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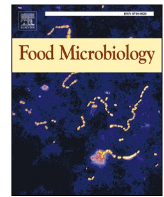

Modulation of the food microbiome by apple fruit processing

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A B S T R A C T

During the early life, introduction to external exposures such as consumption of solid foods contribute to the development of the gut microbiota. Among solid foods, fruit and vegetables are normally consumed during early childhood making them key components of a healthy human diet. The role of the indigenous microbiota of fruits as a source for beneficial gut microbes, especially during food processing, is largely unknown. Therefore, we investigated the apple fruit microbiota before and after processing using functional assays, advanced microscopic as well as sequencing technologies. Apple fruits carried a high absolute bacterial abundance (1.8×10^5 16S rRNA copies per g of apple pulp) and diversity of bacteria (Shannon diversity index = 2.5). We found that heat and mechanical treatment substantially affected the fruit's microbiota following a declining gradient of absolute bacterial abundance and bacterial diversity from shredded > boiled > pureed > preserved > dried apples. *Betaproteobacteriales* and *Enterobacteriales* were the two dominant bacterial orders (51.3%, 20.4% of the total 16S rRNA sequence reads) in the unprocessed apple. Boiling and air drying reduced the microbial load, but an unexpected, substantial fraction of 1/3 of the microbiota survived. Boiling and air drying shifted the microbiota leading to a relative increase in low abundant taxa such as *Pseudomonas* and *Ralstonia* (>2 $\log_2$ fold change), while others such as *Bacillus* decreased. *Bacillus* spp., frequently found in raw fruits, were shown to have specific traits, i.e. antagonist activity against opportunistic pathogens, biosurfactant production, and bile salt resistance indicating a probiotic potential. Our findings provide novel insights into food microbial changes during processing and demonstrate that food microbiome studies need a combined methodological approach. Food inhabiting microbes, currently considered being a risk factor for food safety, are a potential resource for the infant gut microbiome.

1. Introduction

Recently, it was recognized that early life is the most crucial phase for the assembly of the human gut microbiome, which is primarily linked to immune system development and eventually long-term health (Depner et al., 2020; Stewart et al., 2018; Stokholm et al., 2018; Zheng et al., 2020). The development and establishment of the gut microbiome start during pregnancy (Chu et al., 2019). Hereinafter, babies receive their first inoculum of microbiota through the mother via vaginal delivery, and further obtain microbes through the environment and diet (Rodríguez et al., 2015; Tanaka and Nakayama, 2017). During the development of the gut microbiota during early life, introduction to external exposures, i.e. bacterial infections, antibiotic treatment, and food consumption are frequently occurring, which all contribute to microbial changes to take place (Rodríguez et al., 2015). One major factor shaping the gut microbiota during early childhood is the

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composition of the diet. Diverse health benefits were associated with breastfeeding (Ho et al., 2018; Stewart et al., 2018; van den Elsen et al., 2019), however, the exposure and role of the first solid diet is not yet fully understood.

Fruit is known to be an important component of a healthy human diet and, in raw form or processed into purees, is one of the first foods for babies worldwide. Many studies have investigated the role of food intake and diet on the gut microbiome in adults including consumption of a plant based diet (David et al., 2014; Johnson et al., 2019; Meslier et al., 2020; Stewart et al., 2018). Previous studies demonstrated that nutrient and fibre content in the fruit directly influenced the gut microbiome in infants and adults (Leong et al., 2018; Stewart et al., 2018). Fruits are also known to harbor hundreds of thousands to millions of microorganisms (Badosa et al., 2008; Wassermann et al., 2019; Zhang et al., 2020). Moreover, the so-called "edible" microbiome is also important as (i) an additional contributor to the microbial diversity of our gut microbiome, and (ii) as a stimulus for the human immune system (Berg et al., 2015). Some fruit associated bacteria can be resistant to bile salt which are associated with the ability to survive in the gastrointestinal tract, and they can also show antimicrobial activity against human pathogens (Maheshwari et al., 2019). These features are important characteristics for probiotic candidates (Dunne et al., 2001; Lillo-Pérez et al., 2021). Hence, *in vitro* screening of these characteristic is the first step to identify potential properties of probiotics according to FAO/WHO (FAO/WHO, 2002).

Processed fruits are one of the most common first solid foods given to infants. Prior to consumption, fruits are likely processed and exposed to high levels of heat i.e. by boiling or drying, mechanical force or chemical food preservatives. Different ways of processing can affect the nutritional content of fruits that we consume and consequently impact the gut microbiome. However, we lack knowledge about the impacts of food processing on the microbial abundance and diversity of fruits that infants consume during early childhood. What also remains unclear is the extent to which microbial taxa that are affected by food processing as well as the importance of these taxa related to human health.

Therefore, the objectives of this study were to 1) evaluate the impact of apple processing on the apple bacterial community structure, and 2) identify bacterial taxa that are affected by different apple processing methods and their potential functions. We choose apples as our model for this study because apples are one of the most widely consumed raw fruits in the world, and they are especially popular for babies and children (Herrick et al., 2015; Whiteside-Mansell and Swindle, 2019). We subjected apples to different processing methods and analyzed the bacterial community by implementing a polyphasic approach based on culture-dependent and independent experiments, which is generally suggested for holistic microbiome analyses (Berg et al., 2020). The present study provides a basic foundation for further intervention studies to investigate the impact of food processing on the developing infant gut microbiota.

2. Materials and methods

2.1. Experimental design and sample processing

In this study, we used the apple (*Malus domestica* Borkh.) variety 'Royal Gala', which is grown and consumed extensively around the world (Hampson and Kemp, 2003). We obtained the apple samples from one local supermarket in Graz, Austria to limit divergence of other parameters, i.e. different handling or origins, and visually selected those that have similar characteristics i.e. round, red, and similar size. Before processing, apples were washed using sterile water, peeled, and dice cut in a similar size (approx. 2–3 cm each side). Apples were processed differently; namely 1) unprocessed – "raw", 2) shredded using glass grater 3) boiled (100 °C) for 15 min in water, 4) boiled for 15 min and then mashed using sterilized potato masher therein defined as "puree", 5) preserved by aseptically filling the apple puree into a glass jar that had been heated at 85 °C, pasteurized for 10 min, and stored for 7 days at 4 °C and 6) dried – apple samples were cut into semi-circular slices and then air-dried using a food dehydrator at 60 °C for 6 h. The chosen processes are the most common ones to prepare apples for infants (Štěpán et al., 2005). Each process consisted of eight apple replicates. Apples that were exposed to heat treatments, except the preservation treatment, were processed after cooling down at room temperature. Before DNA extraction, apple samples (approx. 10 g) were homogenized in a Stomacher laboratory blender (BagMixer, Interscience, Saint--Nom-la-Bretèche, France) with 10 mL sterile NaCl (0.85%) solution for 3 min. A total of 2 mL of homogenized and processed apple suspensions were centrifuged for 20 min at 16,000 *g* and pellets were used for DNA extraction. Before DNA extraction, the pellets were treated with propidium monoazide (PMA) as described in Wicaksono et al. (2016) to exclude amplification of the dead bacterial fraction during PCR. Total DNA was extracted using the FastDNA SPIN Kit for Soil and the FastPrep Instrument (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's protocol and stored at − 20 °C until PCR reactions were carried out.

2.2. Investigation of the apple associated bacterial bioactivity using different assays

2.2.1. Isolation of bacteria from raw apples

Culturable bacteria were isolated from apple pulp prior the apple processing to further investigate potential beneficial functions of the apple microbiota. A total of 100 μL of the apple suspensions prepared as described above were serially diluted 10-fold and plated on both Reasoner's 2A (R2A) (Carl Roth GmbH + Co. KG; Karlsruhe, Germany) and Nutrient Broth II agar (NA) media (SIFIN, Berlin, Germany) in triplicates. The plates were incubated at 25 °C for 7 days. Single bacterial colonies were picked and subsequently sub-cultured on new NA plates to purify the bacterial isolates. We selected representative bacterial isolates based on differences in colony morphology (shape and color) from each dilution to increase the number of unique isolates. The bacterial isolates were then transferred to 96-well plates containing Nutrient Broth II medium and 30% glycerol for long-term storage and the plates were kept at −70 °C at the Institute of Environmental Biotechnology, Graz University of Technology, Graz, Austria. Prior to functionality assays, each isolate was sub-cultured in 200 μl NB II medium in 96-well plates and incubated at 25 °C for 2 days. We used these liquid cultures as bacterial suspensions for functionality assays that are described below.

2.2.2. Identification of bacterial isolates based on 16S rRNA gene sequencing

We identified all bacterial isolates based on the sequence of their 16S rRNA gene fragments using the primer set 27F and 1492R (Marchesi et al., 1998). Bacterial genomic DNA was extracted by using the Triton method as described in (Kolia-Diafouka et al., 2018). Polymerase chain reactions (PCRs) were carried out in the Whatman Biometra® Tpersonal thermocycler (Biometra 141 GmbH, Göttingen, Germany). The PCR products were then Sanger sequenced at the commercial sequencing provider LGC Genomics (Berlin, Germany). We performed manual quality filtering using BioEdit (Hall, 1999) to remove ambiguous sequences. Further, the quality-filtered sequences were compared against the Silva ribosomal RNA gene database v132 (Quast et al., 2012) using the Basic Local Alignment Search Tool (BLAST (Camacho et al., 2009),). To perform phylogenetic analysis, the sequences were aligned using MUSCLE (Edgar, 2004) and the distance matrices were calculated by maximum-likelihood algorithms in MEGA X (Molecular Evolutionary Genetic Analysis) (Kumar et al., 2018). The resulting phylogenetic tree was visualized using the interactive tree of life software (iTOL (Letunic and Bork, 2019),).

2.2.3. Assessment of antagonistic activity against opportunistic human pathogens

Approximately 5 μL of bacterial suspension in NB II medium were spotted on NA plates pre-inoculated with five human opportunistic pathogens, including *Acinetobacter baumanii* strain 6340276, *Enterococcus faecium* strain 6428631, *Escherichia coli* strain 6402087, *Pseudomonas aeruginosa* strain 6436029, and *Stenotrophomonas maltophilia* strain EA23. The first four bacterial pathogens were obtained from the culture collection of the Department of Internal Medicine, Medical University of Graz whereas *S. maltophilia* is a part of the microbial culture collection of the Institute of Environmental Biotechnology (Graz University of Technology). Inhibition zones on the agar surface were examined after 4 days of incubation at 25 °C. All isolates that produced visible inhibition zones were defined as antagonists of the model pathogens (Supplementary Figs. S1A and S1B).

2.2.4. Screening for biosurfactant-producing bacteria

We performed qualitative screening of biosurfactant-producing bacteria using the drop-collapsing assay as described in (Bodour and Miller-Maier, 1998). In brief, 2 μL mineral oil was placed on each well of the lid of a 96-well microtiter plate and incubated for 2 h to equilibrate. Subsequently, 5 μL of bacterial suspension were added to the mineral oil and visible changes were examined after 1 min. When the drops collapsed, the bacterial isolates were defined as biosurfactant-producing bacteria, while a drop that remained beaded the bacterial isolates were defined as non-biosurfactant-producing bacteria (Supplementary Fig. S1D).

2.2.5. Screening for protease-producing bacteria

We performed qualitative screening of protease-producing bacteria using a 10% skim milk agar (Heirler Bio Magermilchpulver, Heirler Cenovis GmbH, Germany) as described in (Pailin et al., 2001). In brief, 5 μL of bacterial suspension were spotted on the skim milk agar plates. The plates were examined after 4 days of incubation at 25 °C. Bacterial isolates with a translucent zone around the colony were defined as those that can produce proteases (Supplementary Fig. S1C).

2.2.6. Bile salt tolerance

A bile salt resistance assay was performed as described previously in (Prete et al., 2020), except we used NB II medium instead of de Man, Rogosa, and Sharp (MRS) broth medium. In brief, 5 μL of bacterial suspension were added to 200 μl NB II medium with increasing concentrations of bile salts (0%, 0.30%, 1.8% and 3.6% w/v; Thermo Fischer Scientific) in 96-well microtiter plates made of polystyrene. After a 24-h incubation at 25 °C, bacterial growth was assessed turbidimetrically by measuring optical density (OD_{600}) of each well using the Tecan microplate reader Infinite® 200 PRO (Tecan Austria GmbH, Grödig, Austria).

2.3. Microscopic in situ visualization of bacterial colonization in apple pulp

To visualize bacterial colonization in apple pulp prior to the apple processing, unprocessed apple pulp samples were fixed with 4% paraformaldehyde/phosphate-buffered saline at 4 °C overnight before Fluorescent *in situ* Hybridization (FISH) as described previously in (Cardinale et al., 2008). We used various probes, including Cy3-labeled EUB338MIX (Amann et al., 1990; Daims et al., 1999), ATTO488-labeled BET42a (Manz et al., 1992), and Cy5-labeled GAM42a (Manz et al., 1992) to visualize overall bacterial colonization and for specific detection of *Betaproteobacteria* (also recognized as the *Betaproteobacteriales* order in the SILVA v132 database (Quast et al., 2012)) and *Gammaproteobacteria*, respectively. We chose *Betaproteobacteria* and *Gammaproteobacteria* because these taxa were the two dominant bacterial classes in the apple samples according to 16S rRNA gene amplicon sequencing (see Results section). To stain host cell walls, all FISH samples were also treated with Calcofluor White. Furthermore, to visualize bacterial colonization and distinguish between viable and dead bacteria after the apple processing, apple pulps that had been boiled and dried as described above (see Section 2.1), were stained with the LIVE/DEAD™ BacLight™ Bacterial Viability Kit (Molecular Probes). The bacterial colonization in the apple pulp was visualized using a Leica TCS SPE confocal laser scanning microscope (Leica Microsystems, Mannheim, Germany). The resulting confocal stacks from each run were merged to obtain a maximum projection of all channels.

2.3.1. Bacterial quantification in apple pulp

We used quantitative real-time PCR (qPCR) based on SYBR Green fluorescence to assess absolute bacterial abundance after each treatment using the primer pair 515f–806r (Caporaso et al., 2011). The qPCR reaction as well as the required standards were prepared as described in (Köberl et al., 2011). The qPCR reaction mix contained 1 μL extracted DNA, 5 μL KAPA SYBR® FAST qPCR Master Mix (2X) (KAPA Biosystem, USA), 1 μL 10 μM of each primer, and 3 μL ultrapure water. The Unibac-II fragment (Köberl et al., 2011) was subjected to serial dilution (1:10) to obtain a qPCR standard series. Fluorescence quantification was carried out using the Rotor-Gene 6000 real-time rotary analyzer (Corbett Research, Sydney, Australia) with initial denaturing at 95 °C for 10 min, followed by 40 cycles of denaturing at 95 °C for 30 s, annealing at 54 °C for 30 s, and extension at 72 °C for 30 s, and a final melting curve.

2.4. Library preparation and 16S rRNA gene amplicon sequencing

Extracted total community DNA was used for amplification of the 16S rRNA gene V4 and V5 hypervariable region using the primer pair 515f–806r (Caporaso et al., 2011) with the addition of Illumina indexes (barcode sequences) for multiplexing. All PCR reactions were performed in two technical replicates. Peptide nucleic acid (PNA) clamps were added to PCR mix to inhibit amplification of the host plastic and mitochondrial DNA (Lundberg et al., 2013). Polymerase chain reactions (PCRs) were carried out in Whatman Biometra® Tpersonal thermocycler (Biometra 141 GmbH, Göttingen, Germany) with 35 cycles at 94 °C for denaturation for 45 s, 54 °C annealing for 60 s, and 72 °C elongation for 90 s. Purification of amplicons (two technical replicates per biological sample) was done using Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI) and amplicons were pooled in equimolar concentrations. The sequencing of the barcoded amplicons was performed on an Illumina MiSeq (2 × 250 bp paired-end reads) by the sequencing provider Genewiz (Leipzig, Germany). Amplicon sequences were deposited at the European Nucleotide Archive (ENA) under the project number PRJEB48252.

2.5. Bioinformatics and statistical analysis

For 16S rRNA gene amplicon sequencing data, we used cutadapt to remove low-quality reads, primer sequences, and demultiplex the reads according to the assigned barcode (Martin, 2011). The DADA2 algorithm (Callahan et al., 2016) implemented in QIIME2 (Bolyen et al., 2019) was used to quality filter, denoise, and remove chimeric sequences. This process generated representative sequences, called amplicon sequences variants (ASVs), and a feature table. ASVs were further classified using the vsearch algorithm against the SILVA v132 database (Quast et al., 2012; Rognes et al., 2016). Plant-derived sequences i.e., chloroplasts and mitochondria were excluded prior further statistical analyses.

Statistical analysis and visualization of graphs were conducted in R studio v. 2021.09.0 (Allaire, 2012) unless stated otherwise. The Kruskal Wallis test was performed to determine significant differences ($P < 0.05$) of bacterial gene copy numbers per gram of unprocessed and processed apple pulp and followed by Dunn's post-hoc tests for multiple pairwise comparison. The bacterial community analysis was performed using phyloseq and microbiome R packages (Chong et al., 2020; McMurdie

and Holmes, 2013), and the ASV tables and taxonomic classifications were used as the input dataset. Moreover, the 16S rRNA gene dataset was rarefied by randomly selecting subsets of sequences to the lowest number of read counts. Taxonomical composition was visualized using plot bars. Using the rarefied dataset, differences in alpha diversity according to the Shannon diversity index (H′) were determined using the Kruskal Wallis test followed by Dunn's post-hoc tests for multiple pairwise comparison at $P < 0.05$. The rarefied dataset was used to calculate non-metric Bray-Curtis dissimilarity matrices which was then subjected to permutational analysis of variance (PERMANOVA, 999 permutations) to determine significant effects of apple processing on bacterial community structures. A two-dimensional non-metric multidimensional scaling (NMDS) plot was generated to visualize the distance matrices. We used edgeR (Robinson et al., 2010) to identify differentially abundant bacterial genera in unprocessed and processed apple pulps. Bacterial genera were defined significantly different if the $P_{adjusted}$ value was less than 0.1 (Chong et al., 2020).

3. Results

3.1. Bacterial isolates from unprocessed apples are highly diverse and exhibit probiotic properties

A total of 151 representative isolates were isolated from unprocessed apples and selected based on differences in colony morphology (shape and color). We retrieved taxonomical information and evaluated bioactive properties of cultivated bacterial isolates using Sanger sequencing and functionality assays, respectively. The isolates were assigned to nine bacterial orders (Fig. 1A, Supplementary Table S2), including *Bacillales* (37.0%), *Enterobacteriales* (14.5%), *Micrococcales* (20.5%), *Rhizobiales* (7.3%), *Pseudomonadales* (7.3%), *Xanthomonadales* (9.9%), *Sphingomonadales* (1.3%), *Betaproteobacteriales* (1.3%), and *Propionibacteriales* (0.7%). At genus level, the bacterial isolates were assigned to 17 bacterial genera such as *Bacillus* (n = 53 isolates), *Erwinia* (n = 15 isolates), *Pseudomonas* (n = 11 isolates), and *Xanthomonas* (n = 10 isolates).

We performed three *in vitro* tests (Supplementary Fig. S1) to identify potential properties of probiotics i) antagonistic activity against potentially pathogenic bacteria, ii) production of biosurfactants, which can reduce pathogen adhesion to surfaces and iii) bile salt resistance screening as this was shown to correlate with gastric survival *in vivo*. Overall, antagonistic activities against *Acinetobacter baumanii* and *Escherichia coli* were prevalent (n = 46, 30.5%; n = 31, 20.5%, respectively, Fig. 1B, Supplementary Table S2). A total of 20 isolates were able to inhibit both opportunistic pathogens. These isolates were identified as members of the genera *Bacillus* (n = 12), *Pseudomonas* (n = 2), *Erwinia* (n = 2), *Xanthomonas* (n = 2), and *Paenibacillus* (n = 1). The ability to antagonize *Pseudomonas aeruginosa* and *Enterococcus faecalis* was rare. Only one isolate which belongs to *Pantoea* was able to inhibit both opportunistic pathogens. Moreover, no isolates were able to antagonize *Stenotrophomonas maltophilia*. The apple–associated bacterial collection showed a high prevalence (n = 45, 29.8%) of biosurfactant-producing bacteria, where most of the biosurfactant-producing isolates were identified as *Bacillus* spp. (*Bacillales*, Fig. 1A). Protease activity was also prevalent among *Bacillus* isolates although multiple isolates that belong to *Microbacterium* and *Curtobacterium* (*Micrococcales*) also had the same properties. According to the bile salt resistance screening test, more than a half of the isolates from the collection (n = 84, 55.7%) could slowly grow in the media with 0.3% bile salt ($OD_{600} > 0.150$), whereas among them seven isolates were able to grow in the media with 1.8% of bile salt

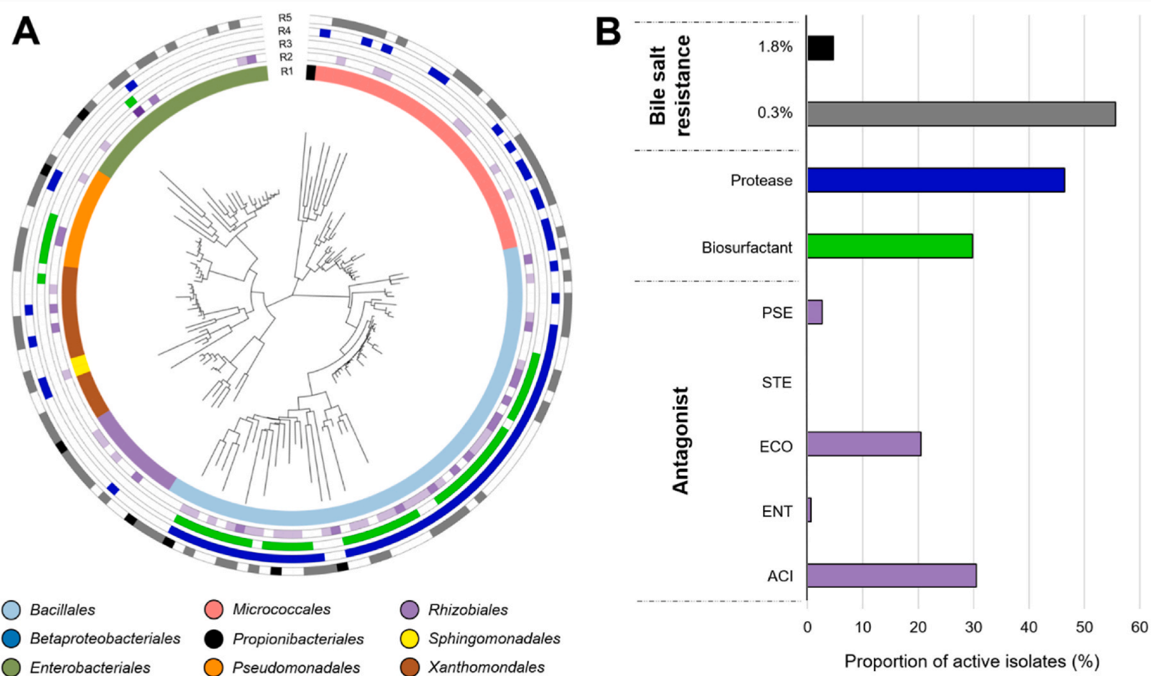

Fig. 1. Phylogenetic tree based on partial 16S rRNA genes of bacteria cultivated from apple pulp (A) and their bioactivities properties (B). The phylogenetic tree was constructed using Fasttree 2–approximately maximum-likelihood trees and visualized using Interactive tree of life software (iTOL). Ring 1 (R1) indicates bacterial taxonomy. Ring 2 (R2) indicates the number of tested opportunistic pathogens that were inhibited by cultivated bacterial isolates from apple pulp (dark purple: three of the tested opportunistic pathogens were inhibited, purple: two of the tested opportunistic pathogens were inhibited and bright purple: one of the tested opportunistic pathogens was inhibited). Ring 3 (R3) indicates presence of biosurfactant production (green). Ring 4 (R4) indicates presence of protease activity (blue). Ring 5 (R5) indicates resistance of bacterial isolates toward bile salt (grey – resistance in 0.3% of bile salt, black-resistance in 0.3 and 1.8% of bile salt). The proportion of cultivated bacterial isolates with bioactive properties in each assay i.e, resistance to bile salt, production of biosurfactants, production of proteases, and antagonism against opportunistic pathogens was determined (B). Each of the opportunistic pathogens tested is labeled with abbreviations (ACI: *Acinetobacter baumanii* strain 6340276, ENT: *Enterococcus faecium* strain 6428631, ECO: *Escherichia coli* strain 6402087, PSE: *Pseudomonas aeruginosa* strain 6436029, and STE: *Stenotrophomonas maltophilia* strain EA23). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(OD_{600} > 0.150). These isolates were assigned to the genera *Bacillus*, *Curtobacterium, Rhizobium, Pseudomonas,* and *Enterobacter*. This finding suggests that apple-associated bacteria have an intrinsic tolerance toward bile salt which is a common characteristic used to screen probiotic strains. Overall, these results demonstrate that bacterial isolates from apples have potential properties as probiotics.

3.2. Bacterial colonization patterns in apple pulp

To confirm and visualize bacterial colonization before and after the processing of apple fruits, we used confocal laser scanning microscopy (CLSM) micrographs in combination with fluorescence *in situ* hybridization (A) and the LIVE/DEAD™ BacLight™ Bacterial Viability Kit. Before apple processing, bacterial colonization could be visualized in the apple pulp (Fig. 2A and B). Colonization of *Gammaproteobacteria* (yellow dots) and *Betaproteobacteria* (pink dots) were differentiable from the other bacterial classes (red dots) (Fig. 2A and B and Supplementary Fig. S2). Before processing of apple fruits, viable bacteria colonizing the fruit pulp were detected (green dots, Fig. 2C). Following the boiling and drying process, relatively low cell densities of viable bacteria (in comparison to apple pulp prior to apple processing) were observed (Fig. 2D and E). Here, we visually showed that boiling and drying did not completely eliminate bacteria.

3.3. Processing of apple fruits affected absolute bacterial abundance, diversity, and community structure

To examine the influence of apple processing on the absolute bacterial abundance in unprocessed and processed apple pulps, we subsequently performed qPCR using samples treated with propidium monoazide (PMA) to mask DNA from dead cells. Absolute bacterial abundance as measured via qPCR in unprocessed apple pulps was determined as 1.8×10^5 16S rRNA copies per g of apple pulp (Fig. 3A). In processed apple pulps, the absolute bacterial abundance was reduced in the range between 63.3% and 86.7% in comparison to the unprocessed apple pulps ($P < 0.001$). A decreasing trend in absolute bacterial abundance was observed from shredded > boiled > pureed > preserved > dried apples indicating that longer heat treatments reduced absolute bacterial abundance (Fig. 3A). A reduction in bacterial diversity was observed in preserved (H' = 1.9) and dried (H' = 1.9) apple pulp when compared to unprocessed apple pulp (H' = 2.5, $P < 0.05$). No significant differences in the bacterial diversity were observed between unprocessed apple pulp when compared with either shredded, boiled, or pureed samples.

Beta diversity analyses based on a Bray-Curtis distance matrix indicated clear clustering between unprocessed and processed apple pulps (Fig. 3C). PERMANOVA analysis revealed that the processing of apple fruits affected the bacterial community structure ($P = 0.001$); this factor explained 30.5% of the bacterial variation in bacterial community

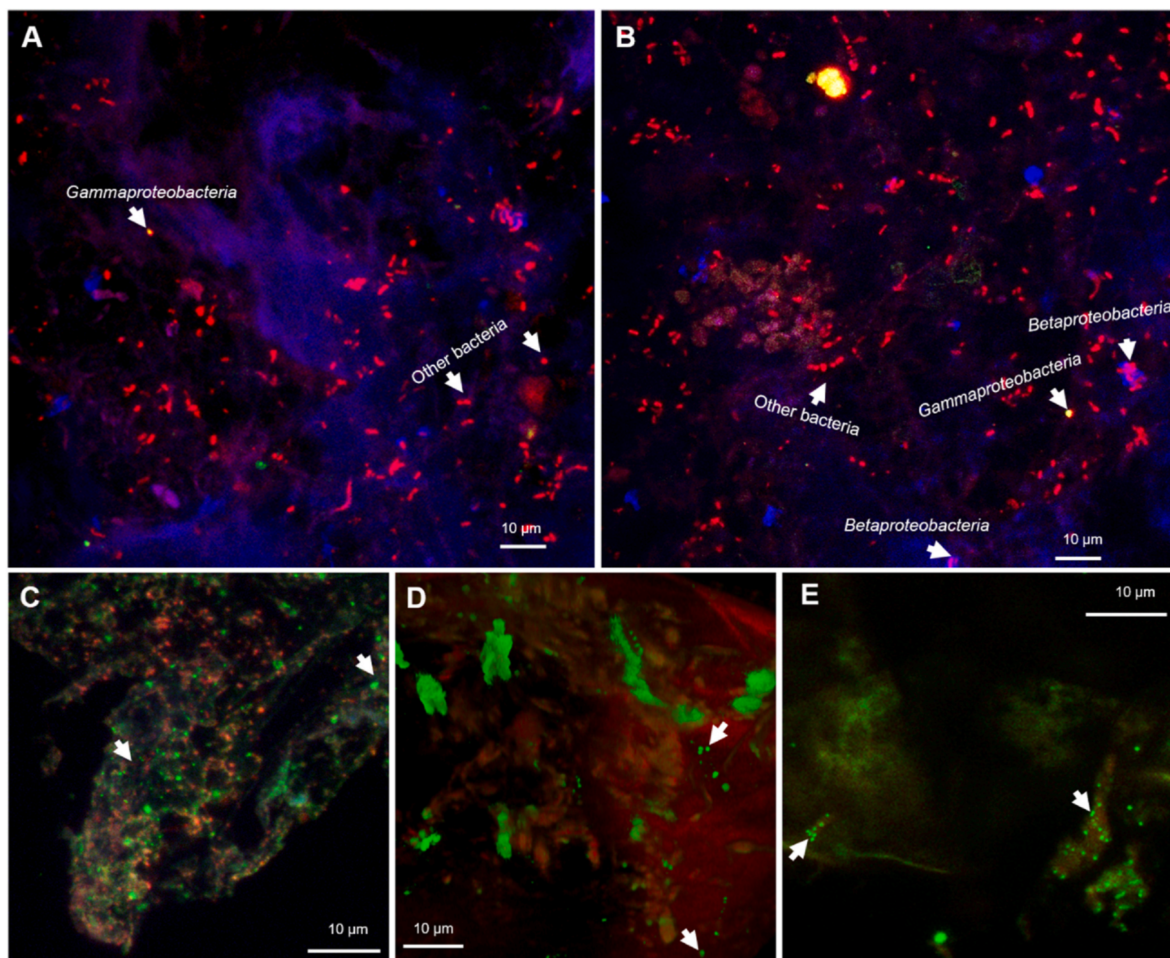

Fig. 2. Confocal laser scanning microscopy (CLSM) micrograph in combination with fluorescence *in situ* hybridization (A–B) and the LIVE/DEAD™ BacLight™ Bacterial Viability Kit (C–E) showing bacterial colonization of apple pulp. Bacteria were stained with FISH probes specific for *Betaproteobacteria* (pink), *Gammaproteobacteria* (yellow) and remaining bacteria of other classes (red) (A and B). Viable bacteria were coloured in green (bright spots) for untreated samples (C) and after apple fruit pulp was boiled (D) and dried (E) indicating that bacteria were alive at the time point of sample collection. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

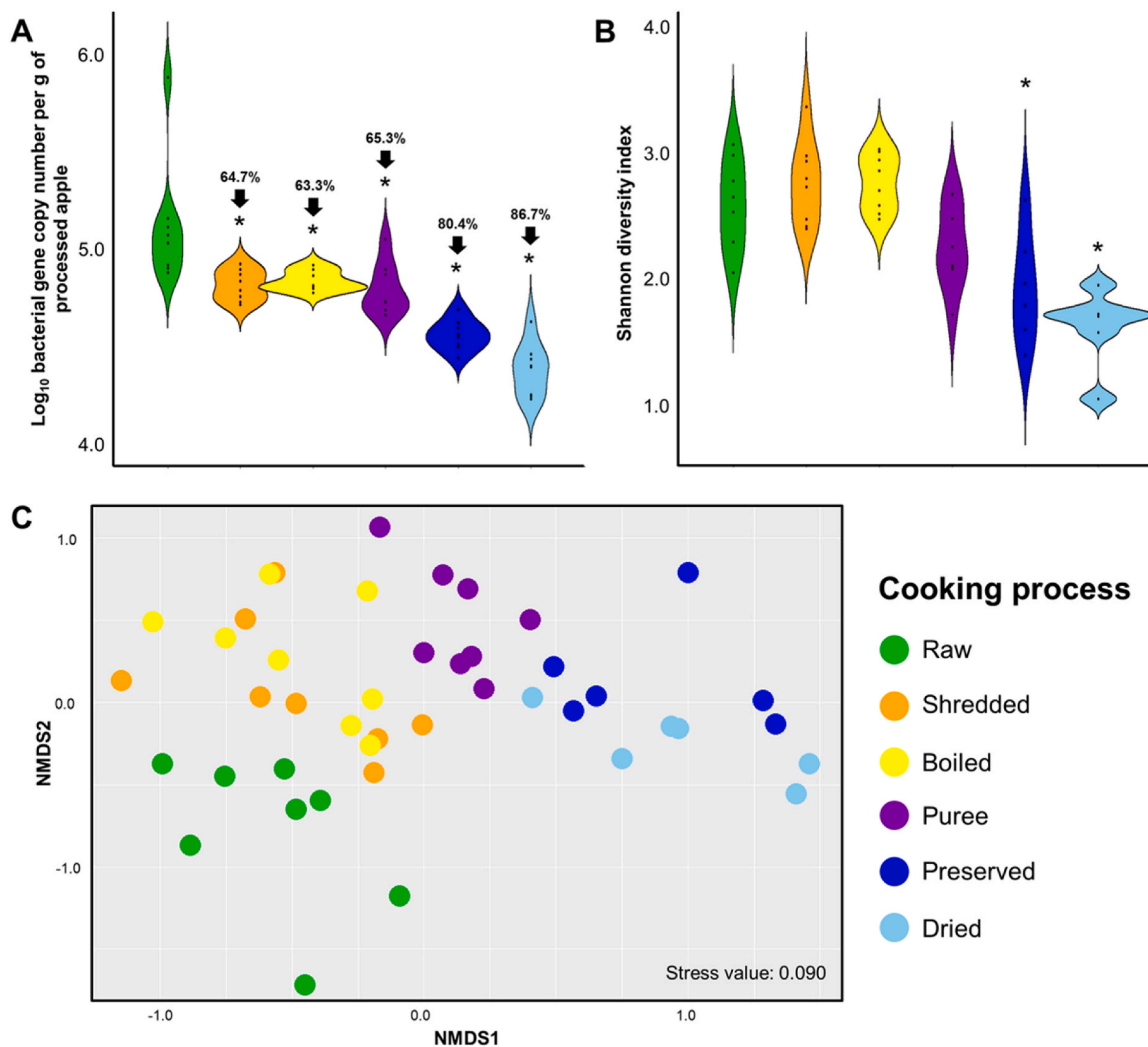

Fig. 3. Absolute bacterial abundance, bacterial diversity and community structure after processing of apple fruits. Absolute bacterial abundance was determined via qPCR (A). Bacterial diversity (B) and community structure (C) were assessed via 16S rRNA gene amplicon sequencing. Significances in bacterial absolute abundance (A) and bacterial diversity (B) were determined with the Dunn's test pairwise comparison with untreated samples and indicated by asterisks, representing $P < 0.05$. Arrows in panel A indicate the relative proportion of bacterial reduction in comparison to untreated samples. Bacterial community clustering was assessed based on a Bray-Curtis distance matrix and visualized in a two-dimensional NMDS plot (C).

structure. Statistical analysis indicated significant differences in bacterial community structure between unprocessed and processed apples ($P < 0.05$). Interestingly, processed apple pulps were ordinated away from the unprocessed apple pulps (Fig. 3C). Overall, we showed that longer heat treatments, i.e. preserving and drying severely reduced absolute bacterial abundance and bacterial diversity in the apple pulp which led to the shift in microbial community structure.

3.4. Processing of apple fruits substantially affects bacterial composition and in particular low-abundant taxa

Given the observed changes in the bacterial community structures, we were interested in identifying specific bacterial taxa highly affected by the processing of apple fruits. *Betaproteobacteriales* and *Enterobacteriales* were the two dominant bacterial orders, contributing an average of 51.3% and 20.4% of the total 16S rRNA sequence reads in the unprocessed and processed apples, respectively (Fig. 4A, Supplementary Table S1). Other bacterial taxa, i.e. *Rhizobiales* (3.9%), *Bacillales* (3.7%), *Micrococcales* (2.4%), *Pseudomonadales* (2.5%), *Sphingomonadales* (2.9%), and *Xanthomonadales* (0.4%) occurred at relatively low abundance in the unprocessed apples. Several rare taxa (<0.4%) such as *Rhodobacterales*, *Caulobacterales*, *Myxococcales*, *Bifidobacteriales* and *Lactobacillales* were also detected. Moreover, a certain overlap with our bacterial culture collection was observed. Therefore, it partially represents dominant and rare taxa of the naturally occurring apple microbiota. .

At the genus level, *Burkholderia*, *Erwinia,* and *Paracoccus* contributed to 65.5% of the total 16S rRNA sequence reads in the unprocessed apples. *Bacillales* occurred in relatively low abundance (3.8%) and their relative abundance gradually decreased in shredded > boiled > pureed > dried apples (Fig. 4B). Relative abundance of *Rhizobiales* also showed a similar pattern, from 3.9% in unprocessed apples to non-detectable in processed apples (Fig. 4A). In contrast, the relative abundance of *Xanthomonadales* gradually increased from shredded < boiled < pureed < dried < preserved apples (Fig. 4A). A striking change was observed in the relative abundance of *Pseudomonadales,* where their relative abundance increased from 2.5% in the unprocessed apples to 11.9% and 24.1% in preserved and dried apples, respectively (Fig. 4A).

Since the preserved and dried apples were the two treatments with the most noticeable changes, we specifically compared bacterial relative abundances in the unprocessed apples with these two treatments. The relative abundances of *Pseudomonas*, *Ralstonia,* and *Reyranella* were

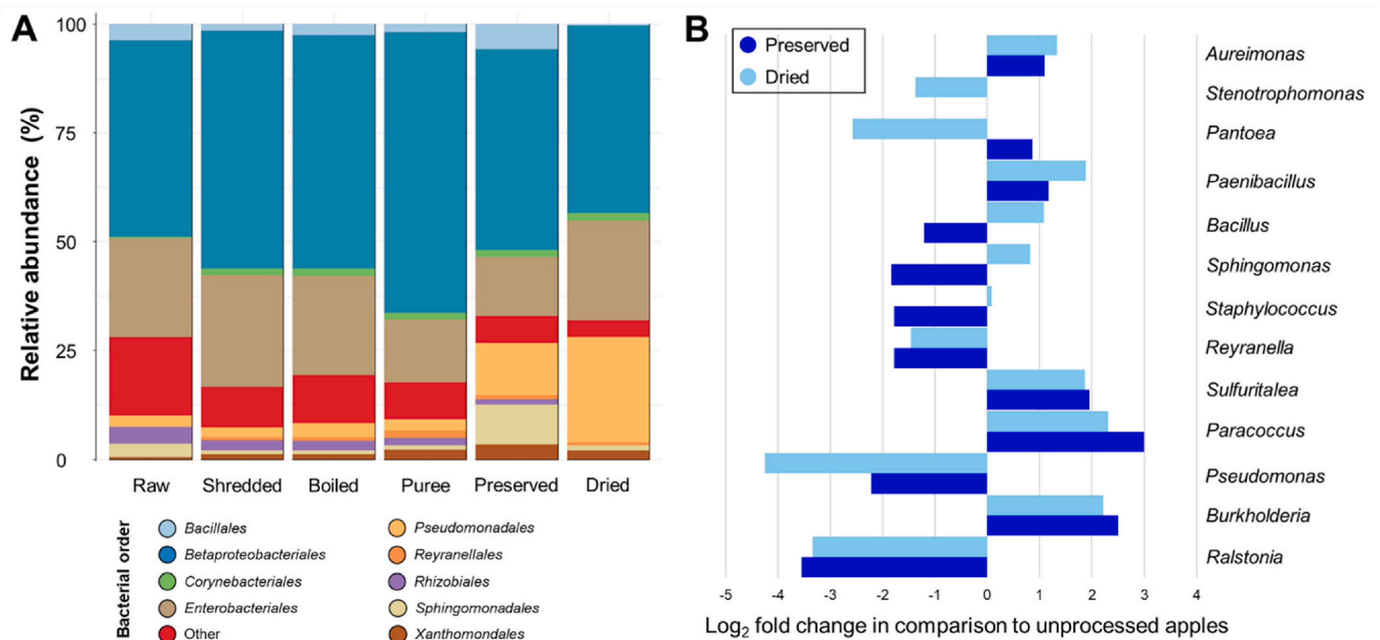

Fig. 4. Relative abundance of the bacterial taxa on order level after different processing of apple fruits (A) and bacterial genera that were enriched/reduced in preserved and dried apples in comparison to untreated apples (B). Bacterial orders with an average relative abundance less than 0.3% and that were detected in at least 50% of total samples were included in "Others" (A). EdgeR was used to identify bacterial taxa that were significantly enriched/reduced ($P < 0.1$). Negative $\log_2$ fold change values indicate that the respective bacterial taxa are more abundant in preserved or dried apples whereas positive values indicate that the genes are more abundant in unprocessed apples.

enriched in the treated apples (Fig. 4B). These taxa were detected in low abundance (3.3, 1.6, and 0.03%) in the unprocessed apples. The relative abundance of *Ralstonia* was enriched by more than a 3 log2 fold change in both preserved and dried apples in comparison to the unprocessed apples. Moreover, there were certain taxa associated with a specific treatment. For instance, increased relative abundances of *Staphylococcus* and *Stenotrophomonas* were associated with preserved and dried apples, respectively. In contrast, the relative abundance of *Paracoccus, Burkholderia, Paenibacillus*, and *Sulfuritalea* decreased in the preserved and dried apples in comparison to the unprocessed apples. Additionally, the relative abundance of *Bacillus*, one of the most frequently recovered and bioactive taxa from the culture-dependent approach, decreased in the dried apple in comparison to the unprocessed apples, but showed the opposite pattern in the preserved apples.

4. Discussion

In the present study, we characterized the structure and function of the apple microbiota and found that certain bacterial members may have beneficial functions for human health. We further demonstrated that apple fruit processing substantially affects bacterial diversity and community structure. While the beneficial role of fruit consumption on the gut microbiota and human health is increasingly recognized (Berg et al., 2015; Henning et al., 2017; Koutsos et al., 2015; Tomova et al., 2019), we showed novel and unexpected insights into how the processing of apple fruits impacts their indigenous microbiota.

New insights are also provided into the microbiota of unprocessed apples because we observed a high occurrence of beneficial bacteria related to human health. The probiotic potential of fruit and vegetable inhabiting lactic acid bacteria is well known (Mabeku et al., 2020; Maheshwari et al., 2019; Xu et al., 2018), but less is known about the beneficial functions of bacteria derived from fresh products. The majority of the cultivated apple bacteria in this study belong to *Bacillus*, *Curtobacterium*, *Erwinia*, *Pseudomonas,* and *Xanthomonas*. *Bacillus*, *Erwinia*, and *Pseudomonas* were also the most abundant bacterial genera as previously observed in a global study focusing on the apple microbiome (Abdelfattah et al., 2021). *Erwinia* was also found to be highly abundant in conventionally managed apples (Wassermann et al., 2019). The selected isolates in the present study exerted activities that might have implications for human health but were mostly present in relatively low abundances (<5%). A high number of isolates (mainly *Bacillus*) was able to inhibit Gram-negative opportunistic pathogens, i.e. *A. baumanii* and *E. coli*. Moreover, to survive in the gastrointestinal tract, bacteria must survive under bile-acid stress (Prete et al., 2020). Although bile concentration in the human intestine can vary, it is generally in a range between 0.2 and 2% (Gunn, 2000; Hofmann, 1998). Hence, we argue that some members of the indigenous apple microbiota, i.e. *Bacillus*, *Curtobacterium, Rhizobium, Pseudomonas,* and *Enterobacter* tolerant towards bile salt in this range, might be able to survive in the gastrointestinal tract. Interestingly, various biofilm-forming probiotic *Bacillus* species have previously shown great potential as probiotic candidates, i.e., therapy for metabolic diseases, including overweight and high cholesterol, preventing diarrhea and increasing immune responses to various infectious agents (Cao et al., 2020; James and Wang, 2019; WoldemariamYohannes et al., 2020). Moreover, we discovered a significant prevalence of biosurfactant- and protease-producing bacteria (mainly *Bacillales*), which are known to efficiently disrupt the biofilm development of opportunistic pathogens (Banat et al., 2014; Primo et al., 2015). Furthermore, there is a growing body of data linking short-chain fatty acids (SCFAs) to improved human health (Chambers et al., 2018; Ríos-Covián et al., 2016). Interestingly, the ability to produce SCFAs was also observed in bacteria that were isolated from different fruits, i.e. *Weissella*, *Leuconostoc*, *Lactobacillus*, and *Enterococcus* (Pabari et al., 2020; Ruiz Rodríguez et al., 2019). As the production of SCFAs can be modulated by the microbiota, identification of this trait could also be included for further screening of probiotic candidates isolated from fruits. Further studies to investigate bacterial pathogenicity are also needed as apples can harbour food-borne pathogens, i.e. *Enterobacter*, *Escherichia-Shigella* and *Klebsiella* (Abadias et al., 2006; Wassermann et al., 2019). In this context, a recently published study showed that

post-harvest storage and transport has a crucial impact on the microbiome and resistome of apples because in that period a significant shift towards *Enterobacteriales* was observed (Wassermann et al., 2022). Taken together, the current study indicated that the indigenous apple microbiota has potential properties as probiotics. We also acknowledge that more research is needed to properly determine their potential health-promoting effects, including a safety evaluation utilizing human trials, as well as the proper dosage, according to WHO guidelines (Joint FAO/WHO Working Group Joint FAO/WHO Working Group, 2002). Moreover, further studies are also needed to investigate which bacteria and how many of them that are actually transferred to the human gut.

In general, processing of apple fruits decreased bacterial diversity and modulated the bacterial community structure substantially. The detection of viable bacteria was quantitively confirmed via qPCR and amplicon sequencing. In this study, we used PMA to exclude amplification of DNA from bacteria whose membrane was damaged during the processing of apple fruits (Nocker et al., 2006). This procedure was successfully applied in many microbiome studies (Mahnert et al., 2019; Vaishampayan et al., 2013) but can have certain limitations in terms of qualitative assessments (Wang et al., 2021). Therefore, we complemented our approach with Fluorescent *in situ* Hybridization and confocal laser scanning microscopy (FISH-CLSM) as well as differential staining to visualize bacterial viability and studied a culture collection in detail. This polyphasic approach allowed us a comparison of the differently treated samples and revealed a comprehensive picture of the apple microbiota. This combination of approaches is also used to overrule the possibility of the presence of contaminations that are commonly found in extraction kits and reagents when dealing with low biomass samples (de Goffau et al., 2018). In addition, it is recommended to always include a no-template control with every sequencing run regardless of whether a PCR product is detectable by gel electrophoresis. It should be noted that although PMA treatment was shown to be appropriate to analyze qualitative shifts in viable bacterial community composition for most settings, it is not always implementable to accurately quantify viable taxa (Wang et al., 2021). Overall, we observed a reduction in absolute bacterial abundance after the fruit processing, especially in treatments with longer heat exposure. This result strengthens the CLSM observations where only a small cluster of bacterial colonies were observed after the boiling and drying treatments. Altogether this study showed that such food microbiome analyses require a combined methodological approach.

The impact of fruit processing on the bacterial community structure in the apple pulp was substantial. Interestingly, the time of exposing apple samples to high temperature seemed to be more important than the temperature itself; drying slowly at 60 °C seemed to affect bacteria more than processing apple fruits for a short while at 100 °C. Bacterial community and diversity structure shifted along the fruit processing. After the shredding process, browning occurred due to the enzymatic oxidation of endogenous phenolic compounds catalyzed by polyphenol oxidase (PPO) (Nicolas et al., 1994). This enzyme is known to play a role as a defense mechanism against bacterial pathogens (Khodadadi et al., 2020; Vanitha et al., 2009) which could induce a change in bacterial community structure when compared to fresh apples. Interestingly, boiled apples were more similar to shredded apples in comparison to pureed, preserved, and dried apples. In practice, boiling apples is meant to soften the apple tissue so that it can be eaten more easily by infants. This unexpected result could be due to the circumstance that heat during the fruit processing did not distribute uniformly. Therefore, no substantial change in the bacterial composition in boiled apples in comparison to shredded apples was observed. In contrast, during the process of making apple puree, the apples were mashed immediately after boiling, and hence the heat was more evenly distributed and the bacterial diversity and composition changed substantially. A longer heat exposure of apples, i.e. preserved and dried apples, was shown to reduce the bacterial density and diversity even more. Overall, the presence of bacteria after exposure to high temperatures suggests that common practices to prepare homemade baby food do not completely eliminate all members of the indigenous apple microbiota but substantially shift the bacterial communities along with longer exposure to heat. These results, however, do not apply to commercially available baby foods if the manufacturing process utilizes temperatures over 100 °C.

Low abundant indigenous taxa were highly affected by the fruit processing. Several studies reported heat-resistant and biofilm-producing *Pseudomonas* and *Ralstonia* as spoilage agents of foods (Carrascosa et al., 2021; Kumar et al., 2019; Machado et al., 2017; Xin et al., 2017). Moreover, this needs further attention because the genus *Ralstonia* has been recognized as an emerging global opportunistic pathogen (Ryan and Adley, 2014). We hypothesize that the increase of the low abundant but more versatile taxa, namely *Pseudomonas* and *Ralstonia,* were due to substantial reductions of more abundant taxa, i.e. *Burkholderia* and *Erwinia,* during the fruit processing. In contrast, the relative abundance of minor but the most bioactive taxa in functional assays, *Bacillus* (*Bacillales*), were also highly affected by the fruit processing. *Bacillus* spp. are recognized as potential probiotics due to their inherent property to produce secondary metabolites, i.e. vitamins and carotenoids, inhibit pathogens and survive in hostile environments (Elshaghabee et al., 2017). Various food processing and preservation methods are meant to reduce bacterial numbers, inhibit growth of potential pathogens, and increase the shelf life of the end-products (Mogren et al., 2018), including processed fruits. Here we demonstrated that although the fruit processing by using high-temperature treatments, i.e. by boiling or drying, is meant to eliminate undesired bacteria, at the same time it also provides a condition for low abundant taxa to either proliferate or diminish with potential health implications, e.g. increase in the proportion of potential pathogens. Recently, storage and transport were shown to influence the apple microbiota and their antimicrobial resistance gene compositions (Wassermann et al., 2022). We suggest that the indigenous fruit microbiota and factors that influence their composition have a significant impact on food quality and safety.

In conclusion, we suggest that raw-eaten fruits are an important source of beneficial bacteria. Furthermore, we could show that the processing of apples also substantially affected the indigenous pulp microbiota, an element that is mostly overlooked. The processing of apple fruits substantially reduced the bacterial load and diversity as well as shifted the bacterial community structure. Our findings imply that consumption of apples is a potential resource of the infant gut microbiota. Hence, any modification of the indigenous fruit microbiota prior to consumption is potentially influencing the gut microbiota composition. Here, we set a foundation for future studies to link the type of fruit processing, i.e. commercially processed and naturally processed, to the gut microbiota development during early childhood. In addition, we highlighted the potential beneficial properties and compatibility with the gastrointestinal environment of various bacteria associated with apples.

Availability of data and materials

Raw sequencing data for each sample used in this study was deposited at the European Nucleotide Archive (ENA) in the FASTQ format and is available under the Bioproject accession number PRJEB48252.

Author's contributions

GB and WAW conceived and designed the study. WAW, AB, and PK performed the experiment. WAW and PK performed bioinformatic analysis. WAW, AB, and GB wrote the manuscript. PK, AS, TC, and WAW analyzed the data. PK, TC, AS, OHL, SV, and HH critically read and commented on the manuscript. GB, AK, and HH are involved in project administration and funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2022.104103.

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