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OBSERVATION

Residual Antibiotics Disrupt Meat Fermentation and Increase Risk of Infection

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ABSTRACT **Fermented sausages, although presumed safe for consumption, sometimes cause serious bacterial infections in humans that may be deadly. Not much is known about why and when this is the case. We tested the hypothesis that residual veterinary antibiotics in meat can disrupt the fermentation process, giving pathogenic bacteria a chance to survive and multiply. We found that six commercially available starter cultures were susceptible to commonly used antibiotics, namely, oxytetracycline, penicillin, and erythromycin. In meat, statutorily tolerable levels of oxytetracycline and erythromycin inhibited fermentation performance of three and five of the six starter cultures, respectively. In model sausages, the disruption of meat fermentation enhanced survival of the pathogens *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium compared to successful fermentations. Our work reveals an overlooked risk associated with the presence of veterinary drugs in meat.**

IMPORTANCE **Antibiotics have for a long time been used as growth promoters in farm animals, and while they are banned as such in Europe, their clinical use in farm animals still accounts for the majority of consumption. Here, we examined how acceptable levels of antibiotics in meat influence fermentation. Our results show that commonly used bacterial starter cultures are sensitive to residual antibiotics at or near statutorily tolerable levels, and as a result, processed sausages may indeed contain high levels of pathogens. Our findings provide a possible explanation for outbreaks and disease cases associated with consumption of fermented sausages and offer yet another argument for limiting the use of antimicrobials in farm animals.**

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Fermented sausages such as salami and pepperoni are commonly considered safe for consumption, and the acidification by lactic acid starter bacteria is one of the main preserving factors. Nevertheless, outbreaks of serious sausage-borne gastrointestinal infections with pathogens such as verocytotoxic (shigatoxic) *Escherichia coli* (VTEC/STEC), *Salmonella*, and *Listeria monocytogenes* do occur regularly (1). For example, in a 2006 outbreak in Norway, 17 people with sausage-borne VTEC infections (10 of them children <9 years old) were treated for renal failure or hemolytic uremic syndrome, with one fatal outcome (2). Although fermented beef products have been identified as potential health hazards (3), it is largely unclear when and why fermented sausages pose a risk to consumers. We tested the hypothesis that statutorily tolerable levels of veterinary antibiotics in meat pose a food safety threat associated with sausages by inhibiting microbial starter cultures and fermentation, thus allowing pathogens to multiply during processing (4). Sausage processors typically initiate fermentation with lyophilized starter cultures that produce large amounts of lactic acid, and the rapidly decreasing pH levels prevent growth of pathogenic bacteria. Such starter bacteria are primarily selected for production purposes. Therefore, most strains of lactic acid bacteria are highly sensitive to antibiotics, and commercial suppliers even take care not to provide strains that carry antibiotic resistance genes (5). Still, antibiotics are used extensively and widely in livestock farming for treatment, control, and prevention of animal diseases as well as for production purposes, e.g., to enhance growth or improve feed efficiency. As a consequence, meat products may contain residual levels of antimicrobial compounds. Food safety regulation in many countries sets maximum tolerable levels of microbial residues. In the European Union (EU), these are called maximum residue limits (MRL) (6), whereas in the United States, the Food and Drug Administration (FDA) has defined tolerance levels (TL), and violations, where the measured antibiotic concentrations in meat exceed the MRL/TL, are registered. National residual monitoring programs showed violations in 0.2 to 1.0% of sampled beef and pork meat in the EU and United States in 2009 (7, 8), with 0.4% of bovine animals and 0.05% of pigs being required to be controlled in the EU (8). Meat samples violating the tolerance levels are withdrawn from the food chain, but not all violations are caught in the monitoring programs. Furthermore, many countries lack monitoring or reporting of sales and data on use of antibiotics (9), and the frequency of inspection of imported meat is low; e.g., in the United States in 2009, 3,872 imported meat samples were analyzed for chemical contamination (7). Shortcomings in the current screening methods are likely to cause underestimation of noncomplying prod-

ucts, and there is a need for development and implementation of more adequate residual screening methods (10).

Here, we evaluated six commercially available starter cultures that are widely used for sausage production (Bactoferm FSC-111, F1, and T-SPX [C. Hansen, Hoersholm, Denmark] and BITEC Advance RD-1, LS-25, and LS-25-2 [Gewürzmüller, Korntal-Münchingen, Germany]). The starter cultures are all a mixture of two or three species, including both lactic acid-producing bacteria, which are important for pH control, and coagulase-negative cocci, which are important for flavor formation. The starter cultures used in this study contain either (i) *Pediococcus pentosaceus* in combination with *Staphylococcus xylosus*; (ii) *Lactobacillus sakei* in combination with *Staphylococcus carnosus*; or (iii) *Lactobacillus curvatus*, *S. carnosus*, and *Kocuria varians*. In broth experiments, these bacteria were evaluated as mixed cultures to mimic conditions during sausage fermentation. We found that under laboratory conditions (de Man, Rogosa, and Sharpe broth [MRS], pH 6.1), all six starter cultures were sensitive to three commonly used veterinary antibiotics (oxytetracycline, erythromycin, and penicillin) (Fig. 1) at or near the levels designated as tolerable (TL or MRL). As expected, acidification correlated well with starter culture growth after 20 h incubation at 25°C. Importantly, in the presence of antibiotics at or close to the tolerable concentrations, only minor acidification occurred, with a final pH of 6, whereas in the absence of antimicrobials, the final pH was 4.3.

To evaluate the impact of residual antibiotics on fermentation, we created standardized 50-g model sausages stuffed in 60-ml plastic syringes (11) using a recipe that resembles those of traditional European sausages (minced beef or pork meat, 3% NaCl, 100 ppm sodium nitrite, 0.7% dextrose, and starter culture, according to the manufacturer's recommendations; 25 g kg meat batter^{-1}). Fermentation was monitored in terms of pH decrease after 48 h of incubation at 25°C. Model sausages (controls) fermented by any of the starter cultures had an average end pH of 4.75 $\pm$ 0.07 after fermentation, whereas spontaneous fermentation in meat without addition of starter cultures resulted in a final pH of 5.45 $\pm$ 0.12 ($n \geq 12$ for each condition). In contrast, we found that with the addition of erythromycin at the MRL concentration, fermentation was significantly reduced in five of the six starter cultures, resulting in an average end pH of 5.16 $\pm$ 0.34. Also, the presence of oxytetracycline at the TL concentration reduced fermentation in three of the six starter cultures, resulting in an average end pH of 4.84 $\pm$ 0.14. It was reported in 1998 that commercial starter cultures are susceptible to low levels of antibiotics like erythromycin, penicillin, tylosin tartrate, and ceftiofur hydrochloride (Excenel), leading to inhibited starter culture performance in meat (12), and this problem seems to be overlooked, as the continuous development of starter cultures has failed to addressed the issue.

The influence of residual antibiotics on pathogen survival and viability was evaluated using reporter strains of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium UK1 that express high levels of bioluminescence (13). Model sausages were inoculated with *E. coli* or *S.* Typhimurium to a level of 2×10^7 to 4×10^7 CFU g^{-1}, as determined by selective plating, by harvesting 10 ml of LB cultures at an absorbance at 600 nm of 1.0 by centrifugation (5,000 $\times$ *g* for 5 min) and resuspending pellets in sterile, saline (0.9%) water before inoculation. Bioluminescence signal intensities were used to measure pathogen viability in relative light units (RLU, in photons s^{-1} cm^{-2}) in a Xenogen IVIS100 imaging

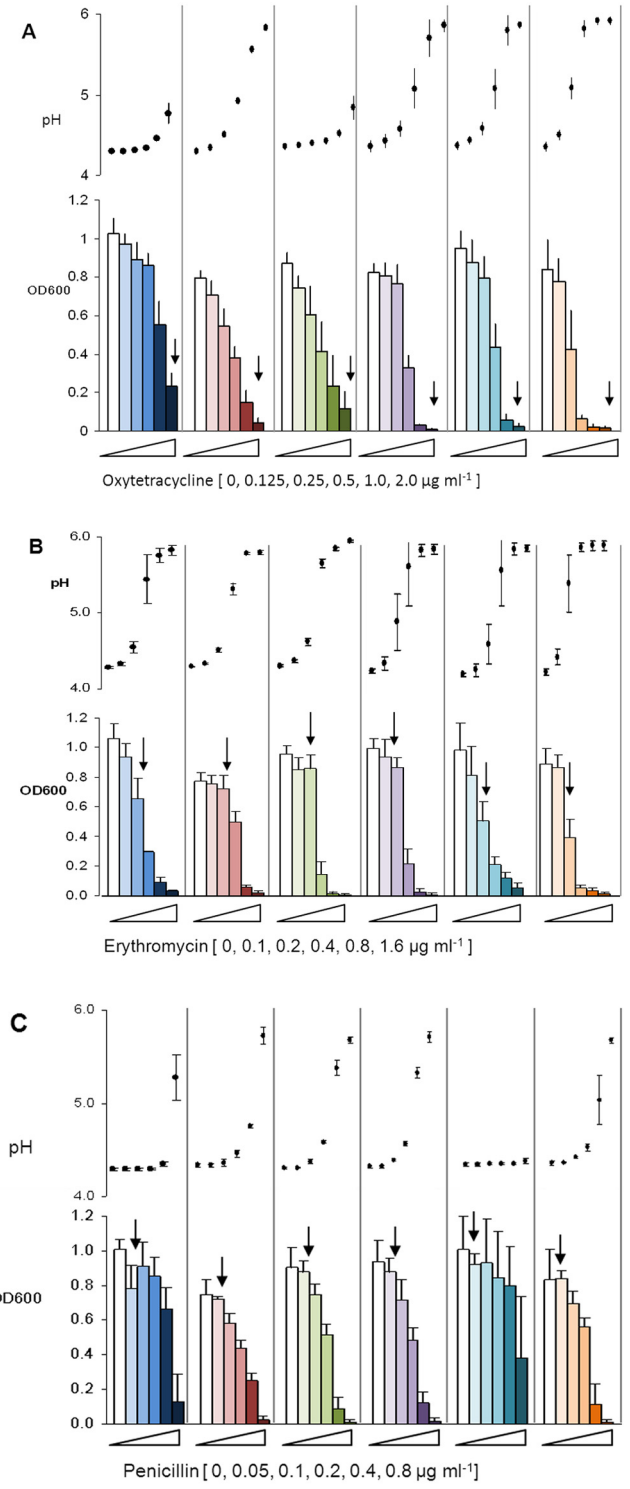

FIG 1 Impact of residual antibiotic concentrations on starter culture growth and acid production. Six commercial starter cultures were inoculated in MRS broth (Oxoid) at an absorbance at 600 nm of 0.05 in the presence or absence of oxytetracycline (A), erythromycin (B), or penicillin (C). After incubation at 25°C for 20 h, absorbance (bars) and pH (dots) were measured. Arrows mark the tolerance levels (TL, set by the FDA) for residual tetracyclines (2 μg ml^{-1}) or the maximal residual limits (MRL, set by the EU) for penicillin (0.05 μg ml^{-1}) and erythromycin (0.2 μg ml^{-1}). Color intensities indicate antibiotic concentrations.

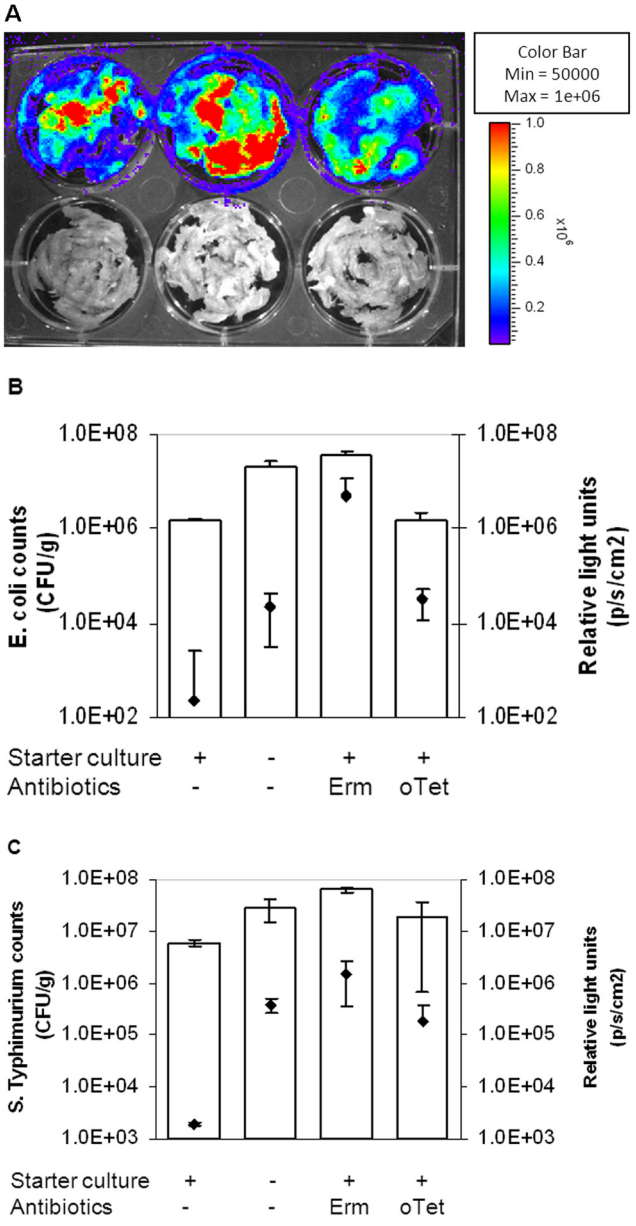

FIG 2 Residual antibiotic impact on pathogen survival and fermentation. (A) Bioluminescent imaging after 48 h of fermentation at 25°C on triplicate 1-g sausage samples inoculated with the *E. coli* O157:H7 reporter strain without (lower row) and with (upper row) the addition of erythromycin (0.4 μg g^{-1}). The color bar indicates bioluminescence signal intensity (in photons s^{-1} cm^{-2}). (B) Selective *E. coli* O157:H7 and (C) *S.* Typhimurium counts (bars) and relative light units (RLU; dots) in model sausages after fermentation (48 h, 25°C). Each bar or dot shows the value for one representative of three biological replicates, measured in three technical replications. Erm, erythromycin (0.4 μg g^{-1}); oTet, oxytetracycline (2.0 μg g^{-1}). Starting counts before fermentation were 2.9 × 10^7 to 3.3 × 10^7 CFU g^{-1} for *E. coli* and 1.7 × 10^7 to 2.5 × 10^7 CFU g^{-1} for *S.* Typhimurium.

system (Xenogen, Alameda, CA) before and after fermentation. Importantly, high-level pathogen bioluminescence was observed only in sausages with residual erythromycin present at twice the MRL (0.4 μg g^{-1}) and not in control sausages without antibiotics (Fig. 2A). Furthermore, in sausages without antibiotics, fermentation reduced the number of pathogens by 1 log unit (accompanied by a 3- to 4-log-unit decrease in bioluminescence), whereas in the presence of erythromycin at twice the MRL (0.4 μg g^{-1}), no reduction neither in pathogen counts nor bioluminescence levels was observed (Fig. 2B and C). This suggests that, at a level close to the MRL, erythromycin can render the starter culture ineffective during sausage processing and enable pathogen survival.

The resistance of starter cultures to veterinary drugs was investigated previously (12, 14), but in contrast to earlier studies, our study shows that starter cultures can be affected by antibiotics at levels that are probable and even tolerable in meat, which highlights the importance for food safety. We conclude that sausages prepared from meat with residual concentrations of antibiotics at or close to levels deemed tolerable by U.S. and EU regulators can lead to full or partial fermentation failures and thus to food products capable of causing serious food-borne infections. There is a general lack of knowledge about the decay of antibiotics in meat or the state of the residuals, and we can only mimic the state of the antibiotics in meat. One of the common methods for qualitative screening of antibiotic residuals in muscle tissue relies on microbiological activity against sensitive bacterial strains (15), but this and other applied detection methods are reported to be insensitive or inadequately accurate (10, 15). Industrial sausage producers typically monitor postfermentation pH levels by sampling sausages at regular intervals. We suggest that fermentation disruption could occur in single batches in a production and that random sampling could leave some fermentation failures undetected. Some smaller sausage producers, including local butcher shops, altogether lack adequate pH control (16, 17). Our results indicate that fermentation during sausage production should be monitored closely to reduce food safety risks. At present, no data on the use of antibiotics in livestock farming worldwide are collected systematically (9). Our work reveals an overlooked risk associated with the presence of veterinary drugs in meat. The results add to a large body of research pointing to adverse effects of antibiotics in food, and this may prompt legislators to reconsider the criteria behind the establishment of tolerance levels.

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