Plant-based meat alternatives and their associated microbial communities

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Abstract

It is still unclear whether vegan meat substitutes are a short-lived trend or become established int the long term. However, they are currently a popular way for flexitarians to reduce their meat consumption without having to give up the pleasure of burgers and Co. Over the last few years, the trend of increasing sales and diversifying the product range has continued, but publication activities in this field are currently limited mainly to market research and food technology topics. The paucity of research results on more microbiological levels has prompted us to investigate vegan meat substitutes from Austrian supermarkets using culture-dependent and culture-independent methods (16S rRNA gene amplicon sequencing) to investigate whether product-characteristic microbial communities can be identified. The examined products showed three different microbial profiles. Based on 16S rRNA gene amplicon sequencing data the majority of the products were dominated by lactic acid bacteria (either *Leuconostoc* or *Latilactobacillus*), and generally had low alpha diversity. *Proteobacteria* dominated the other part of the products, on which the dominance of individual amplicon sequence variants (ASVs) is noticeably weaker and the alpha diversity distinctly higher than in the lactic acid bacteria (LAB) dominated samples. However, the cultivability of the represented genera was lower in these samples, which raises the legitimate question of whether living representatives of these genera are actually found on the final products. In addition to the dominant representatives of the LABs, a high diversity of different *Bacillus*, but also some *Enterobacteriaceae* and potentially pathogenic species were found with the culturing approach. We assume that especially the dominance of the heterofermentative LABs has a high relevance for the product stability and quality and that there is potential for an improved shelf life of the products. The *Enterobacteriaceae* and potential pathogens isolated, were relatively low, but they still demonstrated that these products are certainly suitable nursery for them. Even though all the examined products had to be heated before consumption, we would like to point out that the lack of consumer experience with this type of product (spoilage detection, preparation, storage and shelf life) is relatively low, which is why we believe that further research into product safety, taking these aspects into

account, would be desirable.

Keywords: meat analogue, vegan meat, meat substitute, microbial profile, food safety, plant protein

1. Introduction:

For most of the population of the Western world, meat consumption is an integral part of their diet. An average U.S. American consumed 102 kg and a European Union citizen 69 kg of meat in 2020 (European Commission and Directorate-General for Agriculture and Rural Development, 2020; OECD, 2022). The global meat consumption increased from 24 kg per year and capita in 1990 to 34 kg in 2020 (OECD and Food and Agriculture Organization of the United Nations, 2022; OECD, 2022). Although the OECD estimates that consumption will level off at around 35 kg per year and capita by 2030, the total meat consumption will further increase with population growth (OECD and Food and Agriculture Organization of the United Nations, 2022). This globally growing appetite for meat is linked to livestock farming and consequently plays a major role in the ecological issues we are currently facing, like land degradation, climate change, water pollution and loss of biodiversity (Steinfeld, 2006; Bianchi et al., 2018). Additionally, common industrial animal husbandry influences public health by supporting the spread of antibiotic resistances and vector-borne disease (Economou and Gousia, 2015; Bianchi et al., 2018; Watts et al., 2018). Further, high meat consumption, as practiced in the Western world and increasingly in transition countries, contributes significantly to many widespread common diseases, like coronary heart disease, diabetes mellitus or colorectal cancer and consequently burdens health care systems (Micha et al., 2010; Chan et al., 2011; Parkin et al., 2011; Feskens et al., 2013). These mentioned aspects are main drivers for more and more people in western civilization to change their meat consumption routines (Stoll-Kleemann and Schmidt, 2017; Ploll and Stern, 2020). In a survey, conducted 2021 in ten different European countries, 2% of the participants referred to themselves as vegans, 5% as vegetarians, 3% as pescetarians and 30% as flexitarians (European Union's Horizon 2020 reasearch and innovation programme, b). The last group is characterized, among other things, by the fact that they want to reduce their meat consumption, but do not want to give up the positive experience that comes with it. As main target group, flexitarians account for about 90% of the sales of plant based meat alternatives (Neuhofer and Lusk, 2022). A market research within "The Smart Protein Project" noted a sales value increase of 82% for plant-based meat (vegan and vegetarian) within 2018 and 2020 for Austria (European Union's Horizon 2020 reasearch and innovation programme, a). The sales of this

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product group and the number of different products have increased strongly over the last few years (Curtain and Grafenauer, 2019; European Union's Horizon 2020 reasearch and innovation programme, a). Despite the increased consumption of these products, there have been only a few studies on their microbiological properties. However, these would be important for ensuring food safety, characterizing potential hazards, assessing risks, and for sustainability questions. Since two of the UN's sustainability goals (goal 2 – zero hunger and 12 – responsible consumption and production) affects our eating habits, increased attention should be paid to reducing food waste. As 30% of food products in primary processing does not even reach the consumer, mainly because of microbial spoilage or pathogen contamination, it is essential to improve the knowledge of the microbial communities of our food, which could help to increase the shelf life and reduce the contamination with pathogens. Still largely unanswered is the question if and how a high microbial diversity on food (consisting of living and dead microbiota) can have a positive effect on consumer's health. For example, a high microbial diversity permanently stimulates the innate and adaptive immune system and provides resistance against colonizing pathogens (Crowe et al., 1973; Mackowiak, 1982; Smith et al., 2007). It was also supposed recently, that the loss of microbial diversity including the disappearance of ancestral indigenous microbiota, which is currently happening in western countries, affects human health and contributes to post-modern conditions such as obesity and asthma (Blaser and Falkow, 2009; Vangay et al., 2018). For all these research questions fundamental knowledge on the microbial compositions on food are necessary, but still lacking. Since, little is known on these products, we sampled a selection of plant-based meat alternatives (PBMA) available in Austrian supermarkets to investigate the general microbial community patterns. Further, we described characteristic microbial profiles and compared four groups of the most common product types (pea and soybean based products with either "minced" (minced meat, burgers, etc.) or "fibrous" (meat chunks, Schnitzel, etc.) texture. We hypothesized that products within one group have, based on similar protein processing, more similar communities than between the groups. Since we examined a relatively undescribed product group, we have chosen a combined approach using culture-dependent and culture-independent techniques. This allowed a more complete description of the microbial community than any of the applications could stand on their own, but also raised additional questions and uncertainties that are relevant to future microbial research of (highly processed) foods.

2. Material and Methods

2.1. Sample acquisition

We purchased 32 different PBMA, between July 12 and July 14, 2021, from four supermarket chains in Vienna, Austria. The focus was on pea- and soybean- protein based products with either a minced or a fibrous texture, since they were the most common representatives of PBMA at this time point in Austria. Additional criteria for the selection were, that the products where entirely plant-based (vegan) and do not contain fermented products, like tofu. Beside these characteristics, the samples were different in their composition, packing, shelf life, etc. (Table 1). All samples were transported refrigerated and stored at 4°C until processing. Sample processing took place within the shelf life and latest five days after purchase.

2.2. Sample preparation

In total, 10 g of each sample, representing all layers of a product, were placed in sterile Stomacher®400 classic strainer bags (Seward Ltd, Worthing, United Kingdom) and diluted with 90 ml sterile phosphate buffered saline (PBS, gibco, Bleiswijk, The Netherlands). A BagMixer® 400 CC (Interscience, Puycapel, France) was used to mechanically comminute the samples for 120 s. To remove coarse food particles, the homogenates were centrifuged at 300 rcf (relative centrifugal force) for 2 min at room temperature (RT) using an Eppendorf Centrifuge 5810R and an A-4-62 rotor (Eppendorf Corporate, Hamburg, Germany). The remaining supernatants were transferred to new tubes and centrifuged at 3,000 rcf (30 min at RT). The obtained cell pellets were diluted 1:10 with sterile PBS and were used freshly for bacterial and fungal isolation, or were frozen at -80°C for later DNA extraction.

2.3. Bacterial and fungal isolation

We carried out two sets of cultivation experiments. The first set was done with a general selection of media (Columbia agar (BioMérieux, Marcy l'Etoile, France), Violet Red Bile Glucose agar (VRBG, Biokar diagnostics, Allonne, France), brain heart infusion agar (BHI broth (Biokar diagnostics) + 1.5% agar (bacteriological agar type E, Biokar diagnostics)), plate count agar (PCA, Biokar diagnostics), Rose Bengal Chloramphenicol agar (Biokar diagnostics), and Baird Parker agar (GranuCult™, Merck, Darmstadt, Germany)), to get a broad range of the expected microbial community. The plates were inoculated with a 10^2 dilution and aerobically and semi-anaerobically (using GENbox anaer, BioMérieux, in BD BBL™GasPak™systems, Becton Dickinson, New Jersey, United States) incubated at 37°C. In order to recover high amounts of different species, the plates were incubated for 16-68 hr, depending on colony size and growth density. Samples with

growth densities too high for picking single colonies after 16 hr of incubation were diluted 10^3 or 10^4 in sterile PBS and plated again. For the second media set we used the information from the 16S rRNA gene amplicon sequencing to select specific media and growth conditions in order to isolate representatives of genera we could not isolate under the first culturing conditions. Depending on the sample specific microbial composition we used Luria Bertani Agar (1% tryptone (Oxoid, Basingstoke, UK), 0.5% yeast extract (micro-granulated, Roth, Karlsruhe, Germany), 1% NaCl (Sigma-Aldrich, St. Louis, USA), 2% agar, pH 7.0), nutrient agar (0.5% casein peptone, tryptic digest (Roth), 0.3% beef extract powder (Fluka analytical, Seelze, Germany), 1.5% agar, pH 7.0), trypto-casein soy agar (TSA,Biokar diagnostics), marine agar (marine broth (Roth) + 1.5% agar), corynebacterium agar (1% casein peptone, tryptic digest, 0.5% yeast extract, 0.5% D(+)-glucose (Roth), 0.5% NaCl, 1.5% agar, pH 7.3), De-Man-Rogosa-Sharpe agar (MRS, Oxoid) and pseudomonas agar F (1% tryptone, 1% casein peptone, 0.15% K_2HPO_4, 0.15% (Roth), $MgSO_4$ (Merck), 1% glycerol (Roth), 1.5% agar) and cultivated the samples (dilution 10^2-10^5) aerobically at 25°C for 48 hr. In both cultivation experiments, we selected morphologically unique, single colonies for re-cultivation followed by 16S rRNA gene Sanger sequencing of the pure cultures for identification. DNA was extracted, using a protocol modified after Walsh et al. (Walsh et al., 2013), by lysing pure cultures with 100 μl 0.01 M TRIS/HCl (Trizma®base, Sigma-Aldrich, St. Louis, United States) and 400 μl 2.5% Chelex®100 resin solution (BioRad, Hercules, United States) at 95°C for 10 min, followed by centrifugation with 15,000 rcf for 30 s. The supernatants were subsequently used for 16S rRNA gene PCR, using a final concentration of 200 nM of each of the universal primers from LGC Genomics GmbH (Berlin, Germany; 27F – 5'-GAG TTT GAT C**M**T GGC TCA G-3' and 1492R – 5'-GG**Y** TAC CTT GTT ACG ACT T-3'), 0.025 U/μl Platinum™Taq DNA-Polymerase (Invitrogen™, Vilnius, Lithuania), 1x TaqMan PCR buffer, 2 mM MgCl2, and 250 nM dNTP Mix (Thermo Scientific™, Vilnius, Lithuania). For the PCR a protocol of 95°C for 5 min (Taq activation) followed by 35 cycles of 40 s at 95°C (denaturation) 40 s at 52°C (annealing) and 1 min at 72°C (elongation) was used. For negative control a reaction with ddH_20 as well as a reaction the negative extraction control were performed. In-house *Listeria monocytogenes* DNA served as positive control. All PCR products were checked with a QIAxcel DNA High Resolution Kit (Qiagen, Hilden, Germany) in a QIAxcel Advanced system (Qiagen). Samples without detectable amounts of PCR products were used for an ITS2 gene PCR (200 nM of each of the primers ITS3 – 5'-GCA TCG ATG AAG AAC GCA GC-3' and ITS4 – 5'-TCC TCC GCT TAT TGA TAT GC-3' (White et al. 1990), 0.025 U/μl Platinum™Taq DNA-Polymerase (Invitrogen™), 1x TaqMan PCR buffer, 2 mM $MgCl_2$, and 250 nM dNTP Mix (Thermo Scientific™), with a protocol of 95°C for 5 min (Taq activation) followed by 30 cycles of 40 s at 94°C, 40 s at 56°C and 1 min at

72°C). For cultures, negative in 16S rRNA and ITS2 PCR, we repeated the extraction with the NucleoSpin tissue kit (Machery-Nagel, Düren, Germany), using the manual in combination with the recommendations for hard-to-lyse bacteria. LGC Genomics GmbH purified and sequenced the PCR products in one direction (using 27F primer for 16S rRNA and ITS4 for the ITS2 region). Potential pathogens and unclassified *Enterobactericeae* were further whole genome sequenced with FLO-MIN106 flow cells on a MinION Mk1C (Oxford Nanopore Technologies, Oxford, UK). The library preparation for this approach was done according to the protocol of Oxford Nanopore Technologies ("Ligation sequencing gDNA - native barcoding (SQK-LSK109 with EXP-NBD196)" (Oxford Nanopore Technologies)) using the NEBNext® FFPE DNA repair kit (New England BioLabs® Inc., Ipswich, United States) for DNA repair and end-preparation, NEB Blunt/TA Ligase Master Mix (New England BioLabs® Inc.) and Native Barcoding Kit 96 (EXP-NBD196, Oxford Nanopore Technologies), for native barcode ligation, Adapter Mix II (Oxford Nanopore Technologies) and NEBNext® Quick Ligation Module (New England BioLabs® Inc.), for adapter ligation, Agencourt AMPure XP beads (Beckman Coulter™), for clean-up steps and SQK-LSK109 sequencing kit (Oxford Nanopore Technologies).

2.4. Bacterial quantification and composition

For direct DNA extraction the DNeasy® PowerFood® Microbial Kit (Qiagen) was used. That for, the in PBS stored cell pellets (-80°C) were thawed, centrifuged (3,000 g, 30 min) and resuspended in 450 μl MBL buffer. Deviating from the DNeasy® PowerFood® Microbial Kit Handbook (Qiagen), the lysis step was proceeded in Lysing Matrix A, 2 ml tubes (MP Biomedicals Germany GmbH, Eschwege, Germany). The further steps were processed according to the protocol. For elution, the same 30 μl of ddH_2O was centrifuged twice over the column. Parallel an extraction of ddH_2O was performed as negative extraction control. 16S rRNA gene amplicon library generation and sequencing was performed at the Vienna Biocenter Core Facilities NGS Unit (Vienna, Austria, www.vbcf.ac.at). Sequencing libraries of the 16S rNRA gene (V3/4 region) were prepared based on Illumina 16S Metagenomic Sequencing Library Preparation recommendations. Primers 341F (5'-CCT ACG GG**N** GGC **W**GC AG-3') and 805R (5'-GAC TAC **HV**G GGT ATC TAA TCC-3') (Klindworth et al. 2013) were used together with Illumina adapter sequences (5' CGT CGG CAG CGT CAG ATG TGT ATA AGA GAC AG-3' and 5' GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G-3', respectively) for amplification. Libraries were constructed by ligating sequencing adapters and indices onto purified PCR products using the Nextera XT Sample Preparation Kit (Illumina). Equimolar amounts of each of the purified amplicons were pooled and sequenced on an Illumina MiSeq Sequencer with a 300 bp paired-end read protocol.

2.5. Sequence Processing and Statistics

The data obtained from the MiSeq sequencing went through a QIIME 2™v2021.4.0 (Bolyen et al., 2019) workflow including the integraded demultiplexing, DADA2 denoising and quality control filtering. The taxonomic classification was done with the Scikit-learn algorithm using a pre-trained full-length-uniform-classifier based on the SILVA 138.1 database (Quast et al., 2013; Bokulich et al., 2018; Kaehler et al., 2019; Robeson et al., 2021; Kaehler). After removal of sequences with mitochondrial or chloroplastic origin, further analyses were performed with R (R Core Team, 2021). To estimate the alpha diversities of the samples Hill-Simpson and Hill-Shannon diversity were calculated using the "iNEXT" package (Hsieh et al., 2022), based on 99.5% coverage rarefied samples (Chao and Jost, 2012; Roswell et al., 2021). Group comparisons were done with Kruskal-Wallis tests followed by Bonferroni-alpha-corrected Dunn's tests for pairwise comparisons. For beta diversity analyses the samples were rarefied with 100 iterations depending on a coverage of 99.5% using the package "phyloseq" (McMurdie et al., 2021) and "metagMisc" (Mikryukov, 2022). Based on that we generated distance matrices with Bray-Curtis dissimilarities, Jaccard indices and Jensen-Shannon divergence and use them for graphical (t-distributed stochastic neighbor embedding - tSNE) and statistical (PERMANOVA, LEfse) analysis. For tSNE we used the "Rtsne" package (Krijthe, 2022) with a maximum of 999 iterations, a perplexity of 5 and two initial dimensions, as recommended by Oskolkov (Oskolkov, 2019). The high variability of the products and little knowledge on the underlying production conditions made it impossible to find adequate variables for PERMANOVA, besides the main protein source and the texture. The producing facility as additional variable would be meaningful, but the product assortment is dominated by one company, which made the model design very unbalanced. PERMANOVA was done with the "vegan" package (Oksanen et al., 2022) using "betadisp" to check for homogeneous dispersion and "adonis" functions for PERMANOVA with 999 iterations using main protein source and texture as explanation variables. LEfse was done with the relative abundance data of the coverage rarefied data in combination with "phyloseqCompanion" package (Stagaman, 2022), for data transformation and the LEfse Bioconda tool by Segata et al. (Segata et al., 2011), using the protein source and texture as class, a normalization of 10^6 and a $\log_{10}$ LDA score threshold of 4.0. In parallel, a group comparison for the same features, as examined with LEfse, was done with Kruskal-Wallis tests followed by a Benjamin-Hochberg alpha correction.

The Sanger sequences from the isolates were trimmed using the "SangerRead" function within the "sangeranalyseR" package (Chao et al., 2020) with a Phred score mean quality cutoff of 40 and a sliding window size of 15 bp. Trimmed sequences, with >100 bp length, were classified with the "assignTaxonomy" function of the "dada2" package (Callahan et al., 2016) in R (with kmer size 8 and 50 bootstrap replicates), based

on an RDP (Ribosomal Database Project) Naïve Bayesian Classifier algorithm (Wang et al. 2007). Further, the isolate sequences were assigned to a database generated from the MiSeq data set to connect the culture-based and culture-independent approaches. For figure 1, the isolate sequences of each genus were clustered within each sample, group and producer using the "IdClusters" function from the "DECIPHER" package (Wright, 2016) with a cutoff of 0.06. More clusters within each genus were interpreted as a higher species or strain diversity within each genus.

The whole genome data sequenced with the MinION were trimmed and filtered with Filtlong v0.2.1 (Wick, 2021), assembled with Flye v2.9 (Kolmogorov et al., 2019; Lin et al., 2016) followed by several polishing steps (four repetitions of Racon v1.5.0 (Vaser et al., 2017) and a final step with Medaka v1.6.0 (Oxford Nanopore Technologies, 2022)), before they were used in the TORMES v1.3.0 (Quijada et al., 2019) workflow. All tools were used with default settings if not mentioned otherwise.

3. Results

3.1. Product descriptions

The four groups did not have the same sizes, since the product variety for each group was not large enough. Still, we had an overall of 16 pea-based and 16 soybean-based products (Table 1). Beside the four main properties, the products were quite diverse. Particularly noteworthy is the number of ingredients used per product (4-27, with a total of about 120 different used ingredients within the 32 sampled products) and the large range of shelf life. The products are also different on factors, which might influence the bacterial composition, i.e. pre-heating or freezing steps, as well as packing in modified atmosphere. Most of the products had clear cooking instructions on the labels, including the recommendation for thorough cooking (Table 1). In total, 27 samples were packed in modified atmosphere with unknown composition. Out of 30 samples sold refrigerated (the other two frozen) six products were frozen at any point during the retail chain (Table 1).

3.2. Cultivable microbial communities

In total, 465 colonies were picked and selected for 16S rRNA or ITS2 gene sequencing. Among these, 431 could be classified to the genus level, representing 38 genera in four different phyla (Figure 1). The remaining isolates could only be assigned to family level (n=16, all *Enterobacteriaceae*), were fungi (n=15; *Wickerhamomyces* (n=7), *Issatchenkia* (n=6), *Yarrowia* (n=1), *Dipodascus* (n=1)), or stayed unassigned (n=3). We isolated *Bacillus* from 19, *Leuconostoc* from 18, *Enterococcus* from 12, and *Latilactobacillus*

from 10 samples. Species from the genus *Bacillus*, *Leuconostoc* and *Latilactobacillus* could be isolated from each of the four sample groups. *Enterobacteriaceae*, which are usually surveyed as an additional hygiene criterion, were only found in the pea protein products of a single manufacturer (Figure 1). A selection of these *Enterobacteriaceae* and isolates classified by the RDP Naïve Bayesian Classifier algorithm as potential pathogens (i.e. *Staphylococcus aureus*, *Bacillus cereus* group, *Klebsiella* sp.) were whole genome sequenced with a MinION device. DATA MISSING

3.3. Lactic acid bacteria and gamma-Proteobacteria dominate the 16S rRNA gene amplicon sequences

In total, 28 samples (883,866 sequences; median frequency per sample: 25,627; range: 439-253,681) passed the quality criteria and were processed with QIIME 2. Because we used coverage based rarefaction (with a coverage of 99.5%) and all of the remaining samples meat this coverage, we removed none of them for further analysis. Over all samples, the ASVs were assigned to 25 different Phyla, however, only three Phyla with >3% in at least one of the samples were found (i.e. *Firmicutes* 0.00-0.95%, *Proteobacteria* 0.00-0.31%, *Bacteroidota* 0.00-0.11%). In total, 18 samples were dominated (>50% relative abundance) by *Firmicutes* (10 samples with >90%), while in the other 9 samples *Proteobacteria* is the most abundant Phylum. The most common genera were *Leuconostoc* (detected in 25 samples; 0.03-100.00% rel. abundance), *Latilactobacillus* (detected in 21 samples; 0.02-86.38% rel. abundance), *Pseudomonas* (detected in 20 samples; 0.36-35.25% rel. abundance), *Serratia* (detected in 19 samples; 0.03-8.92% rel. abundance), and Acinetobacter (detected in 17 samples; 0.09-15.40% rel. abundance). The genus Leuconostoc was the most abundant in 13 samples, followed by Latilactobacillus (4 products), and Shewanella (4 products) (Figure 2). Some genera were found proportionally high (>10%) in one or more samples, but could not be isolated (i.e. *Shewanella*, *Xanthomonas*, *Photobacterium*, *Myroides*, *Pediococcus*).

3.4. Protein source and texture are not the main driver for the community pattern

The Kruskal-Wallis tests comparing the alpha-diversity indices (i.e. Hill-Shannon index and Hill-Simpson index) between the four groups (based on proteins source and texture) were significant (p.value = 0.020 and 0.052), but in post-hoc Dunn's test with Bonferroni alpha adjustment only the Hill-Shannon index between groups "pea-fibrous" and "pea-minced" differed significantly (p.value= 0.016 - Supplement Figure xy). The group dispersions were homogenous in all examined distance methods (i.e. Bray-Curtis, Jaccard, JSD). The PERMANOVA showed that texture and protein source significantly affects the microbial composition (Supplement Table 2), but explained only between 15.9371178and 23.4413074% of the total variance. The

variance explanation by the PERMANOVA would increase, if the manufacturer as variable was added to the model, but since the sampling was very unbalanced on that, we avoided this step.

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3.5. 16S rRNA sequencing revealed three distinct community profiles

In the tSNE plot we see three distinct clusters (Figure 3), which we described, based on the dominating genera as *Leuconostocaceae*-, *Latilactobacillus*- and *Proteobacteria*-profiles. The clustering is traceable, when comparing the similarity of the relative abundance patterns of the samples within each cluster. Although there is some clustering, there is no clear separation based on the examined variables (main protein source, status, manufacturer).

ALPHA DIV AND LEFSE FOR PROFILES LEfSe identified 32 discriminative features with an LDA Effect Size >4.0 (Fig 3). This analysis highlighted a predominance of *Leuconostocaceae* (log LDA 5.48) and its classification levels above in pre-cooked pea products. It identified *Proteobacteria* (log LDA 5.33) as most characteristic for raw pea products, mainly based on the predominance of Pseudomonas (log LDA 4.81). Highly discriminant for soy products were some low abundant features. LEfSe calculated and log LDA score of 4.68 for Acetobacter in pre-cooked soy products and of 4.62 for *Flavobacteriaceae* in raw soy products.

4. Discussion

Highly processed food, like the plant-based meat alternatives we examined in this study, brings challenges to microbiologists. The broad range of ingredients (~120 in in the 32 examined products), different processing steps, and a variety of equipment lead to many potential sources for bacterial contamination. The product specific production processes were not available, but up to the present low (LMEC) and high moisture extrusion cooking (HMEC) are the most common commercially used technologies to produce meat textured plant proteins (Dekkers et al., 2018). Both methods are based on an interaction of heat, shear force and pressure, but the conditions for extrusion process depends on the original protein source and the desired final protein structure (Lin et al., 2002; Beniwal et al., 2021; Ferawati et al., 2021) . From the perspective of microbial survival during this process temperature, pressure and time are the most relevant variables. These parameters depend strongly on the process settings and can therefore only serve as a guide. The extruder barrels show temperature gradients with temperatures at 150-170°C in the high-temperature melting zone for HMEC (Schmid et al., 2022), but the extruder die is approximately 15-25°C cooler than that (Lin et al., 2000). The barrel temperature for LMEC is general lower with 120-150°C (Guyony et al., 2022). The

pressures in this barrels is between 1-4 MPa in HMEC and up to 13 MPa in LMEC (Kristiawan et al., 2018; Pietsch et al., 2019; Guyony et al., 2022). Yu et al. showed in different LMEC settings fastest particle residence times (first signs of the tracer) from 10-40 s and extrudate collection times (complete tracer passed) from 60 to 120 s, resulting in mean residence times of 35-87 seconds, depending on feed moisture, screw speed and die diameter (Yu et al., 2014). So far it is described, that vegetative forms of bacteria get inactivated effectively by this process and only spores of bacteria like *Bacilli* and *Clostridia* would survive the extrusion process (Mwangi, 2008; Leutgeb, 2017). However, reactivation of spores during further processing is possible (Filho et al., 2005). This could be one explanation why *Bacillus* was isolated from most of the samples (19/32), while the relative abundance of 16S rRNA gene DNA is relatively low, compared to other genera. We assume, due to this initial extrusion procedure, the main protein is not the source for most of the living bacterial cells we have isolated from the products. Highly probable contamination sources are the addition of ingredients, especially spices and herbs (Sagoo et al., 2009), and production environment.

Based on the microbial distribution patterns from the MiSeq data, we roughly describe three different community profiles. Lactic acid bacteria dominated two of them (14/28 *Leuconostocaceae* dominated samples, 4/28 *Latilactobacillus* dominated samples). The majority of these samples, was dominated by one ASV, which are mostly assignable to an isolate from the same sample, which suggests that these species are actually active in the final product. This is underlined by the high plate counts of these samples. Based on whole genome sequencing of representative isolates the ASVs and isolates could be classified as *Leuconostoc mesenteroides* and *Latilactobacillus sakei*.

!!!nochmal checken, bzw. sequenzieren!!! L. mesenteroides vs. L. citreum UND checken auf Gene zur Bacteriocin produktion!!!

Leuconostoc mesenteroides subsp. an important fermenter for products like kimchi and sauerkraut (Chun et al., 2017), or soybean paste (Zhang et al., 2019). Other positive features of *L. mesenteroides* are potential probiotic properties of some strains and antimicrobial activities against pathogenic bacteria, like *Salmonella typhymurium* and *Listeria monocytogenes* (de Paula et al., 2015; Thangavel and Thiruvengadam, 2019). Nevertheless, *L. mesenteroides* is usually described as common spoiler for meat, meat products and other food, asssociated with off-odors, off-flavours, slime and gas production (Björkroth and Holzapfel, 2006, Casaburi et al. (2015); Lianou et al., 2016). *Leuconstoc* as spoiler is often described in combination modified atmosphere packed products and cold stored products (Hamasaki et al., 2003; Hultman et al., 2015; Pothakos et al., 2014).*Leuconstoc mesenteroides* and the also isolated *Leuconostoc citreum* belong among others to the most isolated lactic acid bacteria from plants and plant-based food (Yu et al., 2020). *Latilactobacillus*

sakei subsp. are similarly versatile. They were described as fermenter particularly for sausages (Vinderola et al., 2019), and vegetable products (Jung et al., 2014), as producer of bacteriocines (Castellano et al., 2017), and as probiotics (Park et al., 2008).

Geeraerts et al., 2020 examined ready to vegetarian, vegan and insect based meat alternative, including products with fermented ingredients, like fermented sour dough. Based on that, they assumed sour dough as main source for *Latilactobacillus sakei*, which they isolated most (Geeraerts et al., 2020). Different to that study, we excluded products with fermented ingredients, but vinegar. Still we found large amounts of lactic acid bacteria in the examined products. Unlike Geeraerts et al., the majority of the samples in ours study were dominated by *Leuconostoc*, but we could not determine this by product characteristics. For kimchi and other plant products is described, that *Leuconostoc*, *Entercoccus* and *Lactococcus*, as generalists act as initial colonizers, followed later by *Lactobacillus*, *Pediococcus* and *Weissella* (Yu et al., 2020). This can be explained by a better adaptability to environmental conditions (e.g. through a large number of accessory genes), but also by the already high population density on the ingredients, while in later stages decreasing pH values inhibits *Leuconostoc* species sensitive to acids (Yu et al., 2020). However, the examined products were very diverse in their shelf life, so it was impossible to conclude, whether *Latilactobacillus* dominated samples, were in a later stage of shelf life than the *Leuconostoc* dominated samples. In vegan meat alternatives, the drop in pH should not be comparable to kimchi (pH 4.0) (You et al., 2017). It ranks in the range of 5.4-6.6 (Geeraerts et al., 2020; Tóth et al., 2021), and thus should not favour *Latilactobacillus sakei*. In general, in both species there are strains that are able to grew at 4°C (Hamasaki et al., 2003; Jung et al., 2014; Zagorec and Champomier-Vergès, 2017), although *Leuconstoc mesenteroides* strains, examined by Comi and Iacumin grew faster at 4°C than *Latilactobacillus sakei* (Comi and Iacumin, 2012). *Leuconostoc* spp., among these *Leuconstoc mesenteroides* and *Leuconstoc citreum* are described to inhibit the growth of *Latilactobacillus sakei* strains (Kim, 2013; Lee et al., 2015), but it was also demonstrated, that *Latilactobacillus sakei* is able to inhibit the growth of *Leuconstoc mesenteroides*, when the are inoculated equally to cooked bacon (Comi et al., 2016).

Overall, we examined nine samples with high relative abundances of *Proteobacteria*, mainly *Pseudomonas*, *Psychrobacter* and *Shewanella*. These three genera are common spoilers in meat and fish products, especially in products with slightly higher pH values, like fish, sea food and poultry (Borch et al., 1996, Odeyemi et al. (2018)). Plant-based meat products are usually also slightly higher in pH (Geeraerts et al., 2020, Tóth et al. (2021)). Although the number of ASVs within a these genera is much higher, compared e.g. with *Leuconostoc* from other samples, what we associate with different sources of contamination, there are still

single ASVs with relatively high abundances, which indicates either a large entry from one source or an active growth on the product at some time point during the production. The most obvious sources would be the main protein, the used water (via biofilms in water reservoirs or water hoses) or biofilms during the process. From the found genera in the MiSeq data, at least 13 are described as biofilm builders in food processing environments (Wagner et al., 2021), among these *Pseudomonas*, *Psychrobacter* and *Shewanella*. The possibility of biofilm formation on the processing equipment is high, since many of the used machines are hard to clean. However, this is contradicted by the fact that among the nine *Proteobacteria*-dominated samples seven had a very similar pattern, but were from six different producers. There are no available studies on the microbial communities of raw soybean or pea proteins, but 16S rRNA patterns of peas or the phyllosphere of soy do not support the thesis, that this kind of contamination is associated with the main protein source. Only sample B4, which is the only sample, that is dominated by *Alphaproteobacteria*, had high similarities to the microbial community of the soy phyllosphere (Vorholt, 2012). However, some of the producer just have a few products in this food segment, so we would not exclude, that they buy already extruded proteins from a large distributor and use it for their products. Although we assume that most *Proteobacteria* detected here are dead in the final product, the collected microbial communities of these products are not irrelevant. On the one hand, it is possible that bacteria, which are metabolically active at some point during the process, contributes to the development of (off-)odors and (off-)tastes, and on the other hand, it can provide information on whether there are contamination inputs in the ingredients or process areas that could be considered in the HACCP concept in the longer term (e.g. preventing biofilm formations).

!!! check Beneficial Microorganisms in food and nutraceuticals chapter *Leuconostoc* spp. as Starters and Their Beneficial Roles in Fermented Foods!!! ...

!!!HERE BACILLUS!!!

Staph aureus

Beside the dominating, spoilage associated genera, we also isolated some *Enterobactericeae*, of which selected isolates where whole genome sequenced and taxonomically assigned to *Leclercia*, *Atlantibacter*, *Citrobacter*, *Escherichia* and *Klebsiella*. This confirms the findings of Luchansky et al., who inoculated beef and vegan burgers with Shiga toxin-producing human pathogens (i.e. *Escherichia coli* (STEC), *Salmonella* and *Listeria monocytogenes*) and showed that they could survive and even grow in vegan burgers (Luchansky et al., 2020). Apart from containing pathogenic species, the *Enterobacteriaceae* family serves as a hygiene indicator. In our sample, all isolated *Enterobacteriaceae* came from products of the same manufacturer.

However, in MiSeq the relative abundances of *Enterobacteriaceae* were very low in all examined samples. Heating kills effectively *Eterobacteriaceae* in these products (Luchansky et al., 2020). All products were labelled with cooking instructions, most of them specific with cooking time in minutes, some only with "heat through before consumption". This final heating step by the consumer is considered the HACCP concept of the manufacturer. While cooking time in minutes is a good guidance for the consumers, "heat through" is in our opinion too imprecise. First, there is a lack of experience with these kind of products, second in contrast to animal meat (products) there is no indicator like color change to determine a sufficiently cooked state. However, this lack of experience with these products is not limited to preparation, but also to spoilage detection, handling of the raw product and shelf life or storage before and after preparation. In total, nine out 32 samples had an expiry date, the others a best before date, leaving the final spoilage detection to the consumer. The odor of the products, we tested, were in general not comparable to the corresponding meat product. Although the best-before date on these products is to be welcomed for reasons of sustainability, it is understandable that consumers are more inclined to discard the products once this date has passed, as they do not trust themselves to make an assessment. With regard to the preparation before heating, the products are to be differentiated. Most of them are "ready-to-heat", so there is no need for handling before the heating step. Additional preparation steps are most likely for vegan mince (mixing and forming steps).In this case, the same kind of kitchen hygiene is appropriate as is recommended for raw meat. Toth et al. concluded in their study that dishes with vegan meat substitutes spoiled faster than their meat counterparts when stored after preparation (Tóth et al., 2021). These findings, together with the generally higher refrigerator temperatures than recommended (James et al., 2017), once again shows the importance of increasing consumer awareness of food handling and storage.

As already mentioned, the microbiological assessment of highly processed foods is not easy. A solely culture-based focus is not ideal, as only divisible, culturable cells are detected. In this case, for example, non-culturable species or viable but nonculturable cells completely elude our observation. The latter are particularly relevant for processed foods, as their emergence is often induced by environmental changes (Zhao et al., 2017). This cell state is described for a long list of human pathogens and other bacteria (Li et al., 2014; Dong et al., 2020). A culture-independent approach alone is also inappropriate, as there is also a large amount of dead cells in these products due to the many process steps, which also leads to a misrepresentation of the microbial ecology of these products. A combined approach, as we have used in this study, complicates the interpretation of the data but provides much information on how to improve future analyses. Overall, it is hard to generalize the results of this study, due to the highly diverse attributes of

this product category. Th sampling design was based on main protein source and texture, since we thought that the main ingredient, and its processing contributes substantially to the product's microbial community. Furthermore, they were the only objectivisable product characteristics, with enough products represented per group for a balanced study design. Although main protein source and texture were significant variables in the PERMANOVA, they only explained about 18% of the model's variance. Adding the manufacturer as additional variable to the PERMANOVA increased the explained variance to 53%. However,the two most frequently represented manufacturers contributed 18 (11+7) out of 32 products in this study, while the other seven manufacturers contributed with a maximum of two samples. For this reason, we consider the sample selection to be too unbalanced to be able to make valid statements under consideration of the manufacturer. Nevertheless, we assume that the production plant has a non-negligible effect on the product's microbial community.

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5. Conclusion

6. Conflict of Interest

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

7. Acknowledgements

We would like to thank Birgit Bromberger and Christoph Eisenreich for their active support in conducting the experiments.

8. Contributions of Authors

FF.R. and E.S. conceived and planned the experiments. FF.R. and M.D. carried out the experiments. FF.R., NM.Q. and E.S. performed the computations. FF.R. wrote the manuscript with input from all authors. All authors provided critical feedback to the research, analysis and manuscript.

9. Tables

Table 1: Showing the labelled attributes of the examined products

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ID	protein source[1]	texture[2]	shelf life[3]	cooking time[4]	no. of ingredients	additional labelling
Manufacturer 01						
A1	pea	minced	9 d to ed (0 d)	7-8 min	17	consume only thoroughly heated; MAP
A4	pea	minced	2 d to ed (0 d)	5-8 min	18	consume only thoroughly heated; MAP
Manufacturer 02						
A2	pea	minced	6 d to bbd (1 d)	3-5 min	14	raw; consume only thoroughly heated; MAP
A3	pea	minced	3 d to bbd (0 d)	4-6 min	13	MAP
A5	pea	minced	14 d to bbd (1 d)	-	13	MAP
A7	pea	minced	13 d to bbd (1 d)	2 min	14	MAP
A8	pea	minced	6 d to bbd (1 d)	3-5 min	15	consume only thoroughly heated; MAP
D2	pea	fibrous	7 d to bbd (1 d)	-	12	MAP
D4	pea	fibrous	28 d to bbd (1 d)	3-5 min	11	MAP
D5	pea	minced	12 d to bbd (1 d)	-	14	pre-heated; MAP
D6	pea	fibrous	14 d to bbd (1 d)	3-5 min	13	MAP
D7	pea	fibrous	12 d to bbd (1 d)	3-5 min	13	MAP
D8	pea	fibrous	28 d to bbd (1 d)	3-5 min	10	MAP
Manufacturer 03						
A6	pea	minced	29 d to bbd (2 d)	3-5 min	6	
B8	soybean	minced	22 d to bbd (2 d)	3-5 min	8	
Manufacturer 04						
B1	soybean	minced	52 d to bbd (3 d)	4-6 min	10	MAP
B3	soybean	minced	10 d to bbd (3 d)	4-6 min	20	MAP
Manufacturer 05						
B2	soybean	minced	5 d to ed (-)	7 min	15	raw; consume only thoroughly heated; frozen once; MAP
B6	soybean	minced	3 d to ed (-)	8-10 min	12	raw; consume only thoroughly heated; frozen once; MAP
B7	soybean	minced	3 d to ed (-)	12 min	21	raw; consume only thoroughly heated; frozen once; MAP
C1	soybean	fibrous	13 d to ed (-)	5 min	6	pre-heated; frozen once; MAP
C2	soybean	minced	18 d to ed (-)	5-7 min	22	pre-heated; frozen once; MAP
C3	soybean	fibrous	10 d to ed (-)	4-7 min	16	pre-heated; frozen once; MAP
C4	soybean	fibrous	24 d to ed (-)	4-7 min	18	pre-heated; frozen once; MAP
Manufacturer 06						

Table 1: Showing the labelled attributes of the examined products *(continued)*

ID	protein source[1]	texture[2]	shelf life[3]	cooking time[4]	no. of ingredients	additional labelling
B4[5]	soybean	fibrous	174 d to bbd (2 d)	6-8 min	4	consume only thoroughly heated; frozen once;
B5[5]	soybean	fibrous	119 d to bbd (2 d)	6-8 min	27	consume only thoroughly heated; frozen once;
Manufacturer 07						
C5	soybean	fibrous	0 d to bbd (-)	4-5 min	14	pre-heated; consume only thoroughly heated; MAP
C6	soybean	fibrous	0 d to bbd (-)	4-5 min	19	pre-heated; consume only thoroughly heated; MAP
C7	soybean	fibrous	0 d to bbd (-)	4-5 min	21	pre-heated; consume only thoroughly heated; MAP
Manufacturer 08						
C8	soybean	fibrous	8 d to bbd (1 d)	5 min	23	consume only thoroughly heated; MAP
Manufacturer 09						
D1	pea	minced	10 d to bbd (1 d)	-	16	MAP
D3	pea	fibrous	7 d to bbd (1 d)	-	16	MAP

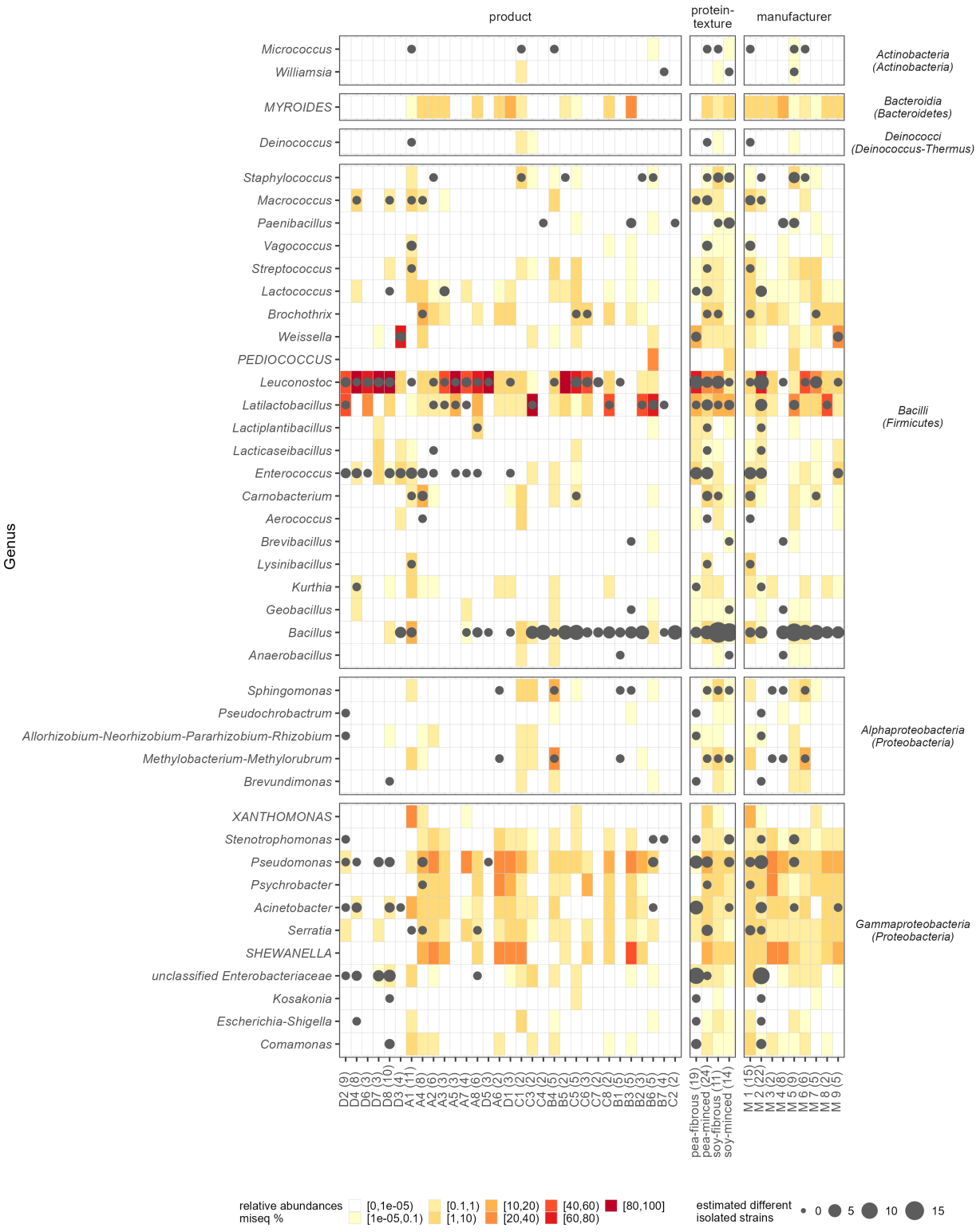
[1] Protein basis of the examined product. Only pea or soybean protein products were selected for the study.
[2] product designation. Products with a minced 'meat' basis (i.e. minced meat, burger, cevapcici, sausages) were additionally classified as 'minced', products immitating pieces of meat or a meat structure (i.e. fillets, steaks, chunks, kebab) were classified as 'fibrous'.
[3] days to expiration date (ed) or best before date (bbd) at sampling. In brackets: consume within x days after opening.
[4] Recommended cooking time. If label said (e.g.) 2 minutes per side, the recommended cooking time were doubled to 4 minutes for this table.

10. Figures

Figure 1: Present isolates per sample, group or manufacturer are represented by dots. Isolates with a genus were clustered based on their 16S rRNA gene sequences. Different clusters represents different strains or species. The higher the number of clusters within a genus, the larger the plotted dot in the figure. The surrounding area is shaded according to the relative abundances in the amplicon sequencing (for the group and manufacturer summary, the mean relative abundance of each included sample is used).

Figure 2: Taxonomy plot based on amplicon sequencing, showing the relative abundances on genus level. The underlying relative frequencies of the ASVs are recognizable as pale yellow lines within a genus. Genera with a maximum value of 3% across all samples were subsumed by color in the next higher taxonomic level. The samples are ordered by main protein source, texture and manufacturer. ASVs with matching isolates (>99% identity), were outlined in red.

Figure 3: **A:** tSNE plot clustered samples to different profiles based on Bray-Curtis dissimilarity (Similar clusters were also found with other distance matrices - see Supplements S4). **B:** Hill-Shannon diversity and **C:** Hill-Simpson diversity comparing these profiles.



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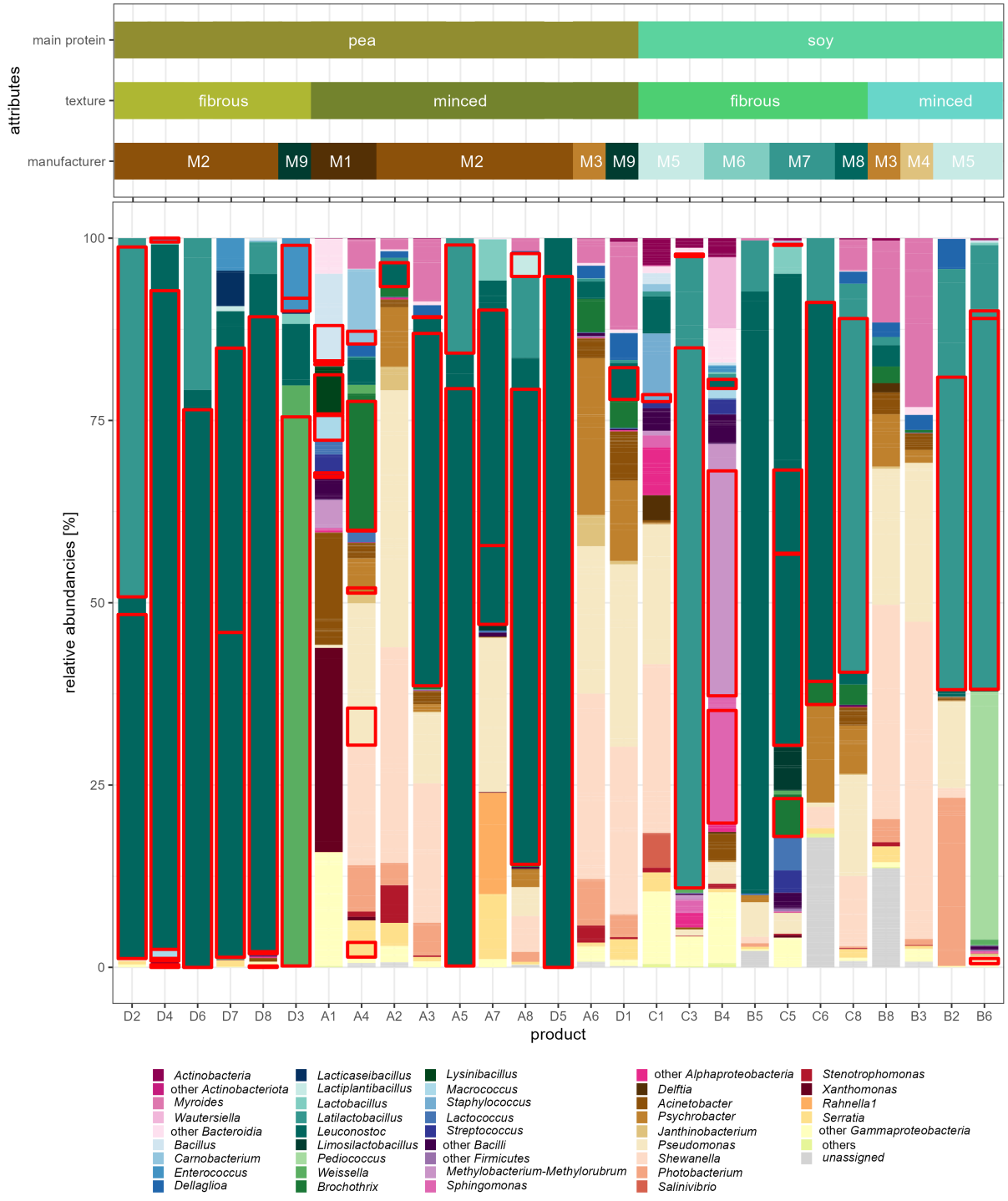

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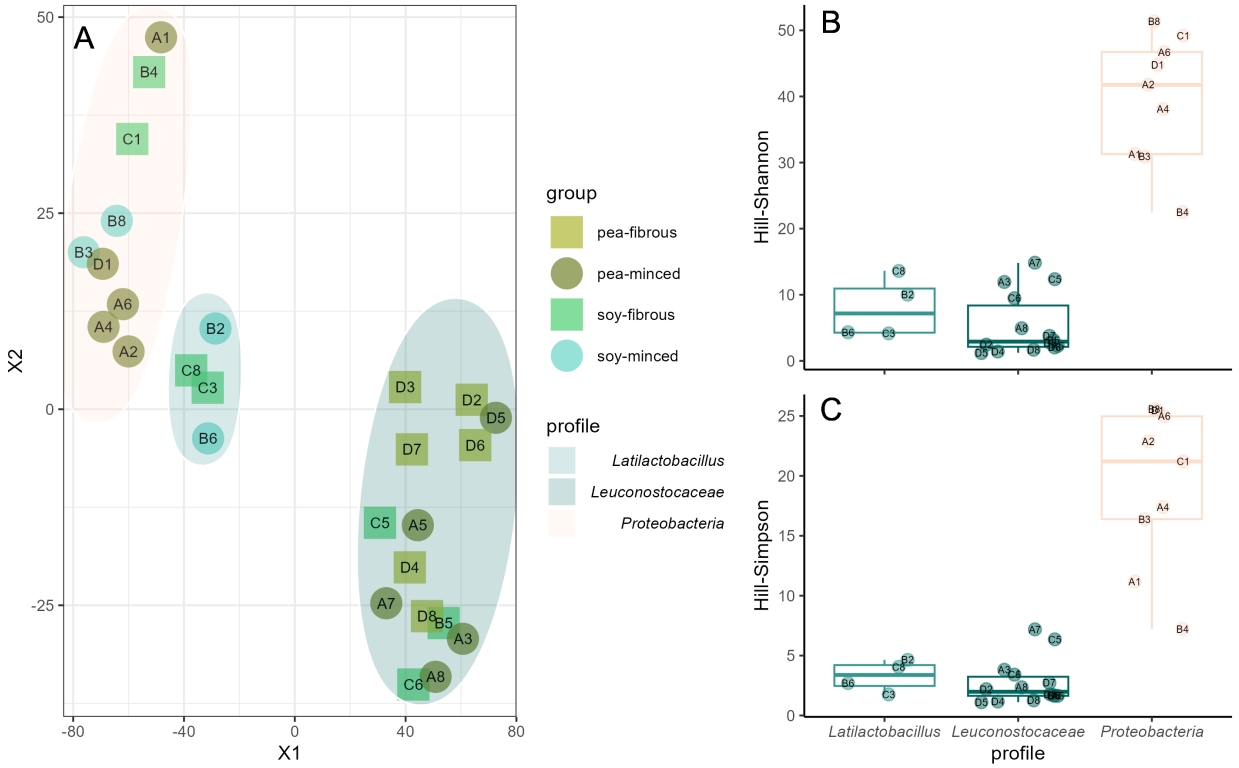

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Supplementary material

11. Supplements

Supplement Table S1:

Supplement S1: Groupwise comparison of the alpha-diversity using Hill-Shannon and Hill-Simpson indices. Hill-Shannon index differed significantly between fibrous and minced pea products (p-val=0.016).

Supplement S2: NMDS plots using different distance methods.

Supplement S3: tSNE plots based on Bray-Curtis dissimilarity, Jaccard distance and Jensen-Shannon divergence. In all three methods, the same clusters form.

Supplement S4: LEfSe per group

Supplement S5: LEfSe per profile.

Table S1: PERMANOVA results based on different distance matrices.

	Df	Sum of Sqs	R2	F	Pr. F
Bray-Curtis					
Permutation test for adonis under reduced model					
Terms added sequentially (first to last)					
Permutation: free					
Number of permutations: 999					
*adonis2(formula = dmlistshort[[i]] ~ protein.source * texture,*					
data = data.frame(sample_data(relab_po)), permutations = 999)					
protein source	1	0.7258	0.0743	2.090	0.030
texture	1	0.8391	0.0859	2.416	0.017
protein source:texture	1	0.2185	0.0224	0.629	0.825
residual	23	7.9874	0.8175		
total	26	9.7708	1.0000		
Permutation test for adonis under reduced model					
Terms added sequentially (first to last)					
Permutation: free					
Number of permutations: 999					
*adonis2(formula = dmlistshort[[i]] ~ texture * protein.source,*					
data = data.frame(sample_data(relab_po)), permutations = 999)					
protein source	1	0.6784	0.0694	1.953	0.041
texture	1	0.8864	0.0907	2.553	0.017
protein source:texture	1	0.2185	0.0224	0.629	0.834
residual	23	7.9874	0.8175		
total	26	9.7708	1.0000		
Jaccard					

Table S1: PERMANOVA results based on different distance matrices. *(continued)*

	Df	Sum of Sqs	R2	F	Pr. F
Permutation test for adonis under reduced model					
Terms added sequentially (first to last)					
Permutation: free					
Number of permutations: 999					
*adonis2(formula = dmlistshort[[i]] ~ protein.source * texture,*					
data = data.frame(sample_data(relab_po)), permutations = 999)					
protein source	1	0.6774	0.0626	1.712	0.041
texture	1	0.7484	0.0691	1.891	0.022
protein source:texture	1	0.3001	0.0277	0.758	0.789
residual	23	9.1035	0.8406		
total	26	10.8295	1.0000		
Permutation test for adonis under reduced model					
Terms added sequentially (first to last)					
Permutation: free					
Number of permutations: 999					
*adonis2(formula = dmlistshort[[i]] ~ texture * protein.source,*					
data = data.frame(sample_data(relab_po)), permutations = 999)					
protein source	1	0.6367	0.0588	1.609	0.065
texture	1	0.7891	0.0729	1.994	0.014
protein source:texture	1	0.3001	0.0277	0.758	0.770
residual	23	9.1035	0.8406		
total	26	10.8295	1.0000		
Jensen-Shannon					
Permutation test for adonis under reduced model					
Terms added sequentially (first to last)					
Permutation: free					
Number of permutations: 999					
*adonis2(formula = dmlistshort[[i]] ~ protein.source * texture,*					
data = data.frame(sample_data(relab_po)), permutations = 999)					
protein source	1	0.3254	0.0874	2.626	0.034
texture	1	0.4556	0.1224	3.676	0.008
protein source:texture	1	0.0918	0.0246	0.740	0.607
residual	23	2.8504	0.7656		
total	26	3.7231	1.0000		
Permutation test for adonis under reduced model					
Terms added sequentially (first to last)					
Permutation: free					
Number of permutations: 999					
*adonis2(formula = dmlistshort[[i]] ~ texture * protein.source,*					
data = data.frame(sample_data(relab_po)), permutations = 999)					
protein source	1	0.3698	0.0993	2.984	0.019
texture	1	0.4111	0.1104	3.318	0.007
protein source:texture	1	0.0918	0.0246	0.740	0.609
residual	23	2.8504	0.7656		
total	26	3.7231	1.0000		

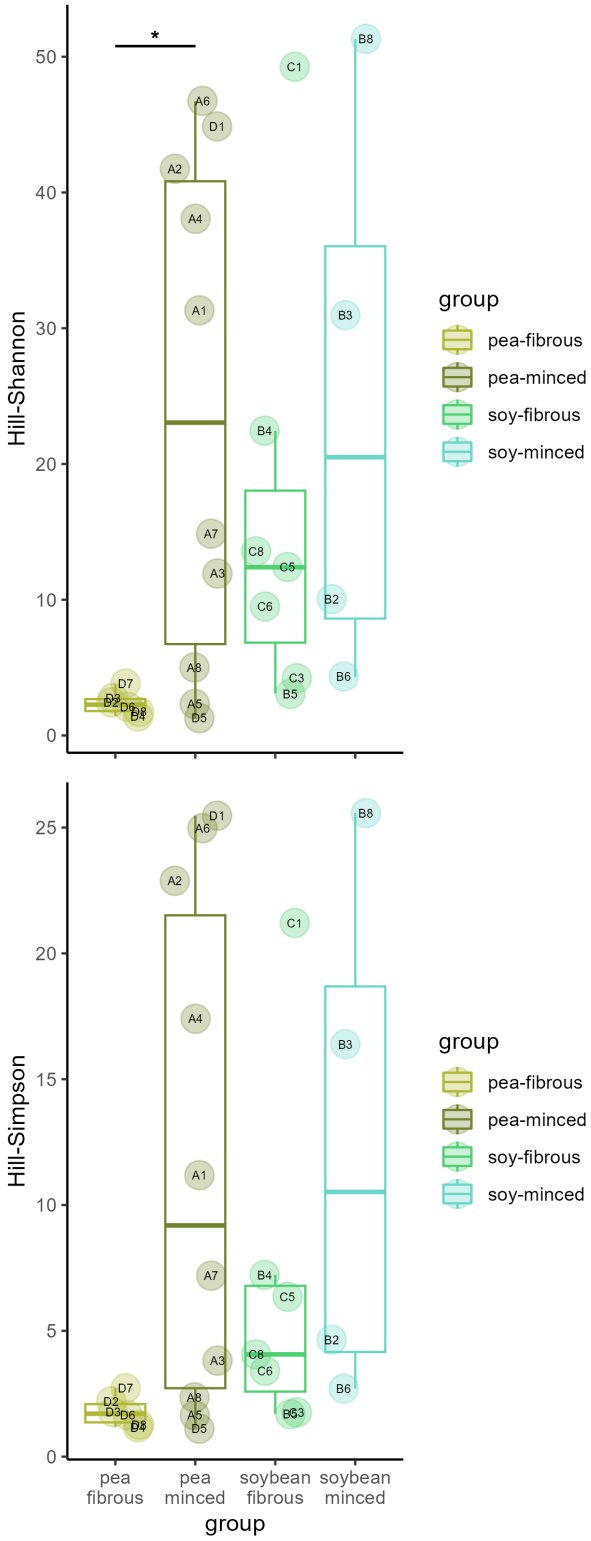

Figure S1: Groupwise comparison of the alpha-diversity using Hill-Shannon and Hill-Simpson indices. Hill-Shannon index differed significantly between fibrous and minced pea products (p-val=0.016).

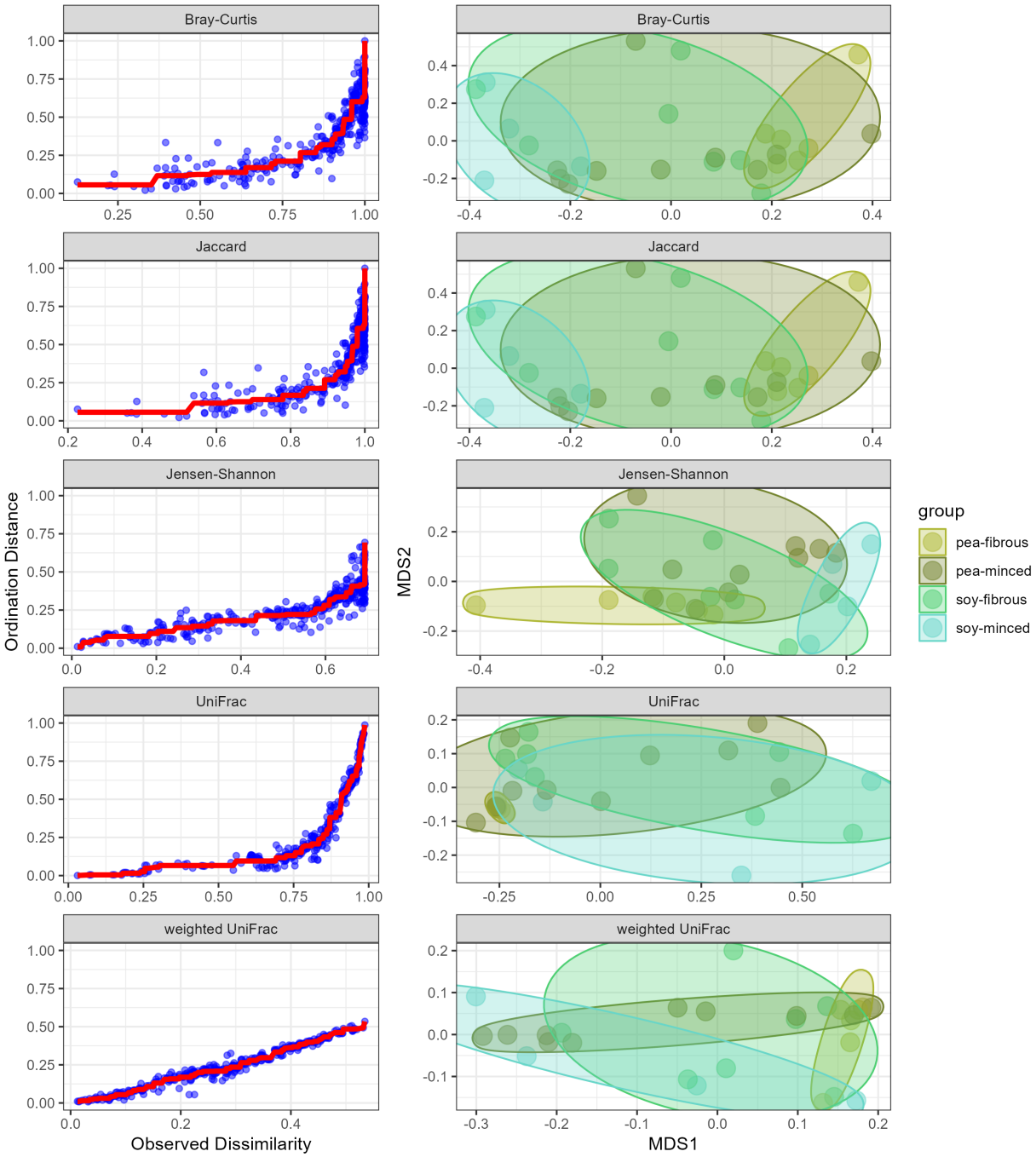

Figure S2: NMDS plots using different distance methods.

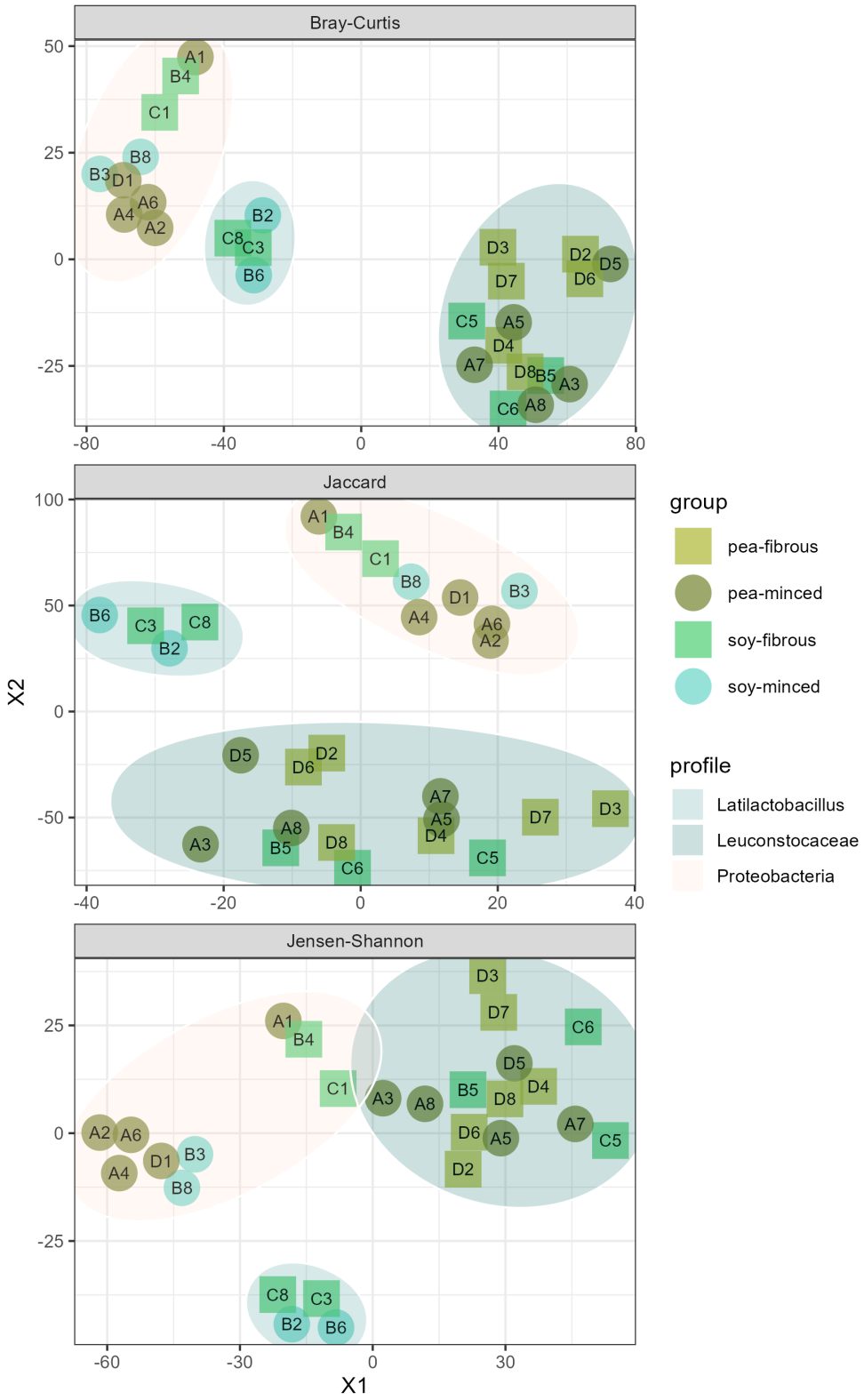

Figure S3: tSNE plots based on Bray-Curtis dissimilarity, Jaccard distance and Jensen-Shannon divergence. In all three methods, the same clusters form.

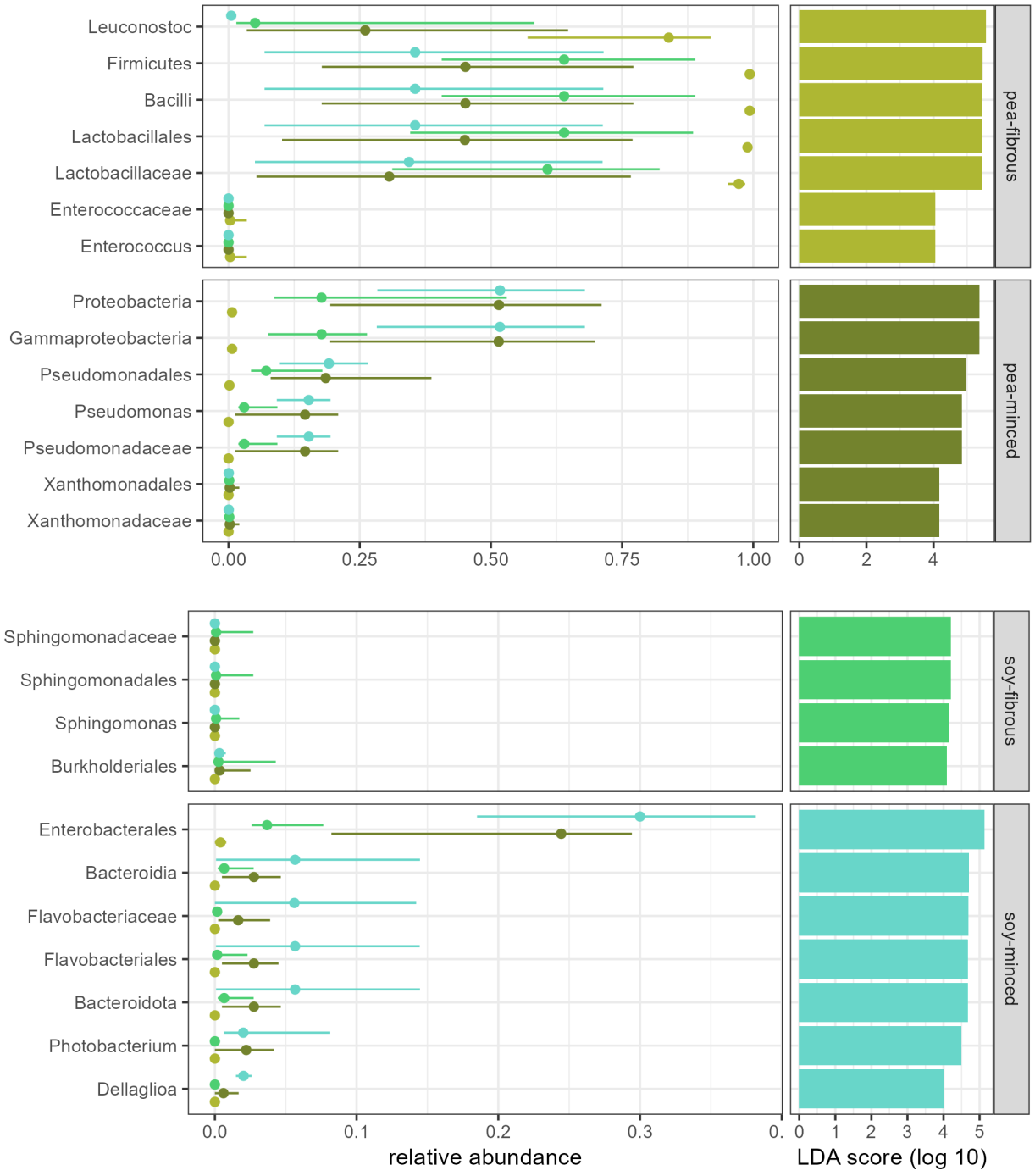

Figure S4: LEfSe per group.

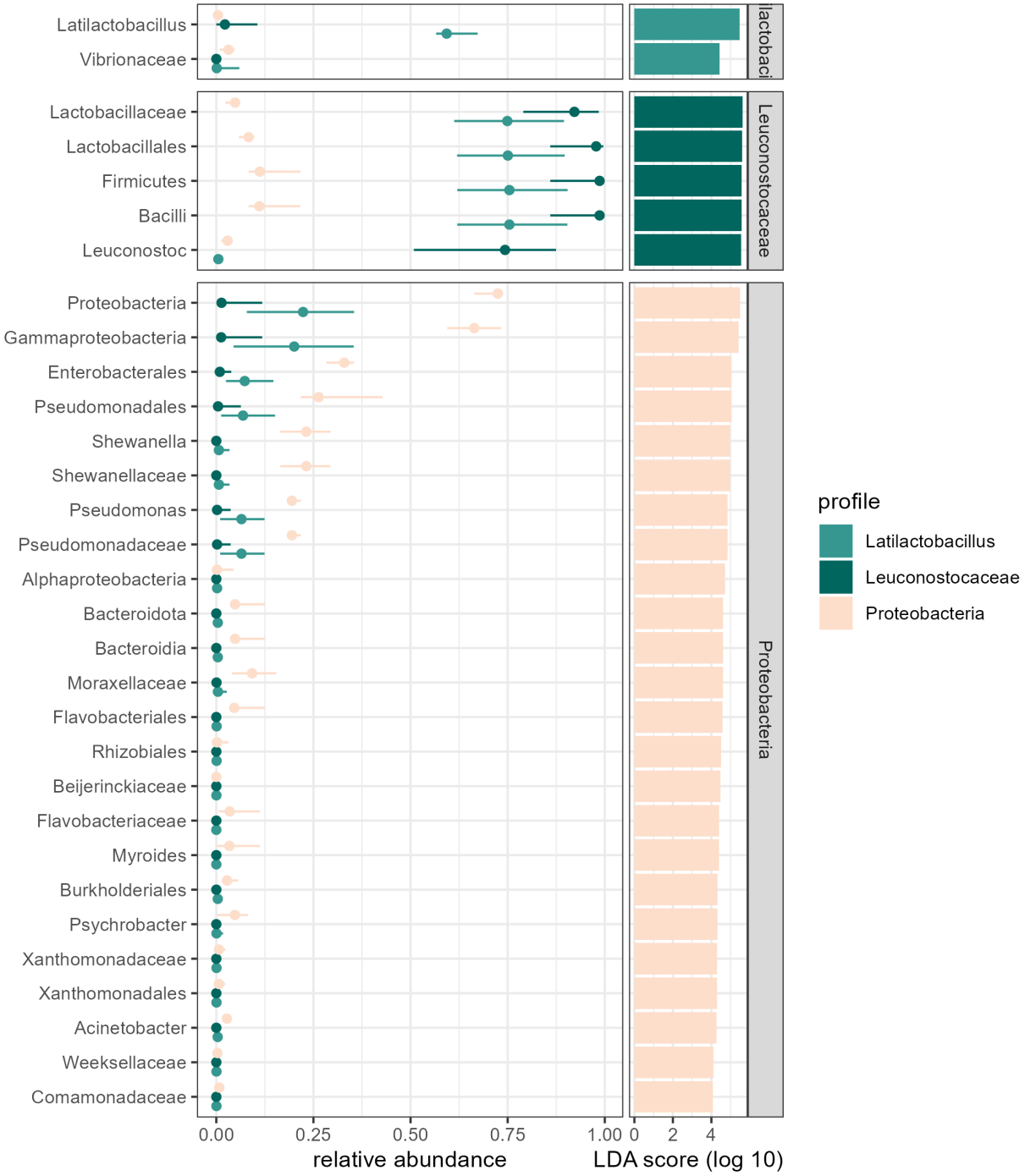

Figure S5: LEfSe per profile.

s 1 - alpha div group s 2 - NMDS s 3 - PERMANOVA res s 4 - LEfSE group s 5 - LEfSe profile

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