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Fitness of Enterohemorrhagic *Escherichia coli* (EHEC)/Enteroaggregative *E. coli* O104:H4 in Comparison to That of EHEC O157: Survival Studies in Food and *In Vitro*

Christina Böhnlein,[a] Jan Kabisch,[a] Diana Meske,[a] Charles M. A. P. Franz,[a] Rohtraud Pichner[a,b]

Department of Microbiology and Biotechnology, Max Rubner-Institut, Federal Research Institute of Nutrition and Food, Kiel, Germany[a]; Department of Nutritional, Food, and Consumer Sciences, University of Applied Sciences, Fulda, Germany[b]

ABSTRACT

In 2011, one of the world's largest outbreaks of hemolytic-uremic syndrome (HUS) occurred, caused by a rare *Escherichia coli* serotype, O104:H4, that shared the virulence profiles of Shiga toxin-producing *E. coli* (STEC)/enterohemorrhagic *E. coli* (EHEC) and enteroaggregative *E. coli* (EAEC). The persistence and fitness factors of the highly virulent EHEC/EAEC O104:H4 strain, grown either in food or *in vitro*, were compared with those of *E. coli* O157 outbreak-associated strains. The log reduction rates of the different EHEC strains during the maturation of fermented sausages were not significantly different. Both the O157:NM and O104:H4 serotypes could be shown by qualitative enrichment to be present after 60 days of sausage storage. Moreover, the EHEC/EAEC O104:H4 strain appeared to be more viable than *E. coli* O157:H7 under conditions of decreased pH and in the presence of sodium nitrite. Analysis of specific EHEC strains in experiments with an EHEC inoculation cocktail showed a dominance of EHEC/EAEC O104:H4, which could be isolated from fermented sausages for 60 days. Inhibitory activities of EHEC/EAEC O104:H4 toward several *E. coli* strains, including serotype O157 strains, could be determined. Our study suggests that EHEC/EAEC O104:H4 is well adapted to the multiple adverse conditions occurring in fermented raw sausages. Therefore, it is strongly recommended that STEC strain cocktails composed of several serotypes, instead of *E. coli* O157:H7 alone, be used in food risk assessments. The enhanced persistence of EHEC/EAEC O104:H4 as a result of its robustness, as well as the production of bacteriocins, may account for its extraordinary virulence potential.

IMPORTANCE

In 2011, a severe outbreak caused by an EHEC/EAEC serovar O104:H4 strain led to many HUS sequelae. In this study, the persistence of the O104:H4 strain was compared with those of other outbreak-relevant STEC strains under conditions of fermented raw sausage production. Both O157:NM and O104:H4 strains could survive longer during the production of fermented sausages than *E. coli* O157:H7 strains. *E. coli* O104:H4 was also shown to be well adapted to the multiple adverse conditions encountered in fermented sausages, and the secretion of a bacteriocin may explain the competitive advantage of this strain in an EHEC strain cocktail. Consequently, this study strongly suggests that enhanced survival and persistence, and the presumptive production of a bacteriocin, may explain the increased virulence of the O104:H4 outbreak strain. Furthermore, this strain appears to be capable of surviving in a meat product, suggesting that meat should not be excluded as a source of potential *E. coli* O104:H4 infection.

The Shiga toxin-producing *Escherichia coli* (STEC) strains, which include enterohemorrhagic *E. coli* (EHEC) strains, are pathogenic *E. coli* strains with the potential to cause severe enteric and systemic disease in humans. These bacteria have accounted for various foodborne infections over recent decades. The pathogenesis of EHEC disease depends on the production of Shiga toxin (Shiga toxin 1 [Stx1] and/or Stx2) in combination with other virulence factors. EHEC strains typically harbor virulence genes for attachment (e.g., intimin genes) or potentiating toxin genes (e.g., subtilase cytotoxin) (1). Non-sorbitol-fermenting (NSF) *E. coli* O157:H7 is recognized as one of the most important foodborne pathotypes occurring worldwide and is associated with human diseases including diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome (HUS). Moreover, sorbitol-fermenting (SF) *E. coli* O157:NM (nonmotile) strains have emerged as important pathogens, because they are associated with a higher incidence of progression to HUS than NSF *E. coli* O157:H7 strains, especially in continental Europe (2–4). The proportion of STEC infections in Europe caused by non-O157 serotypes increased from 45.1% to 51.1% in 2013 (5). Even though the clinical manifestations of non-O157 STEC infections may differ, they can be as virulent as O157:H7 infections.

This was shown to be the case in one of the world's largest outbreaks of HUS, in Germany in 2011. The causative agent was identified as a rare *E. coli* serotype, O104:H4, that exhibited the virulence profiles of both typical Shiga toxin-producing *E. coli* (EHEC) and enteroaggregative *E. coli* (EAEC) (6). The phenotypes expressed by the O104:H4 German outbreak strain included the production of Stx2, aggregative adherence to epithelial cells,

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Address correspondence to Christina Böhnlein, christina.boehnlein@mri.bund.de.

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and the production of extended-spectrum β-lactamase (ESBL) (6, 7). The data suggest that besides enhanced adherence to intestinal epithelial cells, which might facilitate the systemic absorption of Shiga toxin, resistance to antibiotics was also responsible for the apparently augmented virulence potential of the O104:H4 outbreak strain (6). Consumption of sprouted fenugreek seeds was identified as the most likely source of infection for primary outbreak cases (8). Particularly later in the outbreak, person-to-person transmission and foodborne outbreaks associated with infected food handlers also took place (9).

Humans have been shown to be a reservoir both for EAEC strains, including O104:H4 strains, and for SF *E. coli* O157:NM, in addition to other possible, but still unknown, reservoirs (6, 10–13). Several studies have also provided evidence of a reservoir for SF *E. coli* O157:NM in cattle (14–17). Recently, genes characteristic of the O104:H4 German outbreak strain were found in cattle herd samples from one abattoir located near the outbreak epicenter (18). Consequently, like other STEC/EHEC serotypes, both *E. coli* O104:H4 and *E. coli* O157:NM might enter the food chain via cattle as the potential reservoir. After a number of foodborne STEC outbreaks that involved fermented sausages, the European Union considered that minced and/or fermented beef and its products represent a hazard to public health with regard to these pathogens (19). Commonly, the incorporation of sequential or concurrent hurdles consisting of different preservation strategies, such as the addition of preservatives (nitrite or sodium ascorbate), the growth of competitive microbiota, acidification, smoking, and drying, result in safe and stable fermented-sausage products (20). However, a delayed start of fermentation and short curing periods at cold temperatures were identified as the main factors enabling EHEC survival in fermented sausage. These factors were linked to an outbreak of *E. coli* O157:H7 in southern Sweden, where contaminated beef was suspected to be the source of infection (21). Even the issue of cross-contamination, e.g., by asymptomatic infected persons (22), should not be ignored; cross-contamination may also contribute to the presence of STEC/EHEC, including *E. coli* O104:H4 and O157:NM, in fermented sausage or generally, in all foods. Many studies have dealt with the survival and inactivation of *E. coli* O157:H7 in different food matrices, but little research has been carried out so far on the persistence of non-O157 STEC in foods. Rode et al. (23) described the sorbitol-fermenting O157:NM outbreak strain as the STEC strain that showed the greatest ability to survive the conditions in fermented sausages. Only a few studies investigated the survival of the EHEC/EAEC O104:H4 outbreak strain, and these focused mainly on plant foods (24–27). Moreover, the European Food Safety Authority (EFSA) recently expressed the need for further controlled studies that fully quantify the survival characteristics of EAEC in wet and dry substrates under laboratory and natural conditions (28).

In order to gain basic knowledge about the survival and fitness of the EHEC/EAEC O104:H4 strain in food, compared to those of the *E. coli* O157:H7 and sorbitol-fermenting *E. coli* O157:NM outbreak-associated strains, challenge studies were carried out in fermented raw sausages as well in *in vitro* assays under conditions similar to those occurring in the sausage environment. These conditions included decreased pH, the presence of sodium nitrite, and moderate fermentation temperatures. Moreover, the most resistant EHEC isolate that survives the conditions in fermented raw sausages was determined in multistrain cocktail inoculum experiments.

MATERIALS AND METHODS

Bacterial isolates and growth conditions. Two EHEC strains linked to recent outbreaks in Germany were used in the present study. The *E. coli* O104:H4 (MRI collection number E965) and O157:NM (MRI collection number E963) isolates, recovered from HUS patients in 2011 and 2009, respectively, were kindly provided by the Bavarian Health and Food Safety Authority (Oberschleißheim, Germany). *E. coli* O157:H7 EDL933 (ATCC 43895; MRI collection number E135) was used as the reference strain (29). Selected strains of *Enterobacteriaceae* from the MRI strain collection ($n = 25$) served as indicator strains in inhibition assays with *E. coli* O104:H4 (Table 1).

Stock cultures were maintained in cryobeads (Pro-Lab Diagnostics Microbank bacterial and fungal preservation system; bestbion^{dx}, Cologne, Germany) at −70°C. Bacteria were revived in 9 ml of brain heart infusion (BHI; Oxoid, Wesel, Germany) or Trypticase soy broth plus 0.5% yeast extract (TSBY) for 16 h at 37°C. For inoculation experiments with either a single strain or a cocktail of strains, cell concentrations were determined in a Thoma counting chamber and were then diluted to an inoculation level of 3 $\log_{10}$ CFU/g. This was confirmed by plating on sorbitol-MacConkey (SMAC) agar (Oxoid, Wesel, Germany). Colonies were enumerated after the incubation of plates for 24 h at 37°C.

Sausage preparation. Fresh raw materials of high quality were used in order to keep background microbiota at a minimum. Batters for the production of pure beef salami were prepared from lean beef (70%) and abdominal fat (30%) according to a standard recipe (30). Sliced frozen meat and fat ingredients were chopped in a rotating bowl of a meat cutter (ETK 20/1 [10 liters]; E.-Müller, Saarbrücken, Germany) with the addition of sodium ascorbate (0.05%), brine salt (Südsalz GmbH, Heilbronn, Germany) with sodium nitrite (150 ppm), glucose (0.5%), saccharose (0.5%), and pepper (0.3%). Freeze-dried BITEC LS-1 starter culture, comprising *Lactobacillus curvatus*, *Staphylococcus carnosus*, and *Kocuria varians* (Gewürzmüller GmbH, Korntal-Münchingen, Germany), was added at approximately 6 $\log_{10}$ CFU/g to the sausage batter. For challenge experiments, single EHEC strains were either mixed alone into different salami batters or were combined as an EHEC cocktail inoculum that contained equal numbers of each strain (1:1:1) and a final concentration of 3 $\log_{10}$ CFU/g for inoculation. Batters were stuffed into 60-mm synthetic casings (Naturin Viscofan GmbH, Weinheim, Germany). Sausages were adjusted to room temperature for 4 to 5 h before fermentation and ripening in a climate-controlled cabinet (Karl Weiss, Giessen, Germany). The production process included fermentation for 72 h in total with 24 h each at 22°C (relative humidity [RH], 93 to 94%), 21°C (RH, 90 to 92%), and 20°C (RH, 90%). After maturation at 18°C for 72 h (RH, 88%) and 48 h (RH, 85%), sausages remained for an additional 9 days at 17°C (RH, 83 to 85%). According to the standard manufacturer's procedure, the salami products were vacuum packed 18 days after preparation and were stored at ambient temperature. For each inoculation experiment with a single strain or the triple-EHEC-strain cocktail, three salami batches were produced on different days, together with an uninoculated control batch. Three technical replicates were used for chemical and microbiological analyses. Samples were tested on the production day (day 0), during ripening and maturation (days 1, 2, 3, 7, and 14), after vacuum packaging and storage (days 21, 28, and 42), and at the end of the product's shelf life (day 60).

Chemical and microbiological analyses. Control batches were tested in triplicate for pH and water activity (a_w) on each sampling day (see above). For this purpose, a 25-g sample was homogenized in 225 ml deionized water in a laboratory paddle blender (Stomacher 400 Circulator; Seward), and the pH of the homogenate was measured with a pH meter (WTW pH 526). The a_w of sausages was determined with a cryometer (Nagy AWK-20). For comparison, commercial beef salami products made with a similar recipe and similar production periods ($n = 5$) were also tested for pH and a_w.

For microbiological analyses, 25 g of a sausage sample or a commercial beef salami product was added to 225 ml buffered peptone water (BPW)

TABLE 1 Inhibitory activity of *E. coli* O104:H4 in competition assays with selected *Enterobacteriaceae* indicator strains

Indicator strain	Serotype	Collection no.	Source	Inhibition zone[a]
Escherichia coli	O157:H7	E135 (EDL933; ATCC 43895)	Meat (burger)	+++
	O157:H7	E118	Bovine feces	++
	O157:H7	E141	Apple cider	++
	O157:NM	E963	Human feces (HUS)	++
	O157:NM	E148	Human feces (HUS)	++
	O26:H11	E157	Human feces (HUS)	++
	O26:H11	E165	Calf feces	+
	O26:H11	E972	Wild boar meat	++
		E162	Ice cream	+++
		E163	Smear cheese	+
		E164	Raw milk	+++
Salmonella enterica subsp. *enterica*	Typhimurium	S509	Swab (meat plant)	−
	Typhimurium	S692	Bovine feces	−
	Typhimurium	S702 (ATCC 14028)	Heart/liver tissues (chicken)	−
	Enteritidis	S522	Mettwurst sausage	−
	Enteritidis	S575	Lymphatic tissue (pig)	−
	Enteritidis	S630	Meat juice	−
Citrobacter freundii		Ci5	Bovine feces	−
		Ci24	Chicken meat	−
		Ci29	Dry cured ham	−
Hafnia spp.		NE146	Teewurst	−
		NE137	Swab (meat plant)	−
		NE142	Meat	−
Klebsiella oxytoca		Kl31	Ground meat	−
		NE226	Human feces	−

[a] Results of three experiments in a duplicate setup are presented. Diameters of inhibition zones were calculated and are indicated as +++ (>15 mm), ++ (15 to 10 mm), or + (10 to 5 mm); −, no inhibition activity.

and was macerated for 5 min in a stomacher. The homogenate was serially diluted and was spread plated, using an automatic spiral plater (Eddy Jet; IUL Instruments, Königswinter, Germany), onto plate count (PC) agar (Oxoid, Wesel, Germany) for total mesophilic aerobic bacteria (MAB), onto de Man, Rogosa, and Sharpe (MRS) agar (Oxoid, Wesel, Germany) for lactic acid bacteria (LAB), and onto SMAC agar (Oxoid, Wesel, Germany) for EHEC counts. Colonies were enumerated after incubation for 48 h at 30°C (PC agar and MRS agar) and for 24 h at 37°C (SMAC agar). For counts lower than the limit of quantitative detection (10 CFU/g), EHEC strains were recovered by selective or nonselective enrichment in modified Trypticase soy broth supplemented with novobiocin (mTSB-N) (Oxoid, Wesel, Germany) or BPW, respectively. For this purpose, a 25-g sample of raw material (beef and fat) or sausage was homogenized in 225 ml mTSB or BPW and was incubated with shaking at 37°C for 18 h. One loop of enrichment broth was then streaked out onto SMAC agar in order to determine the presence of viable EHEC cells. As a confirmation, multiplex real-time PCR with the CFX96 Touch real-time PCR detection system (Bio-Rad Laboratories GmbH, Munich, Germany) was also used to detect EHEC bacteria, as described by Pavlovic et al. (31) (see below).

In inoculation experiments with the cocktail of EHEC strains, biochemical and molecular traits were used to distinguish between the different EHEC strains. The total EHEC count was determined on SMAC agar, which allowed identification of the colorless colonies as the non-sorbitol-fermenting *E. coli* O157:H7 strain. Red colonies, on the other hand, were picked and were resuspended in 1 ml BPW. After transfer to Brilliance ESBL agar (Oxoid, Wesel, Germany) with a sterile toothpick and incubation for 24 h at 37°C, the isolates that were able to grow on this medium were identified as *E. coli* O104:H4, while those that did not were characterized as *E. coli* O157:NM. At least five colonies from SMAC agar plates from which each of the three EHEC strains could be identified were confirmed as belonging to the respective types by multiplex real-time PCR, which targeted the virulence genes stx_1, stx_2, and *eae* (31). For DNA extraction, a single colony was boiled in 100 μl *Limulus* amoebocyte lysate (LAL) reagent water (VWR, Ismaning, Germany) for 15 min and was centrifuged for 3 min at 9,000 × *g*. The supernatant was stored at −20°C until use. Each PCR mixture (25 μl) contained 2× master mix (DyNAmo Flash Probe qPCR kit; Biozym, Hessisch Oldendorf, Germany) with primers and probes at the concentrations described by Pavlovic et al. (31), as well as 5 μl of template DNA. Thermal cycling and detection were performed using the CFX96 Touch real-time PCR detection system (Bio-Rad, Munich, Germany). Differences in the virulence gene profiles of the different EHEC strains resulted in positive signals for all three virulence genes (stx_1, stx_2, and *eae*) (*E. coli* O157:H7), for stx_2 and *eae* only (*E. coli* O157:NM), or for *eae* only (*E. coli* O104:H4).

***In vitro* survival assays.** The conditions found in raw sausage—decreased pH, the presence of sodium nitrite, and moderate fermentation temperatures—were simulated *in vitro*. For this purpose, assays with *E. coli* O104:H4 and O157:H7 were performed in TSBY adjusted to pH 5.1 with 20% (vol/vol) lactic acid (Sigma-Aldrich, Taufkirchen, Germany) either in the absence or in the presence of 150 mg/liter sodium nitrite (Merck, Darmstadt, Germany). TSBY without any additions was used as a control. Three broth batches—TSBY, TSBY at pH 5.1, and TSBY at pH 5.1 containing 150 mg/liter sodium nitrite—were inoculated with 7 $\log_{10}$ CFU/ml of one of the EHEC strains as determined by a Thoma cell counting chamber (see above). After 0, 18, 42, and 66 h of incubation at 22°C, cell viabilities were assessed using the LIVE/DEAD BacLight viability kit (Invitrogen, Life Technologies GmbH, Darmstadt, Germany). Stock solutions of the two nucleic acid stains used for this method, green fluorescent SYTO 9 dye and red fluorescent propidium iodide, were prepared in 5 ml distilled water (dH_2O) and were stored in aliquots at 4°C according

TABLE 2 Chemical parameters and bacterial cell counts of total mesophilic aerobic bacteria, lactic acid bacteria, and EHEC during the fermentation and storage of beef salami[a]

	Chemical parameter		Bacterial cell count[b] ($\log_{10}$ CFU/g)					
					E. coli			
Day	pH	a_w	MAB	LAB	O157:H7	O157:NM	O104:H4	EHEC cocktail
0	5.54 ± 0.10	0.959 ± 0.002	7.19 ± 0.09	6.95 ± 0.21	3.55 ± 0.26	3.60 ± 0.09	3.06 ± 0.30	3.32 ± 0.36
1	5.56 ± 0.09	0.957 ± 0.006	7.91 ± 0.20	7.85 ± 0.19	3.10 ± 0.30	3.26 ± 0.11	3.10 ± 0.27	2.92 ± 0.09
3	5.12 ± 0.14	0.953 ± 0.003	8.77 ± 0.22	8.83 ± 0.15	2.47 ± 0.25	2.58 ± 0.12	2.37 ± 0.30	2.61 ± 0.36
7	5.00 ± 0.14	0.942 ± 0.005	8.79 ± 0.13	8.87 ± 0.12	2.15 ± 0.18	2.45 ± 0.20	2.28 ± 0.25	2.36 ± 0.32
14	5.03 ± 0.11	0.909 ± 0.01	8.86 ± 0.16	8.83 ± 0.12	1.84 ± 0.32	1.92 ± 0.39	1.65 ± 0.66	2.26 ± 0.35
21	5.05 ± 0.09	0.893 ± 0.015	8.76 ± 0.13	8.79 ± 0.15	1.15 ± 0.40	<1	<1	1.30 ± 0.57
28	5.07 ± 0.11	0.891 ± 0.015	8.67 ± 0.18	8.71 ± 0.18	<1	<1	<1	<1
42	5.10 ± 0.10	0.886 ± 0.014	8.46 ± 0.20	8.60 ± 0.23	<1	<1	<1	<1
60	5.24 ± 0.12	0.876 ± 0.016	8.04 ± 0.51	8.08 ± 0.30	<1	<1	<1	<1

[a] Results are means ± standard deviations for three experiments in a triplicate setup. MAB, total mesophilic aerobic bacteria; LAB, lactic acid bacteria.
[b] The limit of quantitative detection was 1 $\log_{10}$ CFU/g.

to the manufacturer's instructions. Ten milliliters of bacterial suspensions was centrifuged for 15 min at 3,000 × *g*, and the pellet was washed twice in 10 ml dH_2O and was then resuspended in 1 ml dH_2O. Fifteen microliters of the staining stock solution and 15 μl of the bacterial suspension were mixed and were incubated in the dark for 15 min. For fluorescence detection, 5 μl of the stained mixture was placed on a microscope slide under a coverslip, spread with immersion oil, and analyzed using a fluorescence microscope at a magnification of ×100 (Olympus BX60 microscope; BH2-RFL-T3 power supply; U-MWB filter module; band pass 450- to 480-nm exciter filter). The respective numbers of dead, viable, and damaged cells were determined by counting red, green, and orange cells, respectively, in the field of view for 1 min in triplicate, and the percentages of live and dead cells were calculated. All experiments were repeated three times.

Inhibition assays. The inhibitory spectrum of *E. coli* O104:H4 was determined according to the method of Bigwood et al. (32). Briefly, *E. coli* O104:H4 was grown as streaks across the center of TSA (TSB containing 1.5% [wt/vol] agar) plates, and the plates were incubated for 16 h at 37°C. At the same time, indicator microorganisms (Table 1) were grown for 16 h at 37°C in TSB, and 0.1 ml of overnight cultures was inoculated into 10 ml TSB and was incubated for 4 h at 37°C. A 0.1-μl volume was then added to 4 ml soft TSA (0.7% agar) equilibrated at 47°C. The tube was briefly vortexed, and the agar was then poured as an overlay onto the TSA plate with *E. coli* O104:H4 growth. Plates were incubated overnight at 37°C before examination for inhibition zones of the bacterial lawn in the agar overlay. Inhibitory activity was regarded as strong (+++) if the diameter of the zone of clearing measured >15 mm, as moderate (++) if the diameter was 15 to 10 mm, and as weak (+) if the diameter was 10 to 5 mm.

The published genome of *E. coli* O104:H4 strain 2011C-3493 (33) was analyzed *in silico* with the BAGEL automated bacteriocin mining tool (http://bagel2.molgenrug.nl/). BAGEL is a Web server that identifies putative bacteriocin open reading frames (ORFs) in a DNA sequence using novel, knowledge-based bacteriocin databases and motif databases (34).

Statistical analysis. Colony counts were log-transformed, and samples with counts below the quantitative limit of detection (LOD) (<1 $\log_{10}$ CFU/g) that were positive after an enrichment procedure were arbitrarily assigned a value of 0.7 $\log_{10}$ CFU/g (≙5 CFU/g). Samples that were negative in the enrichment procedure were assigned the value of zero. $\log_{10}$ reductions in EHEC counts during sausage production were calculated by subtracting the $\log_{10}$ CFU of EHEC per gram of fermented sausage (day 3, 7, or 14) from the $\log_{10}$ CFU of EHEC per gram of sausage batter on the production day (day 0). Median and mean values, as well as standard deviations, were calculated. EHEC fractions in the inoculation experiments with triple-strain cocktails were determined by dividing the count of non-sorbitol-fermenting bacteria on SMAC agar or Brilliance ESBL agar by the total-colony count on SMAC agar (and multiplying by 100 to determine the percentage). One-way analysis of variance (ANOVA) with SigmaPlot, version 11.0, was used to analyze differences in EHEC reduction rates during salami fermentation and differences in cell viability between strains in correlation with incubation conditions at a significance level (P) of <0.05 (Tukey test) or <0.001 (Holm-Sidak method).

RESULTS

Reductions in EHEC counts during sausage production. All samples of raw material from beef and fat were free of STEC at the beginning of the experiment, as determined after enrichment for STEC. The noninoculated sausages remained negative for STEC throughout storage. The pH of beef salami decreased from 5.54 ± 0.10 to 5.00 ± 0.14 within 7 days and then increased during storage until a pH of 5.24 ± 0.12 was obtained at day 60 (Table 2). In contrast, the a_w gradually decreased from 0.959 ± 0.002 to 0.876 ± 0.016 within 60 days (Table 2). During fermentation and ripening, the products experienced a weight loss of 28.2% ± 1.7% in total. The commercial beef salami products, used as controls ($n = 5$), exhibited pH values ranging from 4.50 to 4.73 and a_w values of 0.874 to 0.886 on the day of purchase, which was approximately 3 to 4 weeks postproduction (data not shown).

Total counts of mesophilic aerobic bacteria (MAB) and lactic acid bacteria (LAB) were ca. 7 $\log_{10}$ CFU/g at day 0 (Table 2). The MAB and LAB counts then increased and reached almost 9 $\log_{10}$ CFU/g on day 3; they stayed at this level up to day 28 and then decreased to approximately 8 $\log_{10}$ CFU/g (Table 2). Counts of 8 $\log_{10}$ CFU/g and 7 $\log_{10}$ CFU/g were determined for MAB and LAB in the commercial beef salami products, respectively.

In inoculation experiments, EHEC counts decreased rapidly within 3 days of sausage fermentation by about 0.75 to 1 $\log_{10}$ unit, to reach counts between 2.4 $\log_{10}$ CFU/g and 2.6 $\log_{10}$ CFU/g (Table 2; Fig. 1). In total, EHEC loads were reduced on day 14 by ca. 1.7 $\log_{10}$ units (*E. coli* O157:H7/NM) or 1.4 $\log_{10}$ units (*E. coli* O104:H4), whereas EHEC counts in fermented sausages inoculated with the three-EHEC-strain cocktail were reduced by only 1 $\log_{10}$ unit (Fig. 1). The log reduction rates of the various single EHEC strains differed significantly from that of the EHEC strain cocktail on day 14 ($P < 0.001$) (Fig. 1). The limit of detection (LOD) by direct plating was reached on day 14 for *E. coli* O157:NM and *E. coli* O104:H4 and on day 21 for O157:H7 and

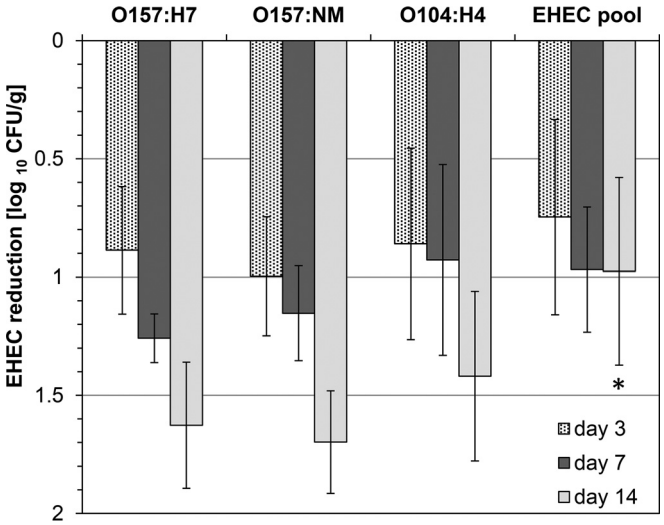

FIG 1 Reduction of EHEC counts in single- and triple-strain inoculation experiments during the fermentation and maturation of raw fermented sausages up to day 14. EHEC cell counts were determined on SMAC agar. Values are presented as the means for three independent experiments with a triplicate setup and are expressed as $\log_{10}$ CFU per gram. EHEC $\log_{10}$ reductions were calculated by subtracting the EHEC count in fermented sausages on day 3, 7, or 14 from the EHEC count in the sausage batter on the day of production (day 0) (with both counts expressed in $\log_{10}$ CFU per gram). The asterisk indicates a significant difference ($P < 0.001$) by the Holm-Sidak method.

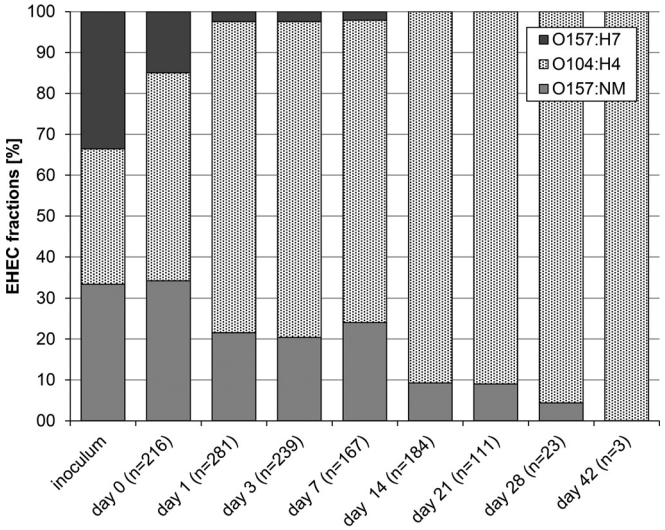

FIG 2 Percentages of occurrence of *E. coli* O157:H7, O104:H4, and O157:NM in the EHEC cocktail used as an inoculum in fermented sausages. Homogenized sausage samples were spread on SMAC agar for quantitative enumeration of EHEC, and isolates were further characterized. Red sorbitol-fermenting isolates were cultivated on ESBL agar for the screening of β-lactamase activity. Further molecular traits (the presence or absence of the virulence markers stx_1, stx_2, and *eae*) were used for identification of the different EHEC strains. Values are averages for three independent experiments; *n*, number of isolates.

the EHEC cocktail of strains (Table 2). After this time point, enrichment was used to detect the presence of EHEC. After 60 days, *E. coli* O157:NM and *E. coli* O104:H4 could be detected by mTSB-N enrichment in 10 of 12 and 6 of 12 samples, respectively, whereas *E. coli* O157:H7 was not detectable in any of the 18 samples tested (Table 3). More-efficient isolation of *E. coli* O104:H4 could be shown for the samples enriched with the nonselective medium BPW (69.7%) than for the mTSB-N enrichment broth samples (24.1%) from day 28 to day 60 (Table 3).

Sorbitol fermentation, β-lactamase production, and molecular detection of the virulence markers stx_1, stx_2, and *eae* were used to monitor strain persistence during the production and storage of fermented sausages that were inoculated with the cocktail of three different strains. The experiments were designed so that each of the EHEC strains occurred in the inoculum cocktail at the same level. After inoculation and adaption for 30 min in the food matrix, *E. coli* O157:H7, O104:H4, and O157:NM strains from the cocktail could be isolated on SMAC agar at incidences of 15%, 51%, and 34%, respectively, on day 0 (Fig. 2). During sausage fermentation, the proportional distribution of the individual strains initially inoculated as a cocktail shifted toward a remarkable dominance of *E. coli* O104:H4, with a decrease in the occurrence of the *E. coli* O157 serotypes. Only 2% of EHEC isolates on SMAC agar were NSF O157:H7 strains on days 1 to 7, and after 14 days, *E. coli* O157:H7 was undetectable (Fig. 2). Levels of *E. coli* O157:NM decreased from 34% to 4% within 28 days, and this serotype could not be detected on day 42. Quantitative detection was accomplished in individual samples on day 42, but only the *E. coli* O104:H4 strain could be identified (Fig. 2). BPW enrichment on day 60 produced positive results for 9 of 12 samples. Phenotypic and molecular analyses of the EHEC isolates from these samples matched only with the corresponding characteristics of the *E. coli* O104:H4 strain.

TABLE 3 Qualitative detection of EHEC by selective or nonselective enrichment during the storage of beef salami

Day	No. of samples positive for the indicated organism(s)/total no. of samples[a] in:				
	mTSB-N			BPW	
	O157:H7	O157:NM	O104:H4	O104:H4	EHEC cocktail
21	ND	11/11	9/12	ND	ND
28	15/15	16/16	5/18	9/9	12/12
42	7/18	18/18	2/18	11/12	12/12
60	0/18	10/12	6/12	3/12	9/12

[a] mTSB-N, modified Trypticase soy broth (selective enrichment); BPW, buffered peptone water (nonselective enrichment). The results of three independent experiments are presented. ND, not done (>1 $\log_{10}$ CFU/g).

Fitness of *E. coli* O104:H4 under food-related laboratory conditions. Raw sausage conditions—decreased pH, the presence of sodium nitrite, and moderate fermentation temperatures—were simulated *in vitro*. Cell viability was analyzed after 0, 18, 42, and 66 h of incubation at 22°C. *E. coli* O104:H4 appeared to be more viable than *E. coli* O157:H7, as indicated by larger proportions of live cells in all samples (97% ± 0.8% versus 93% ± 3.2% [$P < 0.001$]). In control (TSBY) and acidified (TSBY at pH 5.1) broth media, the viabilities of *E. coli* strain O157:H7 and O104:H4 cells were similar over time ($P > 0.05$). The addition of sodium nitrite led to significant differences in viability between *E. coli* O157:H7 and O104:H4 cells ($P < 0.05$) (Fig. 3). After 66 h of incubation, only 86% of *E. coli* O157:H7 cells were alive, while 97% of *E. coli* O104:H4 cells were alive (Fig. 3).

Bacteriocin production by *E. coli* O104:H4. The inhibitory range of *E. coli* O104:H4 (E965) was determined with selected

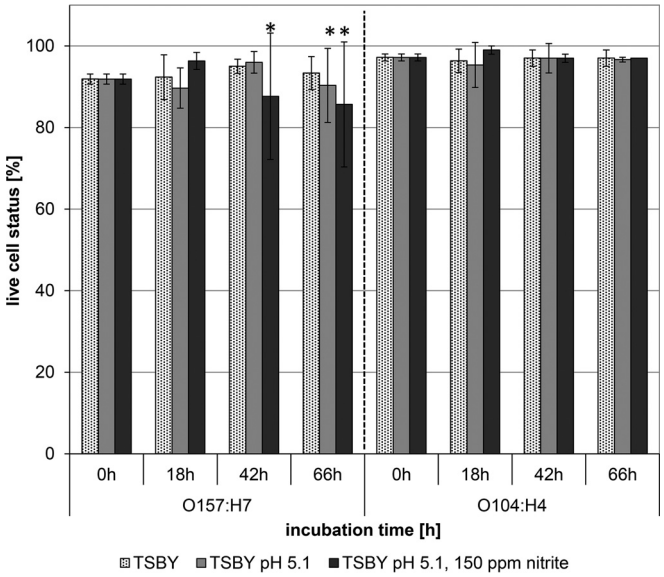

FIG 3 Live-cell status of *E. coli* O157:H7 and *E. coli* O104:H4 under raw sausage conditions of decreased pH and the presence of sodium nitrite *in vitro*. Assays were performed either in Trypticase soy broth plus 0.5% yeast extract (TSBY) as a control, in TSBY adjusted to pH 5.1 with 20% (vol/vol) lactic acid (TSBY pH 5.1), or in TSBY at pH 5.1 with the addition of 150 mg/liter sodium nitrite (TSBY pH 5.1, 150 ppm $NaNO_2$). After 0, 18, 42, and 66 h of incubation at 22°C, cell viabilities were assessed using the LIVE/DEAD BacLight viability kit according to the manufacturer's instructions. The numbers of dead, viable, and damaged cells were determined by counting red, green, and orange cells in a triplicate setup, and the percentages of live, dead, and damaged cells were calculated. Values are averages for three independent experiments. *, $P < 0.05$.

indicator strains of *Enterobacteriaceae* isolated from different sources (Table 1). The antibacterial substance produced had a narrow spectrum of activity. Inhibitory activities ranging from weak to strong could be observed only against strains of the *E. coli* species. Very strong activity was demonstrated against *E. coli* O157:H7 (ATCC 43895). The growth of other species of *Enterobacteriaceae* tested (*Salmonella enterica* subsp. *enterica*, *Citrobacter freundii*, *Klebsiella oxytoca*, and *Hafnia* spp.) was not affected by the presence of *E. coli* O104:H4 (E965).

In silico analysis of the published genome of *E. coli* O104:H4 strain 2011C-3493 (33) with the BAGEL bacteriocin mining tool indicated the existence of potential bacteriocin genes. Two genome areas of interest showed the presence of potential microcin and bottromycin gene loci. A GenBank database search indicated that these genes matched with genes encoding the bacteriocin microcin I47 (MccI47) (results not shown).

DISCUSSION

The beef salami manufactured in this study was representative of products found on the retail market, since the MAB/LAB counts and sausage a_w values that were measured were comparable to those of freshly produced sausages. The commercial products, however, were determined to be slightly more acidic. These differences may be attributed to the use of a different starter culture, the presence of more fermentable sugar and acidifiers (e.g., lactic acid), or the use of smoke in the commercially obtained products.

During the fermentation of beef salami, the reduction rates for *E. coli* O157:H7, O157:NM, and O104:H4 strains and the EHEC cocktail with all three strains (0.89, 1.0, 0.86, and 0.75 $\log_{10}$ unit, respectively) did not differ significantly after 3 days of sausage fermentation. Rode et al. (23) showed that the average reduction in the counts of 11 different STEC strains, mainly outbreak strains, was approximately 0.8 $\log_{10}$ unit after 5 days in dry fermented sausage, with no statistically significant difference in the average reduction between isolates. Overall, the reductions in the counts of the three EHEC isolates during fermentation and maturation up to day 14 ranged from 1.4 to 1.7 $\log_{10}$ units, which were also comparable to the reductions in STEC/EHEC counts observed in other studies on dry fermented sausages (35–39). In our study, no strain-dependent differences in the reduction kinetics of the EHEC strains tested were evident, indicating that the combination of increasing acidity and reduced a_w and redox potential (E_h), together with the presence of preservatives, such as nitrite, and competitive microbiota, was effective at reducing the EHEC load in fermented sausages and, conversely, preventing EHEC growth. Glass et al., comparing the survival of non-O157 STEC strains (O26, O45, O103, O111, O121, and O145 serovars) with that of *E. coli* O157:H7 strains during pepperoni production, showed that O103 and O157 strains exhibited the highest survival rates (40). Consequently, processes suitable for controlling *E. coli* O157 would also be applicable to the inactivation of other STEC strains tested in the study (40). Luchansky et al. (41) investigated the thermal stability of *E. coli* O104:H4 and other STEC strains in ground beef and showed that the cooking times and temperatures effective for inactivating serotype O157:H7 in ground beef were equally effective against the seven non-O157:H7 Shiga toxin-producing strains, including the O104:H4 strain. Our results indicate that *E. coli* O157:NM and *E. coli* O104:H4 are more resistant to raw sausage conditions than *E. coli* O157:H7, since the strains of the former two serovars could be detected by qualitative methods during the whole evaluation period, up to the end of the shelf life on day 60.

According to Rode et al. (23), the sorbitol-fermenting O157:NM outbreak strain showed the greatest ability to survive conditions in fermented sausage. Moreover, Alvarez-Ordóñez et al. (42) confirmed strong acid stress resistance especially for O157:NM strains. These strains are associated with a higher incidence of progression to HUS than *E. coli* O157:H7 strains and have emerged as important pathogens since their first isolation in Germany in 1998 (2–4). It is assumed that such increased resistance might be a key factor for the successful persistence of this clonal type of EHEC and for the serious pathogenicity of this strain in the host (42). Enhanced persistence may also account for the extraordinary virulence potential of the O104:H4 outbreak strain, as indicated by the large number of HUS cases and deaths in 2011. Markland et al. (24) detected low populations of *E. coli* O104:H4 strains on basil plants 10 days postinoculation, while *E. coli* O157:H7 was not found. This finding addresses the hypothesis that certain *E. coli* strains have evolved toward enhanced fitness in adverse environments and thus may indicate that these strains are better adapted to harsh environments (43). The results of our *in vitro* survival assays revealed significant differences in cell viability between *E. coli* O157:H7 and *E. coli* O104:H4, with 86% and 97% live cells in the presence of acidic nitrite, respectively, differences that also point to enhanced fitness of *E. coli* O104:H4 under food-related laboratory conditions.

The adaptation of *E. coli* O104:H4 to multiple adverse conditions occurring in the food matrix has been documented by only a few studies (25–27). A recent study showed increased resistance of

the O104:H4 strain to high pressure after precultivation of the strain in nutrient broth at pH 5 and inactivation in carrot juice (pH 5.1) (27). A comparison between the growth kinetics values observed for *E. coli* O104:H4 in foods and those predicted for *E. coli* O157:H7 by using the U.S. Department of Agriculture Pathogen Modeling Program indicated that *E. coli* O104:H4 grows faster than *E. coli* O157:H7 in broth and in alfalfa and broccoli sprouts at 15°C (25). Yoo et al. (26) compared the growth characteristics of unstressed and stressed O157 or non-O157 STEC strains, including *E. coli* O104:H4 strains, in fresh produce. Their results suggested that sublethal osmotic, acid, or starvation stress may enhance the growth of non-O157 STEC strains on lettuce or cantaloupe, leading to a greater safety risk. Therefore, it is highly recommended to include non-O157 STEC strains in food risk assessments that previously addressed only *E. coli* O157:H7 and to use a STEC strain cocktail composed of several serotypes, including O104:H4. In order to account for differences in survival among strains, challenge studies should generally be conducted using three to five bacterial strains, either individually or in combination (44). However, results might be biased, reflecting the characteristics of the most resistant serotype in the strain cocktail (45). In our study, challenge experiments in fermented sausages were also performed with an EHEC strain cocktail consisting of equivalent cell counts of *E. coli* O157:H7, O157:NM, and O104:H4. The reduction rate of the EHEC strain cocktail up to day 14 was only 1 $\log_{10}$ unit, in contrast to counts of single EHEC strains that showed 1.4 to 1.7 $\log_{10}$ ($P < 0.001$) reduction rates. Differences of <0.5 $\log_{10}$ unit in counts at specific sampling points might result from analytic variabilities due to sampling and measurement errors. A difference of >0.5 $\log_{10}$ unit, however, is considered an appropriate criterion for relevant changes in counts (44). By comparing EHEC counts from strain cocktails with the single EHEC strain counts, differences of >0.5 $\log_{10}$ unit were detected for O157:H7 and O157:NM. The cell counts of the EHEC cocktail and *E. coli* O104:H4 differed by only 0.4 $\log_{10}$ unit, reflecting similar behaviors. Indeed, analysis of the composition of EHEC strains in the cocktail showed that *E. coli* O104:H4 was the dominant representative in all samples of fermented sausage over 42 days of quantitative detection. The dominance of a certain strain in an inoculation cocktail could be also shown by Kagkli et al. (46), who inoculated five strains of *Listeria monocytogenes* individually and as a cocktail in cheese. The strains did not show the same behavior when inoculated individually as when pooled. Specifically, one serotype 4b strain prevailed over the others, and strain interactions in the inoculation cocktail were more or less obvious (46). Levels of *E. coli* O157:H7 were already dramatically reduced shortly after inoculation into the fermented sausage. After 14 days, *E. coli* O157:H7 was undetectable, in contrast to the successful detection of O157:H7 until day 21 in single-strain inoculation experiments. The combination of biochemical trait comparisons and multiplex PCR allowed reliable differentiation of all EHEC strains isolated in this study.

Antagonism between strains in the EHEC cocktail may rely on the production of a bacteriocin(s) or other antimicrobial compounds. The inhibitory activity of *E. coli* O104:H4 (E965) was verified in a inhibition assay against several strains of *Enterobacteriaceae* from different sources, including *E. coli*, *Salmonella enterica* subsp. *enterica*, *Citrobacter freundii*, *Klebsiella oxytoca*, and *Hafnia* spp. Zones of inhibition were limited to the *E. coli* strains tested as sensitive indicator microorganisms, with noticeably strong activity occurring against an O157:H7 strain (EDL933). Bacteriocins are antimicrobial peptides that are generally active against bacteria closely related to the producer. *E. coli* is known to produce two types of bacteriocins, classified by their molecular masses into colicins (25 to 80 kDa) and microcins (10 kDa). The absence of colicin production distinguishes the outbreak strain of 2011 from the serotype O104:H4 (HUSEC41) strain isolated in the year 2001 (47). *In silico* analysis of the published genome of *E. coli* O104:H4 strain 2011C-3493 with the BAGEL automated bacteriocin mining tool, however, revealed the existence of presumptive bacteriocin genes with homology to microcin I47 (MccI47). MccI47 is part of the MccH47 genetic system, which contains all genes necessary for peptide production, posttranslational maturation, secretion, and immunity, as well as genes for a second antibacterial activity of microcin I47, the production of which is detected only under conditions of iron deprivation (48). A microcin gene cluster was previously identified in the *E. coli* O104:H4 German outbreak strain and is absent from the genome of its close relative *E. coli* strain 55989 (49). By using the newly developed GIST (genomic island identification by signals of transcription) method, Huang et al. (50) detected a genomic island, which included the microcin H47 system, in the 2011 German *E. coli* O104:H4 outbreak strain. Hence, it is assumed that the antibacterial activity of *E. coli* O104:H4 (E965) is mediated by microcins. Studies are in progress to identify the gene or gene cluster conferring potential bactericidal activity by constructing a genome library of *E. coli* O104:H4 (E965). Ongoing studies also seek to confirm gene function by transposon mutagenesis and to characterize the bacteriocin expressed. Previous studies have suggested that microcins act as fitness factors, which aid in successful competition with other bacteria during intestinal colonization, and contribute to the virulence of *E. coli* (51–53). Therefore, our results suggested that the secretion of bacteriocins detected in *E. coli* O104:H4 (E965) could be conducive to the high virulence of the O104:H4 outbreak strain.

This study demonstrated that the EHEC O157:NM and O104:H4 outbreak-associated strains survived longer than *E. coli* O157:H7 during the production and storage of fermented raw sausages. The results suggested, furthermore, that *E. coli* O104:H4 is well adapted to multiple adverse conditions encountered in fermented sausages or in food-related laboratory environments. Furthermore, secretion of potential bacteriocins by *E. coli* O104:H4 was indicated by the antagonism of this strain toward others in an EHEC strain cocktail, and this was verified in inhibition assays with several *E. coli* strains, including serotype O157 strains. Consequently, this study strongly suggests enhanced persistence and the possible production of bacteriocins as potential factors which, among others, could account for the increased virulence of the O104:H4 outbreak strain in Germany in 2011.

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