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Growth and survival of *Yersinia enterocolitica* and *Escherichia coli* O157:H7 in fresh sausages

Wachstums und Überlebensrate von Yersinia enterocolitica und Escherichia coli O157:H7 in rohen Bratwürsten

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Summary

We investigated the growth and survival of *Yersinia enterocolitica* and *Escherichia coli* O157:H7 in vacuum-packed fresh sausages at 6 °C. High (400 cfu/g) and low (0.4 cfu/g) multistrain mixtures of *Y. enterocolitica* and *E. coli* O157:H7 were inoculated separately in fresh sausages. Sausages were sampled for microbial counts immediately and at 9 and 12 days after the inoculation, and a five-tube most-probable-number method was used for enumeration. The bacterial counts decreased during storage. Both bacteria survived in sausages with the high inoculum and *Y. enterocolitica* also in sausages with the low inoculum. *Y. enterocolitica* and *E. coli* O157:H7 are regularly found in raw material of fresh sausages and even survival of *E. coli* O157:H7 without growth may present a risk for consumer health.

Keywords: meat product, inoculation study, MPN method

Zusammenfassung

Wir haben Wachstums- und Überlebensrate von *Yersinia enterocolitica* und *Escherichia coli* O157:H7 in vakuumverpackten rohen Bratwürsten bei 6 °C studiert. Die Würste wurden mit hohen (400 KBE/g) und niedrigen (0.4 KBE/g) Konzentrationen von *Yersinia enterocolitica* und *Escherichia coli* O157:H7 separat beimpft. Mikrobielles Wachstum wurde sofort und 9 beziehungsweise 12 Tage nach Beimpfung mittels MPN-Verfahren ermittelt. Die Anzahl der Bakterien hat sich während der Lagerung reduziert. Beide Bakterien haben aber in den mit hohen Konzentrationen beimpften Würsten überlebt und *Y. enterocolitica* konnte auch bei niedrigem Inokulum überleben. *Y. enterocolitica* und *E. coli* O157:H7 werden regelmäßig in Rohmaterialien von Würsten gefunden, wodurch auch das Überleben von *E. coli* O157:H7 ohne Wachstum ein Gesundheitsrisiko darstellt.

Schlüsselwörter: Fleischprodukt, Inokulierungsstudie, MPN-Verfahren

Introduction

Yersinia enterocolitica and Shiga toxin-producin *Escherichia coli* (STEC) cause foodborne illnesses and are common intestinal bacteria of animal hosts. *Y. enterocolitica* strains are divided into subtypes according to biotypes and O antigens; the predominant bioserotype in continental Europe is 4/O3, whereas in England bioserotypes 2/O:9 and 2/O:5 are more common (Fredriksson-Ahomaa et al. 2000, Fredriksson-Ahomaa et al. 2007 , EFSA 2009, Ortiz Martínez et al. 2009, Ortiz Martínez et al. 2010, Ortiz Martínez et al. 2011). The incidence of yersiniosis was 10 cases/100 000 population in Finland in 2009 (Hulkko et al. 2010). Symptoms caused by *Y. enterocolitica* infection vary from bloody diarrhoea to acute mesenteric lymphadenitis and various post-infectious complications (Bottone 1999).

The majority of *E. coli* strains are commensal bacteria, but pathogenic strains also exist. One of the pathogenic forms of *E. coli* is the highly virulent enterohaemorrhagic *E. coli* (EHEC) O157:H7. EHEC causes severe gastrointestinal illness, which can lead to haemolytic-uremic syndrome, kidney failure and even death (Tarr et al. 2005). The infective dose of EHEC can be very low, less than 10 cells (Tilden et al. 1996). The annual incidence rate of EHEC is 0.3–0.9 cases/100 000 population in Finland; 52 % of these cases are due to serogroup O157 (Hulkko et al. 2010).

Y. enterocolitica is a facultatively anaerobic and psychrotrophic bacterium, making it an important bacterium from a food hygiene perspective, (Harrison et al. 2000, Fredriksson-Ahomaa et al. 2010). Prolonged shelf-lives and increased use of the cold chain also give more opportunities for *Y. enterocolitica* to grow in foods. The most common mode of transmission of *Y. enterocolitica* is pork and meat products made of pork (Ostroff et al. 1994, Fredriksson-Ahomaa et al. 2006). Pathogenic strains of *Y. enterocolitica* have been frequently isolated from the tonsils and intestines of clinically healthy pigs (Korte et al. 2004, Fredriksson-Ahomaa et al. 2007, Laukkanen et al. 2009, Ortiz Martínez et al. 2009). The link between *Y. enterocolitica* in pigs and humans remains unclear, but food products containing pork and pig offal are believed to spread the bacterium (Fredriksson-Ahomaa et al. 2001, Fredriksson-Ahomaa et al. 2006, Laukkanen et al. 2010). Pork and meat products made of pork are contaminated with *Y. enterocolitica* (Fredriksson-Ahomaa et al. 1999, Thisted Lambertz et al. 2007).

The main reservoir of EHEC is ruminants, especially cattle and sheep, for which the organism is not pathogenic (Caprioli et al. 2005). In Finland, 1.2 % of cattle studied were EHEC-positive in 2007 (EFSA 2009). The most common mode of primary transmission of EHEC is food (54 %), but animal contact (8 %), water (7 %) and the environment (2 %) have also been reported as modes of primary transmission (Snedeker et al. 2009). Foodborne EHEC cases have been associated with consumption of inadequately cooked beef, minced meat, cured sausage, unpasteurized milk, other dairy products and surface-contaminated fruits and vegetables (Eklund 2005, Sartz et al. 2008, Snedeker et al. 2009). Approximately 19 % of EHEC cases are due to secondary transmission (Snedeker et al. 2009).

Fresh sausages are made from raw meat, salt and spices, and they are not smoked or cooked during manufacturing. These sausages can be vacuum-packaged or packaged in modified atmosphere, and they must be stored refrigerated. Consumers should fully cook them before eating. Good hygienic quality of raw material and good hygienic practices are very important in the manufacture of fresh sausages. *Y. enterocolitica* and EHEC may be present in raw materials, thereby contaminating fresh sausages. In this study, the objective was to investigate the growth and survival of *Y. enterocolitica* and EHEC O157:H7 in vacuum-packaged fresh sausages during storage at 6 °C.

Materials and methods

Bacterial strains

Three strains of *Y. enterocolitica* isolated from pork carcasses; YLUT 78.2 I, YEPT 166.IK and YEPT 179.1C, and three *E. coli* O157:H7 strains; ATCC 43895, ATCC 43889 and ATCC 43894, were used.

Each strain was cultured from frozen stock on blood agar overnight. After incubation, each *Y. enterocolitica* strain was cultured in tryptic soy broth (TSB, Merck KGaA, DE) and incubated at 30 °C overnight, and each *E. coli* O157:H7 strain was cultured into brain heart infusion broth (BHI, Difco Laboratories, BD, US) and incubated at 37 °C overnight. The optical densities were measured (Biophotometer, Eppendorf AG, DE) and dilution series made on plate count agar (PCA, Difco) to determine the bacterial density of both suspensions. Three-strain cocktails of *Y. enterocolitica* and *E. coli* O157:H7 were mixed separately from fresh suspensions and diluted with peptone water (Difco) to obtain the required numbers of cells for inoculations: 0.4 cfu and 400 cfu per one gram of sausage batter for low and high inoculations, respectively.

Manufacture and inoculation of fresh sausages

Fresh sausage batter was prepared by mixing pork meat (75 %) and beef (25 %). The salt and lactate concentrations of the sausage batter were 1.3 % and 3.4 %, respectively. Batter also included nitrite (150 mg/kg), vinegar, spices (mustard seed, coriander, ginger, marjoram and cumin) and a natural flavour (PuraQ® arome NA4V, Purac, NL).

One quarter of the batter was separately inoculated with the low (0.4 cfu/g) and another quarter with the high (400 cfu/g) multistrain mixture of *Y. enterocolitica* and mixed thoroughly (Metos Bear AR40, Wodschow & Co. A/S, DK). The other two quarters of the batter were stored at 2 °C and separately inoculated with the low and high multistrain mixtures of *E. coli* O157:H7 and mixed thoroughly. Immediately after the inoculations, the batter was stuffed (Robot 500, Vemag, DK) into natural casings and vacuum-packaged (SB 415, Turbovac, HFE Vacuum systems B.V., NL) in vacuum bags (CO 80, Wipak, DE), four sausages in each package. After packaging, the sausages were stored at 6 °C.

Microbial analyses

Sausage batter was sampled for microbial counts for *Y. enterocolitica* and EHEC right before inoculation of the pathogen in question. The inoculated sausages were sampled for microbial counts for *Y. enterocolitica* and EHEC on days 0, 9 and 12 after the inoculation. A five-tube most-probable-number method (MPN) was used for enumeration of bacteria (U.S. Food and Drug Administration 2006). At each sampling, two parallel packages inoculated with *Y. enterocolitica* and EHEC were studied.

Y. enterocolitica was isolated with the method of the Department of Food and Environmental Hygiene (DFEH), as previously described by Laukkanen et al.

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(2010), except that for cold enrichment the PMB broth (phosphate-buffered saline with 0.5 % peptone [Bacto, BD, US], 1 % mannitol [Merck] and 0.15 % bile salts [Bacto]) was incubated at 4 °C for 21 days, and cefsulodin irgasan novobiosin agar plates (CIN, Oxoid Ltd, GB) were incubated at 30 °C for 24 h.

Briefly, the 25-g sample was homogenized with 225 ml of PMB broth, and part of the PMB broth was tenfold diluted with irgasan ticarcillin potassium chlorate broth (ITC, Sigma-Aldrich, US). Both PMB and ITC broths were tenfold serially diluted, and each dilution was divided into five tubes to be used for MPN enumeration. ITC broth was incubated at 30 °C for 48 h. After incubation, the ITC broth was spread onto CIN agar plates and incubated at 30 °C for 24 h. In cold enrichment, the PMB broth was incubated at 4 °C for 21 days, and the sample was then alkali-treated (0.5 ml of the PMB broth mixed with 4.5 ml of KOH solution for 20 s) before cultivation on CIN agar. Typical colonies for *Y. enterocolitica* from CIN agar were transferred to trypton soy agar (TSA, Merck). Colonies from TSA medium were confirmed using polymerase chain reaction (PCR) to detect *ail, virF* and *inv* genes, as previously described by Laukkanen et al. (2010), except that annealing temperature was 53°C.

EHEC was isolated according to the method 164/2005 of the Nordic Committee on Food Analysis (NMKL, 2005), except that immunomagnetic separation was not used, and the API® 20E test (BioMérieux sa, FR) and the latex agglutination test (*E. coli* O157 Latex, Oxoid) were used for confirmation. Briefly, the 25-g sample of batter was homogenized with 225 ml of modified trypton soy broth (mTSB, TSB with bile salts [Oxoid] and novobiocin [Sigma]), which was tenfold serially diluted, and each dilution was divided into five tubes to be used for MPN enumeration and incubated at 42 °C for 24 h. The mTSB broth was spread to sorbitol MacConkey agar (Oxoid) with cefixime and tellurite (CT-SMAC) and incubated at 37 °C for 24 h. Typical EHEC colonies from CT-SMAC were transferred to TSA. Colonies from TSA were confirmed as EHEC with the latex agglutination test and the API® 20E test, according to manufacturers' instructions.

Only tubes whose colonies on selective agar (CIN for *Y. enterocolitica* and CT-SMAC for EHEC) were typical and whose confirmation tests were positive for the bacteria studied (PCR for *Y. enterocolitica* and latex agglutination test and API® 20E test for EHEC) were used for enumeration.

Measurement of pH

Measurement of pH of the inoculated fresh sausages was conducted on days 0, 9 and 12 after the inoculation (InoLab 720, WTW GmbH, DE).

Results and discussion

Growth and survival of *Y. enterocolitica* and EHEC were studied in fresh sausages during the storage for 9 and 12 days at 6 °C. Both bacteria survived in high-inoculum sausages and *Y. enterocolitica* also in low-inoculum sausages, but the bacterial counts decreased during storage (Tab. 1). No *Y. enterocolitica* or EHEC was found in raw material. No significant changes were seen in the pH level of sausages during storage (Tab. 2). Mean pH level was 5.88.

TABLE 1: *Cell counts of Yersinia enterocolitica and Escherichia coli O157:H7 in inoculated fresh sausages during storage of 9 and 12 days.*

Inoculum	Enrichment	After inoculation[a])	9 days from inoculation[a])	12 days from inoculation[a])
Y. enterocolitica 0.4 cfu/g	ITC[b])	0.52 (± 0.43)	0.18 (± 0.04)	0.06 (± 0.02)
	PMB[c])	2.1 (± 2.68)	0.15 (± 0.04)	BDL
Y. enterocolitica 400 cfu/g	ITC[b])	182 (± 210.01)	3.9 (± 5.52)	2.3 (± 0)
	PMB[c])	330 (± 0)	6.15 (± 2.33)	1.0 (± 1.41)
EHEC 0.4 cfu/g	mTSB[d])	0.1 (± 0.14)	BDL	BDL
EHEC 400 cfu/g	mTSB[d])	22 (± 12.73)	1.3 (± 0)	1.04 (± 0.37)

[a]): Two-sample mean of the most-probable-number result (cfu/g) of the microbial analysis (standard deviation). [b]): Irgasan ticarcillin potassium chlorate broth. [c]): Cold enrichment in peptone mannitol bile salt broth. [d]): Modified tryptic soy broth. BDL: Below the detection limit.

TABLE 2: *pH values of fresh sausages inoculated with Yersinia enterocolitica and Escherichia coli O157:H7 during storage at 6 °C.*

Inoculum	After inoculation[a])	9 days from inoculation[a])	12 days from inoculation[a])
Y. enterocolitica 0.4 cfu/g	6.02	5.98	5.78
Y. enterocolitica 400 cfu/g	5.96	5.94	5.68
EHEC 0.4 cfu/g	5.95	5.93	5.70
EHEC 400 cfu/g	5.97	5.91	5.67

[a]): Mean of two parallel pH determinations.

Y. enterocolitica was found in all fresh sausage samples, except for the low-inoculum sausages after 12 days using cold PMB enrichment, but the amount decreased during storage. The only significant ($p < 0.05$) decrease was observed in high-inoculum sausages after cold enrichment between the inoculation day and day 9 and the inoculation day and day 12, but not between days 9 and 12. *Y. enterocolitica* is a psychrotrophic bacterium that is capable of growing at refrigerated temperatures. The pH levels of fresh sausages were continuously at a level where *Y. enterocolitica* is able to grow (Fredriksson-Ahomaa et al. 2010). Nitrite levels as high as in fresh sausages (150 mg/kg) have been shown to decrease *Y. enterocolitica* levels below detection levels in fermented sausages (Asplund et al. 1993).

In the low-inoculum sausages, EHEC was below the detection limit on days 9 and 12. In the high-inoculum sausages, all samples were positive for EHEC, but the levels decreased during storage. A significant ($p < 0.05$) decrease in the amount of EHEC in the high-inoculum sausages was observed between the manufacturing day and day 9 and the manufacturing day and day 12, but not between days 9 and 12. EHEC is a mesophile that is unable to grow at refrigerated temperatures. Nitrite levels used in fresh sausages could reduce EHEC, whereas the pH levels of fresh sausages would allow EHEC to survive (Casey and Condon 2000, Marques et al. 2001). EHEC was able to survive during the 12-day storage, indicating that there is no factor that can eliminate EHEC from this kind of unheated, non-fermented fresh sausage product.

Both *Y. enterocolitica* and EHEC are sensitive to heating; thus the most efficient way to reduce the risk caused by these bacteria is proper heat treatment (Doyle and Schoeni 1984, Bottone 1999). Because *Y. enterocolitica*

and EHEC are regularly found in raw material, it is also possible that these bacteria end up in meat products such as fresh sausages. Inadequate heating or cross-contamination during meal preparation represents a risk for consumer health if the sausages have been previously contaminated with *Y. enterocolitica* or EHEC. Therefore, high hygienic quality of raw material and good hygienic practices during manufacturing should be taken into account. Because of the low infective dose of EHEC, it especially presents a public health risk. The responsibility of adequate heat treatment lies with the consumer; thus proper instructions on the package are essential.

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