Survival of *Mycobacterium avium* subsp. *hominissuis* in Homemade Smoked Pork Sausages

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**ABSTRACT**


We assessed the survival of *Mycobacterium avium* subsp. *hominissuis* (MAH) in artificially contaminated homemade smoked sausages prepared from pork meat according to traditional recipes, the effect of storage of such sausages at –20°C for three months on MAH viability and to compare assessment of MAH viability/presence by culture and qPCR. Three isolates of MAH were inoculated into the sausage mixture at concentrations of 10^6 CFU per gram of meat and cold smoked at 40°C for 12 h or hot smoked at 70°C for 6 hours. MAH survived the cold smoking procedure without any significant decrease in viable MAH CFU counts; no viable MAH were detected in the hot smoked sausages. The storage of sausages at –20°C caused a decrease in viable MAH counts of about 1 to 3 log_{10}. Absolute MAH counts determined by qPCR were not significantly reduced by the storage or smoking. The presence of viable MAH in sausages after the cold smoking should be considered as a risk for immunodeficient individuals and children.

**Keywords**: IS1245; pork meat; food safety; zoonosis; heat treatment
and at the end of the storage.

and compare these counts with culture CFU before and quantification of previously published triplex qPCR for the detection smoking of sausages, (ii) procedures that are represented by the cold and hot whether –20°C on the survival of term storage of smoked and non-smoked sausages (iii) to determine the aims of this study were: (i) smoking at 70°C for 6 h and the remaining 5 ones (ii) to use a previously published triplex qPCR for the detection and quantification of MAH regardless of viability and compare these counts with culture CFU before and at the end of the storage.

**MATERIAL AND METHODS**

**Isolates.** For the experimental cotamination of the sausage mixture three different MAH isolates were chosen: MAH-a originated from the submandibular lymph node of a domestic pig (PAVLíK et al. 2007), MAH-b from naturally contaminated peat (MATLOVA et al. 2005) and MAH-c from potting soil purchased in a hobby market. A single colony of each isolate was inoculated into Middlebrook 7H9 liquid media (Difco, Livonia, USA) and was cultured at 37°C for one month. The number of MAH CFUs in the broth was determined by plating on Herrold’s Egg Yolk Medium (HEYM) in triplicates.

**Preparation of the mixture and sausage processing.** Eight kilograms of ready-to-use sausage mixture was bought in a local butchery. It contained minced pork meat with approx. 40% fat content and flavour enhancers (salt with sodium nitrite, sucrose, spice mixture and dextrose). The mixture was split into four parts of 2 kg. The first one was used as a negative control for the whole experiment to prove that MAH was not present in the sausage mixture before the experimental inoculation. The remaining three parts were artificially contaminated with the MAH isolates. Fifty ml of the respective MAH suspension with the concentration 4 × 10^7 CFU/ml was added to each part to obtain the final concentration of each MAH isolate in the mixture at 10^6 CFU/g. Each mixture was precisely homogenised after the addition of the respective MAH isolate. Artificially contaminated and control mixtures were stuffed into a pork gut of a diameter of around 3 cm resulting in sausages with weights from 140 g to 150 g per single sausage. From each mixture, 15 sausages were prepared.

After overnight storage in a refrigerator, 5 sausages from each artificially contaminated mixtures and 5 sausages from control mixtures were subjected to cold smoking at 40°C for 12 h, 5 sausages were smoked at 70°C for 6 h and the remaining 5 ones were not smoked. From the total of 12 groups (smoked vs. non-smoked, artificially contaminated vs. control) one sausage was analysed from each group for the presence and viability of MAH by culture immediately after the smoking procedure. The remaining 4 sausages from each group were stored at –20°C.

**Storage effect on the survival of MAH in the smoked and non-smoked sausages.** In order to
determine the impact of long term storage of sausages at −20°C on the viability of MAH, sausages were stored in the freezer for 14, 30, 60 and 90 days. On each collection interval, a sausage from each group was sampled and then discarded to avoid possible reduction of MAH viability by repeated freezing and thawing.

**Determination of MAH viability in sausages by culture.** For the culture, the inner section of the sausage was sampled, homogenised and decontaminated as described previously (Matlova et al. 2003). Briefly, 1 g of the sample was transferred to a stomacher bag and 7 ml of sterile PBS was added. The bag was sealed and the sample was mechanically homogenised in the stomacher at maximum speed for 2 minutes. The homogenised sample was centrifuged at 5000× g for 10 min and the supernatant was discarded. The pellet was resuspended in 1 ml in the tube. The pellet was resuspended in the rest of the supernatant. In order to determine the number of MAH CFU in each sample, the decontaminated suspension was further diluted 10−2 and 10−4 in PBS. One hundred microliters from undiluted and diluted MAH suspensions were precisely spread on three flasks with HEYM and cultured at 37°C for 2 months. Evaluation of the cultures was performed each week for the first month (in order to see possible contamination) and subsequently at the end of culture.

**Identification of mycobacterial isolates by PCR.** The identity of all isolates was confirmed by a conventional PCR method for the determination of M. avium subspecies (Moravková et al. 2008).

**Quantification of MAH directly in the sausages by triplex qPCR.** A previously reported triplex qPCR assay amplifying insertion sequences IS901 and IS1245 for the simultaneous detection and quantification of MAA and MAH with an internal amplification control was used in order to determine the number of MAH in the non-smoked and smoked sausages regardless of viability (Slana et al. 2010). DNA from the sausages (including control ones) was isolated according to the protocol of DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) modified to improve the yield and purity of the DNA (Slana et al. 2010). The number of MAH in the isolated DNA was assessed according to the gradient of standard plasmids. The recalculation was done according to the assumption that a single MAH cell contains on average 15 copies of IS1245 element. The number of MAH was then recalculated to 1 g of sausage and the experimentally determined efficiency of DNA isolation was taken into account (Slana et al. 2010). Isolated DNA was analysed by triplex qPCR in analytical triplicates. The MAH number in the sausages before storage and at the end of storage (after 90 days) was assessed.

**RESULTS AND DISCUSSION**

**Survival of MAH in sausages after smoking**

Control sausages (not contaminated with MAH) were culture negative. All three isolates of MAH used in this study survived cold smoking at 40°C for 12 h (Figure 1). There was almost no change in MAH CFU numbers compared to the non-smoked sausages. The main risk associated with consumption of meat products contaminated with NTM (including MAH) is that they are generally more thermoresistant than the other mycobacteria (compared to M. bovis; Merkal & Whipple 1980; Pavlas 1997). The viability of MAH in contaminated Wiener sausages was not reduced even in the presence of a high concentration of sodium nitrite and smoked at 50°C for 30 min (Merkal et al. 1979). This shows that even higher temperature, but for shorter time does not reduce MAH viability in smoked sausages.

The efficient temperature that is lethal for MAH in Wiener sausages was determined to be 68.3°C for a moment (Merkal et al. 1979). As it would be expected, there was no growth of MAH observed after hot smoking at 70°C for 6 hours.

There is no information about the absolute numbers of MAH in the meat products. The initial number of MAH used for inoculation of mixtures in this study was chosen with regard to the study where it was found that pigs fed with the peat and reared on the organic farms can have heavy loads of MAH in various organs (Matlova et al. 2005). In further studies, different inoculum sizes should be tested in order to determine whether it has any impact on the survival of MAH during the smoking.
Effect of sausage storage at –20°C on the viability of MAH

During the storage of sausages contaminated with different isolates of MAH at –20°C a reduction of CFU numbers was observed (Figure 1). During the storage, no growth of MAH was observed in hot smoked sausages. After 14 days of storage, there was a slight reduction in CFU numbers in non-smoked sausages. In contrast, cold smoked sausages contaminated with MAH-a and MAH-c isolates showed an almost 3 log₁₀ reduction compared to Day 1. In cold smoked sausages contaminated with MAH-b, the reduction was not so dramatic and was comparable with the non-smoked samples.

After a month of storage in a freezer, the number of CFU in non-smoked sausages contaminated with the isolate MAH-c dramatically decreased by 2 log₁₀. Over the next two months of storage there was an additional 1 log₁₀ reduction. Such a trend was not observed in sausages contaminated with the other two MAH isolates where the overall reduction in viable MAH CFU counts was only 2 log₁₀ and 1 log₁₀ for sausages contaminated with MAH-a and MAH-b, respectively (Figure 1).

In cold smoked sausages no further significant reduction in CFU counts was observed for sausages contaminated with MAH-a and MAH-c and the final number of viable MAH after 90 days of storage remained similar as for that determined after 14 days. An exception to this was cold smoked sausages contaminated with MAH-b, where the CFU reduction continued and ended at 2 log₁₀ after 90 days of storage (Figure 1).

The survival of tuberculous and NTM in smoked and dried sausages has been previously investigated (Savov 1975). In dried meat products M. avium of a non-defined serotype was detectable by culture even after 180 days of storage at 6°C. In smoked sausages viable mycobacteria were isolated until day 170. It can be concluded that although there were differences in CFU numbers among the MAH isolates and the smoked and non-smoked sausages, the general trend of a decrease in MAH CFU counts over the time was observed for all MAH isolates.

Quantification of MAH in sausages by triplex qPCR

Samples of control and MAH contaminated sausages before storage at –20°C and after 90 days of storage were analysed. No MAH was detected in control non-contaminated samples confirming the results of culture. The absolute numbers...
of MAH without consideration of viability were stable for the whole course of the experiment and no significant changes were observed even in hot smoked sausages as well as in the cold smoked and non-smoked ones (Table 1). The overall absolute numbers of MAH CFU from smoked and non-smoked sausages was more than 2 log₁₀ lower than absolute numbers of MAH obtained by a triplex quantitative PCR assay. Similar results were observed with MAA and MAH when tissue samples from artificially infected pigs were tested by culture and triplex qPCR simultaneously (Slana et al. 2010) and in M. a. paratuberculosis when a sample is regarded as culture positive when it contains more than 10³ of M. a. paratuberculosis cells as determined by qPCR (unpublished observation). What should be considered is the fact that triplex qPCR revealed that the absolute number of MAH cells in the samples did not change during the smoking and storage; the role of dead but intact mycobacterial cells (otherwise the DNA would be degraded in such a complex matrix as the meat is) in foodstuff should not be neglected.

Foodborne zoonoses originating from pork meat also now include mycobacteria. Statistical analysis of 440 articles about foodborne zoonoses reveals that although the presence of non-specified mycobacteria in meat is rather rare, the severity scores for mycobacterial infection is very high and comparable with infections with Listeria monocytogenes or Clostridium botulinum. Cases of mycobacterial infections originating from pork meat are frequently associated with systemic infections, hospitalization and even with death (Fosse et al. 2008).

**CONCLUSIONS**

In conclusion, from the food safety point of view traditional rustic cold smoking of sausages and their consumption could represent a potential risk for consumer health. Although the smoking itself should have antibacterial effects and was applied for 12 h, the viability of MAH was not reduced. Storage of the sausages in a freezer showed a negative impact on MAH viability in the sausages, but the reduction in viability was not complete. On the other hand, hot smoking of at least 70°C proved to be sufficient for the efficient eradication of viable MAH in sausages. Comparison between qPCR and MAH isolation suggested that molecular methods may be useless for evaluating the decontamination procedures because when it was used there were no changes in cells’ number. With regard to human health, there is no information about the possible influence of intact dead mycobacterial cells on the immune system of immunocompromised individuals or children.

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