microbial biotechnology

Special Issue Article

Autochthonous fungi are central components in microbial community structure in raw fermented sausages

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Summary

Raw meat sausage represents a unique ecological niche rich in nutrients for microbial consumption, making it particularly vulnerable to microbial spoilage. Starter cultures are applied to improve product stability and safety as well as flavour characteristics. However, the influence of starter cultures on microbial community assembly and succession throughout the fermentation process is largely unknown. In particular the effect on the fungal community has not yet been explored. We evaluate the microbiological status of four different raw meat sausages using high-throughput 16S rRNA gene and ITS2 gene sequencing. The objective was to study temporal changes of microbial composition during the fermentation process and to identify potential keystone species that play an important role within the microbial community. Our results suggest that fungi assigned to the species *Debaryomyces hansenii* and *Alternaria alternata* play a key role in microbial community dynamics during fermentation. In addition, bacteria related to the starter culture *Lactobacillus sakei* and the spoilage-associated genera *Acinetobacter*, *Pseudomonas* and *Psychrobacter* are central components of the microbial ecosystem in raw fermented sausages. Elucidating the exact role and interactions of these microorganisms has the potential to have direct impacts on the quality and safety of fermented foods.

Introduction

The production of dry fermented sausages has a long history, with origins dating back to ancient Greek and Roman cultures. Without the ability to keep food at refrigerated temperatures, food preservation through means of salting and curing of meat has been an essential tool to avoid spoilage of food on long journeys. For example, Caesar supplied his legions with dry fermented sausages to keep their vigour and strength on their enduring conquests in Gaul (Pederson, 1971).

Today we know that the indigenous microbiota from the production environment colonizes dry sausages in the process of fermentation, contributing to the organoleptic properties and the extended shelf life of the final product (Talon and Leroy, 2007). In modern sausage manufacturing, starter cultures are often added at the beginning of the ripening process to guarantee product safety and consistent flavour development (Hammes and Hertel, 1998). The most common starter cultures are lactic acid bacteria (LAB) and coagulase-negative staphylocci (CNS) (Aquilanti et al., 2016). LAB are added to establish an acidic environment through the fermentation of sugars into lactic acid that should prevent growth of potentially adverse bacteria. CNS possess mainly proteolytic and lipolytic properties and are involved in nitrate reduction, developing the characteristic colour and flavour (Aquilanti et al., 2007; Sánchez Maina and Stavropoulou, 2017; Franciosa et al., 2018). Additionally, *Pediococcus* spp. are sometimes added as protective cultures against specific pathogens because

of their production of pediocin, a potent anti-listerial bacteriocin (Papagianni and Anastasiadou, 2009; Porto *et al.*, 2017). Naturally, yeasts and other fungi also play a role in sausage fermentation, yet only a few fungi associated with the genera *Debaryomyces*, *Candida* and *Penicilium* are commonly used as starter cultures (Mendonça *et al.*, 2013; Franciosa *et al.*, 2018; Laranjo and Potes, 2019). In general, our understanding of the role of fungi during sausage fermentation is limited since most research efforts to-date have been focused on the bacterial population (Połka *et al.*, 2014; Greppi *et al.*, 2015; Ferrocino *et al.*, 2018). Yet, fungi have been perceived as potential health and safety concerns due to their production of mycotoxins in the past (Holck *et al.*, 2017). Therefore, more studies on the fungal diversity in dry fermented sausages are imperative to gain a complete picture of the potential microbial functions and interactions taking place during a natural fermentation process. In fact, fermentation is a dynamic and complex biological process performed by a multitude of microorganisms that constantly compete for resources and interact with each other in synergistic and/or antagonistic ways, influencing the quality and safety of the final product (Aquilanti *et al.*, 2007; Hall *et al.*, 2018; Senne de Oliveira Lino *et al.*, 2021). Elucidating the succession and temporal dynamics of microbial communities throughout the fermentation process is important to identify microorganisms that contribute to specific properties of the final product or may have been unnoticed as potentially adverse.

Here we provide a comprehensive overview of the bacterial and fungal community throughout ripening of four different dry fermented sausages with various ripening times and added starter cultures. We used high-throughput gene sequencing of the 16S rRNA gene and the ITS2 gene to gain broad insights into the overall microbial community composition during the fermentation process. In general, we expand our knowledge on microbial dynamics during sausage fermentation regarding both fungi as well as bacteria. Our goal was to identify specific bacteria and fungi that consistently correlate with many other species, indicating a central role in the ecosystem of fermented meat sausages. Applying ecological association network analysis, we were able to determine several potential keystone species that were associated to both kingdoms.

Results

We monitored the fermentation process of four different raw meat sausages with regard to temporal changes of the microbial community throughout the ripening process. The bacterial community was investigated by sequencing the 16S rRNA gene and the fungal community by sequencing the ITS2 gene, which are universal among bacteria and fungi respectively. After stringent quality filtering, an average of 26 163 bacterial and 35 494 fungal sequences per sample remained that were used for downstream statistical analysis.

Bacterial diversity decreases, but fungal diversity remains stable throughout ripening

Overall, sausages showed a higher bacterial than fungal diversity (Fig. 1). At the beginning of ripening, 149.71 ± 44.57 bacterial amplicon sequence variants (ASVs) and 25.25 ± 9.21 fungal ASVs were observed. Similarly, Shannon and Simpson diversity indices were higher for bacteria (Shannon: 4.52 ± 0.34, Simpson: 0.98 ± 0.01) than for fungi (Shannon: 2.07 ± 0.43, Simpson: 0.76 ± 0.10) at the start of ripening. The bacterial diversity then decreased over the course of ripening in all four analysed sausage types while the fungal diversity remained stable or even increased. For example, the longest ripened sausage (Sausage B2: 42 days of ripening) initially had 155.08 ± 26.14 bacterial and 18.08 ± 7.24 fungal ASVs at the start versus 61.50 ± 0.71 bacterial and 18.42 ± 14.14 fungal ASVs at the end of ripening. A linear regression analysis supported a negative relationship of bacterial diversity and ripening time whereas fungal diversity was positively related to ripening time (Fig. 1). Furthermore, the decrease of the Shannon and Simpson diversity indices over time for bacterial diversity indicated that a few bacterial species are outgrowing and suppressing the rest of the bacterial community, thus reducing the overall diversity. In contrast, the fungal diversity increased over time as more species were observed at the later time points, while the community evenness remained stable until the end of ripening.

The degree of community differentiation is higher in the fungal community compared to bacteria

A core sausage microbiome was identified looking at the differentiation among the microbial communities of the different sausage types. In detail, 47 of 292 ASVs (16.1%) of the more abundant (> 0.1% relative abundance) bacterial community were shared among all sausage types while 114 out of 292 ASVs (39.04%) occurred in both facilities (Fig. 2A). In contrast, 95 ASVs (32.53%) were only found in facility A and 83 ASVs (28.43%) were unique to facility B. The 47 bacterial ASVs that were found to be part of the sausage core microbiome were associated to six different genera, including the starter cultures *Lactobacillus* and *Staphylococcus*, as well as the spoilage-associated genera *Brochothrix*, *Lactobacillus*, *Staphylococcus*, *Photobacterium*,
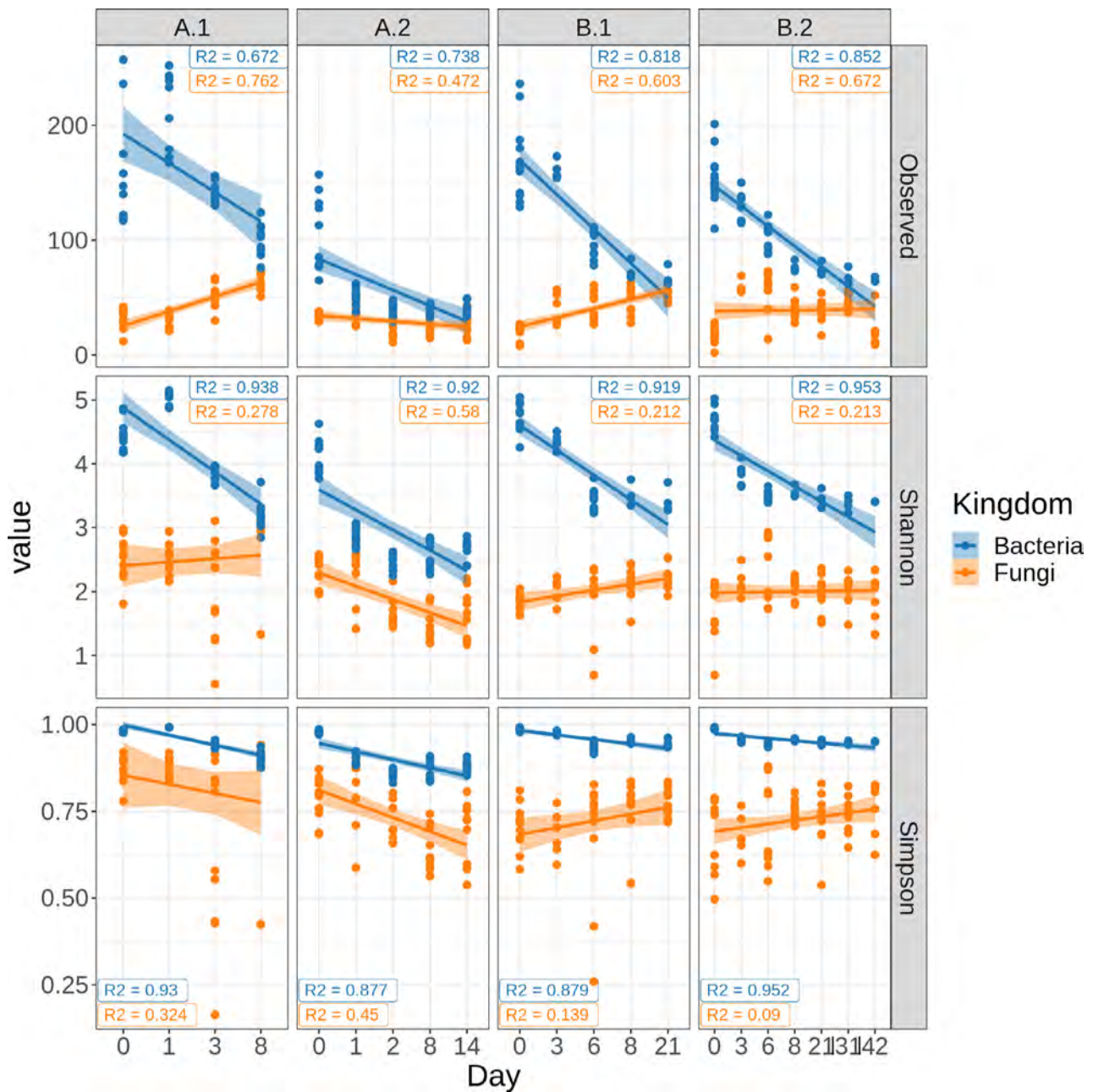
Fig. 1. Alpha diversity indices of 16S rRNA and ITS gene libraries from different sausages over ripening time. Dots represent values from individual samples, lines represent regression lines of linear models, and shaded areas indicate the confidence interval (0.95).

Pseudomonas and *Psychrobacter* (Appendix S1). The shared proportion of the fungal community was smaller. Here, 11 of 165 ASVs (6.7%) were shared between all sausage types and 45 ASVs (27.27%) were common in both facilities. Facility A harboured 74 intrinsic ASVs (44.84%) and 46 ASVs (27.87%) were only found in facility B. The core fungal microbiome consisted of *Alternaria alternata*, *Mycosphaerella tassiana*, *Cladosporium cladosporioides*, *Candida zeylanoides*, *Tausonia pullulans*,

Debaryomyces hansenii and *Vishniacozyma victoriae* (Appendix S2).

Beta diversity analysis revealed major shifts in the microbial community structure within the first days of ripening in all four different sausage types (Fig. 2B). After this initial shift, the community changed only marginally over the rest of the ripening time. Notably, the shifts in community composition were consistent among biological replicates indicating an analogous
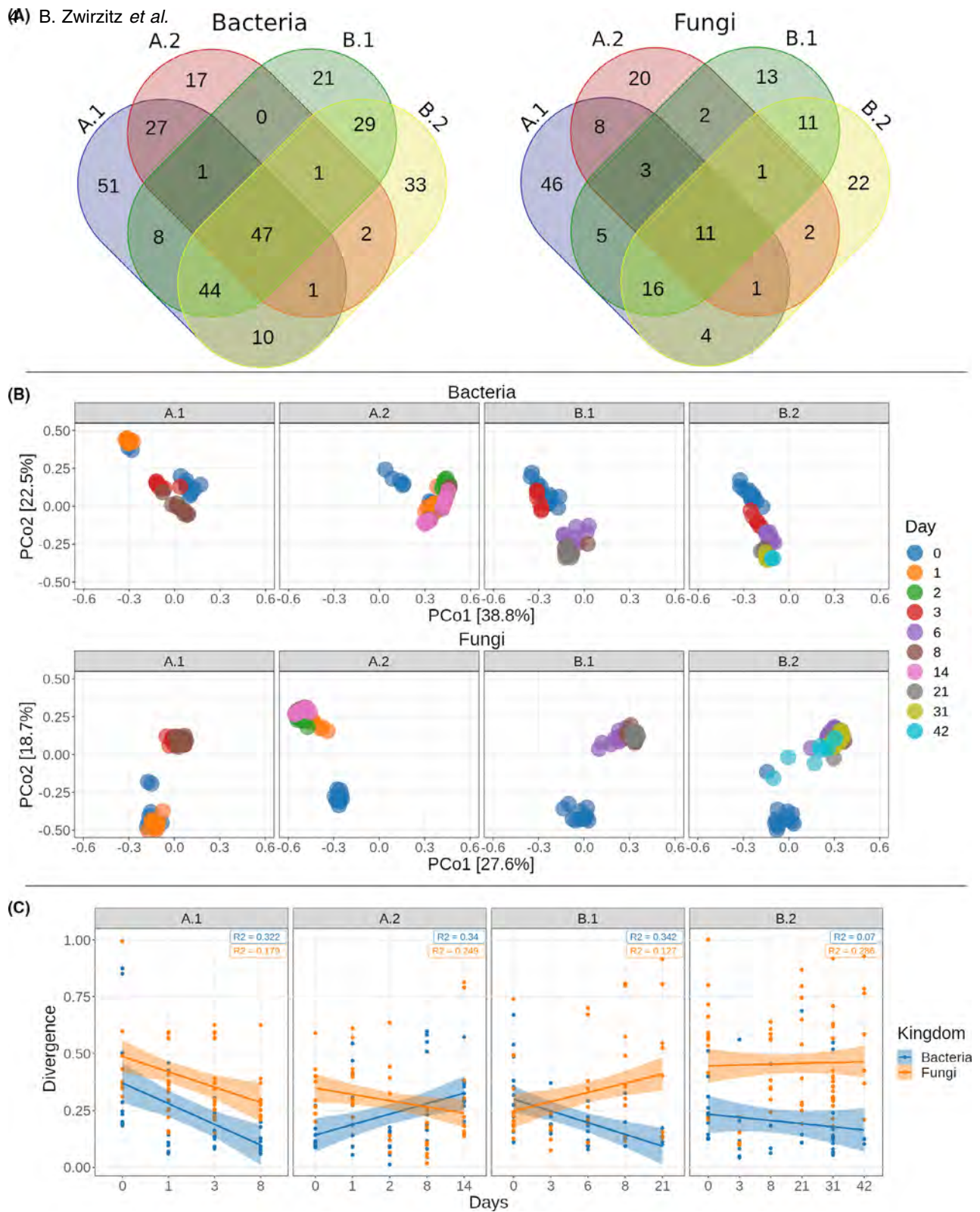
Fig. 2. Beta diversity analysis of bacterial and fungal microbiomes based on 16S rRNA and ITS gene libraries.
A. Venn diagrams indicating shared amplicon sequence variants with a minimum relative abundance of 0.1% across the four different sausages.
B. Principal coordinates analysis based on Bray–Curtis dissimilarities. Dots represent values from individual samples with colours expressing samples from different days of ripening.
C. Divergence quantified as the average dissimilarity of each sample from the group mean. Dots represent values from individual samples, with lines representing regression lines of linear models, and shaded areas indicate the confidence interval (0.95).

development of the microbial community over time in all sausages from one type. In the long-ripened sausages from facility B, the overall heterogeneity in bacterial community composition (Divergence) decreased over time, whereas it increased for the fungal community (Fig. 2C). The microbial communities in the short-ripened sausages from facility A showed inconsistent development. Here, the heterogeneity of the microbial community decreased over time for both the bacterial and the fungal part of the community in sausage A.1, whereas the bacterial divergence increased in sausage A.2. Overall, these results suggest that the fungal community gets more heterogeneous with increasing ripening time, while the bacterial community tends to become more homogeneous as ripening progresses.

Temporal changes of the most abundant bacterial genera and fungal species during sausage ripening

The starter cultures that were added to the sausages before the filling process dominated the microbiome from day 0 on (Fig. 3). Sausages from facility A were inoculated with *Lactobacillus (L.) sakei*, *Staphylococcus (S.) carnosus* and *Kocuria (K.) salsicia*. In particular,

Lactobacillus was present at high abundances already at the beginning of ripening and continued to increase until the end of ripening (A.1: 45.7% → 86.8%; A.2: 38.4% → 53.5%). In contrast, *Staphyloccous* and *Kocuria* had lower starting abundances (A.1: 16.4% and 1.4% respectively; A.2: 41.0% and 5.2%) and did not proliferate as successfully as *Lactobacillus* over the course of ripening. Similarly, *Lactobacillus* was also thriving compared to *Staphylococcus* in sausages from facility B (B.1: 10.1% → 60.5%; B.2: 9.9% → 54.2%). In these sausages, *Pediococcus (P.) acidilacti* was added instead of *K. salsicia* as a protective culture against *Listeria monocytogenes*. The successful establishment of a *P. acidilacti* population could be observed until the end of ripening in both analysed sausages. Since *L. monocytogenes* was not found in any of the samples (also from facility A), we cannot draw conclusions about the effectiveness of the protective capacities of *P. acidilacti* towards *L. monocytogenes*.

Potential spoilage organisms in the genera *Pseudomonas*, *Brochothrix*, *Psychrobacter* and *Acinetobacter* were present at various abundances at the beginning of ripening in all analysed sausages. However, their numbers decreased significantly over the course of ripening,
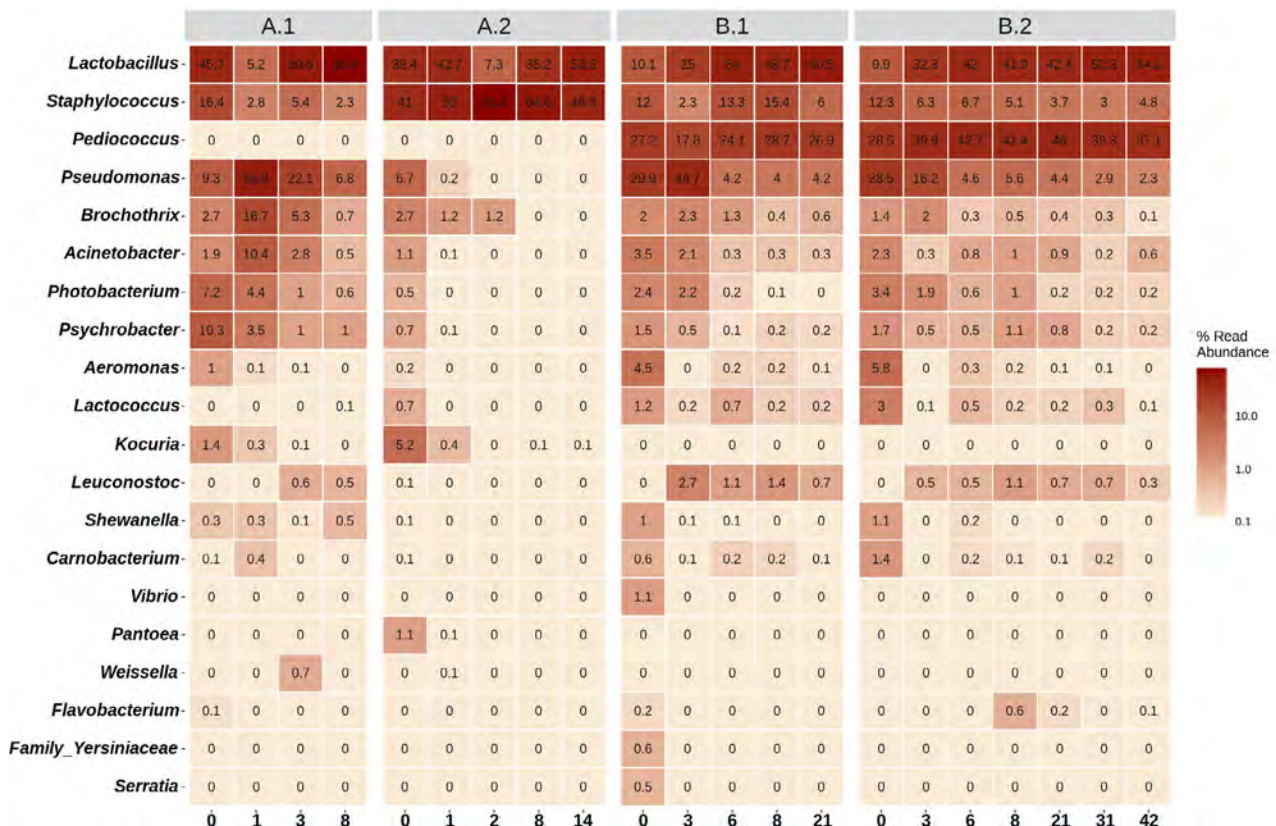

Fig. 3. Heatmap showing the relative abundance of the 20 most abundant bacterial genera over ripening time in days. Samples were grouped by days and split by sausage type. Relative abundance was normalized by total sum scaling and the mean for each sample group is depicted.

demonstrating the effectiveness of the starter cultures to suppress growth of other potentially harmful microorganisms. This was especially true for sausage A.2, in which all non-starter bacteria were reduced to levels below 0.1% relative abundance after 8 days of ripening. *Leuconostoc* was the only other genus next to the added starter cultures that managed to proliferate throughout ripening in sausages A.1, B.1 and B.2.

While the bacterial community in the sausages was mostly dominated by the starter cultures, the fungal community was more diverse and varied more between the sausages. Sausage A.2 was the only sausage that had fungi (*Penicillium nalgoviensis* and *Penicillium candidadum*) sprayed onto it at the beginning of the ripening process. This was also recovered by the sequencing data, which found fungi of the genus *Penicilium* to be the most abundant fungi in sausage A.2 but not in the other sausages (Fig. 4). The most abundant fungi overall were *Calophoma sandfjordenica*, *Itersonilia perplexans*, *Alternaria alternata* and *Debaryomyces hansenii*. *Calophoma sandfjordenica* was predominant in sausages

from facility B and to a lesser extent in sausage A.1. In contrast *Alternaria alternata* was more abundant in sausages from facility A compared to facility B. Notably, *Debaryomyces hansenii* showed a very consistent abundance pattern across all four analysed sausages, namely an initial increase until the mid-ripening phase followed by a decrease towards the end of ripening. This arch-shaped pattern was directly converse to *Itersonilia perplexans,* which had higher abundances at the beginning of ripening, then decreased shortly after, before it increased in abundance again until the end of ripening. *Candida zeylanoides*, another highly abundant yeast commonly found in sausages, significantly decreased during ripening, whereas *Mycosphaerella tassiana* increased from start to end of ripening.

Potential keystone species during sausage fermentation

Cross-domain networks of microbial communities revealed a wide array of potential interactions between individual microbial community members in raw
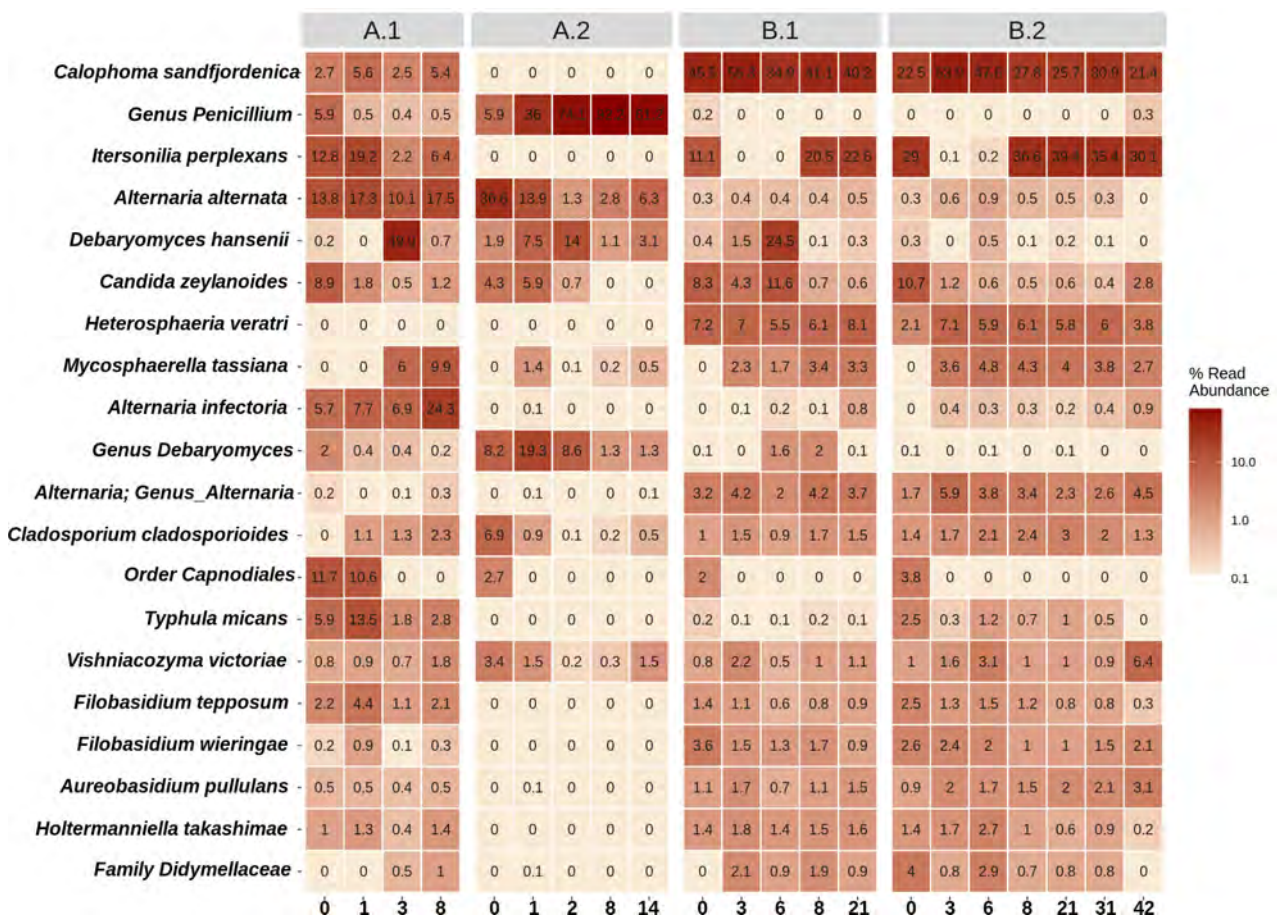

	A.1				A.2					B.1					B.2						
Calophoma sandfjordenica	2.7	5.6	2.5	5.4	0	0	0	0	0	95.5	79.5	84.9	81.1	40.2	22.5	83.9	47.1	27.8	25.7	80.9	21.4
Genus Penicillium	5.9	0.5	0.4	0.5	5.9	36	74.5	80.5	61.5	0.2	0	0	0	0	0	0	0	0	0	0	0.3
Itersonilia perplexans	12.8	19.2	2.2	6.4	0	0	0	0	0	11.1	0	0	20.5	22.6	29	0.1	0.2	36.6	39.4	35.4	30.1
Alternaria alternata	13.8	17.3	10.1	17.5	36.6	13.9	1.3	2.8	6.3	0.3	0.4	0.4	0.4	0.5	0.3	0.6	0.9	0.5	0.5	0.3	0
Debaryomyces hansenii	0.2	0	19.9	0.7	1.9	7.5	14	1.1	3.1	0.4	1.5	24.5	0.1	0.3	0.3	0	0.5	0.1	0.2	0.1	0
Candida zeylanoides	8.9	1.8	0.5	1.2	4.3	5.9	0.7	0	0	8.3	4.3	11.6	0.7	0.6	10.7	1.2	0.6	0.5	0.6	0.4	2.8
Heterosphaeria veratri	0	0	0	0	0	0	0	0	0	7.2	7	5.5	6.1	8.1	2.1	7.1	5.9	6.1	5.8	6	3.8
Mycosphaerella tassiana	0	0	6	9.9	0	1.4	0.1	0.2	0.5	0	2.3	1.7	3.4	3.3	0	3.6	4.8	4.3	4	3.8	2.7
Alternaria infectoria	5.7	7.7	6.9	24.3	0	0.1	0	0	0	0	0.1	0.2	0.1	0.8	0	0.4	0.3	0.3	0.2	0.4	0.9
Genus Debaryomyces	2	0.4	0.4	0.2	8.2	19.3	8.6	1.3	1.3	0.1	0	1.6	2	0.1	0.1	0	0	0.1	0	0	
Alternaria; Genus_Alternaria	0.2	0	0.1	0.3	0	0.1	0	0	0.1	3.2	4.2	2	4.2	3.7	1.7	5.9	3.8	3.4	2.3	2.6	4.5
Cladosporium cladosporioides	0	1.1	1.3	2.3	6.9	0.9	0.1	0.2	0.5	1	1.5	0.9	1.7	1.5	1.4	1.7	2.4	3	2	1.3	
Order Capnodiales	11.7	10.6	0	0	2.7	0	0	0	0	2	0	0	0	0	3.8	0	0	0	0	0	0
Typhula micans	5.9	13.5	1.8	2.8	0	0	0	0	0	0.2	0.1	0.1	0.2	0.1	2.5	0.3	1.2	0.7	1	0.5	0
Vishniacozyma victoriae	0.8	0.9	0.7	1.8	3.4	1.5	0.2	0.3	1.5	0.8	2.2	0.5	1	1.1	1	1.6	3.1	1	1	0.9	6.4
Filobasidium tepposum	2.2	4.4	1.1	2.1	0	0	0	0	0	1.4	1	0.6	0.8	0.9	2.5	1.3	1.5	1.2	0.8	0.8	0.3
Filobasidium wieringae	0.2	0.9	0.1	0.3	0	0	0	0	0	3.6	1.5	1.3	1.7	0.9	2.6	2.4	2	1	1	1.5	2.1
Aureobasidium pullulans	0.5	0.5	0.4	0.5	0	0.1	0	0	0	1.1	1.7	0.7	1.1	1.5	0.9	2	1.7	1.5	2	2.1	3.1
Holtermanniella takashimae	1	1.3	0.4	1.4	0	0	0	0	0	1.4	1.8	1.4	1.5	1.6	1.4	1.7	2.7	1	0.6	0.9	0.2
Family Didymellaceae	0	0	0.5	1	0	0.1	0	0	0	0	2.1	0.9	1.9	0.9	4	0.8	2.9	0.7	0.8	0.8	0
	0	1	3	8	0	1	2	8	14	0	3	6	8	21	0	3	6	8	21	31	42

% Read Abundance: 10.0 / 1.0 / 0.1

Fig. 4. Heatmap showing the relative abundance of the 20 most abundant fungal species over ripening time in days. Samples were grouped by days and split by sausage type. Relative abundance was normalized by total sum scaling and the mean for each sample group is depicted.

fermented sausages (Appendix S3). Positive and negative associations were detected within and across kingdoms. In all four sausage types, fungi were found to play a central role in ecological network organization with some species showing a high connectivity and centrality within the networks (Appendix S3). For example, ASVs associated with the genus *Debaryomyces* (F5 and F8) or the species *Alternaria alternata* (F11 and F393) had a high node degree and betweenness centrality in more than one sausage type, indicative for potential general keystone species within sausage ripening ecosystems (Fig. 5). On the other hand, some fungal species, for example, *Candida zeylanoides* (F1), *Mycosphaerella tassiana* (F10), *Typhula micans* (F39), *Calophoma sandfjordenica* (F224) and *Filobasidium wieringae* (F33) were identified as potential keystone species in one of the four sausage types, but not in the others. Potential bacterial keystone species were ASVs associated with the genera *Acinetobacter* (B3181), *Lactobacillus* (B1510, B1512, B1520, B1524, B1535), *Pseudomonas* (B26, B40, B41, B101, B130), *Psychrobacter* (B3277, B3280, B3287) and exclusively in sausage B.2 *Brochothrix* (B1276) and *Lactococcus* (B1304). Standard nucleotide blast revealed that the potential keystone ASVs associated with the genus *Lactobacillus* are all different oligotypes of *L. sakei* with different relative abundance patterns over time (Appendix S4).

Discussion

Microorganisms are responsible for the development of many organoleptic properties during the fermentation process of raw meat sausages. Bacterial starter cultures are usually added at the beginning of the ripening process to ensure a microbiologically safe product with consistent flavour and smell. Recent studies have focused on the bacterial composition of various sausages using high-throughput 16S rRNA gene sequencing (Połka et al., 2014; Greppi et al., 2015; Rebecchi et al., 2015; Quijada et al., 2018). In this study, we show that in addition to the bacterial community, the fungal community is also an integral part of the microbiome of raw fermented meat sausages, and is more diverse than previously thought. Regarding overall diversity patterns, we found that the fungal community was more stable and more heterogeneous compared to the bacterial community over the course of ripening. A decline in bacterial diversity has been observed in the past and was linked to a
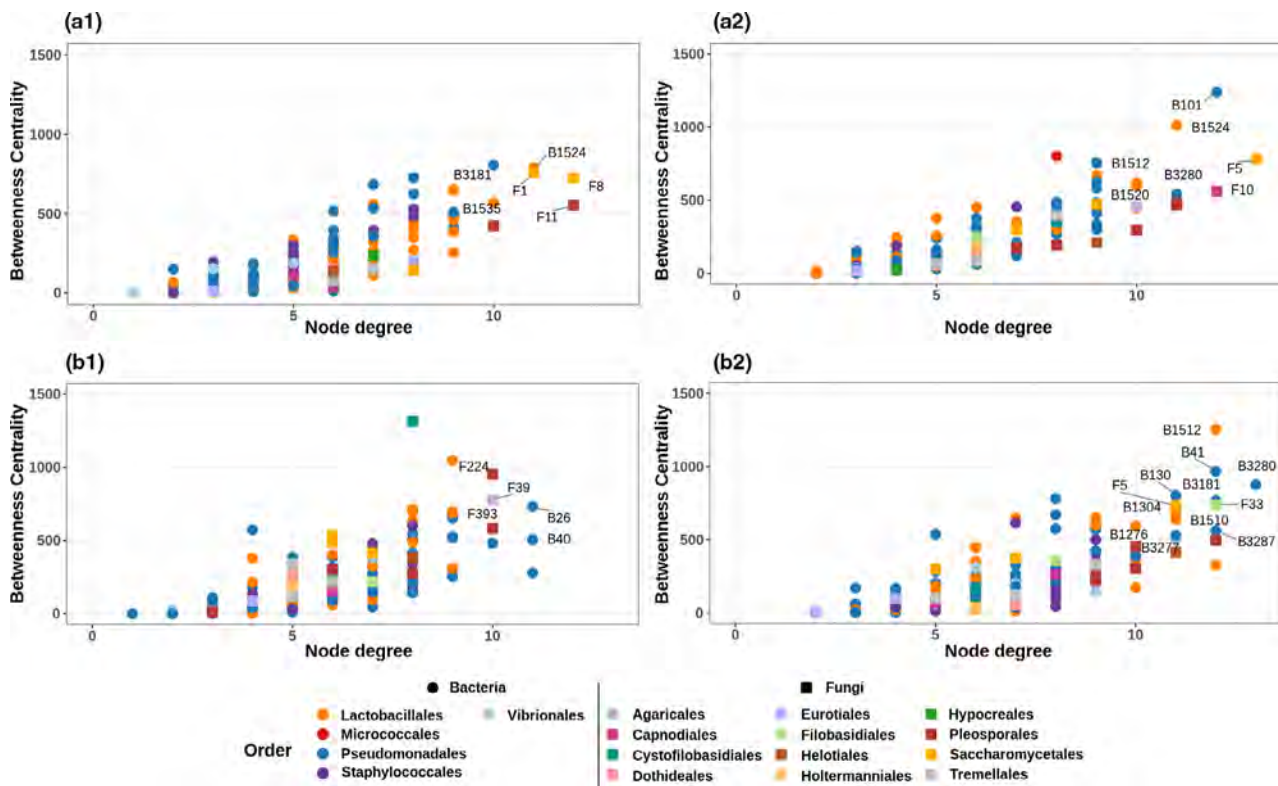

Fig. 5. Betweenness centrality vs. node degree of all amplicon sequence variants (ASVs) in the ecological association networks. Nodes with high betweenness centrality and high node degree represent hubs in the network, indicating potential keystone species. Bacterial ASVs (circles) and fungal ASVs (squares) were coloured based on Order affiliation. ASVs that had a minimum betweenness centrality of 500 and a minimum node degree of 10 are highlighted.

drop in pH and water activity during the fermentation process (Wang and Wang, 2019). On the contrary, many fungi are xerophilic, therefore adapt well to the reduction of a_w, explaining their stability up to the later stages of ripening (López Díaz *et al.*, 2002). The majority of the fungal population was intrinsic to the different facilities, indicating a large autochthonous fungal community that is facility specific. Still, we identified a common core microbiome across multiple sausage types from different facilities and revealed consistent temporal changes of certain species over ripening time. This demonstrates that similar basic communities exist across different sausage types regardless of added starter culture compositions, or other factors like geographical origin, or differences in added spices, size and ripening time. On the other hand, the fluctuations over time show that the fermentation process is dynamic and that assessments of the microbial community at multiple time points are essential in order to gain a comprehensive picture of the microbial processes taking place during ripening.

In all analysed sausages, the decrease in bacterial diversity along with the relative abundance data of the most abundant genera shows the effectiveness of the starter cultures to proliferate and outgrow other bacteria (Talon *et al.*, 2008). The only bacterial genera that could be detected at the end of the ripening time were *Pseudomonas, Aeromonas, Brochothrix, Acinetobacter, Psychrobacter, Photobacterium, Lactococcus* and *Shewanella*. These genera are commonly found in various types of raw fermented sausages and include known meat spoilage organisms (Cocolin *et al.*, 2013; Cardinali *et al.*, 2018; Odeyemi *et al.*, 2020). Seeing similar succession patterns across various sausage types produced in different facilities also signifies consistent and critical interactions. Elucidating these interactions is imperative for unravelling the complexity of microbial ecosystems in general and fermented foods can be used as model systems to investigate them (Wolfe *et al.*, 2014). Here, we point out potential keystone species that have many possible interactions within and across kingdoms using ecological network analysis. While microbial network analysis can be a great tool for exploratory data analysis and hypothesis generation, the detected associations require further experimental validation due to several limitations and challenges in network construction (Faust and Raes, 2012; Röttjers and Faust, 2018; Faust, 2021). Thus, we propose to test the potential keystone species and their interactions that have been identified in this study in future studies, by means of experimental co-cultivations and leave-one-out or challenging experiments. In that regard, the fungi *Debaryomyces hansenii* and *Alternaria alternata* as well as bacteria associated with the genera *Pseudomonas, Acinetobacter, Lactobacillus* and *Psychrobacter* are of particular interest.

Alternaria alternata is a common plant pathogen, but has been detected in various meat products before (Dorn-In *et al.*, 2013; Wen *et al.*, 2021). Our data reinforce that *Alternaria* species are a common member of the raw fermented meat microbiota. *Alternaria* spp. produce mycotoxins that have been found to affect the growth of human gut bacterial strains, impairing a healthy gut microbial community (Crudo *et al.*, 2021). In addition, *Alternaria alternata* produces a range of potent allergenic proteins that can induce or enhance respiratory allergic diseases (Gabriel *et al.*, 2016). Over the last decade, *Debaryomyces hansenii*, a halotolerant yeast, has come into focus for many researchers due to its probiotic and nutritional effects, as well as its high biotechnological potential (Prista *et al.*, 2016; Angulo *et al.*, 2020). However, there is also increasing evidence that it is involved in the inflammation of mucosal tissue and Crohn's disease (Jain *et al.*, 2021). Further studies are necessary to investigate possible implications of these fungi on human health and the human gut microbiome upon ingestion of them through sausage consumption.

The different oligotypes associated with *L. sakei* that we detected in the different facilities indicate that despite starter culture addition indigenous bacteria of the same species still play an important role in sausage fermentation. Quijada *et al.* (2018) have observed different *Lactobacillus* populations in traditionally (without the use of starter cultures) manufactured sausages from Spain and inferred that they are likely responsible for characteristic organoleptic properties of the products from different companies. Our data suggest that similar processes, although to a lesser extent, also occur in sausages produced with starter cultures. As mentioned before, the other potential keystone bacteria, *Pseudomonas, Acinetobacter, Brochothrix* and *Psychrobacter,* have been found to be the dominant microorganisms in other sausages and meat products and include potentially harmful or spoilage-associated bacteria. (Połka *et al.*, 2014; Kamilari *et al.*, 2021; Zhang *et al.*, 2021). Thus, it is imperative to better understand the factors driving community assembly and growth of these bacteria throughout ripening. By deciphering the effects of early colonizing species and metabolic cooperations between functional groups, we can understand the drivers of succession and avoid growth of potentially harmful microorganisms through countermeasures. For example, once we understand the specific role of *Pseudomonas* in the microbial ecosystem of the sausage, additional starter cultures could be added that perform a similar function, but without the spoilage potential.

Conclusion

In summary, we show that the role of fungi during raw sausage fermentation has been underestimated until

now. Their overall prevalence and centrality in ecological network analysis demonstrate the importance of a holistic approach (i.e. whole microbial community analysis not just bacteria) to elucidate microbial processes and interaction patterns throughout sausage ripening. We have identified several potential keystone species (fungi and bacteria) central to the microbial ecosystem of raw fermented sausages. Additional experiments are necessary to validate the exact interaction processes between individual species in order to link the patterns we see in the data presented here to actual processes. Unveiling the mechanisms underlying interactions within microbial communities will lead to safer and more flavourful products.

Experimental procedures

Sausage manufacturing, experimental design and sampling

Four different types of raw meat sausages were collected from two different facilities in Austria in November 2018 (Facility A) and April 2019 (Facility B). Both facilities used similar production practices. Meat was minced and mixed with rest of the ingredients in a cutter before being stuffed into natural bovine casings. The sausages were then dried for about 24 h after which they were transferred to the ripening room where they were ripened under a controlled climate. One facility provided short-ripened sausages (A.1 ripened for 8 days and A.2 ripened for 14 days) and the other facility provided long-ripened sausages (B.1 – 21 days and B.2 – 42 days). A detailed list of ingredients for each sausage type can be found in Appendix S5. All sausages were inoculated with starter cultures during the mincing process. The sausages from facility A were inoculated with *Lactobacillus sakei*, *Staphylococcus carnosus* and *Kocuria salsicia*. Sausage A.2 was additionally sprayed with a protective mould culture (*Penicillium nalgoviensis* and *Penicillium candidadum*) before ripening started. The sausages from facility B were inoculated with *Lactobacillus sakei, Staphylococcus carnosus* and *Pediococcus acidilacti*. Samples for sausage A.1 were taken on day 0 (Day of production), 1, 3 and 8 (Post production). Sausage A.2 was sampled on day 0, 1, 2, 8 and 14. Sausages from facility B were sampled on day 0, 3, 6, 8 and 21 and sausage B.2 additionally at day 31 and 42. At each sampling time point, 10 biological replicates were taken per sausage type, resulting in a total of 210 samples. Sampling was performed over the course of the day, spreading the replicates over several batches. The sausages were transported to the lab as a whole und cooling conditions, where a 200 mg piece was cut out of the centre with sterile scalpels and stored in 2 ml Eppendorf tubes at −80 °C until DNA extraction.

DNA extraction and sequencing

The DNA was extracted from 200 mg of sausage using the DNeasy PowerSoil Kit (Qiagen GmbH, Hilden, Germany) according to manufacturer instructions. The elution step of the protocol was modified; instead of 200 μl AE buffer, two times 50 μl DEPC-treated water was used. Negative controls (DEPC-treated sterile water), one for each used kit, were also extracted together with the regular samples. The DNA concentration of the samples was measured with the Qubit dsDNA HS Assay Kit and QUBIT 2.0 Fluorometer (Invitrogen, Thermo Fisher Scientific, Oregon, USA).

16S rRNA gene and ITS2 gene amplicon library generation from total DNA and sequencing on a MiSeq Illumina platform after library preparation was performed at the Vienna Biocenter Core Facilities NGS Unit (www.vbcf.ac.at). The bacterial community was studied by the amplification of the V3–V4 region of the 16S rRNA gene with a 300-bp paired-end read protocol. The PCR reactions were performed as described in Klindworth *et al.* using the forward primer 341f 5′-TCGTCGGCAGCGTCA GATGTGTATAAGAGACAG and the reverse primer 785r 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG (Klindworth *et al.* 2013). The fungal community was assessed by the amplification of the ITS2 region (300–400 bp), with the primers ITS3-5′-GCATCGATGAAGAA CGCAGC-3′ and ITS4-5′-TCCTCCGCTTATTGATATGC-3′ (White *et al.* 1990). Raw sequence data is available in the European Nucleotide Archive under accession number PRJEB46804.

Bioinformatics and data analysis

First, primers were removed using CUTADAPT v2.1 (Martin, 2011). Then, the bacterial and fungal sequences were processed independently, using the DADA2 v1.14.1 pipeline in R v3.6.3 (R Core Team, 2015; Callahan *et al.*, 2016). Briefly, low quality sequences were filtered using 'filterAndTrim' with a maximum number of expected errors of 2 and trimming set at a length where the quality score dropped below 30. After learning the error rates, samples were dereplicated and the dada2 sample inference algorithm was run with default parameters. Then, forward and reverse reads were merged and ASV tables were constructed. At this point, ASV tables from individual lanes were merged into one table (one for 16S rRNA gene data and one for ITS2 gene data). Chimeric sequences were removed using the 'removeBimeraDenovo' command with the consensus method. The remaining reads were then used for taxonomic assignment. Bacterial ASVs were classified to the SILVA rRNA database SSU 138 and fungal ASVs were classified to the UNITE database v8.0 (Quast *et al.*, 2013; Nilsson *et al.*,

2019). Possible contaminant ASVs were identified and removed with the R package DECONTAM v1.6, using a prevalence-based contaminant identification with a threshold of 0.5, which classifies all sequences that are more prevalent in negative controls than in positive samples as contaminants (Davis *et al.*, 2018). Furthermore, samples with low read depth (less than 1000 reads) and ASVs with less than 5 reads were excluded from analysis. Finally, uncharacterized taxa and taxa identified as Chloroplast or Mitochondria were removed.

In-depth microbial community analysis was performed in the R environment. Alpha diversity indices were calculated using a dataset rarefied to the minimum sample size (1056) in VEGAN v2.5.6 and linear regression models were fitted with a y ~ x formula for bacterial and fungal communities separately (Oksanen *et al.*, 2015). Venn diagrams of shared ASVs with a minimum relative abundance of 0.1% were determined with a webtool available under: http://bioinformatics.psb.ugent.be/webtools/Venn/.   Beta diversity was assessed with a principal coordinates analysis based on Bray–Curtis distances and relative abundances of bacterial genera and species were calculated and illustrated as heatmaps in AMPVIS2 v2.5.1 (Andersen *et al.*, 2018). The overall community heterogeneity across samples within one group (Divergence) was calculated with the 'divergence' function in the MICROBIOME v1.9.19 R package (Lahti and Shetty, 2017). Ecological association networks were constructed using SPIECEASI v1.0.7 with the extension for cross-domain associations developed by Tipton *et al.* (Kurtz *et al.*, 2015; Tipton *et al.*, 2018). Since the removal of samples with less than 1000 reads omitted different samples in the bacterial and fungal datasets, only samples that were common in both datasets ($n = 157$; A.1 = 30, A.2 = 48, B.1 = 37, B.2 = 42) were used for network construction. Additionally, ASVs with a prevalence of $\leq 33\%$ of the samples and a relative abundance $\leq 0.01\%$ were filtered out to increase the sensitivity of the analysis (Berry and Widder, 2014). Individual networks for each sausage type were calculated using the Meinshausen-buhlmann's neighbourhood selection method and a StARS variability threshold of 0.05. Network properties were determined with the IGRAPH v1.2.5 package following a script for microbial network analysis published by Kerdraon *et al.* (Csárdi and Nepusz, 2006; Kerdraon *et al.*, 2019).

Acknowledgements

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contributions

BZ, SW, MW, and ES conceived and designed the study. Sampling was performed by BZ, ST, and BS. Laboratory work was done by BZ and ST. Data analysis and statistics were performed by BZ. BZ and ES wrote the manuscript. All authors read and approved the final manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.
Appendix S1
Appendix S2
Appendix S3
Appendix S4
Appendix S5