American Journal of Microbiology

Editorial

The Food Additives Nitrite and Nitrate and Microbiological Safety of Food Products

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In Germany, raw sausages are typically produced according to a technology referred to as hurdle technology (Leistner, 2000). The combination of several stressful conditions should successfully inhibit growth and survival of pathogenic bacteria, which can be regularly found in raw pork or beef material (Mataragas *et al.*, 2008; Rhoades *et al.*, 2009). Hurdles applied during raw sausage fermentation are the appearance of a competitive flora and product acidification by fermentation, reduction of water activity by drying of the product or by the addition of salt, the reduction of oxygen tension by vacuum packaging or the addition of ascorbate and the addition of preservatives like sorbate or nitrite and nitrate curing salts. Nitrate is converted by the nitrate reducing starter cultures to nitrite, the actual inhibitory compound. The use of nitrate and nitrite as additives has positive as well as negative aspects (Cammack *et al.*, 1999). Disadvantages are that nitrite itself is toxic in high concentrations and that during heating of the products carcinogenic nitrosamines might be formed. Furthermore it is a food additive that is required to be labeled. Because of these negative aspects, the health-conscious consumer wants products without nitrite or nitrate curing salts. On the other hand, the addition of nitrate or nitrite curing salt to the raw sausages has also positive side effects, e.g., it contributes to the development of the typical red curing color and the curing flavor. Several studies also suggest a positive impact of the uptake of dietary nitrate, especially via consummation of vegetables, on human health, e.g., lowering of blood pressure (Hord *et al.*, 2009). Furthermore, the addition of the curing salt is thought to inhibit growth and survival of unwanted bacteria. Therefore, the restricted addition of nitrite or nitrate to raw sausage products is allowed in the European Union for conservation purposes (EU regulation 1333/2008). The raw sausage manufacturers are now faced with a dilemma. One the one hand, they have to address the consumers concern about food additives and on the other hand, they have to guarantee the microbiological safety of the products. Open questions are: Does the addition of nitrite curing salt indeed inhibit growth and survival of pathogenic bacteria? And if yes, what are the molecular mechanisms behind this observation? How do the pathogens adapt their transcriptional profile to nitrite stress? A better understanding of the inhibitory effect of these additives might help to reduce their amount to the amount that is needed to receive a microbiologically safe product: As much as necessary, as little as possible.

While several studies already described the inhibitory effect on pathogens in several meat products, e.g., ham (Horsch *et al.*, 2014) and Frankfurters (Jackson *et al.*, 2011), scientifically based systematic studies that describe the inhibitory effect of nitrite on pathogenic bacteria in raw sausage products are rare. It was reported recently, that nitrite inhibits growth of *Salmonella* Typhimurium in short ripened spreadable raw sausages (Mühlig *et al.*, 2014a). Furthermore, it was shown that nitrite inhibits growth and survival of *Staphylococcus aureus* and *Listeria monocytogenes* in several types of raw sausages, whilst the growth and survival of the lactic acid bacteria from the starter cultures was not influenced by the presence of nitrite (Kabisch *et al*., 2012 (German) and own unpublished data). Therefore, it is not recommended to produce raw sausages without curing salt. A reduction of the nitrate or nitrite concentration to a concentration that still warrants the microbiological safety of the product would be an alternative. For such an approach, a deeper understanding of the effectiveness of the inhibitory impact of nitrite on growth and survival of pathogens is required.

Therefore, the inhibitory effect of nitrite on *S.* Typhimurium was studied on the molecular level. Under acidic conditions during raw sausage fermentation nitrite is converted to several derivatives, like nitrous acid, peroxynitrite, nitric oxide (NO) and others, which are supposed to be the actual inhibitory agents (reviewed in Cammack *et al.*, 1999). Indeed, it has been shown *in vitro*, that NO inhibits growth of *S.* Typhimurium (Park *et al.*, 2011). As *S.* Typhimurium is also exposed to NO in its natural environment during host colonization, it is not surprising that it produces several well characterized NO detoxification systems, namely the flavohemoglobin (HmpA), the flavorubredoxin (NorV), and the nitrite reductase (NrfA) (Arkenberg *et al.*, 2011; Prior *et al.*, 2009). However, challenge assays with the wild type and the deletion mutants Δ*hmpA*, Δ*norV* and Δ*nrfA* did not indicate an essential function of any of these systems (alone) in withstanding nitrite stress in short-ripened spreadable raw sausages. It is possible, that in raw sausages the deletion of one of the NO detoxification systems can be compensated by the expression of the other two systems, a hypothesis that cannot be disproven at the moment. Another explanation might be, that it is not NO (alone) that is the antibacterial nitrite derivative, a hypothesis for which further indications exist. Based on transcriptional data in combination with screening of a mutant library for an acidified nitrite sensitive phenotype, it was speculated that under acidic conditions the presence of nitrite leads to a decreased intracellular pH compared to that of cells grown only under acidic stress. This hypothesis was further confirmed by fluorescence measurements of a mutant expressing a pH sensitive GFP variant (EGFP) (Mühlig *et al.*, 2014b). Therefore, it was concluded that intracellular acidification is another antibacterial effect of acidified nitrite. Under acidic conditions a fraction of nitrite might be converted to nitrous acid. Nitrous acid, an uncharged molecule, can then diffuse across the bacterial membrane. In the near neutral cytoplasm, nitrous acid is converted to nitrite and a proton. The release of free protons leads to intracellular acidification. Such an inhibitory effect of acidified nitrite has first been described for yeasts (Mortensen *et al.*, 2008). The observations from *S.* Typhimurium indicate that intracellular acidification is not an effect specific to yeasts, but a more general mechanism. Whether such an intracellular acidification is indeed involved in the inhibitory effect of nitrite in the raw sausage product is currently not known. If the formation of nitrous acid (an equilibrium reaction, depending on the nitrite concentration and the pH), however, indeed plays a role in inhibiting growth and survival of *S.* Typhimurium in the raw sausages, it is tempting to speculate, that a similar inhibitory effect can be observed in the presence of lower nitrite concentrations when the pH of the product is lowered. The use of alternative starter cultures that leads to a faster acidification of the product could therefore help to reduce the amount of nitrite and still warranting the microbiological safety of the product.

Acknowledgement

This research project was supported by the German Ministry of Economics and Technology (via AiF) and the FEI (Forschungskreis der Ernährungsindustrie e.V., Bonn), Project AiF 16908 N and by the Förderverein für Fleischforschung e.V, Kulmbach.

Author's Contributions

Stefanie Müller-Herbst: Designed the research plan, organized the study, planned the experiments, coordinated the data-analysis and wrote the manuscript.

Anna Mühlig: Planned and performed the molecular-biological *in vitro* experiments and analyzed the data.

Jan Kabisch: Planned and performed the challenge experiments and analyzed the data.

Rohtraud Pichner and Siegfried Scherer: Designed the research plan, organized the study, planned the experiments and coordinated the data-analysis.

Ethics

The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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