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Research Note

Population Dynamics of the Constitutive Biota of French Dry Sausages in a Pilot-Scale Ripening Chamber†

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ABSTRACT

The population dynamic of constitutive biota on 84 samples belonging to two different types of French fermented dry sausages during the ripening process in a pilot-scale ripening chamber was investigated. Samples were analyzed in three steps of their production: fresh product, first drying stage, and finished product. In addition, 180 strains of lactic acid bacteria were identified using a miniaturized biochemical procedure of characterization. In general, the number of lactic acid bacteria that evolved during the ripening process of French dry sausages increased during the first days of the process after which the number of these organisms remained constant at approximately 8 log CFU/g. *Lactobacillus sakei* and *Pediococcus pentosaceus,* bacteria added as starter, were the dominant species. *Pediococcus urinaeequi, Pediococcus acidilactici*, and particularly *Lactobacillus curvatus* were also present. Finally, we have to take into account that the controlled conditions of the pilot plant generally contribute to the homogenization of the behavior of the starter biota.

In the south of Europe, the production processes used in the Mediterranean area (France, Italy, and Spain, among others) are generally different from those used in northern Europe. Traditional Mediterranean products have a ripening step, which allows the development of molds and yeasts on the surface *(11).* The Dry-sausages Ripening Improvement Project (DRIP), financed by the European Union, performs research into systems to improve the techniques for preparing matured sausages, with special attention being paid to the steps of drying and ripening *(2).* One of the aims of our study was to determinate the microbial behavior of the products elaborated under pilot-scale ripening chamber conditions.

The lactic acid bacteria (LAB) commonly used in starter cultures belong to the species *Lactobacillus sakei, Lactobacillus curvatus, Lactobacillus plantarum, Pediococcus acidilactici,* and *Pediococcus pentosaceus.* Starter cultures may also include the gram-positive, catalase-positive cocci *Kocuria varians* (formerly *Micrococcus varians*), *Staphylococcus carnosus* subspp. *carnosus* and *utilis*, and *Staphylococcus xylosus.* At the end of the fermentation period, LAB are generally the dominant biota *(11)*, whereas microstaphylococci are a major component of the secondary microflora of fermented dry sausage *(5).*

The ability of LAB to lower the pH of the sausage by producing acid from sugars leads to the development of desirable organoleptic properties, prevents the growth of pathogens, and ensures the stability and safety of the final product *(1).* Historically, fermented meat products have been considered innocuous *(9).* To improve the fermented dry sausage elaboration process, the microorganisms that participate in the process must be known and controlled. This article describes the population dynamics of LAB for two types of French fermented dry sausages during the ripening process in a pilot-scale ripening chamber. Also, 180 LAB strains were identified.

MATERIAL AND METHODS

Sampling. Two different types (A and B) of French fermented dry sausages from a pilot-scale ripening chamber were investigated. Two lots of each type (A1, A2, B1, and B2) were analyzed in three steps of their production: fresh product (0 days), first drying stage (7 days), and finished product (30 days for type A and 60 days for type B). The sausage of type A had the following composition: lean, 76.5%; fat, 20%; curing salts and spices, 2.6%; sugars, 0.9%; and starter culture, *L. sakei, S. carnosus*, and *S. xylosus*. The sausage of type B had the following composition: lean, 74.1%; fat, 21%; curing salts and spices, 3.1%; sugars, 1.8%; and starter culture, *P. pentosaceus, S. carnosus*, and *S. xylosus*. The ingredients were mixed in a cutter until the particle size was 7 mm. The mixture was filled into natural casings with a diameter of 55 to 60 mm (type A) or 100 mm (type B). After stuffing, the products were fermented and air dried. Drying was performed under pilot-scale ripening chamber conditions (75 to 80% relative humidity and 12 to 14°C temperature), which were controlled during the entire process. A total of 84 samples were investigated: 2 sausages × 2 lots × 3 samples × 7 replicates.

For microbiological analysis, the casing was aseptically removed, and 10-g samples of each sausage were taken aseptically, transferred to sterile pouches, and homogenized for 2 min with

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90 ml of sterile buffered peptone water (0.1% [wt/vol]; Oxoid, Unipath Ltd., Basingstoke, England) as diluent using a stomacher (Lab Blender, model 4001, Seward Medical, London, England). Appropriate dilutions of the sample homogenates were prepared and inoculated in duplicate in growth media to estimate LAB counts.

Isolation and enumeration of LAB. The LAB count was verified on deMan Rogosa Sharpe (MRS) agar (Oxoid) incubated under anaerobic conditions at 30°C for 72 h. The results were expressed as log CFU per gram. A total of 180 LAB colonies were selected from the counting plates (representative colonies of each sample of finished product were randomly selected). These 180 strains were subcultured in MRS agar (Oxoid) with subsequent inoculation of pure cultures in MRS broth, incubated under anaerobic conditions at 30°C, and maintained at 4°C for further characterization. Working cultures were also kept at −23°C in MRS broth (Oxoid) with 50% glycerol (Merck, Darmstadt, Germany).

Physiological and biochemical identification. Colonies on MRS agar were examined for cell morphologic features, Gram reaction, and catalase test. Only the gram-positive, catalase-negative isolations *(15)* were further identified. The carbohydrate fermentation was determined according to Sharpe *(18)* but using the miniaturized method described by Jayne-Willians *(10)* and adapted by Schillinger and Lücke *(17)*. The carbohydrates included were L (+) arabinose, D (+) cellobiose, galactose, glucose, inuline, lactose, maltose, mannitol, D (+) melezitose, melibiose, D (+) raffinose, ribose, saccharose, D (+) trehalose, and D (−) sorbitol. The test sugars were added to the basal medium (MRS without glucose and meat extract but with 0.004% bromocresol red) as filter-sterilized solutions to a final concentration of 0.5 (wt/vol). The miniplates were incubated at 30°C under anaerobic conditions and were investigated after 2 and 5 days. Growth at pH 3.9 was tested according to Santos et al. *(15)*. Growth in the presence of 7 and 10% (wt/vol) NaCl was performed in MRS broth for 5 days *(15)*. Growth at different temperatures was observed in MRS broth after incubation for 3 days at 15 and 45°C and 7 days at 4°C *(15, 17)*. Hydrolysis of arginine was tested in modified MRS broth without meat extract but with 0.3% (wt/vol) arginine and 0.2% (wt/vol) sodium citrate (Merck) replacing diammonium citrate (Merck) *(17)*. Ammonia was detected after 3 days of incubation using the Nessler reagent (Merck). Gas CO_2 production from glucose was observed in MRS broth containing Durham tubes without ammonium citrate *(15, 17)*. The production of H_2S was determined on Rogosa agar (Merck) *(8)*. The production of H_2O_2 was tested on MRS agar supplemented with 0.75% (wt/vol) manganese dioxide (Sigma-Aldrich Co., Madrid, Spain) and 0.5% (wt/vol) xantam gum (Sigma) and incubated under anaerobic conditions at 30°C for 4 days. Production of dextrane (slime) from sucrose was determined on MRS agar in which glucose was replaced by 5% (wt/vol) sucrose *(7)*. The production of acetoin was investigated using the Voges-Proskauer test, according to Reuter *(12)*.

Statistical analysis. Analysis of variance of data was performed using SAS statistical software (SAS Institute Inc., Cary, N.C.).

RESULTS AND DISCUSSION

In sausage type A, the starter culture includes a population of *L. sakei* of approximately 6 log CFU/g. However, the LAB numbers detected in the fresh product are slightly higher, reaching 7.78 and 7.03 log CFU/g for lots A1 and A2, respectively. Total LAB numbers probably included not only the starter bacteria added but also the population coming from the raw meat mixture, which is naturally contaminated with many kinds of organisms *(11)*. For this type of fermented dry sausage (lot A1), the higher LAB numbers are found in ripened product (after 30 days), whereas for lot A2, important growth is observed after 7 days, slightly decreasing those values later.

The differences in LAB numbers were statistically significant in fresh product and finished product for lots A1 ($P < 0.01$) and A2 ($P < 0.05$). Furthermore, an increasing evolution of these counts above all in the first step of the ripening process was observed. Cocolin et al. *(4)* reported similar results in Italian fermented sausages. On comparing the counts obtained in both lots at different steps of the process, we reported statistically significant differences for fresh product ($P < 0.05$) and first drying stage ($P < 0.05$), whereas for finished product these differences are not statistically significant. This can be explained by the fact that although at the beginning of the process the products could be of higher variability, the end product becomes more homogeneous at the final step.

In sausage type B, the initial population of LAB was approximately 6 log CFU/g (6.05 and 5.98 for lots B1 and B2, respectively), an increasing trend in LAB numbers was also observed (higher than all at the beginning of the process), and statistically significant differences between initial and final counts were found for lot B1 ($P < 0.001$) and lot B2 ($P < 0.01$). González-Fernández et al. *(6)* reported a similar LAB evolution in Spanish fermented dry sausage, with a stronger increase in counts on the first days of ripening. In this way, similar to our results, counts in fresh product were slightly higher than 6 log CFU/g and close to 8 log CFU/g at the end of the process.

Statistically significant differences between counts obtained in both lots at the different steps of the ripening process were not found. This can be explained by the more homogeneous mixture of raw meat in type B. However, in the product after fermentation, the differences appear to be significant in both lots ($P < 0.01$) and the finished products ($P < 0.05$).

Regarding the identification of the strains isolated from finished product to determinate genera and species (Table 1), results of analyses were interpreted according to the different taxonomic studies *(3, 8, 16, 17, 19)*. As in the study by Reuter *(13)*, lactobacilli seem to be especially difficult to identify, because current methods are based on the properties of organisms isolated from different environments and the high variability of these strains in their ability to ferment carbohydrates.

All of the strains tested fermented glucose without producing gas. This is one of the main criteria used as discriminatory by Schillinger and Lücke *(17)*. For these authors, 89% of the strains of *L. sakei* grow at pH 3.9. In our case, only a 28% growth occurred under these conditions (Table 1). These results are similar to those obtained by Samelis et al. *(14)*.

On the other hand, results obtained for acetoin produc-

TABLE 1. *Biochemical characteristics of LAB isolated from finished fermented dry sausages (% of strains positive for each character)*[a]

	Type A					Type B							
	Lot A1			Lot A2		Lot B1				Lot B2			
	Lactobacillus sakei	*L. curvatus*	*Pediococcus acidilactici*	*L. sakei*	*L. curvatus*	*P. pentosaceus*	*L. sakei*	*L. curvatus*	*P. urinaeequi*	*L. pentosaceus*	*L. sakei*	*L. curvatus*	*P. acidilactici*
Gram reaction	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase test	−	−	−	−	−	−	−	−	−	−	−	−	−
Fermentation of:													
Arabinose	10	−	37	38	−	+	28	−	58	64	45	2	28
Celobiose	63	79	+	21	65	64	40	+	+	90	34	68	+
Galactose	90	+	+	74	15	91	+	+	+	88	23	82	+
Inuline	−	−	−	−	−	28	−	15	−	37	−	−	−
Lactose	18	−	72	16	−	63	60	30	50	46	−	−	18
Maltose	5	16	−	16	−	75	20	66	+	64	−	−	−
Mannitol	−	−	−	−	−	18	−	−	43	−	−	−	−
Melezitose	−	−	−	−	−	−	−	15	−	−	−	−	−
Melibiose	28	−	−	85	−	64	+	−	−	55	+	−	−
Raffinose	−	−	+	−	−	90	−	−	+	+	−	−	+
Rhamnose	−	−	10	−	−	46	−	−	−	38	−	−	18
Ribose	+	+	+	+	+	+	+	+	+	+	+	+	+
Saccharose	35	42	−	28	52	−	40	66	+	−	45	45	−
Sorbitol	−	−	−	−	−	−	−	−	−	−	−	−	−
Trehalose	95	45	15	96	52	72	+	+	50	82	98	68	7
Xilose	−	−	+	−	−	27	−	−	43	28	−	−	+
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at pH 3.9	9	−	+	21	57	28	20	−	−	81	22	23	+
Growth at 7% NaCl	82	+	+	48	43	85	80	+	80	90	67	61	+
Growth at 10% NaCl	82	73	−	37	29	36	80	63	−	28	80	60	−
Growth at 4°C	50	43	−	63	43	18	20	34	34	14	80	61	−
Growth at 15°C	82	43	−	58	57	73	+	+	30	33	34	43	−
Growth at 45°C	82	43	+	58	72	81	+	+	30	33	84	35	+
Arginine hydrolysis	91	+	+	81	15	55	90	−	−	73	63	35	+
CO_2 production	−	−	−	−	−	−	−	−	−	−	−	−	−
H_2S production	−	−	−	−	−	−	−	−	−	−	−	−	−
Acetoin production	−	−	−	−	−	−	−	−	−	−	−	−	−
H_2O_2 production	91	+	+	32	29	64	20	+	50	67	23	57	+
Dextrane production	−	−	−	−	−	9	−	−	−	18	20	17	−

[a] +, 100%; −, 0%.

tion were null. Hugas *(8)* found a proportion of 17 and 14% for *L. sakei* and *L. curvatus*, respectively, whereas Schillinger and Lücke *(17)* reported 57 and 45% in each case. Other authors found some differences for lactose and maltose fermentation. *L. sakei* ferments lactose in 18% and maltose in 14%, whereas *L. curvatus* ferments lactose in 20% and maltose in 80% *(17).* In our work, the strains showed different behavior, which was similar to the results of Santos et al. *(16).* Also similar to the results described by these authors were the results of melobiose fermentation by *Pediococcus* spp. Furthermore, according to Hugas *(8),* this carbohydrate can show differences between *L. sakei* and *L. curvatus,* because only *L. sakei* ferments it.

Dextrane and H_2S production is used in discriminatory tests to differentiate lactobacilli. Most of the lactobacilli strains do not produce dextrane *(16),* whereas Schillinger and Lücke *(17)* report production in a low percentage (16% for *L. sakei* and 14% for *L. curvatus*). From the samples tested in this work, only strains of one lot (type B) produce dextrane. None of the acid lactic strains (pediococci included) produced H_2S *(16).* On the contrary, Schillinger et al. *(17)* reported 66% of strains of *L. sakei* and 77% of *L. curvatus* as H_2S producers.

In sausage type A, the predominant species was *L. sakei* (74%) in both lots. However, in lot A1, *L. curvatus* was isolated in 23% and *P. acidilactici* in 3%. In lot A2, 26% of the strains isolated consisted of *L. curvatus* (Table 2). Although the predominant species was that included in the starter culture, other species from initial biota of raw meat mixture were also isolated.

In sausage type B, there is a greater variety of species identified. In lot B1, the main percentages correspond to *P. pentosaceus* (52%), *L. sakei* (24%), *L. curvatus* (14%), and *Pediococcus urinaeequi* (10%). In lot B2, *P. pentosaceus*

TABLE 2. *Occurrence of LAB isolated from finished fermented dry sausages*

Lot and LAB	Occurrence (%)
A1	
Lactobacillus sakei	74
L. curvatus	23
Pediococcus acidilactici	3
A2	
L. sakei	74
L. curvatus	26
B1	
P. pentosaceus	52
L. sakei	24
L. curvatus	14
P. urinaeequi	10
B2	
P. pentosaceus	43
L. sakei	26
L. curvatus	26
P. acidilactici	5

(43%), *L. sakei* (26%), *L. curvatus* (26%), and *P. acidilactici* (5%) are the most frequently isolated species (Table 2).

For Montel *(11),* the *Pediococcus* genus is only found in sausages when it is added as a starter culture. We confirmed this in our study with dry sausage type A, which had isolations of *L. sakei, L. curvatus*, and *P. acidilactici.* According to Santos et al. *(16),* the absence of *Pediococcus* can be due to its inhibition as a consequence of an effective competence by *L. sakei* and *L. curvatus.* Furthermore, in our case these two microorganisms are part of the starter culture. In fact, in sausage type B, *P. pentosaceus* is present in a higher percentage than *L. sakei* and *L. curvatus.* However, that percentage is only approximately 50%, clearly lower than expected because of its presence as part of the starter culture.

In conclusion, in general, the number of LAB that evolved during the ripening process of French dry sausages increased during the first days of the process after which the number of these organisms remained constant at approximately 8 log CFU/g. *L. sakei* and *P. pentosaceus,* bacteria added as starter, were the dominant species. *P. urinaeequi, P. acidilactici*, and particularly *L. curvatus* were also present. Finally, we have to take into account that the controlled conditions of the pilot plant generally homogenize the behavior of the starter biota.

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