Usually in traditional Italian sausages  
322 Enterococci grow during the first days of fermentation, but their concentration never  
323 exceeds 3.2 log CFU/g at the end of ripening (Comi et al. 2000, 2005; Comi and Iacumin,  
324 2013). It is possible to speculate that in the spoiled samples starters cultures could not  
325 inhibit the growth of enterococci and enterobacteriaceae, resulting in defects. Also  
326 contaminated raw meat, kept at 8 °C, could be a cause of the production of biogenic amines  
327 by Enterobacteriaceae and enterococci (Bover-Cid et al., 2000). The spoiled goose sausages  
328 in the present study were produced with different batches of goose meat compared to the  
329 unspoiled sausages, and this could further explain the spoilage. The raw meat used could not  
330 be analysed, however, considering that both the lots of sausages were produced with the  
331 same recipe and technology, it is possible to speculate that the batch of meat used for the  
332 spoiled sausages could have been dubious and more contaminated than the batch of meat  
333 used for the unspoiled sausages. The lack of tyramine, spermine and spermidine do not

334 represent a novelty. Indeed, the presence or absence of the different types of biogenic  
335 amines appears to depend on the microorganisms that grow in the product, and this has been  
336 confirmed in many studies by various authors (Montel et al., 1999; Parente et al., 2001;  
337 Gardini et al., 2008). In the spoiled goose sausages, the presence of only two types of  
338 biogenic amines was confirmed by the *in vitro* test, in which a large number of  
339 *Enterococcus* strains were able to produce histamine and cadaverine, but only a few  
340 tyramine, spermidine, spermine and putrescine .

341 Both the spoiled and unspoiled sausages were properly dried as demonstrated by the pH and  
342  $A_w$  values, by the LAB and CNCPC concentration, that were not significantly different ( $p >$   
343  $0.05$ ) and these values should be regarded as normal for meat products (Tjener et al., 2003;  
344 Comi et al., 2000, 2005; Gounadaki et al., 2008). In particular the pH remained high despite  
345 the concentration of acidifying bacteria (MRS counts) and was similar to that usually found  
346 in sausages without defects (Coppola et al., 1998; Comi et al., 2000, 2005).

347 The presence of a higher concentration of TVB-N in the spoiled goose sausages ( $208.3 \pm$   
348  $9.5$ ) than in the unspoiled ( $p < 0.05$ ) was demonstrated by the presence of mould mycelium  
349 between the meat and casings. All the isolated mould species were able to produce either  
350 TVB-N and acetic acid *in vitro*, and this confirms the higher amount of TVB-N present in  
351 the spoiled goose sausages and consequently the ammonia smell of the ripening area and of  
352 the sausages. The ammonia smell is due to the high TVB-N concentration, which in well-  
353 ripened Italian sausages is typically less than  $100 \text{ mg N}/100 \text{ g}$  (Cattaneo et al., 2003; Comi  
354 and Iacumin, 2013), as found in the unspoiled goose sausages. However, it is also possible  
355 that enterococci, Enterobacteria, LAB and CNCPC could have worked together with moulds  
356 in TVB-N production, considering that CNCPC and LAB can metabolize amino acids and  
357 produce TVB-N, as it has been demonstrated by various authors (Seefeldt and Weimer,  
358 2000; Joffraud et al., 2001; Comi and Iacumin, 2013).

359 Moulds could also play an important role on biogenic amines production of enterococci.  
360 Their proteolytic activity releases amino acids (Kamenic et al., 2014; Trigueros et al., 1996),  
361 which are the main precursors for biogenic amines production.

362 The volatilome of both the goose sausages was almost similar. The analysis was performed  
363 on both sausages, and the data demonstrated that organic acids, alcohols, hydrocarbons,  
364 ketones and esters are related to intense bacterial activity and to fresh meat. In particular,  
365 only 9 types of compounds of the spoiled sausages were present at higher concentrations  
366 than those found in the unspoiled sausages. Conversely, many authors have found much  
367 more volatile compounds between unspoiled and spoiled pork meat sausages (Meyner et al.,  
368 1999; Comi et al., 2000; Luongo et al., 2001; Tjener et al., 2003; Cantoni et al., 2005). In the  
369 spoiled goose sausages, there was a strong presence of acetic acid derived from LAB and  
370 moulds. The total amount of ketones, alcohols and volatile fatty acids in the spoiled was  
371 higher than that in the unspoiled goose sausages. As expected, the concentrations of some  
372 individual molecules produced by fermentation or oxidation were increased in the spoiled  
373 products. However, differences in the concentrations of volatile organic compounds between  
374 the spoiled and unspoiled samples only partially influenced the off-odor, conversely TVB-N  
375 and acetic acid concentration did it, as demonstrated by sensorial analysis.

376 A total of 10 carboxylic acids were detected, and these compounds can all originate from the  
377 activity of lipolytic enzymes. The concentrations of acetic acid, 3-methylpentanoic, butanoic  
378 acid and 3-methylpentanoic acid in the spoiled products significantly differed from the  
379 unspoiled samples ( $p < 0.05$ ). Acetic acid can also originate from the metabolism of sugars  
380 and lipids by moulds (Motilva et al., 1993; Comi and Iacumin, 2013). Alcohol compounds  
381 result from aldehydes reduction, sugar fermentation, oxidative decomposition of lipids and  
382 Strecker degradation of amino acids (Ardò, 2006; Smit et al., 2009; Flores et al., 1997). The  
383 concentrations of 2-butanol, 2-pentanol and 1-propanol significantly differed between the  
384 spoiled and unspoiled goose sausages ( $p < 0.05$ ). Nevertheless, their presence did not cause



385 any pungent and alcoholic characteristics in the spoiled goose sausages. Only 2 aldehydes  
386 were detected, and their concentrations did not significantly differ in the spoiled and  
387 unspoiled samples ( $p > 0.05$ ). The low number of aldehydes can be explained by their  
388 reduction into alcohols or their oxidation into carboxylic acid (Comi and Iacumin, 2013;  
389 Flores et al., 1997). Ketones (2-butanone and 2-pentanone) concentrations were significantly  
390 different ( $p < 0.05$ ), but they did not lead to unpleasant solvent smells (Flores et al., 1997;  
391 Ardò, 2006). Among the hydrocarbons found, 2,4-dimethylexane, 3-ethylhexane and  
392 ethylbenzene concentration was significant different between the spoiled and unspoiled  
393 products ( $p < 0.05$ ). In the unspoiled products, a great proportion of hydrocarbons were not  
394 transformed into aldehydes and ketones, and this was confirmed by the lower concentration  
395 of both the compounds with respect to hydrocarbons. Only one ester was detected, and its  
396 concentration was significant different in the spoiled and in unspoiled goose sausages ( $p <$   
397  $0.05$ ). However, the lack of esters is unusual because esters are produced by the  
398 fermentation of LAB, CNCPC and other bacteria (Stahnke, 1994). Finally no sulfur and  
399 pyrazine compounds were detected.

400

## 401 **Conclusion**

402 The growth of enterococci and Enterobacteriaceae caused the production of high  
403 concentrations of histamine and cadaverine. Indeed, both the amines were responsible for  
404 the headaches, facial flushing and bright red rashes in some of the panelists. EFSA (2011)  
405 has declared that a food is safe if it contains less than 50 mg/kg of histamine, whereas up to  
406 400 mg/kg in food is considered absolutely unsafe (Silla Santos, 1996; Ienistea, 1973).  
407 Despite significant differences in the levels of many volatile compounds between the spoiled  
408 and unspoiled goose sausages, it appeared that the off-odor perceived through the “needle  
409 probing” technique and the off-flavor perceived by tasting were mainly due to the high  
410 concentration of TVB-N. In addition, the high concentration of acetic acid produced a

411 perception of a light vinegar taste. The *in vitro* test demonstrated that moulds grown between  
412 the meat and casing in the spoiled products, produced a high TVB-N concentration.  
413 Consequently, moulds were the main organisms responsible of the off-odor of the spoiled  
414 sausages and enterococci and Enterobacteriaceae for the production of biogenic amines.  
415 Finally, it could be concluded that the control of both microbial groups in the raw meat will  
416 permit the production of safe goose sausages.

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### 557 **Conflict of interest**

558 None of the authors of this paper has a financial or personal relationship with other people  
559 or organisations that could inappropriately influence or bias the content of the paper.

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**Table 1: Microbial characteristics of unspoiled and spoiled goose sausages.**

<b>Microorganism</b>	<b>Unspoiled</b>	<b>Spoiled</b>
PCA counts	6.0 ± 0.1a	5.7 ± 0.2b
MRS counts	8.6 ± 0.2a	8.6 ± 0.1a
Yeasts	2.0 ± 0.3a	2.3 ± 0.2a
Moulds	2.1 ± 0.1a	5.1 ± 1.5b
Enterococci	3.1 ± 0.3a	6.7 ± 0.2b
<i>Escherichia coli</i> *	< 10	< 10
Enterobacteriaceae	2.1 ± 0.1a	4.0 ± 0.4b
CNCPC <sub>1</sub>	6.1 ± 0.2a	5.8 ± 0.3a
CCPPC* <sub>2</sub>	< 10	< 10
Clostridia H <sub>2</sub> S+*	< 10	< 10

581 Legend: PCA: Plate Count Agar; MRS: De Man Rogosa Sharpe; Data in  
582 log CFU/g - \* CFU/g; CNCPC<sub>1</sub>: Coagulase Negative Catalase Positive  
583 Cocci; CCPPC<sub>2</sub>: Coagulase Positive Catalase Positive Cocci; Data represent  
584 the means ± standard deviations of the total samples; Mean with the same  
585 letters within the same lane (following the values) are not significantly  
586 differently (P< 0.05).

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**Table 2: Physico-chemical parameters of unspoiled and spoiled goose Sausages.**

<b>Parameter</b>	<b>Unspoiled</b>	<b>Spoiled</b>
pH	6.3 ± 0.2a	6.3 ± 0.1a
Aw	0.92 ± 0.01a	0.92 ± 0.01a
TVB-N <sup>^</sup>	80.2 ± 10.1a	208.3 ± 9.5b
Histamine	5.6 ± 1.8a	415.26 ± 115.01b
Putrescine	< L.O.D.	< L.O.D.
Cadaverine	32.1 ± 3.2a	339.3 ± 31.4b
Spermine	< L.O.D.	< L.O.D.
Spermidine	< L.O.D.	< L.O.D.
Tyramine	< L.O.D.	< L.O.D.
L*	38.2 ± 6.0a	36.3 ± 4.1a
a*	16.0 ± 1.2a	17.6 ± 1.6a
b*	1.2 ± 0.4a	1.7 ± 0.9a

598 Legend: Data TVB-N: <sup>^</sup>Total Volatile Basic Nitrogen mg N/100 g;  
599 Biogenic amines: mg/kg; < L.O.D.: Limit of quantitation (1.7 to 22.5 µg/L);  
600 Data represent the means ± standard deviations of the total samples; Mean  
601 with the same letters within the same lane (following the values) are not  
602 significantly differently (P< 0.05).  
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**Table 3: Strains of Enterococci isolated and biogenic amines production**

<b>Biogenic amines</b>	<i>E. faecium</i>	<i>E. faecalis</i>
Hystamine	70	30
Putrescine	6	4
Spermine	6	4
Spermidine	6	4
Cadaverine	60	25
Tyramine	10	10
Total isolated	70	30

618 Legend: Number of positive strains  
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629 **Table 4: Identification of the strains isolated from the spoiled goose sausages and their**  
630 **production of TVB-N and acetic acid.**  
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<b>Identification</b>	<b>No. of isolates</b>	<b>TVB-N/acetic acid/biogenic amines production</b>	<b>Source<sup>b</sup></b>
<i>Penicillium nalgiovense</i>	85	+/+/-	JQ434685.1
<i>Penicillium chrysogenum</i>	8	+/+/-	JQ434684.1
<i>Penicillium viridicatum</i>	7	+/+/-	JQ388751.1

Legend: TVB-N, total volatile basic nitrogen; +, positive production; - < LOD; <sup>b</sup>The accession number of the closest related species found by a BLAST search.

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**Table 5: Volatile compounds of spoiled and unspoiled goose sausages**

RT	Compounds	Unspoiled (n=10)	s.d.	Spoiled (n=10)	s.d.
<b>Aldehydes</b>					
22,82	dodecanale	0.16	0.02a	0.11	0.03a
24,2	Benzaldehyde	0.01	0.01a	0.02	0.01a
	<b>total</b>	<b>0.19</b>		<b>0.14</b>	
<b>Ketones</b>					
2,35	acetone	0.28	0.13a	0.30	0.03a
3,29	2-Butanone	1.56	0.48a	2.91	0.07b
11,62	2-Pentanone	1.93	0.15a	0.38	0.02b
14,4	2-Heptanone	0.26	0.11a	0.52	0.18a
18,03	2-nonanone	9.20	0.40a	9.88	0.32a
20,21	3-methyl-2-heptanone	0.08	0.01a	0.05	0.02a
	<b>total</b>	<b>13.30</b>		<b>14.04</b>	
<b>Esters</b>					
17,30	Formic acid pentylester	0.09	0.01a	0.17	0.02b
	<b>total</b>	<b>0.09</b>		<b>0.17</b>	
<b>Hydrocarbons</b>					
1,56	2,4-Dimethylexane	50.23	0.33a	42.69	0.02b
2,27	3-Ethylexane	0.67	0.07a	1.32	0.07b
4,72	2,2,4,6-Methylheptane	1.02	0.10a	0.09	0.13a
8,22	Octane	0.06	0.03a	0.09	0.03a
11,41	ethylbenzene	0.11	0.03a	0.02	0.03b
12,48	Benzene	10.09	0.46a	11.40	1.80a
17,64	1-methylbenzene	0.36	0.01a	0.44	0.12a
	<b>total</b>	<b>62.54</b>		<b>56.05</b>	
<b>Alcohols</b>					
6,96	2-Butanol	1.85	0.40a	3.62	0.19b
7,55	2-Pentanol	0.46	0.14a	0.72	0.03b
10,70	2-Methyl-1-propanol	0.19	0.04a	0.24	0.02a
13,31	1-Propanol	0.64	0.03a	0.98	0.09b
15,79	3-Methyl-1-Butanol	0.86	0.01a	0.79	0.22a
20,35	1-Exanol	0.49	0.02a	0.38	0.06a
25,25	2,3-Butanol	0.06	0.01a	0.09	0.02a
	<b>total</b>	<b>4.55</b>		<b>6.82</b>	
<b>Volatile fatty acids</b>					
22.52	Acetic acid	6.11	0.03a	10.40	0.02b
23,41	2-Methylpentanoic	0.11	0.02a	0.06	0.01b
24,57	Propanoic acid	0.24	0.00a	0.29	0.05a
26,32	2-Methylpropionic	0.04	0.01a	0.08	0.03a
26,49	Butanoic acid	0.10	0.02a	0.19	0.04b
27,35	3-Methylpentanoic acid	0.09	0.01a	0.17	0.02b
28,56	Diethylacetic acid	0.05	0.01a	0.07	0.02a
30,65	Hexanoic acid	0.07	0.01a	0.07	0.07a

<b>32,19</b>	2-Ethylheptanoic acid	0.02	0.01a	0.01	0.01a
<b>33,27</b>	Octanoic acid	0.01	0.01a	0.01	0.01a
	<b>total</b>	<b>6.85</b>		<b>11.24</b>	
	<b>Miscellanea</b>				
<b>5,78</b>	Acetonitrile	0.24	0.12a	0.10	0.14a
<b>16,54</b>	Furan	0.06	0.01a	0.10	0.05a
<b>30,91</b>	2-Methoxyphenol	0.05	0.01a	0.07	0.07a
<b>32,73</b>	3-Methylphenol	0.01	0.01a	0.01	0.01a
	<b>total</b>	<b>0.36</b>		<b>0.27</b>	

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**Legend:** Data (mean of 10 samples) expressed in µg/Kg; Sum of compounds; RT: Retention time. Data represent the means ± standard deviations (S.D.) of the total samples; Mean with the same letters within a row (following the values) are not significantly differently (P< 0.05).