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253

Influence of Indigenous Starter Cultures on the Free Fatty Acids Content During Ripening in Artisan Sausages Produced in the Basilicata Region

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Summary

The influence of indigenous starter cultures on the free fatty acids content during ripening of »salsiccia«, a typical dry fermented sausage produced in the Basilicata region, was studied. Three batches of »salsiccia« were produced using different starter mixtures (*Lactobacillus sakei* G20 and *Staphylococcus xylosus* S81; *L. sakei* G20 and *S. xylosus* S142; *L. sakei* G20 and *S. xylosus* S206), while the control batch was produced without a starter. The amounts of free fatty acids present in the samples at the end of the ripening period were not significantly different, suggesting that the lipolytic enzymes naturally occurring in meat could play a predominant role in the free fatty acids release. Oleic and linoleic acids were present in the highest concentrations, while only small quantities of short chain fatty acids were detected, with acetic acid being the most representative one.

Key words: lipolysis, free fatty acids, ripening, sausages, starter

Introduction

Artisan sausages show a wide microbial and organoleptic variability, and in order to standardise their characteristics, the utilisation of starter cultures has been proposed (*1,2*). However, due to the importance of microbial lipolytic activity, and to avoid any undesirable flavour variability, the selection of indigenous starter cultures is of the utmost importance in order to obtain the desired and typical characteristics of artisan sausages (*3*). Starter cultures usually consist of lactic acid bacteria, fundamental agents of the fermentation process, associated with staphylococci or micrococci, that are considered responsible for flavour development in dry fermented sausages, mainly through their lipolytic activity (*4–6*). Montel *et al.* (*7*) have reviewed the importance of microstaphylococci and lactic acid bacteria for the aroma development, reporting that *S. xylosus* and *S. carnosus* can produce esters and other important aromatic compounds; furthermore, staphylococci, through their high nitrate reductase and catalase activities, can also prevent the formation of off-flavours. Microstaphylococci can also contribute to the stabilisation of colour during ripening, and to the decomposition of peroxides (*8*).

In sausages, lipolysis has been reported to be due to tissular enzymes (fat and lean tissues) (*7*) or to the activity of microorganisms, and microstaphylococci are generally considered to be the main cause for lipid break-

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down in fermented sausages (*9,10*). However, Sorensen and Jakobsen (*11*) reported that microstaphylococci have little lipolytic activity in the conditions normally found in sausages.

»Salsiccia« is a popular and artisan dry fermented sausage produced in the Basilicata region, made of minced pork meat mixed with fat, salt, spices, with the addition of dry pepper powder, and stuffed into natural casings. The sausages are then dried and left to ripe for 2–4 weeks (*12*). Recently, several authors have studied the microflora of the »salsiccia« (*3,12–14*) and microstaphylococci have been found to be present in high concentrations, ranging from 10^6 to 10^8 ufc/g. Among 51 strains of Gram-positive, coagulase-negative cocci isolated from the samples of »salsiccia«, *Staphylococcus xylosus* was the dominant species and only five strains tested for lipolysis of pork fat resulted in being positive (*15*).

Free fatty acids (FFA) resulting from the lipolytic processes play an important role in the development of the typical flavour in dry fermented sausages, and are, in addition, precursors of esters, aldehydes, ketons, lactones and alcohols, that can contribute considerably to the final sensory profile of the sausages (*10,16–18*).

Due to the importance of the FFA fraction for the development of the typical flavour, in this work the influence of indigenous starter cultures on the FFA content in »salsiccia« produced in the Basilicata region was investigated.

Materials and Methods

Starter preparation

The starter utilised in the production of the »salsiccia« was prepared from strains isolated from artisan sausages of the Basilicata region (Southern Italy), and selected on the basis of their lipolytic activity, determined according to the method proposed by Berry (*19*). The strains are part of the microorganism collection of the University of Basilicata.

Cultures of *Lactobacillus sakei* G20, grown in MRS (Man Rogose Sharp) agar, and of *Staphylococcus xylosus* S81, S142 and S206, grown in BHI (Brain Heart Infusion), were centrifuged and the resulting pellets were washed with a physiological solution (0.85 % NaCl) before being suspended in 7.5 % aqueous solution of lactose.

Sample preparation

From 20 kg of pork meat kept refrigerated at 2 °C, four batches of sausages (5 kg each) were produced with a standard formulation differing only in the starter cultures added. The sausages were prepared by comminuting the meat in a manual grinder (diameter of the plate holes, 5 mm) and adding the following ingredients: NaCl (2.5 %), sodium nitrite (E 250) (160 mg/kg), potassium nitrate (E 252) (160 mg/kg), dextrose (0.4 %), milk powder (0.76 %), ascorbic acid (E 300) (0.12 %), fine minced pepper (0.1 %) and dill seeds (0.2 %). The ingredients were mixed in a vacuum kneading machine and the mixture was then stuffed into artificial casings (40

mm diameter) after being inoculated with different starters at the concentration of 10^6 cfu/g of raw material. The following batches were prepared: (A) control, no starter added; (B) meat inoculated with *L. sakei* G20 and *S. xylosus* S81; (C) meat inoculated with *L. sakei* G20 and *S. xylosus* S142; (D) meat inoculated with *L. sakei* G20 and *S. xylosus* S206. Afterwards, the sausages, about 400 g each, were transferred into a drying chamber and then kept at 15 °C and 80–85 % of relative humidity for 15 days until the end of ripening.

Samples for the analyses were taken immediately after stuffing and after 2, 7 and 15 days of ripening.

Microbiological analyses

Sampling was carried out according to the procedure described by Amato *et al.* (*12*). Lactic acid bacteria (LAB) were isolated on MRS, microstaphylococci on Mannitol Salt Agar (MSA), Enterobacteriaceae on Violet Red Bile Agar (VRBA).

FFA determination

Lipid extraction procedure

Lipid extraction was performed according to the method of Lencioni *et al.* (*20*) modified as follows: 1 g of sausages was put in a screw-capped glass centrifuge tube and a mixture of 4 g anhydrous Na_2SO_4, 1 mL of MeOH and 0.3 mL of 0.44 M TCA was added. The mixture was then extracted three times with 3 mL of diethyl ether/hexane (1:1) mixture. Each time the slurry was heated at 40 °C, shaken for 5 min using a Vortex mixer and clarified by a short centrifugation (2 min, 2500 x g, 4 °C). Lipid extracts were combined and brought up to volume with hexane in a 50-mL volumetric flask before the successive step of FFA purification. Each extraction was made in triplicate.

Isolation of FFA

Separation of FFA fraction from the other lipid classes was obtained by utilising aminopropyl SPE columns (500 mg) (Supelco, Milan, Italy). After conditioning the column with 10 mL of hexane, the whole lipid extract was loaded onto the column and eluted drop by drop. The neutral lipids were washed out by using 4 mL of chloroform/hexane (1:1) mixture, and the FFA fraction was then eluted using 4 mL of diethyl ether containing 2 % formic acid. After the recovery of the FFA fraction, the column was washed with 10 mL of methanol and reconditioned with 10 mL of hexane before being reutilised.

Gas chromatographic analysis

FFA analysis was conducted using a Carlo Erba Mega 5160 gas chromatograph (Carlo Erba, Milan, Italy) equipped with a FID and a column injector. The sample (1 µL) was injected onto a Nukol wide-bore column (15 m ×0.53 mm ID) having a film thickness of 0.5 µm (Supelco). The carrier gas (helium) flow rate was set at 20 mL/min, the detector temperature at 220 °C and detector attenuation at 10. The oven temperature was programmed to rise from 90 to 195 °C by 8 °C/min and total analysis time was 50 min.

Different FFA were tentatively identified and quantified using the external standard method, comparing the areas of the peaks with those obtained by injecting a standard solution containing all the acids with even carbon number from 2:0 to 18:3 and 3:0 acid. Starting from a stock solution in hexane, containing about 0.5 mg/mL of each acid, five different working solutions were prepared and utilised to build the calibration curve at following ratios: 1/2.5, 1/5, 1/10, 1/25 and 1/50.

Each sample was analysed in duplicate and the peak areas were calculated by a Carlo Erba Mega Series integrator.

pH determination

The pH determination was performed on each sample by inserting a spear tip electrode of 3 mm diameter (Orion Research, Beverly, USA), connected to a pH-meter 8517 (Hanna Instruments, Padova, Italy), in three different positions of each sausage and the result was expressed as the mean of the three determinations.

Reagents

All the chemicals were of the suitable analytical grade and purchased from Carlo Erba. The FFA standards were obtained from Fluka (Milan, Italy) and substrates of microorganisms growth from Oxoid (Unipath Ltd, Basingstoke, Hampshire, England).

Statistical analysis

The data were elaborated by the statistical package Statistica for Windows 5.0 (Statsoft Inc., Tulsa, OK, USA).

Results and Discussion

The concentration of Enterobacteriaceae, lactic acid bacteria (LAB) and microstaphylococci during the ripening process of »salsiccia« is shown in Fig. 1. Initially, the Enterobacteriaceae level (10^3–10^4 cfu/g) was similar in all the batches, but then in the inoculated samples the number of these microorganisms decreased sharply and after 7 days of ripening no longer viable cells of Enterobacteriaceae were detected. Conversely, in the control, the Enterobacteriaceae were present even after 7 days and only at the end of ripening their number was undetectable. This different behaviour could be ascribed to the presence of the LAB utilised in the starter culture; in fact, several species of lactobacilli have shown the capability of inhibiting the growth of many organisms involved in food spoilage (*21*). Besides the initial difference due to the addition of a starter, lactic acid bacteria counts were not different in the control and in the inoculated samples throughout the whole ripening period, reaching a final concentration of 10^8 cfu/g.

Microstaphylococci counts in the inoculated samples did not show substantial differences during ripening, remaining in the range of 10^7–10^8 cfu/g. In the control, these microorganisms were found at much lower concentrations, and only after 7 days their number reached values similar to those of the other batches.

The measured pH values during ripening did not vary greatly among batches, although, as expected, the
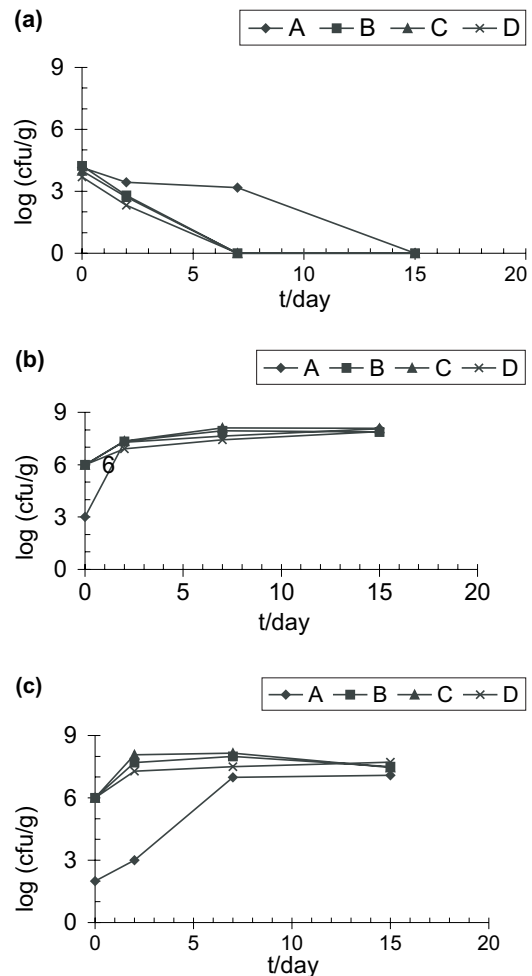

Fig. 1. Evolution of microflora during ripening of salsiccia.
a) Enterobacteriaceae; b) Lactic acid bacteria; c) Microstaphylococci A = control; B = salsiccia with *L. sakei* G20 and *S. xylosus* S81; C = salsiccia with *L. sakei* G20 and *S. xylosus* S142; D = salsiccia with *L. sakei* G20 and *S. xylosus* S206

drop in the first days of fermentation was more rapid and pronounced in the samples with added starter cultures. Initially, the measured pH of all batches was around 5.9 and then decreased to values around 5.0 after 7 days of ripening; a slight increase of pH was then observed after 14 days, with values around 5.4 in all the samples. This could be ascribed to the production of ammonia and other basic compounds arising from proteolytic activity. These results are within the wide interval of values recorded for this type of product, with pH ranging from 4.5 in American and Spanish salami, to values greater than 6.0 in Italian and Hungarian salami (*17*).

In Table 1 the concentration of total long chain FFA found in the experimental »salsiccia« samples during ripening is presented. In all the batches there was a progressive increase of the FFA production over time, but some differences could be pointed out among the treatments. Samples A, C and D showed initially an almost linear increase of the FFA content; however, after 7 days the amount of FFA increased sharply, reaching a final concentration of about four folds higher than that recorded previously. Conversely, in the samples inocu-

Table 1. Evolution of total long chain FFA content (mg/kg) during ripening in »salsiccia«

Batches	Time/day			
	0	2	7	15
A	n.d.	220.4 ± 11.3	613.2 ± 13.2	2180.4[a] ± 41.3
B	n.d.	96.4 ± 4.6	399.5 ± 16.8	1145.3[b] ± 36.7
C	14.3 ± 1.2	208.3 ± 3.6	623.4 ± 21.6	2189.7[a] ± 42.8
D	16.7 ± 0.9	136.3 ± 0.6	558.2 ± 19.2	2133.2[a] ± 32.6
LSD	–	–	–	381

A = control; B = salsiccia inoculated with *L. sakei* G20 and *S. xylosus* S81; C = salsiccia inoculated with *L. sakei* G20 and *S. xylosus* S142; D = salsiccia inoculated with *L. sakei* G20 and *S. xylosus* S206; Values are the mean of three determinations and are reported with the standard deviation; LSD: least significant difference; values with different superscripts are significantly different (p<0.05); n.d.: not detected

lated with *L. sakei* G20 and *S. xylosus* S81, the rate of FFA production was more regular during the whole ripening time, and the final FFA concentration resulted in about the half the amount of that measured in the other samples.

While bacteria belonging to the genus *Kocuria* or *Staphylococcus* are considered to be the main cause for lipid breakdown in dry fermented sausages (*9,10*), in the »salsiccia« produced without the addition of starter the final FFA concentration was not statistically different from those measured in the inoculated samples C and D. Similar results have been reported in the literature: aseptically prepared batches of sausages inoculated with lipolytic strains of microstaphylococci had an FFA content similar to that found in non-inoculated batches, where the lipolytic processes could not be attributed to the microstaphylococci, present at low levels (*22–24*). Moreover, Molly *et al.* (*25*) studying lipolysis in Belgian sausages showed that the addition of antibiotics to meat did not change the amount of FFA formed, whether in the presence of lipolytic micrococcaceae or not. Only for »salsiccie« of batch B, inoculated with *L. sakei* G20 and *S. xylosus* S81, the total FFA content was significantly different from that measured in the other sausages, reaching a lower final value of about 1150 mg/kg (Table 1). Low FFA concentration found in these samples could be related to the amine oxidase activity of *S. xylosus* S81 (*26*); the amine oxidase activity has been shown to increase the catalase activity (*27*) and, therefore, the fatty acids could be oxidised to lower molecular weight moieties (aldehydes, ketones, alcohols, *etc.*) by the O_2 deriving from the hydrogen peroxide produced during the amine oxidation.

In Fig. 2 relative percentages of the long chain FFA after 15 days of ripening are presented. In comparison with the control sample, the addition of starters did not cause substantial modifications of the relative percentages of FFA. Oleic acid was the predominant fatty acid in all the samples, as reported in the literature for other dry fermented sausages at comparable ripening times (*17,28*), present at levels between 41.9 and 44.2 %, followed by linoleic (20.0–21.6 %), palmitic (19.4–21.0 %) and stearic acid (8.5–9.7 %). Myristic and palmitoleic acids were the FFA found in the smallest proportions, with percentages ranging from 1.6 to 2.2 % and from 4.6 to 5.4 %, respectively.

The evolution of the short chain FFA content during the ripening process is shown in Table 2. The amounts of short chain FFA found in the samples during the
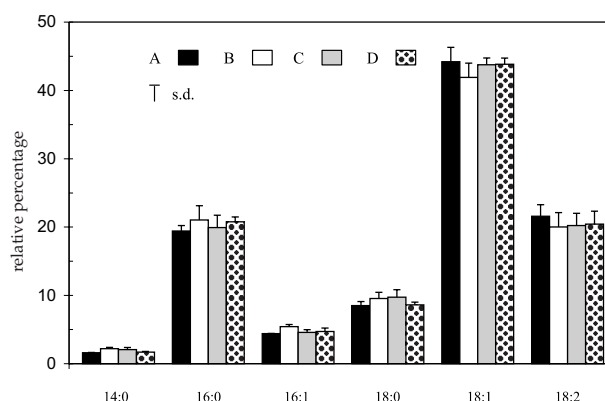

Fig. 2. Long chain FFA in salsiccia: relative percentages after 15 days of ripening. Values are the mean of three determinations. s.d.: standard deviation. A = control; B = salsiccia with *L. sakei* G20 and *S. xylosus* S81; C = salsiccia with *L. sakei* G20 and *S. xylosus* S142; D = salsiccia with *L. sakei* G20 and *S. xylosus* S206

whole ripening period were low, and this could be ascribed to the fact that in salsiccia, as in other dry sausages, there is a lack of an adequate substrate for the release of these FFA *via* lipolysis, because meat and subcutaneous adipose tissue glycerides are mainly esterified with long-chain fatty acids (*16*). Furthermore, short chain FFA are mainly produced by heterofermentative processes (*29*), but also carbohydrate metabolism, lipolysis and aminoacid catabolism may contribute to the formation of these acids (*30*).

Acetic acid was the FFA produced in the highest amounts during ripening and these data are in agreement with those reported in literature for similar products (*2,17,28,31–33*), where the amount of acetic acid increased noticeably throughout the whole experimental period. However, while its final concentration was similar in the sausages of batches A, B and D, in the samples inoculated with *L. sakei* G20 and *S. xylosus* S102 its level resulted to be about 30 % higher than that found in the other batches. Acetic acid content has been reported to be weakly correlated with acid odour (*7*), but Stanke (*34*) found a positive correlation between this moiety and a sourish note to salami odour; furthermore, Berdagué *et al.* (*4*) indicated that acetic acid contributes to dry sausage aroma.

All the other short chain FFA were either not detected or found in very low amounts during ripening. Following an initial decrease of concentration below the

Table 2. Evolution of short chain FFA content (mg/kg) during ripening

FFA*	Batches **	Time/day			
		0	2	7	15
2:0	A	151.1 ± 7.3	176.5 ± 5.3	270.4 ± 7.2	330.3 ± 14.5
	B	35.2 ± 3.5	142.7 ± 6.2	241.7 ± 10.9	397.2 ± 4.3
	C	169.5 ± 6.6	280.2 ± 7.4	360.5 ± 18.1	474.7 ± 4.3
	D	161.1 ± 7.3	202.2 ± 6.5	300.3 ± 8.2	357.7 ± 11.5
3:0	A	n.d.	n.d.	19.5 ± 1.0	11.5 ± 1.1
	B	15.3 ± 0.4	n.d.	n.d.	14.6 ± 0.6
	C	n.d.	n.d.	11.5 ± 0.8	12.1 ± 0.9
	D	n.d.	n.d.	12.2 ± 0.9	10.5 ± 0.6
4:0	A	n.d.	n.d.	14.4 ± 0.8	16.5 ± 0.4
	B	14.2 ± 0.5	n.d.	16.1 ± 0.6	14.7 ± 0.8
	C	13.2 ± 0.7	17.2 ± 0.9	15.4 ± 0.2	14.3 ± 0.5
	D	14.3 ± 0.5	11.4 ± 0.6	15.7 ± 0.4	17.2 ± 0.8
6:0	A	n.d.	n.d.	15.5 ± 0.6	12.2 ± 0.4
	B	9.4 ± 0.8	n.d.	10.1 ± 0.3	13.9 ± 0.9
	C	9.2 ± 0.6	n.d.	16.3 ± 0.9	10.6 ± 0.6
	D	9.7 ± 0.4	n.d.	12.6 ± 0.7	10.5 ± 0.4
8:0	A	n.d.	n.d.	n.d.	22.4 ± 0.8
	B	n.d.	n.d.	n.d.	11.9 ± 1.2
	C	n.d.	n.d.	n.d.	18.6 ± 0.9
	D	n.d.	n.d.	n.d.	13.9 ± 0.7
10:0	A	n.d.	n.d.	n.d.	20.4 ± 0.8
	B	n.d.	n.d.	n.d.	15.7 ± 1.1
	C	n.d.	n.d.	n.d.	20.3 ± 0.9
	D	n.d.	n.d.	n.d.	18.6 ± 0.6

FFA*: Values are mean of three determinations and are reported with the standard deviation
Batches**: A = control; B = salsiccia with *L. sakei* G20 and *S. xylosus* S81; C = salsiccia with *L. sakei* G20 and *S. xylosus* S142; D = salsiccia with *L. sakei* G20 and *S. xylosus* S206; n.d.: not detected

minimum detectable amount, butyric and caproic acids were found in all the samples after 7 days of ripening, and afterwards their levels did not show major changes. Only in the case of butyric acid a tendency to the decrease of concentration could be pointed out during the second week of ripening, and this is consistent with the data reported by other authors (*2,17,31,32*) for similar products.

It has been observed (*17*) that in systems in which lactic acid bacteria develop great activity, such as vacuum-packed meats, accumulation of *n*-butyric acid is more closely associated with the lipases from meat than with microbial activity. In dry fermented sausages butyric acid is, therefore, probably formed by the activity of meat endogenous lipases on short chain fatty acid glycerides during the fermentation phase when pH and temperature conditions are optimum.

The concentration of propionic acid was negligible during the first week, while the presence of octanoic and decanoic acids was detectable only at the end of the ripening period. In any case, no major differences in the short chain FFA concentration profile could be pointed out among the different treatments. Despite a higher absolute content of the FFA, Zalacain *et al.* (*2*) reported a similar relative distribution among the short chain FFA in dry fermented sausages after 15 days of ripening.

Conclusions

The data have shown that in artisan sausages, such as »salsiccia«, the addition of different starter cultures did not increase the FFA release from the matrix. Taking also into account the different species of fatty acids (short and long chain, saturated and unsaturated) no influence of the starter cultures, even if of indigenous origin, could be pointed out and the results suggest that the lipolytic processes may be mainly due to the activity of the native lipases present in meat.

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Utjecaj nativnih starter kultura na udjel slobodnih masnih kiselina tijekom zrenja domaćih kobasica proizvedenih u regiji Basilicata

Sažetak

Ispitan je utjecaj nativnih starter kultura na udjel slobodnih masnih kiselina tijekom zrenja tipične suhe fermentirane kobasice proizvedene u talijanskoj regiji Basilicata. Proizvedene su tri šarže kobasica koristeći različite smjese starter kultura (*Lactobacillus sakei* G20 i *Staphylococcus xylosus* S81; *L. sakei* G20 i *S. xylosus* S142; *L. sakei* G20 i *S. xylosus* S206), a za kontrolu je proizvedena kobasica bez starter kulture. Količina slobodnih masnih kiselina u uzorcima na kraju vremena zrenja nije se bitno razlikovala, što bi mogao biti dokaz da lipolitički enzimi, prirodno prisutni u mesu, imaju odlučujuću ulogu u oslobađanju slobodnih masnih kiselina. Najviše je bilo uljne i linolne kiseline, a nađene su samo male količine kratkolančanih masnih kiselina od kojih je najznačajnija octena kiselina.