APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 2008, p. 2740–2750 Vol. 74, No. 9
0099-2240/08/$08.00+0 doi:10.1128/AEM.02267-07
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Modeling the Aminogenic Potential of *Enterococcus faecalis* EF37 in Dry Fermented Sausages through Chemical and Molecular Approaches[▽]

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Received 5 October 2007/Accepted 10 February 2008

Amino acid decarboxylase activity of the *Enterococcus faecalis* strain EF37 was monitored during fermentation and ripening of a traditional dry fermented sausage from Northern Italy (Salame Veronese) by means of microbiological, chemical, and molecular approaches in relation to three technological factors: fermentation temperature, sodium chloride concentration, and amount of glucose added to the meat mixture. Besides the analytical determination of tyramine and phenylethylamine accumulation and the counts of enterococci, the presence and quantification of the tyrosine decarboxylase gene (*tdc*) and its mRNA transcript were also investigated by using real-time PCR. According to the mathematical models obtained, all of the three factors studied were statistically significant and microbiologically relevant for the early development of enterococci, although the fermentation temperature had a more relevant influence on the enterococcal viable cells of the ripened product. Sodium chloride concentration was the most determinant factor of the final tyramine and 2-phenylethylamine accumulation and also of the levels of *tdc* present in the final product. In contrast, an effect of glucose concentration on *tdc* expression was observed in the last period of ripening. Moreover, increasing amounts of sodium chloride and decreasing fermentation temperature resulted in a reduced *tdc* expression. This is the first time that bacterial tyrosine decarboxylase potential is directly examined through a molecular approach in a fermented meat. The quantification of *tdc* and its transcript can help to elucidate the critical steps and factors during food manufacturing at which bacterial aminogenesis is possible, thus allowing researchers to propose technological measures to control decarboxylase activities.

Enterococci are known as ubiquitous bacteria that can be commonly found within the microbiota colonizing the gastrointestinal tracts of animals and humans, as well as in a variety of food products at variable loads. Interest in this bacterial genus has increased in recent decades due to both their positive and negative traits in relation to human health and food technology (11, 13). In addition to their potential as probiotic organisms, their contribution to the development of the sensorial traits when used as adjunct starter cultures for cheese manufacture has been repeatedly reported (9). Some strains have also been shown to exhibit bioprotection due to bacteriocinogenic activity against some food-borne pathogens such as *Listeria monocytogenes* and *Staphylococcus aureus*, as well as against slime-producing lactic acid bacteria (2, 14). In addition, enterococci are important components of the microbiota involved in the ripening of fermented sausages (14, 15). However, their occurrence in foods is highly controversial since enterococci have been described as spoilage microorganisms and also as indicators of fecal contamination (10). Moreover, food-borne enterococci are known to harbor resistances to different antibiotics (25), and they are recognized as powerful tyrosine-decarboxylating microorganisms (6, 20, 21). The tyrosine decarboxylase enzyme of enterococci has recently been demonstrated to also decarboxylate phenylalanine, although to a lower extent than tyrosine (22).

Tyramine, the product resulting from tyrosine decarboxylation, is usually the main amine found in fermented meat products (28) and the most difficult to avoid. Phenylethylamine results from phenylalanine decarboxylation, and it is commonly present in fermented meat products containing high quantities of tyramine (1, 8). Due to their vasoactive properties, tyramine and phenylethylamine are regarded as undesirable compounds that may cause health disruptions to sensitive consumers, especially to those under treatment with certain drugs, such as mono-amine oxidase inhibitors (27). Even though some bacteria present in fermented foods (lactobacilli and staphylococci) have been reported to be able to decarboxylate tyrosine, high tyramine accumulation has also been associated with high numbers of enterococci (1). Indeed, the relationship between enterococci and tyramine accumulation has been well documented for cheese. The minimum suggested cell concentration for enterococci to detect tyramine during cheese manufacturing was 10^7 CFU/g (16). Leuschner et al. (20) demonstrated the ability of enterococci to grow in cheese and milk up to high cell numbers, yielding tyramine-rich cheeses. The competitiveness of enterococci has also been reported in a fermented meat environment, being often detected in fermented sausages at loads as high as 10^5 CFU/g (14). Tradi-

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▽ Published ahead of print on 22 February 2008.

TABLE 1. Experimental design used in this study

Run	NaCl concn (%)	Glucose concn (g/kg)	Temp (°C)
1	0	0	15
2	5	0	15
3	0	1.4	15
4	5	1.4	15
5	0	0	25
6	5	0	25
7	0	1.4	25
8	5	1.4	25
9	2.5	0.7	20
10	2.5	0.7	20
11	2.5	0.7	20

TABLE 2. Results of viable counts of enterococci and tyramine content in the experimental design runs at the four sampling times of dry fermented sausages

Run	Enterococci (log CFU/g) at day:				Tyramine (mg/kg DM) at day:			
	3	5	19	30	3	5	19	30
1	6.00	7.00	6.34	7.81	1.80	73.17	205.99	207.04
2	5.30	5.89	3.26	5.00	–[a]	–	3.69	1.57
3	6.65	7.51	6.28	6.98	–	70.55	135.28	173.99
4	5.65	5.71	5.18	5.04	–	–	4.80	0.35
5	9.04	9.04	8.08	8.95	84.22	116.60	172.40	231.44
6	6.00	5.00	5.20	5.18	–	3.00	14.49	4.22
7	8.30	8.53	7.75	8.99	94.23	159.12	242.23	268.22
8	6.00	5.95	5.59	5.18	–	–	5.35	1.99
9	8.59	7.49	7.32	7.20	7.60	87.71	134.52	138.25
10	8.46	7.76	7.74	7.04	15.37	108.29	150.78	143.50
11	9.08	7.60	6.43	7.18	10.12	90.47	129.03	104.47

[a] –, not detectable.

tional low-acid fermented sausages, spontaneously fermented (without starter culture) and ripened at low temperatures, are especially advantageous niches for enterococci (3). Indeed, their resistance to environmental stresses, such as food preservation hurdles (salt, nitrite, low water activity, low pH, etc.), makes them able to survive and even grow during food fermentation (13). However, such environmental factors may modify their metabolic activity, including tyraminogenic activity, as has been demonstrated in laboratory media (4, 12). Recently, the occurrence of a tyrosine decarboxylase gene (*tdc*, coding for tyrosine decarboxylase enzyme) in gram-positive bacteria associated with fermented foods has been examined, and a real-time PCR procedure for the quantification of *tdc*, as well as its expression both in pure cultures and meat products, has been developed (29). In the present study, the tyrosine decarboxylase activity was monitored during the fermentation and ripening of Salame Veronese, a traditional dry fermented sausage from Northern Italy, inoculated with the tyraminogenic strain *Enterococcus faecalis* EF37. The study was carried out by means of microbiological, chemical, and molecular approaches in relation to three variables: the fermentation temperature, the NaCl concentration, and the amount of glucose added to the meat mixture. Moreover, the accumulation of phenylethylamine was also considered, as a possible result of the activity of the same enzyme toward a similar aromatic amino acid. In addition to the analytical determination of tyramine and phenylethylamine accumulation, the presence and quantification of *tdc* and its mRNA transcript were also investigated by using real-time PCR. The use of an experimental design allowed us to obtain mathematical models to describe the concomitant effects of the three variables on the aminogenic potential of *E. faecalis* EF37 in fermented sausages.

MATERIALS AND METHODS

Bacterial strain, cultural methods, and DNA extraction. The tyrosine decarboxylase-positive strain *E. faecalis* EF37 (12) was used to inoculate the meat batter utilized for sausage manufacture (see below). The inoculum was prepared from a frozen (−80°C) stock culture grown twice on MRS broth (Oxoid, Milan, Italy) for 18 h at 30°C.

Genomic DNA of *E. faecalis* EF37 was isolated from 2 ml of a late-exponential-phase culture by using a QIAamp DNA minikit (Qiagen, Inc., Valencia, CA) and was quantified by using a UV spectrophotometer (GeneQuant Pro Calculator; Amersham Pharmacia Biotech, Piscataway, NJ).

Experimental design. The tyrosine decarboxylase activity during sausage fermentation and ripening was tested in relation to three selected variables: fermentation temperature (the temperature of the first 3 days after stuffing), NaCl concentration (% [wt/wt]), and glucose added (g/kg). The values of these variables were modulated according to the experimental design shown in Table 1.

Sausage manufacture and sampling. The dry fermented sausage Salame Veronese was manufactured by a local producer in Verona (northern Italy) according to the traditional procedure and recipe, except for the selected variables modified according to each run of the experimental design. Minced meat raw materials, consisting of ca. 75% pork meat and 25% bacon, were mixed with spices and condiments consisting of red wine (6%), in which garlic was macerated overnight, curing salts (0.1%), black pepper (0.2%), nutmeg (0.02%), and cloves (0.04%). No starter cultures were used, but the mixture was inoculated with the tyraminogenic strain *E. faecalis* EF37 at a level of $\sim10^5$ cells/g. The homogenized mixture was stuffed into natural pork casing, and the resulting sausages (8 cm in diameter and weighing ca. 500 to 650 g) were hung into three different climatic chambers under controlled temperature for 3 days to promote fermentation. Thereafter, all sausages were placed in the same chamber at 15°C and ripened for 4 weeks. Sausages not inoculated with *E. faecalis* EF37 were used as a control. In particular, they were prepared using the central condition of the experimental design (i.e., fermentation temperature of 20°C, NaCl concentration of 2.5%, and glucose added at 0.7 g/kg).

Sausages from each run of the experimental design were sampled in duplicate at selected times during the fermentation/ripening process, i.e., the 3rd, 5th, 19th, and 30th days after stuffing.

Microbiological analysis. Enterococci were enumerated by pour plating in kanamycin-esculin-azide agar (Oxoid, Ltd., Milan, Italy) at 37°C for 24 h from the appropriate decimal dilutions of the sausage samples after aseptic removal of

TABLE 3. Results of real-time PCR quantification of *tdc* and *tdc* mRNA transcript in the experimental design runs at the four sampling times of dry fermented sausages

Run	*tdc* (log *tdc* copies/ng total DNA) at day:				*tdc* mRNA transcript (log *tdc* transcript copies/μg total RNA) at day:			
	3	5	19	30	3	5	19	30
1	2.21	2.95	3.59	4.95	–[a]		1.89	2.00
2	1.19	2.04	1.95	1.34	–	–	0.53	0.48
3	2.61	3.33	3.74	3.63	–	1.07	3.04	2.51
4	1.41	1.84	1.94	0.29	–	–	0.72	0.48
5	5.13	5.29	5.12	5.43	2.01	2.37	3.33	3.57
6	0.92	1.74	2.12	0.78	–	–	0.72	0.48
7	4.96	5.03	5.16	5.44	2.44	1.57	3.21	4.37
8	1.46	2.00	2.21	1.17	–	–	0.60	0.48
9	4.26	3.69	4.07	4.01	–	1.17	2.96	1.99
10	3.98	3.86	4.32	4.41	–	0.89	3.15	2.12
11	3.76	3.36	3.83	4.17	–	2.13	3.06	2.66

[a] –, not detectable.

TABLE 4. Coefficients of the final models obtained for viable counts of enterococci and tyramine content at the four sampling times of dry fermented sausages

Variable(s)[a]	Enterococci at day:				Tyramine at day[c]:		
	3	5	19	30	5	19	30
Constant	5.501	6.655	5.393	5.707	–19.308	193.300	91.568
Salt	–[b]	–	–0.461	–	–	–36.382	–
Glucose	6.364	2.575	4.034	–	128.546	–	–
Temp	–	–	–	0.130	6.014	–	6.352
Salt^2	–	–	–	–	–	–	–3.438
Glucose^2	–4.512	–1.728	–2.637	–0.789	–87.112	–65.801	–
Temp^2	0.005	0.003	0.004	–	–	–	–
Salt × glucose	–	–	–	–	–	–	–
Salt × temp	–0.019	–0.024	–	–0.031	–1.056	–	–1.311
Glucose × temp	–	–	–	0.047	–	4.451	–
R^2	0.948	0.943	0.879	0.988	0.966	0.948	0.978
F test	27.469	25.023	10.874	120.413	42.440	42.953	105.664
P	0.076	0.00069	0.00648	0.00001	0.00016	0.00007	<0.00001

[a] R^2 and Fisher test results (with the relative P value) are also reported. Salt, NaCl concentration; Glucose, glucose concentration; Temp, fermentation temperature.
[b] –, coefficient with $P < 0.05$ excluded from the final model.
[c] The existence of too many undetectable values of the dependent variable did not allow us to obtain a model for tyramine at day 3.

the casing and homogenization in a Stomacher Lab-Blender (model 400; Cooke Laboratories, Alexandria, VA) for 2 min.

Biogenic amine analysis. For tyramine and phenylethylamine determinations, sausage samples were extracted with trichloroacetic acid according to the method of Moret and Conte (24), and a dansyl chloride derivatization was performed according to the method of Eerola et al. (7); thereafter, biogenic amines were quantified by means of high-performance liquid chromatography as described by Lanciotti et al. (18). Biogenic amines content of sausage samples throughout the fermentation and ripening are referred to dry matter (DM) to avoid confusion due to the concentration effect of the drying process.

Molecular analysis. Molecular analysis for the detection of *tdc* and its mRNA transcript in sausages was conducted as described by Torriani et al. (29) with minor modifications. Briefly, for nucleic acid extraction from samples, 20 g of sausage was homogenized in 20 ml of TE buffer (10 mM Tris plus 1 mM EDTA [pH 8]). Two aliquots (1.5 ml) of supernatant were transferred into two tubes containing glass beads: one for DNA and the other for RNA extraction. After centrifugation (12,000 × *g*, 4°C, 10 min), the pellets were resuspended in 500 μl of TE containing lysozyme (2.5 mg/ml), followed by incubation at 37°C for 30 min. After centrifugation, the pellets were resuspended in 500 μl of breaking buffer (2% Triton X-100, 1% sodium dodecyl sulfate, 100 mM NaCl, 10 mM Tris [pH 8], 1 mM EDTA) and 25 μl of proteinase K, and a 65°C treatment was performed for 60 min. Nucleic acids were purified by chloroform extraction and precipitated with 2 volumes of absolute ethanol. For RNA, 55 μl of 3 M sodium acetate was also added. After centrifugation, the DNA and RNA pellets were dried and resuspended in 30 μl of sterile water and diethyl pyrocarbonate-treated water, respectively. Contaminating DNA in the RNA preparation was removed by DNase treatment.

Synthesis of cDNA was carried out by using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA) according to the manufacturer's recommendations in a GeneAmp PCR System 2400.

Amplification, detection, and real-time analysis were performed by using a GeneAmp 7000 sequence detection system (Applied Biosystems). Real-time PCRs were set up with Sybr green PCR master mix with 0.2 μM concentrations of the oligonucleotide primers TYR3f (5′-CGTACACATTCAGTTGCATGGCAT-3′) and TYR4r (5′-ATGTCCTACTTCTTCTTCCATTTG-3′), which produce a 171-bp fragment, and the template DNA (~100 ng) or cDNA (generated from ca. 200 to 300 ng of total RNA). The thermal cycle program consisted of an initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 20 s, 58°C for 30 s, and 72°C for 45 s.

Tenfold dilutions of a previously constructed recombinant plasmid (29) containing the 171-bp *tdc* insert ranging from 3 to 3 × 10^8 copies were used for real-time PCR to create the standard curve and used as quantification standards for *tdc* and *tdc* transcript in sausage samples. Three replicates of each sample, standards, and positive and negative controls were processed in each PCR run: a positive control with DNA from *E. faecalis* EF37, a negative control with no template, and a DNase control with DNase-treated RNA.

Statistical analysis. Second-order polynomial response surface models were fitted to each of the response variable with the statistical package Statistica (Statsoft, Inc., Tulsa, OK) version 6.1; a stepwise procedure was chosen in order to generate models containing only significant terms ($P < 0.05$) with satisfactory determination coefficients (R^2) and adequate probability (P) levels of the F test. Response surfaces were drawn by keeping constant at the central value of the experimental design the independent variables not shown in the graphs (i.e., fermentation temperature of 20°C, NaCl concentration of 2.5%, and glucose added at 0.7 g/kg).

RESULTS

The results of the enterococcus counts, tyramine accumulation, and the quantification of *tdc* and its mRNA transcript in the different runs of the experimental design are shown in Tables 2 and 3. These data were used for construction of mathematical models, using polynomial quadratic equations, predicting the behavior of each dependent variable as a function of independent factors (fermentation temperature, amount of NaCl, and glucose added). In order to simplify the models, only terms of the equations with significance higher than 95% were considered, according to a stepwise procedure.

Growth of enterococci. The final models for viable counts of enterococci obtained at the four sampling times are reported in Table 4. All of the three independent variables influenced the growth of enterococci in dry fermented sausages, since they were present in all of the models. The equations were highly significant as indicated by the multiple determination coefficient (R^2) and the P value of the F test. Figure 1 shows the response surfaces for enterococci after 3 days from stuffing, according to the model showed in Table 4. In each figure, the independent variable not present was kept constant at the central value of the experimental design (i.e., NaCl at 2.5%, fermentation temperature of 20°C, and glucose at 0.7 g/kg).

Many of the conditions adopted allowed a considerable growth of enterococci. After the first 3 days of fermentation at different temperatures, the growth of enterococci was maximum when the glucose added to the meat mixture was ca. 0.8 to 1.0 g/kg. The least growth of these microorganisms was observed at the extreme levels of glucose (i.e., 0 and 1.4 g/kg). The decrease in the temperature of fermentation,

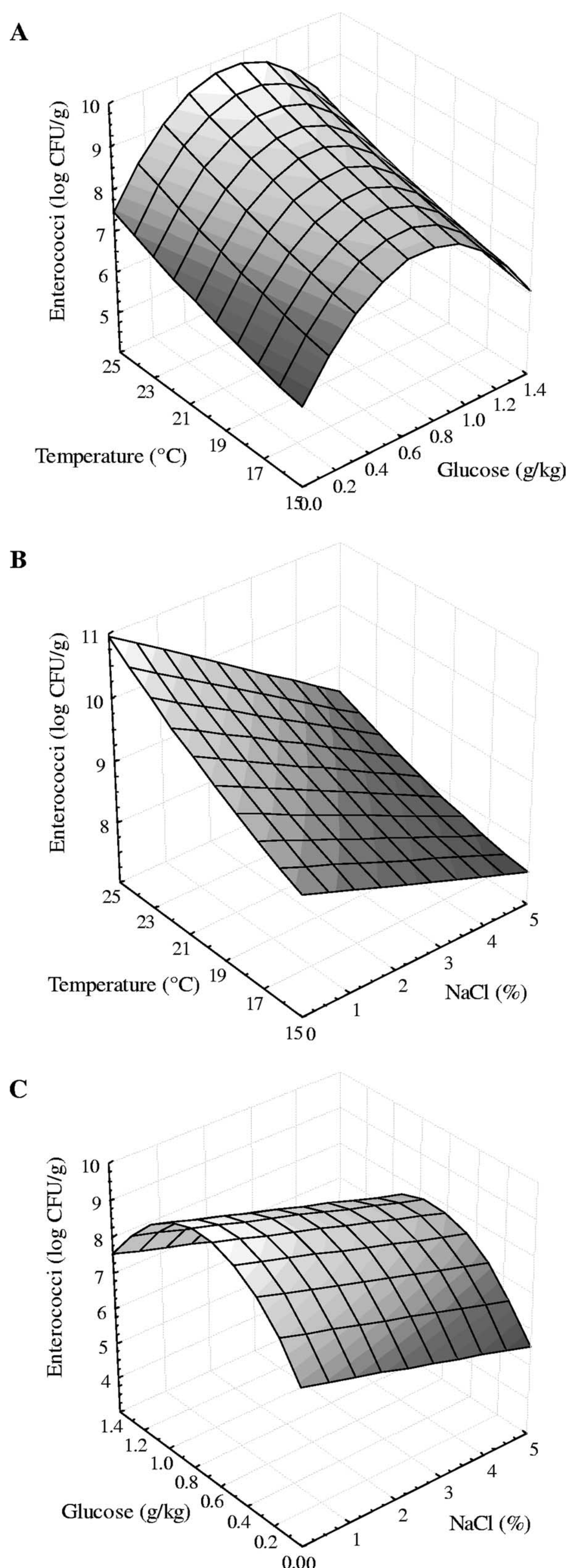


FIG. 1. Response surface graphs corresponding to *Enterococcus* counts (log CFU/g) in dry fermented sausages after 3 days of fermentation, showing the combined effects of fermentation temperature and glucose amount (A), fermentation temperature and salt amount (B), and glucose amount and salt amount (C).

within the range considered, led to lower counts, and the same effect was observed when the NaCl concentration was increased.

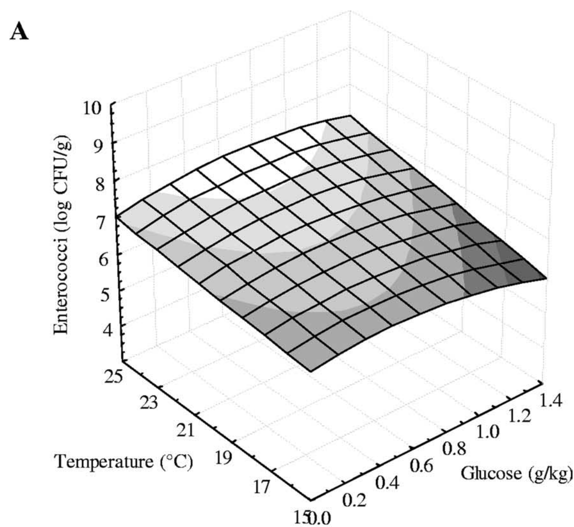
During ripening, the initial fermentation temperature and NaCl and glucose concentrations influenced the counts of enterococci similarly, although the effects were less pronounced. The response surfaces relative to the model obtained after 19 days of ripening are presented in Fig. 2. The influences of glucose and NaCl concentration were strongly reduced, while the contribution of fermentation temperature was still marked at the end of ripening. All of the conditions allowed enterococci to reach counts higher than 10^5 CFU/g after 30 days.

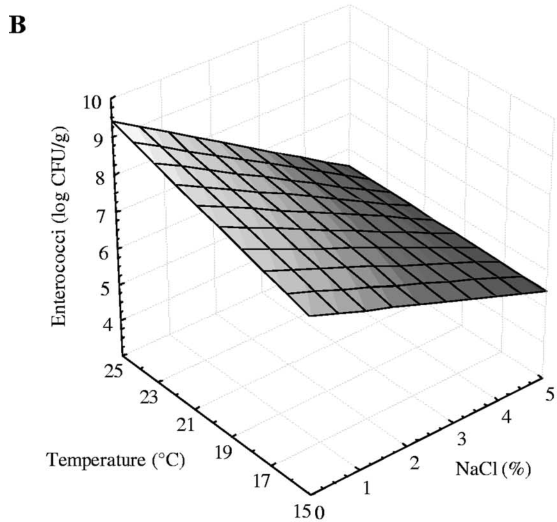
Biogenic amine accumulation. Noticeable differences were observed among the different conditions concerning tyramine accumulation in dry fermented sausages. Tyramine content was already remarkable after 3 days of fermentation in the runs of the experimental design characterized by the absence of glucose and by the higher fermentation temperature (i.e., runs 5 and 7, with 84.2 and 94.2 mg/kg DM, respectively) and in the three repeated central points (7.6, 15.4, and 10.1 mg/kg DM in runs 9, 10, and 11, respectively). Nevertheless, the runs positive for this amine were too few to allow us to obtain a model at the end of the fermentation period. In contrast, highly significant models were fitted for tyramine after 5, 19, and 30 days from stuffing (Table 4).

Figure 3 shows the response surfaces for tyramine content after 5 days of ripening. Important influences were foreseen by the model in relation to the independent variables. Similar to the counts of enterococci, an initial glucose concentration ranging from 0.8 and 1.0 g/kg maximized tyramine accumulation. The decrease in fermentation temperature had a less pronounced effect, inducing a diminution of tyramine accumulation, especially at the lower NaCl concentration. In contrast, NaCl concentration had the most marked influence. In fact, the highest salt concentration reduced the presence of tyramine to negligible levels.

The decisive role of NaCl concentration was even more evident at the end of ripening, and no marked difference was observed in tyramine accumulation at days 19 and 30, since it is deducible from the raw data reported in Table 2. Figure 4 represents the surface response obtained in sausages ripened for 30 days. The initial fermentation temperature had a weaker effect, and the influence of glucose was negligible, whereas the most critical factor determining final tyramine accumulation in ripened sausages was the NaCl concentration, since the content of tyramine ranged from undetectable ($<$1 mg/kg DM) at 5% NaCl to values higher than 200 mg/kg DM in the absence of salt.

Marcobal et al. (22) demonstrated that *E. faecium* and *E. faecalis* possess a gene coding for an enzyme able to perform L-phenylalanine and L-tyrosine decarboxylation. In our study, this ability was revealed in sausages in the latter period of ripening (30 days), in which 2-phenylethylamine accumulated in detectable amounts (from 2.6 to 19.3 mg/kg DM) in seven runs of the experimental design. The model describing the presence of this amine in relation to the variables considered was significant (R^2 = 0.952, F = 19.788, P = 0.0026) and was calculated as follows:





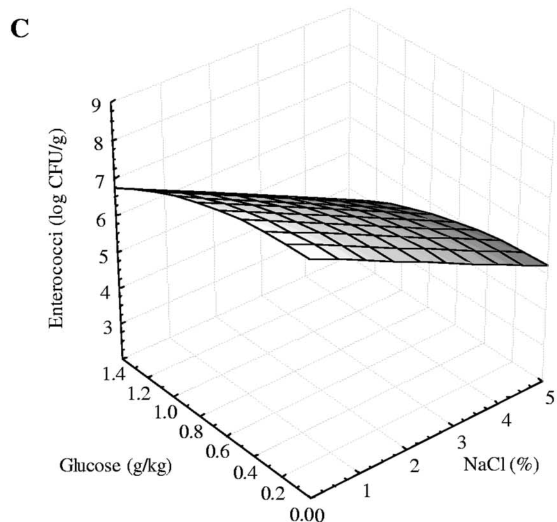
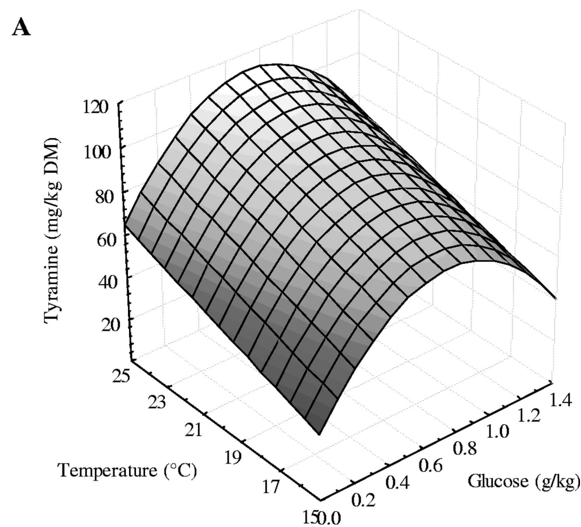
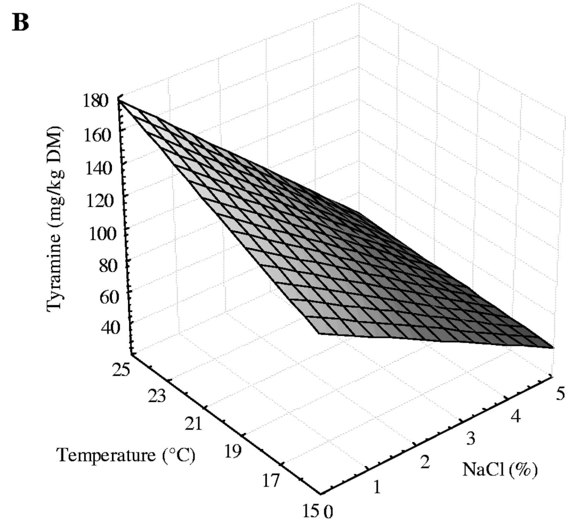


FIG. 2. Response surface graphs corresponding to *Enterococcus* counts (log CFU/g) in dry fermented sausages at the end of ripening, 19 days, showing the combined effects of fermentation temperature and glucose amount (A), fermentation temperature and salt amount (B), and glucose amount and salt amount (C).





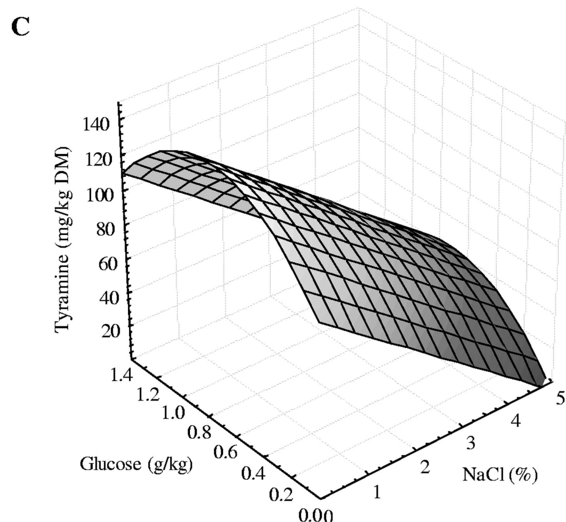


FIG. 3. Response surface graphs corresponding to tyramine accumulation (mg/kg DM) in dry fermented sausages after 5 days of fermentation, showing the combined effects of fermentation temperature and glucose amount (A), fermentation temperature and salt amount (B), and glucose amount and salt amount (C).

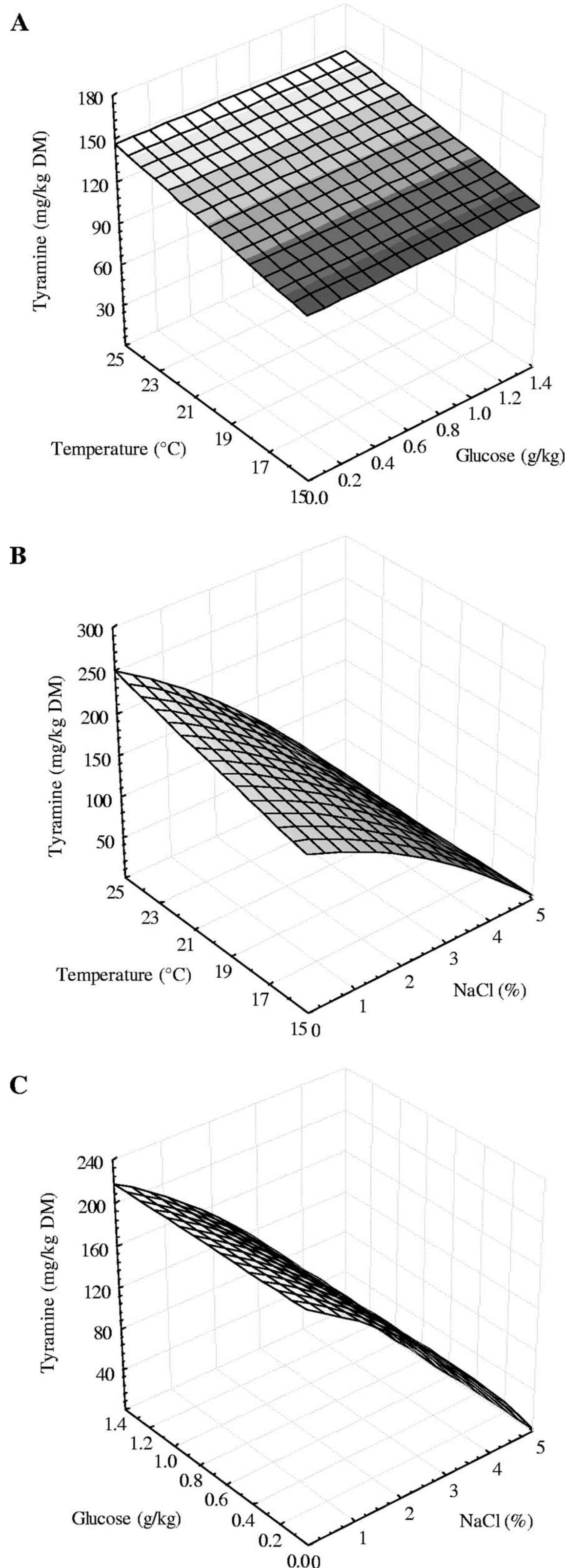


FIG. 4. Response surface graphs corresponding to tyramine accumulation (mg/kg DM) in dry fermented sausages at the end of the ripening, 30 days, showing the combined effects of fermentation temperature and glucose amount (A), fermentation temperature and salt amount (B), and glucose amount and salt amount (C).

$$\text{2-phenylethylamine (mg/kg DM)} = -5.729 + 39.420 \times [\text{glucose}] + 0.741 \times [\text{NaCl}]^2 - 27.649 \times [\text{glucose}]^2 + 0.037 \times [\text{temperature}]^2 - 0.293 \times [\text{salt}] \times [\text{temperature}]$$

The surfaces obtained from the model are presented in Fig. 5, which demonstrates an important influence of the glucose initially added with the maximum amine accumulation in correspondence of the intermediate sugar concentration (0.6 to 0.8 g/kg), an inhibiting action of NaCl at a concentration higher than 2%, whereas an increase of the initial fermentation temperature leads to a higher 2-phenylethylamine accumulation.

Even if the contribution of wild microflora to biogenic amine accumulation cannot be excluded, it can be considered irrelevant compared to the aminogenic activity of *E. faecalis* EF37. In fact, the presence of biogenic amines after 30 days of ripening in the same type of sausages (equivalent to those of the central points) but not inoculated with the selected strain was 47.4 mg/kg DM for tyramine, whereas phenylethylamine was under the quantification limit (data not shown).

Quantification of *tdc*. After the first 3 days of fermentation of dry fermented sausages, specific DNA sequences encoding for the tyrosine decarboxylase enzyme (expressed as the log *tdc* copies/ng total DNA) were detected in the same runs of the experimental design in which tyramine was found (Table 3). The amounts of *tdc* were proportional to the tyramine detected; in fact, 5.13 and 4.96 log *tdc* copies/ng total DNA for runs 5 and 7, respectively, and 4.26, 3.98, and 3.76 log *tdc* copies/ng total DNA, respectively, for the central points of the experimental design were detected. Statistically significant models were obtained for the copy number of *tdc* after 3, 5, 19, and 30 days (Table 5). The surface responses relative to the *tdc* levels after 3 days from stuffing, which were significantly affected by the same variables influencing the independent variables after 5 days, were drawn in Fig. 6. The effects of the three variables on the occurrence of *tdc* were very similar to that observed for enterococci and tyramine after the same ripening period. Glucose amount comprised between 0.8 and 1.0 g/kg maximized the accumulation of *tdc*, which was limited by the increase of NaCl concentration, even if the effect was slightly reduced at the higher concentration, as indicated by the nonlinear plot depending on the presence of the quadratic term of the variable NaCl in the equation. The increase in the initial fermentation temperature favored the number of *tdc*, but it had no practical effect when the highest NaCl concentration was considered.

The effects of the initial fermentation temperature and glucose added became negligible after 19 days (data not shown) and especially after 30 days (Fig. 7) of ripening. Indeed, at the end of ripening the level of *tdc* was dependent mainly on the amount of NaCl added, which markedly decreased the cumulative occurrence of *tdc* particularly at a concentration higher than 2%. The same qualitative and quantitative predominant effect of NaCl was also observed for tyramine accumulation.

Expression of *tdc*. The data concerning the amount of *tdc* mRNA transcripts, expressed as log *tdc* copies/μg total RNA, could be modeled only in dry fermented sausages after 19 and 30 days of ripening, in which enough runs gave detectable

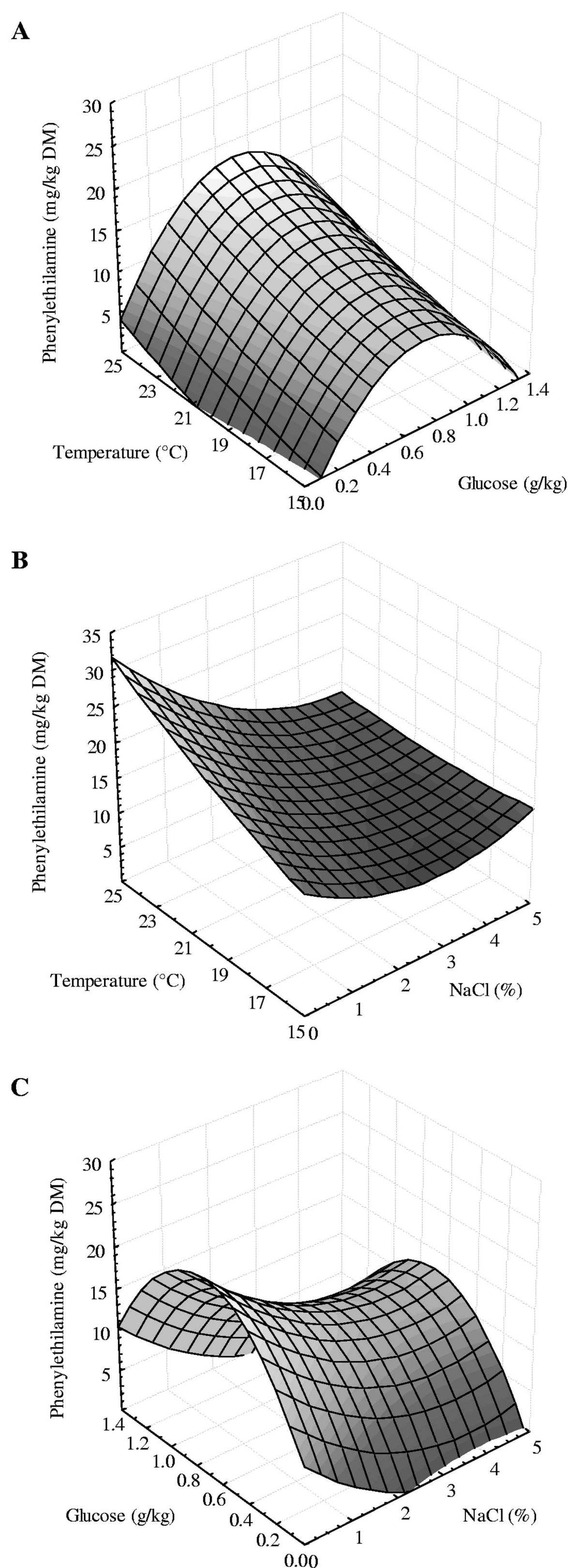


FIG. 5. Response surface graphs corresponding to 2-phenylethylamine accumulation (mg/kg DM) in dry fermented sausages at the end of the ripening (30 days), showing the combined effects of fermentation temperature and glucose amount (A), fermentation temperature and salt amount (B), and glucose amount and salt amount (C).

quantities (Table 5). In the sausages analyzed after 3 days of fermentation, the *tdc* transcript was found only in runs 5 and 7, in which tyramine and *tdc* were detected at the higher concentrations. After 5 days the transcript was detected also in other samples, in which remarkable amounts of tyramine were found. Unlike tyramine and *tdc*, the maximizing effect of intermediate amounts of glucose added to the meat mixture could be observed also in the last period of ripening. Figure 8 represents the surface responses after 19 days of ripening. Increasing the amount of NaCl and a decrease in fermentation temperature resulted in reduced values of *tdc* mRNA transcript, as already observed for enterococci. The situation was analogous after 30 days (data not shown), even if the effect of glucose was less pronounced.

DISCUSSION

Spontaneous nonstarter lactic acid bacteria are recognized as the main responsible for tyramine production in fermented sausages (1). In particular, enterococci are considered among the most active tyrosine decarboxylating bacteria and have also been demonstrated to decarboxylate phenylalanine (6, 22, 28).

All of the three factors studied here (fermentation temperature, NaCl concentration, and amount of glucose added) were statistically significant and microbiologically relevant for the early development of enterococci during the first 3 days of fermentation. At the end of ripening, the amount of glucose and NaCl added to the meat mixture showed a less pronounced effect on the growth of enterococci (although statistically significant for the general model). The temperature at which the initial fermentation was carried out in the first 3 days had a more relevant influence, even if for the remaining part of ripening (27 days) the temperature was the same for all of the sausages. A difference of nearly 2 log units was found between the concentration of enterococci reached in sausages fermented at 15 and 25°C after stuffing. Therefore, although all of the conditions applied allowed a growth of enterococci up to levels higher than 10^5 CFU/g, the initial fermentation temperature is the most critical modulating factor of the final enterococcus counts. The different fermentation temperature not only was fundamental for influencing the enterococcal population dynamics in the first days but also had a decisive role in their evolution during the latter stage of ripening.

Previous in vitro experiments with the same *E. faecalis* strain (12) demonstrated that the higher NaCl concentration determined the lower tyramine accumulation. However, in that case, the extent of growth was the main biological feature influencing the total tyramine production by *E. faecalis* EF37, probably in relation to the short incubation period (72 h). In contrast, in the in vitro experiments (in MRS broth) described by Marcobal et al. (23) with the strains *L. brevis* CECT4669 and *E. faecium* BIFI-58, the maximal extent of growth and tyramine production were affected by different factors, and the optimal cell growth did not always result in high tyramine production levels.

In the present study, detectable amounts of tyramine were found after 3 days of fermentation in the conditions allowing also the highest counts of enterococci. Under real sausage fermentation conditions after 30 days of ripening, the most critical factors determining the final counts and final tyramine

TABLE 5. Coefficients of the final models obtained for the levels of *tdc* and *tdc* mRNA transcript at the four sampling times of dry fermented sausages

Variable(s)[a]	*tdc* (log *tdc* copies/ng total DNA) at day:				*tdc* mRNA transcript (log *tdc* transcript copies/μg total RNA) at day[c]:	
	3	5	19	30	19	30
Constant	0.798	1.990	1.459	5.109	1.680	1.350
Salt	–[b]	–	0.708	0.530	–	–
Glucose	7.140	3.506	–	–2.758	4.077	1.822
Temp	–	–	0.147	–	–	–
Salt^2	0.121	0.077	–0.135	–0.211	–	–
Glucose^2	–4.972	–2.481	–	–	–2.772	–1.136
Temp^2	0.007	0.0050	–	–	0.002	0.004
Salt × glucose	–	–	–	–	–	
Salt × temp	–0.055	–0.042	–0.025	–0.014	–0.022	–0.027
Glucose × temp	–	–	–	0.129	–	–
R^2	0.989	0.982	0.991	0.993	0.959	0.969
F test	92.034	53.664	162.503	141.5493	35.073	46.631
P	0.00006	0.00024	<0.00001	0.00002	0.00027	0.00012

[a] R^2 and Fisher test results (with the relative *P* value) are also reported. Salt, NaCl concentration; Glucose, glucose concentration; Temp, fermentation temperature.
[b] –, coefficient with $P < 0.05$ excluded from the final model.
[c] The existence of too many undetectable values of the dependent variable did not allow us to obtain a model for tyramine at days 3 and 5.

content were different (i.e., the initial fermentation temperature and NaCl concentration, respectively), which would indicate that not only the number of enterococci but also the activity of decarboxylases under given conditions is far more important. The relationship between increasing NaCl concentration and reduced biogenic amine accumulation has already been observed in Portuguese sausages (26) and in Feta cheese (30), although it was not clear whether the effect of NaCl was due to the inhibition of aminogenic microorganisms, decarboxylase activity, or proteolytic activity.

After 5 days, the model describing tyramine accumulation was similar to that of the counts of enterococci at day 3, as indicated by the shapes of the graphs in Fig. 2 and 1, respectively. This observation is reinforced by the linear correlation between tyramine amounts and enterococcus counts after 5 days ($R = 0.863$, $F_{1,9} = 26.32$, $P < 0.001$). Likewise, the quantification of *tdc* after the same ripening time also showed a good correlation with enterococcus counts ($R = 0.941$, $F_{1,9} = 70.07$, $P < 0.00001$). In contrast, the *tdc* transcript was detectable only in 6 of the 11 runs of the experimental design, which were again those characterized by the highest tyramine concentration.

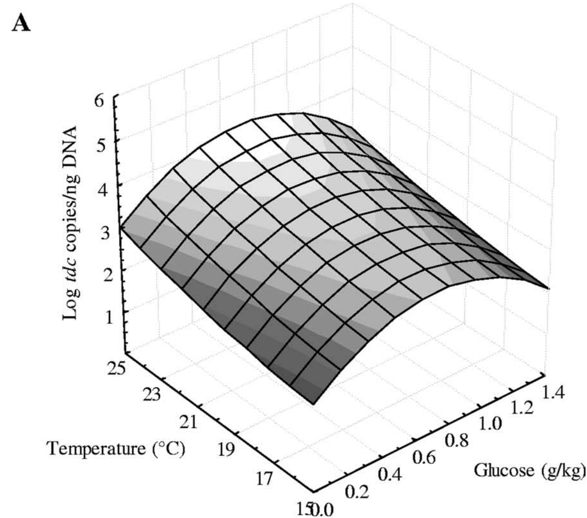
In the second part of ripening, the effects of the variables on tyramine accumulation and *tdc* levels were similar. A good correlation was found between these data after 30 days ($R = 0.952$, $F_{1,9} = 87.05$, $P < 0.00001$). However, the influence was different with respect to that shown in the first part (during fermentation), since the most important variable was NaCl concentration, whereas glucose and fermentation temperature had a limited influence. These two latter variables maintained a relevant importance on the *Enterococcus* counts and revealed a strong influence on the models obtained for *tdc* transcript.

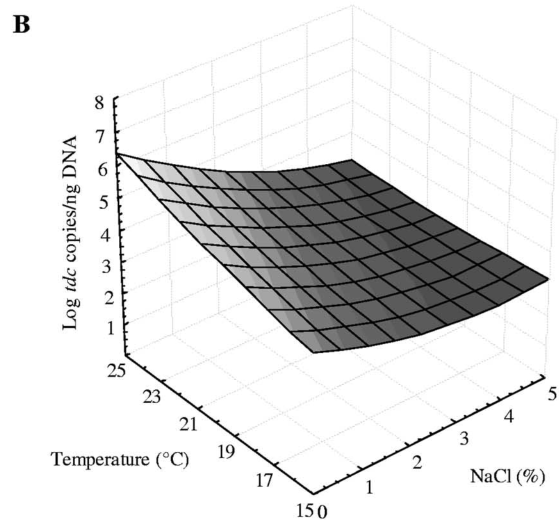
Temperature can influence both the growth of tyraminogenic population and the decarboxylase activity (28). However, in the present study, given that different temperatures were applied only in the first 3 days of fermentation, the effects of its variation were limited to the selection of the microbial population which, in turn, influenced the tyraminogenic activity throughout all of the ripening period.

Even if the amounts of phenylethylamine were lower than those of tyramine, the shapes of the surface response graphics are similar to those obtained for the *tdc* mRNA transcript after the same ripening time. As suggested by Joosten (17), at the end of ripening tyrosine could become a limiting substrate for the activity of decarboxylase enzyme and was substituted by phenylalanine that is decarboxylated to 2-phenylethylamine, even with a reduced efficiency.

The quantification of *tdc* transcripts (from the RNA extracts) seems to be less sensitive than that of *tdc* (from the DNA extracts), since the data could be modeled only after 19 days of ripening, and they did not correlate with the tyramine content. However, it is the result of the expression of the corresponding gene at a given time, which is unstable, meaning that it does not accumulate in the food matrix. Moreover, RNA extraction usually results in a lower recuperation in comparison with that of DNA, even when all of the precautions are taken into account. An activity of this enzyme related to the actual *Enterococcus* counts is also evident after 30 days of ripening. This activity is directed more toward phenylalanine than tyrosine decarboxylation. In fact, no relevant differences were observed between tyramine content after 19 and 30 days, whereas phenylethylamine accumulates in sausages only in this late period.

Several molecular methods for the detection of biogenic amine-producing bacteria on foods have been described (19). However, to our knowledge this is the first time that bacterial tyrosine decarboxylase potential has been directly examined via a molecular approach in a fermented meat product. The quantification of the *tdc* gene and its transcript would indicate the overall tyraminogenic potential evolving during ripening, and the tyramine content would be the result of the ultimate real events. It is obvious that the most important issue, from a practical point of view, is the presence of the tyramine content in the final product, from which the actual intake and its





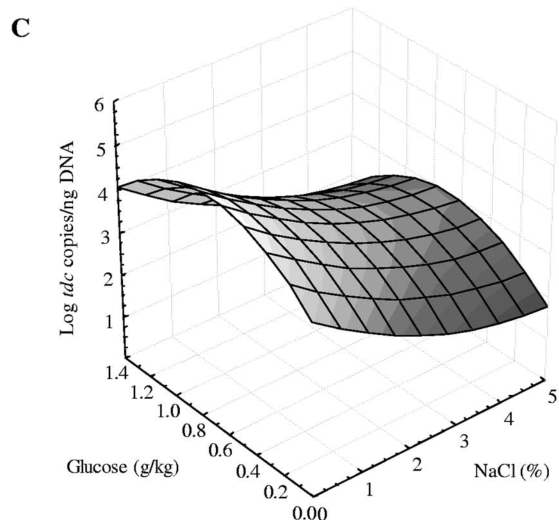
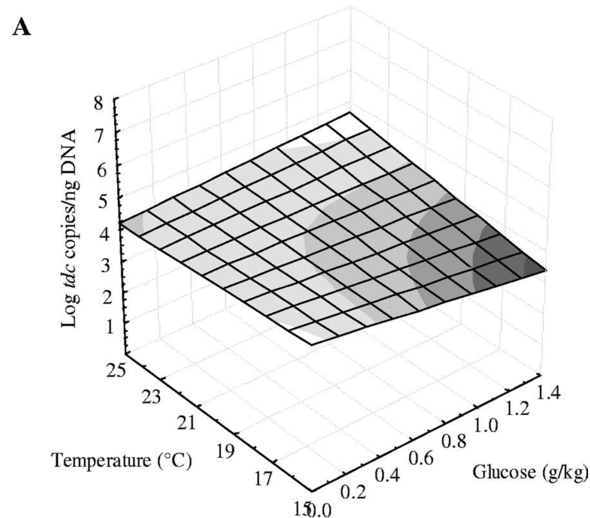
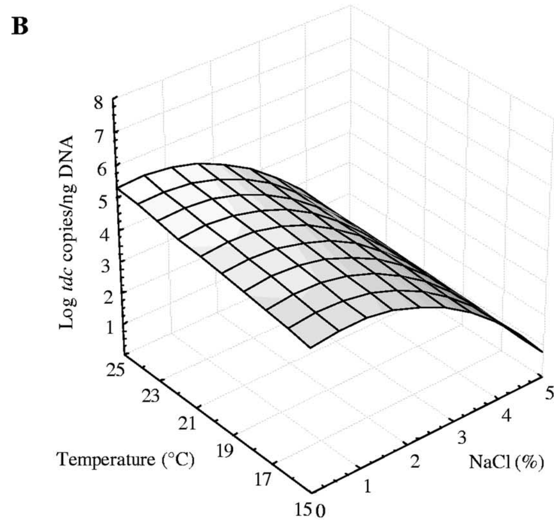


FIG. 6. Response surface graphs corresponding to the levels of *tdc* (log copies/ng total DNA) obtained by real-time PCR in dry fermented sausages after 5 days of fermentation, showing the combined effects of fermentation temperature and glucose amount (A), fermentation temperature and salt amount (B), and glucose amount and salt amount (C).





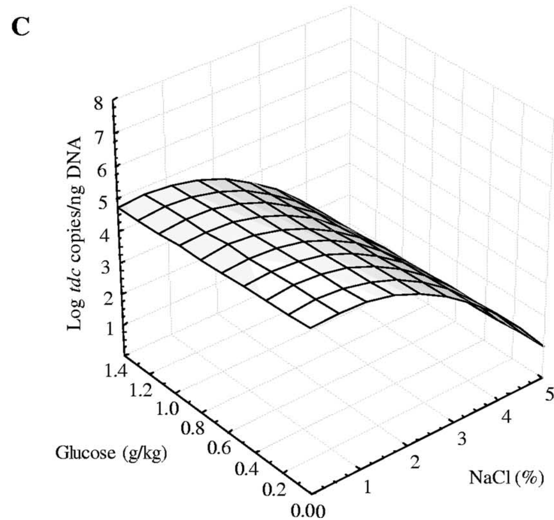


FIG. 7. Response surface graphs corresponding to the levels of *tdc* (log copies/ng total DNA) obtained by real-time PCR in dry fermented sausages at the end of the ripening (30 days), showing the combined effects of fermentation temperature and glucose amount (A), fermentation temperature and salt amount (B), and glucose amount and salt amount (C).

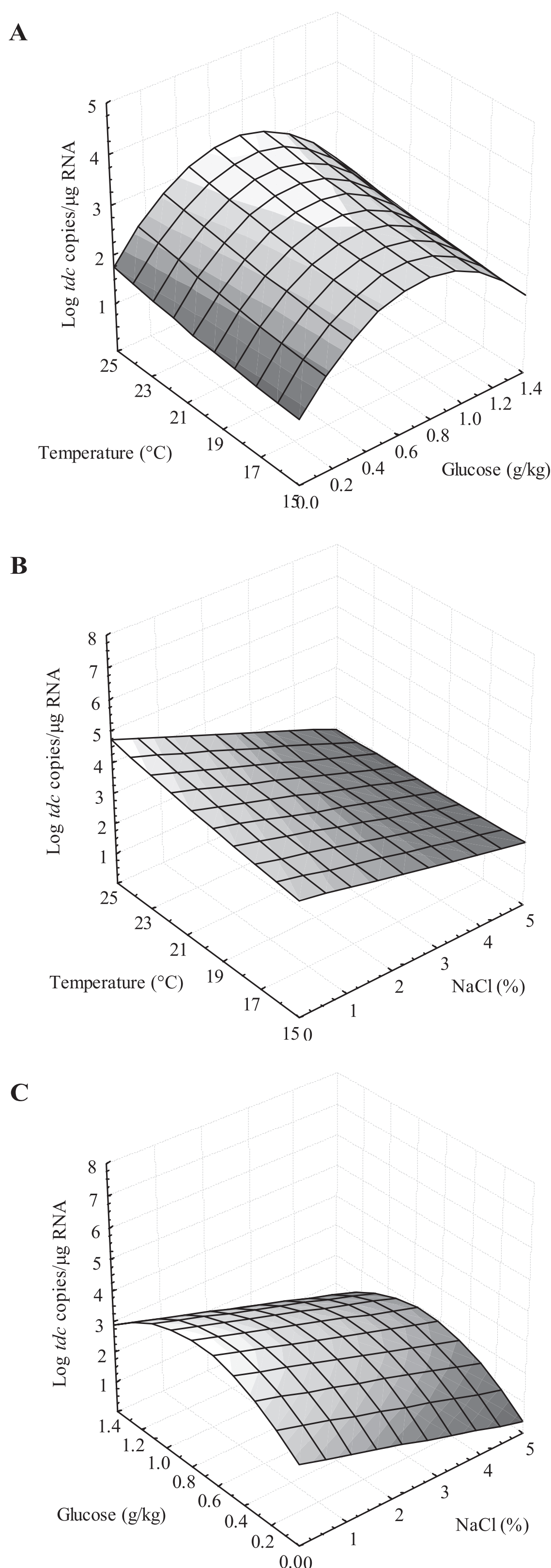


FIG. 8. Response surface graphs corresponding to the levels of *tdc* transcript (log copies/µg total RNA) obtained by real-time PCR after 19 days of fermentation, showing the combined effects of fermentation temperature and glucose amount (A), fermentation temperature and salt amount (B), and glucose amount and salt amount (C).

potential health risks can be inferred. From these results it seems that there is a strict correlation between gene quantification and tyramine accumulation, which suggests that most of the potential actually exists. Moreover, the activity of decarboxylases could continue independent of the integrity of the bacterial cells responsible for their biosynthesis. On the other hand, in efforts to understand the mechanisms and the influencing factors of biogenic amine production in foods, nonaccumulative parameters (nonspecific such as *Enterococcus* counts and more specific such as *tdc* mRNA transcript) are also important. In this sense, if in a given sampling time some conditions are particularly favorable for increasing gene expression, such information may help to explain the results of the biogenic amine presence in the later stages of the process.

There are cases (5) in which the biogenic amine accumulation in a fermented food product is hardly associable with the decarboxylase activities observed in vitro of the bacteria isolated from the matrix and sometimes vice versa. The in situ quantification of *tdc* and its transcript bypasses this incongruence, since it shows the real potential of the microbiota to produce tyramine and 2-phenylethylamine. Therefore, the suitability of the molecular approach described here seems to offer valuable advantages in helping to elucidate the critical steps associated with aminogenesis during sausage manufacture and the factors favoring decarboxylase activity with the final aim of adopting technological measures able to control it.

In conclusion, the advantages deriving from the application of molecular protocols directly to food products is emphasized by their capacity to estimate the tyrosine decarboxylase activities of microorganisms associated with specific manufacturing technology. This should prove to be a rapid and universal method for the technological evaluation of the safety of the processing conditions. The detection of *tdc* transcripts, even if less sensitive than the quantification of the relative gene, can help to elucidate the critical steps during sausage manufacturing at which the environmental conditions allow the bacterial decarboxylase enzyme activities, resulting in the progressive accumulation of tyramine and 2-phenylethylamine.

ACKNOWLEDGMENTS

This research was financially supported by the Italian Ministry of University and Research. S.B.-C. acknowledges the funding support of the Ramon y Cajal Program of the Spanish Ministry of Science and Technology, as well as a grant from the AGAUR–Generalitat de Catalunya (2004BE 00132).

REFERENCES

1. **Ansorena, D., M. C. Montel, M. Rokka, R. Talon, S. Eerola, A. Rizzo, M. Raemaekers, and D. Demeyer.** 2002. Analysis of biogenic amines in northern and southern European sausages and role of flora in amine production. Meat Sci. **61:**141–147.
2. **Aymerich, M. T., M. Garriga, J. Ylla, J. Vallier, J. M. Monfort, and M. Hugas.** 2000. Application of enterocins as biopreservatives against *Listeria innocua* in meat products. J. Food Prot. **63:**721–726.
3. **Aymerich, M. T., B. Martín, M. Garriga, and M. Hugas.** 2003. Microbial quality and direct PCR identification of lactic acid bacteria and nonpathogenic staphylococci from artisanal low-acid sausages. Appl. Environ. Microbiol. **69:**4583–4594.
4. **Beutling, D.** 1996. Effect of sodium chloride and nitrite on the potential of tyramine formation of *Enterococcus faecalis* var. *liquefaciens*. Fleischwirtschaft **76:**1054–1056.
5. **Bover-Cid, S., T. Hernández-Jover, M. G. Miguélez-Arrizado, and M. C. Vidal-Carou.** 2003. Contribution of contaminant enterobacteria and lactic acid bacteria to biogenic amine accumulation in spontaneous fermentation of pork sausages. Eur. Food Res. Technol. **216:**477–482.

6. **Bover-Cid, S., M. Hugas, M. Izquierdo-Pulido, and M. C. Vidal-Carou.** 2001. Amino acid-decarboxylase activity of bacteria isolated from fermented pork sausages. Int. J. Food Microbiol. **66:**185–189.
7. **Eerola, S., R. Hinnkkalnen, E. Linfors, and T. Hirvi.** 1993. Liquid chromatographic determination of biogenic amines in dry sausages. J. AOAC **76:**575–580.
8. **Ferreira, V., J. Barbosa, S. Vendeiro, A. Mota, F. Silva, M. J. Monteiro, T. Hogg, P. Gibbs, and P. Teixeira.** 2006. Chemical and microbiological characterization of alheira: a typical Portuguese fermented sausage with particular reference to factors relating to food safety. Meat Sci. **73:**570–575.
9. **Foulquié-Moreno, M. R., P. Sarantinopoulos, E. Tsakalidou, and L. De Vuyst.** 2006. The role and application of enterococci in food and health. Int. J. Food Microbiol. **106:**1–24.
10. **Franz, C. M., W. Holzapfel, and M. E. Stiles.** 1999. Enterococci at the crossroads of food safety? Int. J. Food Microbiol. **47:**1–24.
11. **Franz, C. M., M. E. Stiles, K.-H. Schleifer, and W. H. Holzapfel.** 2003. Enterococci in foods: a conundrum for food safety. Int. J. Food Microbiol. **88:**105–122.
12. **Gardini, F., M. Martuscelli, M. C. Caruso, F. Galgano, M. A. Crudele, F. Favati, M. E. Guerzoni, and G. Suzzi.** 2001. Effects of pH, temperature, and NaCl concentration of the growth kinetics, proteolytic activity, and biogenic amine production of *Enterococcus faecalis*. Int. J. Food Microbiol. **64:**105–117.
13. **Giraffa, G.** 2002. Enterococci from foods. FEMS Microbiol. Rev. **26:**163–171.
14. **Hugas, M., M. Garriga, and T. Aymerich.** 2003. Functionality of enterococci in meat products. Int. J. Food Microbiol. **88:**223–233.
15. **Klein, G.** 2003. Taxonomy, ecology, and antibiotic resistance of enterococci from food and the gastro-intestinal tract. Int. J. Food Microbiol. **88:**123–131.
16. **Joosten, H. M. L. J., and M. D. Northolt.** 1987. Conditions allowing the formation of biogenic amines in cheese. 2. Decarboxylative properties of some non-starter bacteria. Neth. Milk Dairy J. **41:**259–280.
17. **Joosten, H. M. L. J.** 1988. Conditions allowing the formation of biogenic-amines in cheese. 3. Factors influencing the amounts formed. Neth. Milk Dairy J. **41:**329–357.
18. **Lanciotti, R., F. Patrignani, L. Iucci, M. E. Guerzoni, G. Suzzi, N. Belletti, and F. Gardini.** 2007. Effects of milk high pressure homogenization on biogenic amine accumulation during ripening of ovine and bovine Italian cheeses. Food Chem. **104:**693–701.
19. **Landete, J. M., B. de las Rivas, A. Marcobal, and R. Muñoz.** 2007. Molecular methods for the detection of biogenic amine-producing bacteria on foods. Int. J. Food Microbiol. **117:**258–269.
20. **Leuschner, R. G. K., R. Kurihara, and W. P. Hammes.** 1999. Formation of biogenic amines by proteolytic enterococci during cheese ripening. J. Sci. Food Agric. **79:**1141–1144.
21. **Marcobal, A., B. de las Rivas, E. García-Moruno, and R. Muñoz.** 2004. The tyrosine decarboxylation test does not differentiate *Enterococcus faecalis* from *Enterococcus faecium*. Syst. Appl. Microbiol. **27:**423–426.
22. **Marcobal, A., B. de las Rivas, and R. Muñoz.** 2006 First genetic characterization of a bacterial β-phenylethylamine biosynthetic enzyme in *Enterococcus faecium* RM58. FEMS Microbiol. Lett. **258:**144–149.
23. **Marcobal, A., P. J. Martín-Álvarez, M. V. Moreno-Arribas, and R. Muñoz.** 2006. A multifactorial design for studying factors influencing growth and tyramine production of the lactic acid bacteria *Lactobacillus brevis* CECT 4669 and *Enterococcus faecium* BIFI-58. Res. Microbiol. **157:**417–424.
24. **Moret, S., and L. S. Conte.** 1996. High-performance liquid chromatographic evaluation of biogenic amine in food. An analysis of different methods of sample preparation in relation to food characteristics. J. Chromatogr. **729:**363–369.
25. **Rizzotti, L., D. Simeoni, P. Cocconcelli, S. Gazzola, F. Dellaglio, and S. Torriani.** 2005. Contribution of enterococci to the spread of antibiotic resistance in the production chain of swine meat commodities. J. Food Prot. **68:**955–965.
26. **Roseiro, C., C. Santos, M. Sol, L. Silva, and I. Fernandes.** 2006. Prevalence of biogenic amines during ripening of a traditional dry fermented pork sausage and its relation to the amount of sodium chloride added. Meat Sci. **74:**557–563.
27. **Shalaby, A. R.** 1996. Significance of biogenic amines to food safety and human health. Food Res. Int. **29:**675–690.
28. **Suzzi, G., and F. Gardini.** 2003. Biogenic amines in dry fermented sausages: a review. Int. J. Food Microbiol. **88:**41–54.
29. **Torriani, S., V. Gatto, S. Sembeni, R. Tofalo, G. Suzzi, N. Belletti, F. Gardini, and S. Bover-Cid.** 2007. Rapid detection and quantification of tyrosine decarboxylase gene (*tdc*) and its expression in gram-positive bacteria associated with fermented foods by PCR-based methods J. Food Prot. **71:**93–101.
30. **Valsamaki, K., A. Michaelidou, and A. Polychroniadou.** 2000. Biogenic amine production in Feta cheese. Food Chem. **71:**259–266.