International Journal of Food Microbiology 129 (2009) 264–270

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Fate of acid-resistant and non-acid resistant Shiga toxin-producing *Escherichia coli* strains in experimentally contaminated French fermented raw meat sausages

M.P. Montet [a,*], S. Christieans [b], D. Thevenot [a], V. Coppet [b], S. Ganet [a], M.L. Delignette Muller [a], L. Dunière [a], S. Miszczycha [a], C. Vernozy-Rozand [a]

[a] Unité de Microbiologie Alimentaire et Prévisionnelle, Université de Lyon — Ecole Nationale Vétérinaire de Lyon 69280, Marcy l'étoile, France
[b] Association de Développement Des Industriels de la Viande, 2 rue Chappe, 63039 Clermont-Ferrand cedex 2, France

ARTICLE INFO

Article history:
Received 1 July 2008
Received in revised form 22 September 2008
Accepted 2 December 2008

Keywords:
Shiga-toxin *Escherichia coli*
French sausages
Acid resistance
Survival

ABSTRACT

Both pathogenic and nonpathogenic *E. coli* exhibit a stress response to sublethal environmental stresses. Several studies have reported acid tolerance and survival characteristics of *E. coli* O157:H7 in foodstuffs, but there are few reports about the tolerance of non-O157 serogroups (STEC) to organic acids in foods.
The purpose of this study was to examine the effect of the manufacturing process of French fermented raw meat sausages on the growth and survival of acid-resistant (AR) and non-acid resistant (NAR) STEC strains. The six strains, 3 AR and 3 NAR, were inoculated separately into raw sausage mixture at a level of 10^4–10^5 CFU/g. A total of 19 batches of sausages were manufactured. A rapid and similar decrease in the number of both AR and NAR STEC strains, from less than 1 to 1.5 $\log_{10}$ CFU/g, was observed during the first 5 days of fermentation at 20–24 °C. This rapid decrease was followed by a more gradual but continuous decrease in STEC counts after drying at 13–14 °C, up to day 35. The STEC counts were <10 CFU/g after 35 days for the NAR strains and the same concentration for the AR strains on the best before date (day 60). It was not possible to detect any NAR STEC after 60 days. The present study shows that the process used in the manufacture of French sausages results in a complete destruction of NAR STEC strains after 60 days, but it does not have the same effect on the AR STEC strains.

1. Introduction

Escherichia coli O157:H7 was first recognized as a human enteric pathogen in 1982 and it is still a common cause of severe gastrointestinal illness. In the last decade infections caused by *E. coli* O157:H7 have emerged as a major public health concern in North America and in Europe. This pathogen is considered to be a common cause of haemorrhagic colitis (HC), haemolytic-uraemic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) in humans. Most outbreaks and sporadic cases of HC and HUS have been attributed to O157:H7 Shiga-toxin producing *E. coli* (STEC) strains (Blanco et al., 2001; Chapman et al., 2001). However, non-O157:H7 *E. coli* infections are, in certain geographic regions, considered to be at least as important as *E. coli* O157:H7, but they may often be under-diagnosed (Johnson et al., 2006). As an example, in continental Europe infections by non-O157 STEC, such as O26:H11/H−, O91:H21/H−, O103:H2, O111:H−, O113:H21, O117:H7, O118:H16, O121:H19, O128:H2/H−, O145:H28/H−, and O146:H21, are more common and are frequently associated with severe illness in humans (Nataro and Kaper, 1998; Blanco et al., 2001; Eklund et al., 2001). Consequently, there is growing concern about the risk to human health associated with non-O157 STEC serotypes (Johnson et al., 1996; Tarr and Neill, 1996; Beutin et al., 1998). More precisely, non-O157 STEC serotypes (especially O26, O103, O111, O145, O113, O174, O8, O121 and O45) are increasingly being associated with infections causing bloody diarrhea, HUS, and non-bloody diarrhea.

Fermented meat products had long been thought to be relatively safe due to their low pH, their low water activity (a_w), and the presence of curing salts (nitrite and nitrate). Fermentation and drying have been reported to reduce the numbers of *E. coli* O157:H7 by 1–2 $\log_{10}$ units (Glass et al., 1992; Hinkens et al., 1996; Faith et al., 1997; Riordan et al., 1998). However, certain *E. coli* O157:H7, reported to be acid tolerant, were able to survive the processing of dry fermented sausages (Glass et al., 1992). *E. coli* O157:H7 possesses a high acid resistance or inducible acid tolerance response (Conner and Kotrola, 1995; Lin et al., 1996) and, thus, it can survive in acidic foods, such as apple cider (Miller and Kaspar, 1994), mayonnaise (Zhao and Doyle, 1994) and salamis (Getty et al., 2000). In December 1994, dry cured salami was implicated as the source of serotype O157:H7 in a disease outbreak in Washington State (Food Chemical News, 1994). Also in 1994, dry-cured salami was implicated in outbreaks of serotypes

* Corresponding author. Unité de Microbiologie Alimentaire et Prévisionnelle — Ecole Nationale Vétérinaire de Lyon, 1, avenue Bourgelat, F-69280 Marcy l'Etoile, France. Tel.: +33 4 78 87 25 50; fax: +33 4 78 87 25 54.
E-mail address: mp.montet@vet-lyon.fr (M.P. Montet).

0168-1605/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijfoodmicro.2008.12.002

O157:H7 (Tilden et al., 1996), O157:H− (Ammon et al., 1999) and O111:H− (Paton et al., 1996). More recently an outbreak of STEC O26:H11, caused by fermented beef sausage, occurred in Denmark (Ethelberg et al., 2007). These outbreaks suggest that the acid tolerance of the non-O157 STEC strains, like the O157 STEC strains, enables these bacteria to survive in moderately acidic food. Acid tolerance is defined by the growth of log-phase cultures at a moderately low pH (pH 5.5 to 6.0) inducing mechanisms of survival in more extreme acid conditions of pH 2.5 (Goodson and Rowbury, 1989). A similar phenomenon, termed the "acid tolerance response," has been shown in *S. typhimurium* (Foster and Hall, 1990; Foster, 1993).

Moreover, there are no published experiments evaluating the growth or survival of non-O157 STEC in fermented meat. The purpose of the present work is to address the question of whether the acidic resistance confers an ecological superiority. This potential ecological superiority of STEC strains was evaluated by the ability of acid-resistant (AR) and non-acid-resistant (NAR) STEC strains to survive the fermentation and drying process of French fermented raw meat sausages. For this purpose, the ideal experiment would have been to use "pathogenic" STEC strains belonging to the five major serogroups, i.e.: O157, O26, O111, O103 and O145. However, due to the link with human disease, this type of experiment and the manufacture of sausages must be carried out in safety level/P3 laboratories, which are lacking in France. Consequently, the experimentation we designed to achieve our goal used non-O157 STEC strains belonging to serogroups not implicated in published outbreaks.

2. Materials and methods

2.1. STEC strains

A collection of 62 STEC stains, isolated during previous French epidemiological studies, were tested. With the aim of evaluating their ability to survive exposure to acid, we used the protocol described by Castanie-Cornet et al. (1999) in which four AR mechanisms were tested:

- AR1: oxidative system (glucose-repressed system)
- AR2: system depending on the presence of glutamate
- AR3: system depending on the presence of arginine
- AR4: system depending on the presence of lysine

Three AR STEC strains and three NAR STEC strains were selected from this previous study (data not shown). The survival rate of the strains in the presence of glucose and amino acids is indicated in Table 1. One spontaneous nalidixic acid-resistant mutant was selected *in vitro* for each AR and NAR STEC strain selected previously by plating STEC on BHI agar, containing nalidixic acid (0.1 to 10 μg/ml) in increasing concentrations, using the protocol described by Truong et al. (1997). One rifampicin and one spectinomicin mutant were selected using the same protocol. The antibiotic susceptibility of these AR or NAR mutants is given in Table 1. To follow the growth of these strains during the fermented raw meat sausage process, spontaneous antibiotic-resistant derivatives (nalidixic acid, rifampicin and spectinomycin) were isolated. We carefully checked that the chosen resistant derivatives were neither affected in their growth rate nor in their acid-resistance properties (data not shown).

The acid-resistance of the antibiotic-resistant clones was compared to that of the parental strains. No difference was noted between the clones and the parental strains. These clones, whose AR level was shown to be stable over time, were used in the present study.

2.2. Inoculation procedure

The strains which demonstrated either AR or NAR in the step described above were cultivated in 10 ml Luria Bertani broth (LB) and incubated at 37 °C for 24 h. Then 100 μl was transferred into 10 ml of LB broth and incubated at 37 °C for 24 h. Each subculture (10 μl) was transferred again into 10 ml of LB and incubated at 17 °C for 24 h to obtain cold-adapted strains. Finally, 250 μl of subcultures were transferred into 50 ml of LB broth and incubated at 12 °C until they reached the end of exponential growth. A previous experimental study, using the same protocol, enabled us to determine the growth curve of each strain (with 3 replicates) and to standardize the culture level using a calibration curve that showed the relationship between optical density (OD) at 600 nm and cell counts (data not shown). Following optical density (OD) measurements, the cold adapted inocula were diluted in Peptone Water Broth (MRD-T, bioMèrieux, France) in order to inoculate the sausage mixture to a level of 10^4–10^5 CFU/g.

The six STEC strains were inoculated separately into raw sausage mixture, and in triplicate. A total of 19 batches of sausages were manufactured. One batch, consisting of non-inoculated sausages, served as negative controls.

2.3. Cleaning and disinfecting operations

Between the production of each batch of sausages, cleaning and disinfecting operations were applied to the equipment and utensils used (table, knife, mixing and stuffing machines).

A pre-rinse was performed for 5 min, using water, to remove the fat and organic materials. A mechanical action (5 min) was applied to the equipment in order to get rid of all the particles from the small spaces and narrow openings. An inter-rinse (5 min) was applied before the

Table 1
Virulence factors and acid resistance of STEC strains used for the artificial contamination of raw mixture sausages

	STEC strains			Virulence factors				Acid-resistance (average of 3 repetitions)			
	Strains	Serotyping[a]	Origin	*eae* gene[b]	stx_1 gene[b]	stx_2 gene[b]	Shiga-toxin producing[c]	AR1% of survival	AR2% of survival	AR3% of survival	AR4% of survival
AR STEC strains	ANR 245A1$_{Rif}$	OntH8	Raw milk cheese	N	P	P	P	60	76	0	0
	ANR V1$_{Spec}$	O166:H28	Environment	N	N	P	P	80	1	0	0
	ANR V10$_{Nal}$	O11:H43	Environment	N	P	N	P	67	0	0	0
NAR STEC strains	ANR 42A$_{Nal}$	O6:H1	Raw milk cheese	N	P	P	P	0.7	0	0	0.2
	ANR 418A$_{Rif}$	O6:H10	Raw milk cheese	N	P	N	P	0	0	0	0
	ANR 346A$_{Spec}$	O174:H8	Raw milk cheese	P	P	P	P	0	0	0	0

AR1: oxydative system (glucose-repressed system), AR2: system depending on the presence of glutamate, AR3: system depending on the presence of arginine, AR4: system 8 depending on the presence of lysine.
Rif: Rifampicin resistance, Spec: Spectinomicin resistance, Nal: Nalidixic acid resistance.
[a] Ont: O type not corresponding to any serogroup between O1 and O 174.
[b] P, positive; N, negative.
[c] P production.

cleaning and disinfecting operation, which consisted of the application of a single alkaline chlorinated product (bactegil, SODEVI, Clermont-Ferrand, France) for 30 min at a 10% concentration. A post-rinse was carried out to remove this product.

2.4. Dry sausage manufacturing process

Sausages were manufactured according to a standardized process from French salting plants.

The raw sausage mixture had the following composition: cut pork meat (82%) and pork fat (18%), in order to obtain products with 20% fat; nitrate (0.3 g/kg); salt (26 g/kg); pepper (1.5 g/kg); and sugar (4.5 g/kg of dextrose). Although nitrite is added in the fermented sausage formulation of other countries to protect the sausages against pathogenic bacteria, nitrite is not used in the French formulations. Indeed, in the case of a long fermentative process, such as is used for fermented dried sausages, *Staphtylococcus* reduces nitrate to nitrite resulting in protection against *Clostridium botulinum*. Starter cultures were added to the formulation at a level of 10^6 CFU/g (Texel Dried SA-201) (Rhodia Food, Dange´-Saint-Romain, France). This optimized freeze-dried starter culture compound is composed of Lactic Acid Bacteria (LAB) (*Lactobacillus sakei*), *Staphylococcus carnosus* and *Staphylococcus xylosus*.

The whole sausage-making process was carried out in a climate controlled room between 10 and 15 °C. Frozen fat and meat pork (100 kg) were pretempered at −5 °C for 48 h. Afterwards meat was minced in a cutter (Fatosa, C200, cutter 200 L) (Technocarne, F-67802 Hoenheim Cedex) until coarse pieces of about 10 mm in diameter were produced. Fat, pre-cut into 10 mm cubes, was then added. Cutting continued until particles were about 2 mm in diameter. The minced meat was then transferred to a mixing machine in which all the ingredients were added and mixed for 5 min. The mixture obtained was divided into 19 batches of 4.5 kg and kept at 0 °C overnight. Twenty five milliliters of a single strain of STEC were inoculated into each batch just prior to the stuffing phase. After mixing, the raw material was stuffed into natural casing (pork intestine, 30–40 mm diameter), initially washed in a 0.5% acetic acid solution, to achieve a final weight of about 450 g. Surface fungi (Texel Dried Neo 2000) were then applied to the sausages which were subsequently hung in a climate controlled chamber for fermentation and drying.

During the initial period (5 days), the temperature and relative humidity were gradually reduced from 24 °C and 96–94% RH (relative humidity) to 20 °C and 88–93% RH. During the drying period (30 days), the climate-controlled chamber was maintained at 13–14 °C and 80–82% RH. The weight loss was approximately 40%. When they were ready for consumption, the sausages were kept at 10–15 °C until they reached the best before date (day 60).

2.5. Sampling, counting and detection of STEC

For each batch, a total of 7 sausages were manufactured (one sausage for each sampling time i.e. 0, 1, 2, 5, 15, 35 and 60 days).

Duplicate samples, for enumeration of STEC and other analyses, were taken from the sausage mixture and the stuffed sausages (day 0), from sausages at the beginning, the middle and the end of the fermentation stage (days 1, 2, 3 and 5), in the middle of the drying stage (day 15), at the end of the drying stage (day 35) and on the best before date (day 60). A 25 g portion, aseptically removed from raw meat, the sausage mixture or from the central portion of each sausage, was diluted (1:10) in 225 ml of Buffered Peptone Water (BPW, bioMérieux, Marcy l'Etoile, France). Further decimal dilutions were made with sterile peptone salt (3.5 M disodium phosphate, 1 M peptone, 5 M sodium chloride, 1.5 M potassium dihydrogen phosphate) (Oxoid, Basingstoke, Hampshire, UK) and homogenized for 40 s in a stomacher. Aliquots, from appropriate serial dilutions, were spread using spiral plating (WASP Spiral plating, AES Laboratory, Bruz, France) onto Luria Bertani Agar (LB-A) media composed of (per liter) 10 g of tryptone (Becton Dickinson, Le Pont-De-Claix, France), 5 g of yeast extract (Duchefa Biochemie, Saint Ismier, France), 10 g NaCl (Sigma-Aldrich, France) and 7 g of agar (Merck Sharp & Dohme, Paris, France). LB-A plates were supplemented with rifampicin (100 μg/ml) (Sigma-Aldrich, France), spectinomicin (100 μg/ml) (Sigma-Aldrich, France) or nalidixic acid (40 μg/ml) (Sigma-Aldrich, France), when appropriate, and incubated at 37 °C for 24 h. Enumeration of the colonies was performed with an automatic colony counter EC2 easy count 2 (AES Laboratory, Bruz, France). An enrichment of 24 h at 37 °C in BPW (bioMérieux, Marcy l'Etoile, France) was used for samples in which no STEC cells were detected by direct plating. Presumptive colonies were confirmed by PCR, as described by Read et al. (1992), targeting the *stx* genes with the use of the following degenerate primers: ES149: 5′ CGA AAT CCC CTC TGT ATT TGC C 3′ (22 bp) and ES151: 5′ GAG CGA AAT AAT TTA TAT GT 3′ (20 bp). These primers amplified a conserved sequence of the stx_1 and stx_2 genes, generating a 323 bp fragment. The raw material (pork meat, pork fat and natural casing) was tested for the presence of STEC at the beginning of the procedure, using the PCR targeting the *stx* gene, as described by Read et al. (1992).

2.6. Physical–chemical measurements

The pH levels of the sausages were determined using a penetration electrode (Bioblock scientific pH meter 330; Bioblock scientific, Illkirch, France).

The a_w values were measured using an a_w cryometer (Aqualab, Washington, USA) in the non-inoculated sausages only.

2.7. Statistical analysis

An analysis of variance (ANOVA) was carried out in order to check whether the pH changed significantly during any stage of the processing and to determine if the differences observed between the AR and NAR STEC strains were significant.

The biphasic inactivation curves were modeled by a bilinear model, assuming two different inactivation constants defined as the slope of the inactivation curve (when activity is plotted in logarithmic scale versus time), k_1, during the initial period of the manufacturing process (first 5 days), and k_2 during the second period (days 6 to 60). The

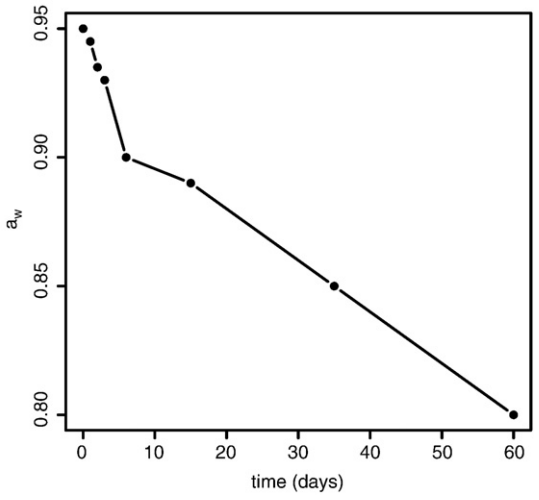

Fig. 1. Water activity values in the non inoculated sausage during the sausage's manufacturing process.

microbial density N[CFU/g] was thus described by the following equation:

$$\log_{10}(N) = \begin{cases} \log_{10}(N_0) - \dfrac{k_1 \times t}{\ln(10)} & \text{for } t \leq 5 \\ \log_{10}(N_0) - \dfrac{k_1 \times 5 + k_2 \times (t-5)}{\ln(10)} & \text{for } t > 5 \end{cases}$$

where N_0[CFU/g] is the microbial density at the beginning of the first period and t is the time [days].

The model was globally fitted to each survival curve using the nls function of the R software (R Development Core Team., 2007). Confidence limits (at 2.5% and 97.5%) were computed for both inactivation constants.

3. Results

3.1. Physicochemical characteristics of sausages

The pH changed significantly during the stages D15, D35 and D60 ($p<0.05$). During the first 5 days of fermentation, and for all sausages, the pH decreased from 5.83±0.02 to 4.99±0.02 (Table 1), and the water activity decreased from 0.95 to 0.90 (Fig. 1). The pH values of sausages remained stable from the middle of fermentation (day 3) to the middle of the drying period (day 15) and reached 4.98±0.02 to 4.95±0.05 (Table 2). The water activity decreased (0.93, 0.90 and 0.89) on the 3rd, 6th and 15th days. From the middle of drying to the end of drying (day 15 to day 35) the pH increased from 4.95±0.05 to 5.22±0.03 and the water activity decreased from 0.89 to 0.85. The pH values increased during storage from 5.22±0.03 to 5.46±0.10 (day 35 to day 60), and the water activity decreased to 0.80 when the weight of the sausages decreased by about 30%.

3.2. Survival of AR and NAR STEC strains during sausage processing

No acid-resistant STEC were detected prior to the challenge testing in the raw materials, i.e. in the pork meat, in pork fat and in natural casing. In addition, a previous study confirmed the effectiveness of the cleaning and disinfecting operations applied between the preparation of the different batches (data not shown).

The counts of STEC in the sausage mixture and stuffed sausages (day 0), sausages during fermentation (days 1, 2, 3) up to the end of fermentation (day 5), in the middle of the drying stage (day 15), at the end of the drying stage (day 35) and on the best before date (day 60) were as follows. A rapid and similar decrease in the number of AR and NAR STEC strains, of less than 1 to 1.5 $\log_{10}$ CFU/g, was observed during the 5 first days of fermentation at 20–24 °C. This rapid decrease was followed by a more gradual but continuous decrease observed from the beginning of drying at 13–14 °C until day 35. More precisely, this decrease was from 0.5 to 2.5 $\log_{10}$ CFU/g for the AR strains and from 2 to 4 $\log_{10}$ CFU/g for the NAR STEC strains. The counts of STEC were less than 10 CFU/g after 35 days for NAR STEC and AR STEC strains and on the best before date (day 60) for AR STEC. It was not possible to detect any NAR STEC after 60 days. However, at day 35, the differences observed between AR STEC (ANR245A1$_{Rif}$, ANRV1$_{Spec}$ and ANRV10$_{Nal}$) and NAR STEC (ANR346A$_{Spec}$ and ANR418A$_{Rif}$) were significant (p values<0.05). A single data point at a particular sampling time indicates that very close results were obtained for the 3 different experiments (Fig. 2).

The biphasic inactivation curves of AR and NAR STEC strains are shown in Fig. 3. The two periods, period 1 from day 0 to day 5 and period 2 from day 5 to day 60, were shown to be linked to two different inactivation constants. The value of the constant was higher for the first period (compared to that of the 2nd period) for each STEC strain, and a higher inactivation constant was measured for the NAR STEC strains during the 2nd period compared to that of the AR STEC, during the same period. The inactivation constants in each of the two periods are given with 95% confidence intervals.

Table 2
pH values during fermentation, drying and storage of the French fermented raw meat sausages

		Day 0	Day 5	Day 15	Day 35	Day 60
AR STEC strains	ANR 245A1$_{Rif}$	5.84 ± 0.02[a]	4.97±0.01[a]	4.93±0.03[a]	5.23±0.01[a]	5.56±0.01[a]
	ANR V1$_{Spec}$	5.86±0.04[a]	5.00±0.04[a]	4.93±0.03[a]	5.24±0.02[a]	5.42±0.06[a]
	ANR V10$_{Nal}$	5.84±0.02[a]	4.98±0.01[a]	4.96±0.05[a]	5.22±0.02[a]	5.50±0.04[a]
NAR STEC strains	ANR 42A$_{Nal}$	5.81±0.01[a]	5.02±0.03[a]	4.97±0.05[a]	5.24±0.01[a]	5.44±0.06[a]
	ANR 418A$_{Rif}$	5.81±0.01[a]	5.00±0.02[a]	5.02±0.05[a]	5.19±0.02[a]	5.53±0.02[a]
	ANR 346A$_{Spec}$	5.82±0.02[a]	4.97±0.02[a]	4.88±0.05[a]	5.17±0.01[a]	5.28±0.01[a]
		5.83±0.02	4.99±0.02	4.95±0.05	5.22±0.03	5.46±0.10

Mean (±) standard deviation.
[a] Values are average of three trials.

4. Discussion

The ability of serotype O157:H7 STEC isolates to survive in acidic foods has been described but little is known about non-O157 STEC serogroups. The aim of the present study was to examine the ability of three AR and three NAR non-O157 STEC strains to withstand the salt, acid, and temperature stresses encountered during the fermentation period, drying and 25 days of storage at 15 °C of French sausages. The non-O157:H7 strains chosen were not from the top 5 in the list of virulent STEC serogroups (O157, O111, O26, O145, O103) since their manipulation, including inoculation of the meat and manufacture of the sausages, required P3 safety rooms that were not available for these experiments. Since there is no published data on the survival of non-O157:H7 strains in fermented sausages, we will refer to data concerning O157 STEC in the discussion of our results.

As a rule, fermented raw meat sausages, as prepared in InnoViandes laboratory, are subjected to relatively high drying temperatures (>24 °C) in order to enhance growth of the added lactic bacteria and a decrease in pH. This results in accelerated texture and flavour development and increased stability as a consequence of the low pH (4.5–5.0). More precisely, the manufacture of dry fermented raw meat sausages is divided into 3 phases, the formulation (mixing of ingredients), the fermentation, and the drying period followed by storage. During the drying process, several microbiological, physico-chemical and biochemical changes take place which are responsible for the sensory quality and safety of this kind of product.

The pH and a_w average values of sausages studied in these experiments decreased from 5.83 to 4.95 and from 0.95 to 0.89, respectively, by the middle of the drying period. During the fermentation step (period 1) of the French sausage preparation a rapid decrease in the bacterial count of AR and NAR STEC strains was followed by a more gradual, but continuous, decrease observed during drying and storage at 10–15 °C (period 2). This is in agreement with other studies which have also reported the same log unit reductions in *E. coli* O157:H7 at the end of fermentation (Glass et al., 1992; Hinkens et al., 1996; Riordan et al., 1998; Muthukumarasamy and Holley, 2007). Several studies on the survival of *E. coli* O157:H7 in the non-heated dry sausage manufacturing process have shown that the counts of *E. coli* O157:H7 could be reduced by 1–3 log units (Clavero and Beuchat, 1996; Hinkens et al., 1996; Calicioglu et al., 1997; Faith et al., 1997; Faith et al., 1998; Yu and Chou, 1998; Erkkila et al., 2000; Chacon et al., 2006). Muthukumarasamy et al. (2007), observed a decrease in the number of *E. coli* O157:H7 by one log unit during the fermentation step, by 0.7 log unit at the end of drying and by 1.7 log CFU/g after 27 days of drying in the sausage processing. The isolation of *E. coli*,

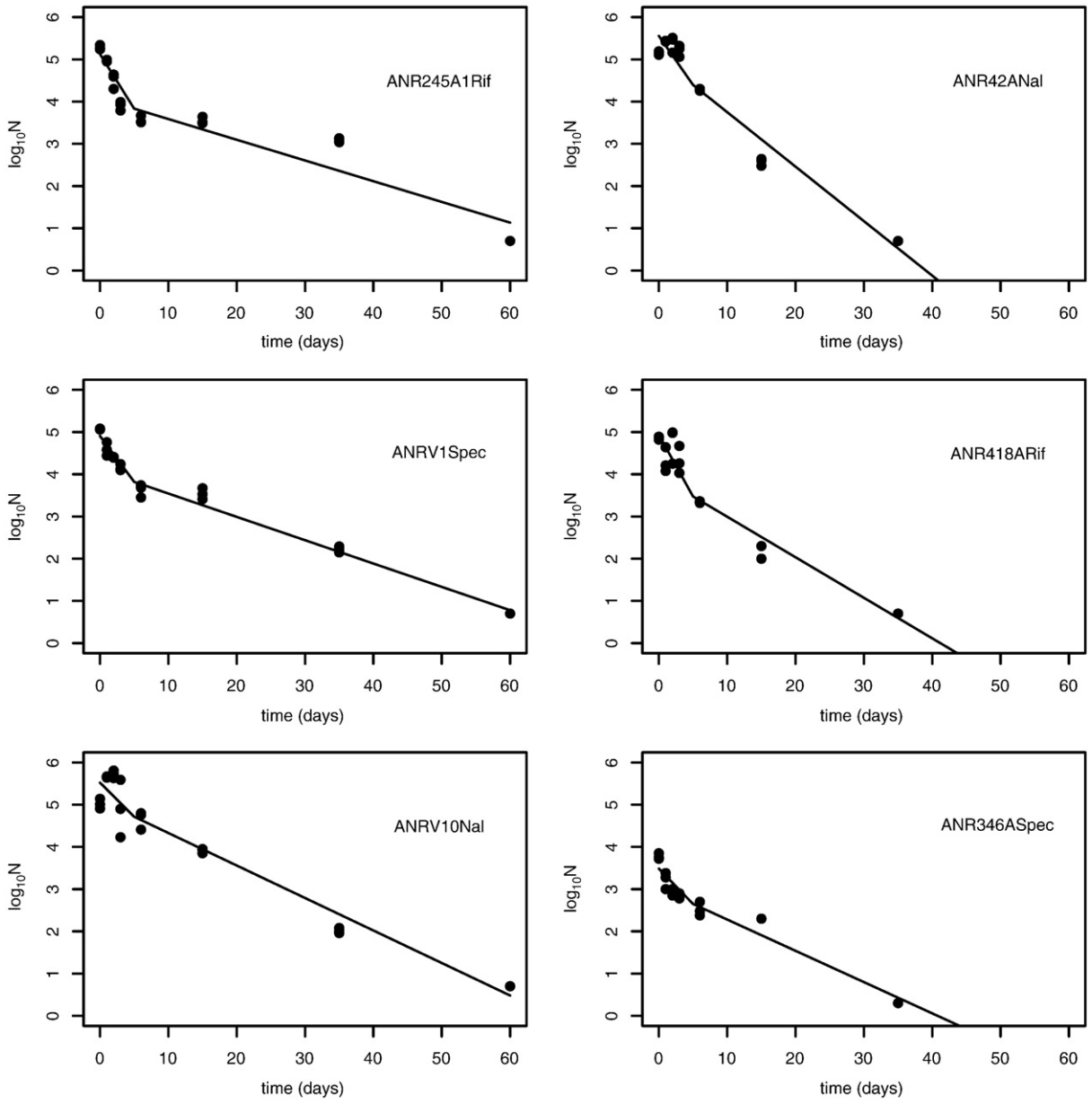

Fig. 2. Counts as $\log_{10}$ (N signifies CFU/g) of AR and NAR STEC strains during the French sausage manufacturing process. Sampling times: day 0, sausage mixtures, in the stuffed sausages; days 1, 2, 3, sausages at the beginning, the middle and the end of the fermentation period; day 5, sausages in the middle of the drying stage; day 35, sausages at the end of the drying stage; day 60, sausages at the best before date. Each curve shows the average values obtained from three independent experiments and a single data point at a particular sampling time indicates very close results.

usually in the late stages of fermentation, can be related to the high resistance that this species exhibits to adverse conditions which continue throughout the drying period (Varnam and Evans, 1991). In the fourth week of drying of industrial semi-dry fermented sausages the only species isolated was *E. coli* (Castano et al., 2002).

From the 6th day until the end of storage (60 days) (period 2), the pH increased slightly from 4.99 to 5.46 and a_w decreased slightly from 0.90 to 0.80. The increase of the pH could be explained by proteolysis and the action of some microorganisms, such as the yeasts which consume lactic acid (Díaz et al., 1997).

Surprisingly, it was during this drying and storage step (period 2) that we observed a difference between AR and NAR STEC strains and not during the fermentation stage (period 1). The change in the inactivation constants between these two periods might be due to a change in the environmental factors. It is noteworthy that the only physico-chemical parameter that changed markedly was the temperature of storage, with a decrease from 20–24 °C in period 1 to 10–15 °C in period 2. The change of temperature might explain the biphasic appearance of the inactivation curves from period 1 to period 2. Other studies have mentioned a similar biphasic decline of *E. coli* O157:H7 STEC strains due to the initial rate of death being followed by a slower second phase decline (Clavero and Beuchat, 1996; Shadbolt et al., 1999). More precisely, Shadbolt et al. (1999) observed that, as the water activity was lowered the magnitude of the first phase inactivation consistently increased (influenced by osmotic shock). According to Ross et al. (2008) the longer second phase appeared to be independent of pH or water activity but instead dominated mainly by temperature.

Shadbolt et al. (2001), also described a biphasic inactivation kinetics consisting of a rapid first phase of death followed by a slower second phase, the second lethal stress (SLS). When cultures initially exposed to an a_w of 0.90 experienced an SLS of pH 3.50, the authors

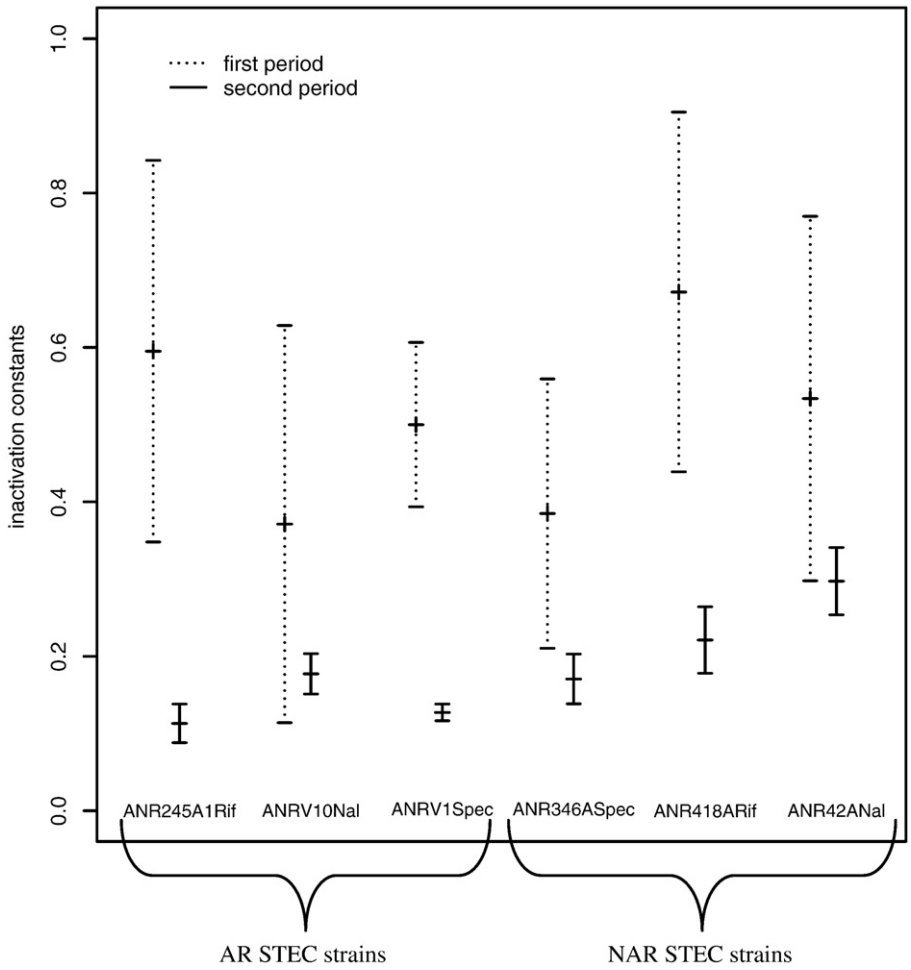

Fig. 3. Inactivation constants in each of the two periods with 95% confidence intervals. The slopes of the inactivation curve are given for the following 2 periods: period 1: day 0 to day 5; and period 2: day 5 to day 60.

observed a second rapid inactivation period before a subpopulation of more resistant cells emerged. This subpopulation was able to survive for approximately 50 h after the introduction of the SLS. In contrast, *E. coli* first exposed to a pH of 3.50 were inactivated rapidly to levels below the limits of detection when exposed to an SLS of a_w 0.90. Their results suggest that the mechanisms for pH and a_w are different and that the sequence and timing of stresses are very important. An acid stress may place a large energy demand on the cell and they also hypothesized that this energy demand greatly sensitizes the cell to successive treatments, such as a_w. Cross-protection is referred to as the ability of one stress condition to provide protection against other stresses. Microbial adaptation responses to one stress can lead to enhanced resistance to a different stress (Cheville et al., 1996; Riordan et al., 1998).

In this study the AR and NAR STEC strains were exposed to pH, a_w and temperature shock stresses. AR STEC strains, in contrast to NAR STEC strains, appear to have stress resistance systems that are able to enhance their survival during the drying step (period 2). Taking into account these elements non-AR STEC could be also sensitive for low a_w environments since they seemingly die off more quickly during the second phase where the pH is relatively high, and even non-lethal.

The survival of AR STEC during manufacture of fermented sausages could be compared to that of *E. coli* O157:H7 in the same foodstuff since O157:H7 are well known for their acid tolerance. Colombo (1998) showed that *E. coli* O157:H7 survived in Milano salami during the 60-day storage period when the initial population was 5.31 log CFU/g.

Though the fermentation and/or the drying of the product can reduce the risk of the presence of pathogens, in many cases they do not eliminate them completely. The present study shows that the manufacturing process of French fermented raw meat sausages results in a full destruction of NAR STEC strains after 60 days but does not have the same effect on the AR STEC strains. Even though non pathogenic STEC strains have been used in these experiments due to safety level requirements, we suggest that AR pathogenic STEC strains, such as those belonging to the 5 majors serogroups (O157, O26, O103, O111 and O145), could survive the fermenting process as well, a hypothesis that has been confirmed by outbreaks associated with fermented meat sausages. Because of the low infective dose and the high mortality rate associated with *E. coli* O157:H7 and non-O157 Shiga-like toxin producing *E. coli*, the presence of even occasionally low numbers of pathogens at the time of consumption cannot be tolerated. Consequently, efforts should be made to reduce fecal contamination at the abattoir level, by improving hygiene standards and control procedures.

Acknowledgements

This research was supported by funds from the National Agency of the Research (ANR-05-PNRA-021(2005) and InnoViandes.

References

Ammon, A., Petersen, L.R., Karch, H., 1999. A large outbreak of hemolytic uremic syndrome caused by an unusual sorbitol-fermenting strain of *Escherichia coli* O157: H. Journal of Infection and Diseases 179, 1274–1277.

Beutin, L., Zimmermann, S., Gleier, K., 1998. Human infections with Shiga toxin-producing *Escherichia coli* other than serogroup O157 in Germany. Emerging Infectious Diseases 4, 635–639.

Blanco, J., Blanco, M., Blanco, J.E., Mora, A., Alonso, M.P., González, E.A., Bernárdez, M.I., 2001. Epidemiology of verocytotoxigenic *Escherichia coli* (VTEC) in ruminants. In: Duffy, G., Garvey, P., McDowell, D. (Eds.), Verocytotoxigenic *Escherichia coli*. Food and Nutrition Press Inc., Trumbull, Conn., pp. 113–148.

Calicioglu, M., Faith, N.G., Buege, D.R., Luchansky, J.B., 1997. Viability of *Escherichia coli* O157:H7 in fermented semidry low temperature-cooked beef summer sausage. Journal of Food Protection 60, 1158–1162.

Castanie-Cornet, M.P., Penfound, T.A., Smith, D., Elliott, J.F., Foster, J.W., 1999. Control of acid resistance in *Escherichia coli*. Journal of Bacteriology 181, 3525–3535.

Castano, A., Garcia Fontan, M.C., Fresno, J.M., Tornadijo, M.E., Carballo, J., 2002. Survival of Enterobacteriaceae during processing of Chorizo de cebolla, a Spanish fermented sausage. Food Control 13, 107–115.

Chacon, P.A., Muthukumarasamy, P., Holley, R.A., 2006. Elimination of *Escherichia coli* O157:H7 from fermented dry sausages at an organoleptically acceptable level of microencapsulated allyl isothiocyanate. Applied and Environmental Microbioliogy 72, 3096–3102.

Chapman, P.A., Cerdan Malo, A.T., Ellin, M., Ashton, R., Harkin, 2001. *Escherichia coli* O157 in cattle and sheep at slaughter, on beef and lamb carcasses and in raw beef and lamb products in South Yorkshire, UK. International Journal of Food Microbiology 64, 139–150.

Cheville, A.M., Arnold, K.W., Buchrieser, C., Cheng, C.M., Kaspar, C.W., 1996. rpoS regulation of acid, heat, and salt tolerance in *Escherichia coli* O157:H7. Applied Environmental Microbiology 62, 1822–1824.

Clavero, M.R.S., Beuchat, L.R., 1996. Survival of *Escherichia coli* O157:H7 in broth and processed salami as influenced by pH, water activity and temperature, and suitability of media for its recovery. Applied and Environmental Microbiology 62, 2735–2740.

Colombo, S., 1998. Survival of E. coli O157:H7 in salami. Notiziario 11, 2–3.

Conner, D.E., Kotrola, J.S., 1995. Growth and survival of *Escherichia coli* O157:H7 under acidic conditions. Applied and Environmental Microbiology 61, 382–385.

Díaz, O., Fernandez, M., De Fernando, G.D.G., de la Hoz, L., Ordoñez, J.A., 1997. Proteolysis in dry fermented sausages: the effect of selected exogenous proteases. Meat Science 46, 115–128.

Eklund, M., Scheutz, F., Siitonen, A., 2001. Clinical isolates of non-O157 Shiga toxin-producing *Escherichia coli*: serotypes, virulence characteristics, and molecular profiles of strains of the same serotype. Journal of Clinical Microbiology 39, 2829–2834.

Erkkila, S., Venalainen, M., Hielm, S., Petaja, E., Puolanne, E., Mattila-Sandholm, T., 2000. Survival of *Escherichia coli* O157:H7 in dry sausage fermented by probiotic lactic acid bacteria. Journal of the Science of Food and Agricuture 80, 2101–2104.

Ethelberg, S., Smith, B., Torpdahl, M., Lisby, M., Boel, J., Jensen, T., Mølbak, K., 2007. An outbreak of verocytotoxin-producing *Escherichia coli* O26:H11 caused by beef sausage, Denmark 2007. Eurosurveillance 12.

Faith, N.G., Parniere, N., Larson, T., Lorang, T.D., Luchansky, J.B., 1997. Viability of *Escherichia coli* O157:H7 in pepperoni during the manufacture of sticks and the subsequent storage of slices at 21, 4 and −20 degrees C under air, vacuum and CO2. International Journal of Food Microbiology 37, 47–54.

Faith, N.G., Parniere, N., Larson, T., Lorang, T.D., Kaspar, C.W., Luchansky, J.B., 1998. Viability of *Escherichia coli* O157:H7 in salami following conditioning of batter, fermentation and drying of sticks, and storage of slices. Journal of Food Protection 61, 377–382.

Food Chemical News, 1994. Unusual E. coli strain causes foodborne illness in Montana.

Foster, J.W., 1993. The acid tolerance response of *Salmonella typhimurium* involves transient synthesis of key acid shock proteins. Journal of Bacteriology 175, 1981–1987.

Foster, J.W., Hall, H.K., 1990. Adaptive acidification tolerance response of *Salmonella typhimurium*. Journal of Bacteriology 172, 771–778.

Getty, K.J.K., Phebus, R.K., Marsden, J.L., Fung, D.Y.C., Kastner, C.L., 2000. *Escherichia coli* O157:H7 and fermented sausages. Journal of Rapid Methods and Automation in Microbiology 8, 141–170.

Glass, K.A., Loeffelholz, J.M., Ford, J.P., Doyle, M.P., 1992. Fate of *Escherichia coli* O157:H7 as affected by pH or sodium chloride and in fermented, dry sausage. Applied and Environmental Microbiology 58, 2513–2516.

Goodson, M., Rowbury, R.J., 1989. Habituation to normally lethal acidity by prior growth of *Escherichia coli* at a sub-lethal acid pH value. Letters in Applied Microbiology 8, 77–79.

Hinkens, J.C., Faith, N.G., Lorang, T.D., Bailey, P., Buege, D., Kaspar, C.W., Luchansky, J.B., 1996. Validation of pepperoni processes for control of *Escherichia coli* O157:H7. Jounal of Food Protection 59, 1260–1266.

Johnson, R., Clarke, R.C., Wilson, J.B., Read, S.C., Rahn, K., Renwick, S.A., Sandhu, K.A., Alves, D., Karmali, M.A., Lior, H., McEwen, S.A., Spika, J.S., Gyles, C.L., 1996. Growing concerns and recent outbreaks involving non-O157:H7 serotypes of verotoxigenic *Escherichia coli*. Journal of Food Protection 59, 1112–1122.

Johnson, K.E., Thorpe, C.M., Sears, C.L., 2006. The emerging clinical importance of non-O157 Shiga toxin-producing *Escherichia coli*. Clinical Infectious Diseases 43, 1587–1595.

Lin, J., Smith, M.P., Chapin, K.C., Baik, H.S., Bennett, G.N., Foster, J.W., 1996. Mechanisms of acid resistance in enterohemorrhagic *Escherichia coli*. Applied and Environmental Microbiology 62, 3094–3100.

Miller, L.G., Kaspar, C.W., 1994. *Escherichia coli* O157:H7 acid tolerance and survival in apple cider. Journal of Food Protection 57, 460–464.

Muthukumarasamy, P., Holley, R.A., 2007. Survival of *Escherichia coli* O157:H7 in dry fermented sausages containing micro-encapsulated probiotic lactic acid bacteria. Food Microbiology 24, 82–88.

Nataro, J.P., Kaper, J.B., 1998. Diarrheagenic *Escherichia coli*. Clinical Microbiology Reviews 11, 142–201.

Paton, A.W., Ratcliff, R.M., Doyle, R.M., Seymour-Murray, J., Davos, D., Lanser, J.A., Paton, J.C., 1996. Molecular microbiological investigation of an outbreak of hemolytic-uremic syndrome caused by dry fermented sausage contaminated with Shiga-like toxin-producing *Escherichia coli*. Journal of Clinical Microbioliogy 34, 1622–1627.

R Development Core Team., 2007. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. ISBN: 3-900051-07-0. Vienna, Austria, (http://www.R-project.org).

Read, S.C., Clarke, R.C., Martin, A., De Grandis, S.A., Hii, J., McEwen, S., Gyles, C.L., 1992. Polymerase chain reaction for detection of verocytotoxigenic *Escherichia coli* isolated from animal and food sources. Molecular and Cellular Probes 6, 153–161.

Riordan, D.C., Duffy, G., Sheridan, J., Eblen, B.S., Whiting, R.C., Blair, I.S., McDowell, D.A., 1998. Survival of *Escherichia coli* O157:H7 during the manufacture of pepperoni. Journal of Food Protection 61, 146–151.

Ross, T., Zhang, D., McQuestin, O.J., 2008. Temperature governs the inactivation rate of vegetative bacteria under growth-preventing conditions. International Journal of Food Microbiology.

Shadbolt, C.T., Ross, T., McMeekin, T.A., 1999. Nonthermal death of *Escherichia coli*. International Journal of Food Microbiology 49, 129–138.

Shadbolt, C., Ross, T., McMeekin, T.A., 2001. Differentiation of the effects of lethal pH and water activity: food safety implications. Letters in Applied Microbiology 32, 99–102.

Tarr, P.I., Neill, M.A., 1996. Perspective: the problem of non-O157:H7 Shiga toxin (Verocytotoxin)-producing *Escherichia coli*. Journal of Infection and Diseases 174, 1136–1139.

Tilden, J.J., Young, W., McNamara, A.M., Custer, C., Boesel, B., Lambert-Fair, M.A., Majkowski, J., Vugia, D., Werner, S.B., Hollingsworth, J., Morris, J.G.J., 1996. A new route of transmission for *Escherichia coli*: infection from dry fermented salami. American Journal of Public Health 86, 1142–1145.

Truong, Q.C., Nguyen Van, J.C., Shlaes, D., Gutmann, L., Moreau, N.J., 1997. A novel, double mutation in DNA gyrase A of *Escherichia coli* conferring resistance to quinolone antibiotics. Antimicrobial Agents and Chemotherapy 41, 85–90.

Varnam, A.H., Evans, M.G., 1991. In: Varnam, A.H., Evans, M.G. (Eds.), Foodborne Pathogens. An Illustrated Text. Wolfe Publishing Ltd, Aylesbury, England.

Yu, C., Chou, C., 1998. Fate of *Escherichia coli* O157:H7 in Chinese-style sausage subjected to different packaging and storage conditions. Journal of the Science of Food and Agricuture 78, 486–490.

Zhao, T., Doyle, M.P., 1994. Fate of enterohemorrhagic *Escherichia coli* O157:H7 in commercial mayonnaise. Journal of Food Protection 57, 780–783.