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Metabolomics workflow for quality control of differently-processed pre-cooked chicken fillets

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ABSTRACT

The contents and profiles of small molecules in a food can provide information about quality-related properties. Processing methods and deterioration during storage, e.g. from bacterial proliferation and degradation, might also lead to changes in the metabolome, which can be determined by mass spectrometry-based metabolomics. By measuring as many metabolites as possible in differently treated pre-cooked chicken fillets in an untargeted approach, we studied individual and combined effects of vacuum packaging (VP), soluble gas stabilisation (SGS), high pressure processing (HPP), and microwave volumetric heating (MW) on the quality and shelf-life of the finished product. The extensive dataset was processed using an optimised workflow of consecutive software tools with stringent statistical analysis to prevent over-interpretation, which is an inherent risk of metabolomics data. Our results showed the predominant influence of VP on storage quality since SGS, HPP, and MW did not have the potential to extent shelf-life.

1. Introduction

Surveillance of food quality and safety is essential for consumer satisfaction and confidence. Food products have to meet expectations with regard to appearance, smell and taste as well as being non-hazardous and hygienic. Relevant national and international authorities have issued numerous guidelines implementing quality and safety policies, and compliance with rules is monitored by food manufacturers and controlled by inspections. The required food analyses are, however, in need of methods that are either capable of determining specific chemical contaminants or allow mapping the contents for the detection of changes in quality. The state-of-the-art technology for both requirements is mass spectrometry (MS)-based metabolomics, performed either in targeted mode for the quantitation of specific analytes or untargeted (i.e. measuring as many non-volatile metabolites in the food as possible) (Kim, Kim, Yun, & Kim, 2016).

The large chemical variability of the molecules in a food matrix, including proteins, amino acids, carbohydrates, lipids, phenol derivates, and others, usually with molecular weights below 1,500 Da, makes the characterisation of the entire metabolome unattainable; however, comparisons of well-defined sample groups, e.g., derived from differently-treated food products, can provide information on differences in metabolite profiles (Argyri, Papadopoulou, Nisiotou, Tassou, & Chorianopoulos, 2018; Beleggia et al., 2011; Lopez-sanchez et al., 2015). Furthermore, the annotation of specific metabolites by matching analytical parameters to online repositories and allocating specific functions and metabolic pathways might lead to the identification of process-related changes.

Metabolomic analysis generates vast amounts of data. Appropriate, sufficiently discriminant statistical data processing is a prerequisite for avoiding over-interpretation and for discovering significant and relevant differences (Li et al., 2021). The assessment of the comprehensive experimental data and connection to biological reactions in food products is challenging but could offer valuable insight into quality-related changes. The use of metabolic profiling in food quality assessment is still only at its beginning, and optimal workflows for sampling, metabolite extraction, and analysis, as well as data processing and evaluation, would be valuable tools to increase the credibility of the results.

In the present study, we applied MS-based metabolomics on differently-processed pre-cooked chicken fillets stored refrigerated for

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different time periods. Chicken meat is the most used meat product worldwide, and global consumption continues to increase (Eurostat, 2019). This is mainly due to the relatively low price, multiple cooking uses, and considerable nutritional value. However, chicken products are easily perishable, and raw chicken meat has a very short shelf-life as a result of bacterial proliferation and deterioration by lipid oxidation and protein degradation, which are typical for food containing high amounts of polyunsaturated fatty acids and proteins (Xiao, Zhang, Lee, Ma, & Ahn, 2011). The related changes in metabolite contents usually lead to safety concerns and a sensory quality loss by, e.g., the development of off-odours (Jayasena, Ahn, Nam, & Jo, 2013). Chicken meat-colonising microflora includes well-known pathogens in poultry farming such as *Salmonella* spp., *Listeria* spp. and *Campylobacter* spp. (Argyri et al., 2018; Morales, Calzada, Rodríguez, De Paz, & Nuñez, 2009; Morey, Singh, & McKee, 2012; Zeinali, Jamshidi, Khanzadi, & Azizzadeh, 2015), but also typical spoilage bacteria such as *B. thermosphacta*, *E. coli, Carnobacterium* spp. and lactic acid bacteria (LAB) (Apostolou, Papadopoulou, Levidiotou, & Ioannides, 2005; Göksoy, James, & Corry, 2000). Metabolomic analysis can thus assist in understanding, evaluating and controlling meat-spoiling reactions by identifying the origin and metabolic function of small molecules that significantly contribute to decay development (Wen, Liu, & Yu, 2020).

Numerous food preservation technologies have been devised to ensure a supply of enough and safe chicken meat of good quality. Packaging, pre-treatments, and different processing methods are typically applied to chicken meat to inhibit microbial growth and reduce oxidative changes in stored products (Ahmed et al., 2015). The access to oxygen influences the microflora composition on the meat and the velocity and extent of enzymatic reactions so that packaging has a central role in product protection. Fresh, pre-cooked, or grilled chicken meat can be packaged in air-permeable plastics or impermeable vacuum packaging (VP) material, which forms an evacuated skin around the food. VP can be applied with or without applying modified atmosphere packaging (MAP) that adds a mixture of carbon dioxide (CO_2) and nitrogen (N_2) to the product (Dang, Rode, & Skipnes, 2021; Tsironi, Ntzimani, & Taoukis, 2019).

Food packaging has been developed to protect foods from environmental stressors such as air, light, moisture, or pathogens to repel microflora and preserve food freshness (Coles & Kirwan, 2011). Packaging methods such as VP and MAP as well as pre-treatment by soluble gas stabilisation (SGS), i.e., the diffusion and dissolution of CO_2 into the product under low temperature and pressure before packaging, have shown to be efficient in extending the shelf-life of meat and meat products (Dang et al., 2021; Tsironi et al., 2019). The use of SGS can reportedly reduce microbial spoilage and prevent package collapse while conserving the physicochemical and sensorial qualities of the food (Dang et al., 2021). In contrast, industrial food processing is a general term for methods used to prepare, convert, or modify food resources into consumables with improved properties (Kim et al., 2016). The primary purposes are ensuring food safety and optimising food textures, tastes, flavours, and colours to make a palatable and attractive product. Moreover, processing can lead to an enhancement of the nutritional value of the food product.

Many processing technologies involve heating, drying, blending and fermenting steps that can considerably affect the content and character of food. More modern approaches attempt to conserve the food composition and appearance as much as possible, thus restricting the loss of valuable nutrients and give the product an untreated, natural look. Among these gentler methods, high pressure processing (HPP) and microwave volumetric heating (MW) have become more often used in recent years. It was shown that pre-cooked chicken fillets treated with HPP, a method for cold-pasteurisation under high pressure, resulted in reduced pathogen proliferation (Morris, Brody, & Wicker, 2007). There are, however, contradicting reports concerning the effects on lipid oxidation, which appeared to be decreased below a pressure of 300 MPa, but increased above this threshold (Dang et al., 2021). MW, also called microwave volumetric heating because the microwave radiation penetrates throughout the material and generates evenly distributed heat, is a thermal technology that has successfully been used for the pasteurisation of poultry products. Short-time processing (<2 min) at 2,450 MHz has been shown to eliminate *Salmonella* and *Listeria* spp., leading to a considerable extension of shelf-life (Dang et al., 2021). However, drawbacks of the methodology are changes in the surface appearance and the generation of lipid oxidation products in the treated meat. MW is nevertheless much used due to its energy efficiency, broad applicability, and low maintenance.

The individual and combined effects of VP, SGS, HPP, and MW on the microbiological and physiochemical shelf-life of pre-cooked chicken fillets have been studied by our group in a recent study (Dang et al., 2021). The experiments showed the effectivity of HPP and MW in microbiota control, eradicating *E. coli* and *Listeria* spp., thereby allowing a LAB population to grow during an extended storage period of 119 days. Food quality attributes, including meat colour, texture, pH, and drip loss, changed only marginally, although lipid oxidation increased significantly at later time points.

Using this outcome as a reference point, the present study had the aim to determine by metabolomic analysis if the findings were mirrored by corresponding changes in the chicken meat metabolome. Since the content of small molecules in a food is closely related to its organoleptic and nutritional properties, metabolomics has the potential to reveal quality-related shifts. By applying a stream-lined and stringent metabolomic workflow for the analysis of chicken fillets that had been processed by pre-cooking and combinations of SGS, VP, HPP and MW, we established reliable conditions that allowed the evaluation of possible treatment-related effects on product quality and shelf-life.

2. Material and methods

2.1. Reagents

LC-MS grade water and acetonitrile (MeCN) (Fisher Scientific, Oslo, Norway), high-performance liquid chromatography (HPLC)-grade methanol (MeOH) (Romil, Cambridge, UK), chloroform (p.a.; Merck, Darmstadt, Germany), and ethanol (EtOH, p.a.) and ammonium carbonate (Fluka, Steinheim, Germany) were used for sample preparation and analysis. Phosphate-buffered saline (PBS, pH 7.5 at 25 °C, 0.01 M) was prepared from ready-to-use tablets (Oxoid, Basingstoke, UK).

2.2. Chicken samples

Fresh fillets cut from the *m. pectoralis major* were obtained from five chickens (two fillets per chicken, 200 g each) provided by Den Stolte Hane AS (Nærbø, Norway). The fillets were kept at 1 °C in Nofima's food technology facility and processed within 2 h after slaughtering.

2.3. Experimental design

An outline of the experimental design is presented in Fig. 1, and the processing methods used have been described previously (Dang et al., 2021). In brief, the chicken breast fillets were oven-cooked for 13 min at 130 °C, 13 min at 115 °C, and cooled to 1 °C. One fillet from each chicken was divided into two parts, of which one was pretreated with SGS and one not (NSG). Samples were produced by cutting the meat with a sharp knife into 5 mm-thick slices weighing 5 g. NSG samples were directly vacuum-packaged (VP) in bags, of which five bags were stored in a cold room (4 °C) immediately after packaging, while from the remaining ten bags, five were subjected to HPP and five to MW before cold storage. The SGS samples (15 bags) were treated with 100% CO_2 at 1 °C and kept under this condition for 18 h, before they were processed further in the same way as the NSG sample set. Major processing steps are described in more detail below.

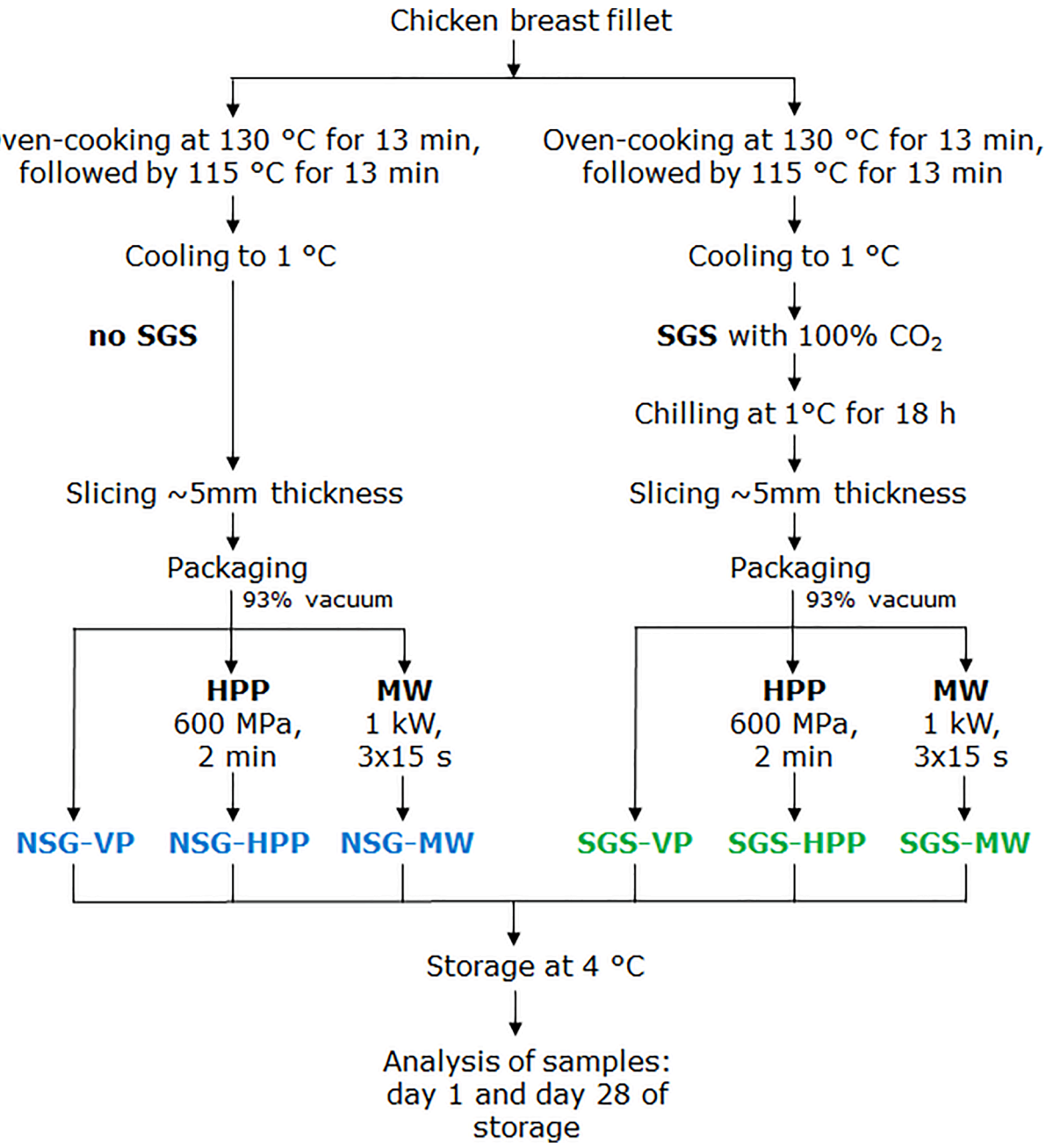

Fig. 1. Overview of chicken fillet treatments. SGS: soluble gas stabilisation; VP: vacuum packaging; HPP: high pressure processing; MW: microwave volumetric heating. Six treatments groups were produced and analysed by metabolomics.

2.3.1. Pre-cooking of chicken fillets

After removing the skin, the fresh chicken breast fillets were placed on racks (8 fillets/rack) for cooking in a convection oven (model Combi FCV/E10, Zanussi Professional, Pordenone, Italy). The cooking parameters (temperatures of oven and chicken, and relative humidity) were recorded (**Supplementary** Fig. 1). The relative humidity inside the oven fluctuated between 10 and 55% during cooking. The core temperature of the chicken fillets was about 90 °C at the end of the cooking period. They were subsequently quickly cooled to 1 °C in a − 20 °C freezer on a stainless steel tray and stored until the subsequently described processing steps were performed.

2.3.2. Soluble gas stabilisation (SGS)

SGS was performed according to the method of Rotabakk, Birkeland, Jeksrud, & Sivertsvik (2006). Pre-cooked and cooled chicken breast fillets were placed on a stainless steel tray (35 × 29 × 5 cm). The tray was placed inside a heat-sealed 20 μm polyamide/70 μm polyethylene bag (70 × 50 cm, Star-pack produktie B.V., Waalwijk, The Netherlands). The atmosphere in the bag was evacuated with a Webomatic SuperMax (Webomatic Maschinenfabrik GmbH, Bochum, Germany), and the bag was flushed with 100% food-grade CO_2. The atmosphere in the bags immediately after sealing was 97.1% CO_2, 0.27% O_2, and 2.63% N_2, whereas it was 98.1 ± 0.8% CO_2, 0.1 ± 0.1% O_2, and 1.8 ± 0.7% N_2 after cooling for 18 h at 1 °C.

2.3.3. Vacuum packaging (VP)

Cut chicken slices were placed individually in 80 μm thick standard Sous vide plastic bags (Arne B. Corneliussen AS, Oslo, Norway) that can withstand heat up to 120 °C for 1 h. The bags were vacuum-packaged (93% vacuum) with a Webomatic SuperMax.

2.3.4. High pressure processing (HPP)

HPP of vacuum-packaged samples was performed at ambient temperature with a lab-scale high-hydrostatic pressure unit QFP 2L-700 (Avure Technologies Inc., Columbus, OH, USA) as previously described (Patterson, McKay, Connolly, & Linton, 2010). Distilled water was used for pressure transmission. The come-up time was approximately 100 s for 600 MPa, and decompression was immediate. The duration of the treatment (2 min) did not include the come-up time. The 600 MPa/2-min condition represents a typical industrial application and was selected based on previous findings (Patterson et al., 2010), which showed that *Listeria monocytogenes* and pressure-resistant *Weissella viridescens* were reduced by this treatment to levels below the detection limit (<1.7 and < 1 $\log_{10}$ colony-forming units/g, respectively).

2.3.5. Microwave volumetric heating (MW)

Vacuum-packaged samples were heated in a laboratory microwave autoclave (Gigatherm AG, Flawil, Switzerland) operated at 2,450 MHz, 1 kW and 2.5 bar (0.25 MPa) pressure (Patterson et al., 2010), in three 15 s cycles with 10 s intervals. Subsequently, the samples were immediately cooled on ice and stored at 4 °C until analysis (Dang et al., 2021).

2.4. Metabolomic analysis by liquid chromatography high-resolution mass spectrometry (LC-HRMS).

2.4.1. Sample homogenisation

Frozen, vacuum-packaged pre-cooked chicken fillet samples were thawed on ice. The bags were subsequently opened, and aliquots of 50 mg were cut out, transferred to 2 mL Precellys® tubes (VWR, International GmbH, Germany), and mixed with extraction solvent in a 1:3 ratio. A Precellys® 24-bead-based homogeniser (VWR, International GmbH, Germany) was used with ceramic beads for homogenisation in three cycles of 20 s at 5,500 rpm with 30 s breaks (as suggested by the manufacturer). The homogenates were transferred to new tubes and stored on ice. The remaining material was flushed out of the Precellys® tubes by adding a few μL extraction solvent (about 10 % of the sample volume) and combined with the first fraction.

2.4.2. Optimisation of sample extraction

The optimal extraction method for the pre-cooked chicken samples was determined in initial experiments considering broad metabolite coverage as the main selection criterion. Samples were either homogenised using EtOH-PBS (85:15, v/v) or MeOH-water (1:0.9, v/v) as the extraction solvent. In the case of the EtOH-PBS extraction, the combined homogenisation fractions were directly centrifuged at 10,000 g for 5 min, and supernatants were collected and stored at − 80 °C until analysis. Alternatively, the samples were homogenised in MeOH-water and mixed with chloroform (50:50, v/v) for 30 s, placed on ice for 30 s, and centrifuged at 1,800g for 10 min at 4 °C. After keeping the samples at room temperature for 5 min, the upper hydrophilic layer was transferred into a fresh tube and stored at − 80 °C until analysis. Quality control samples (QC) for the untargeted LC-HRMS and LC-HRMS/MS analyses were prepared using the one-step extraction method (EtOH-PBS) by pooling 10 μL aliquots of all samples.

2.4.3. Untargeted LC-HRMS and LC-HRMS/MS metabolomics

Analyses were performed by LC-HRMS using a Vanquish Horizon UHPLC coupled to a Hybrid Quadrupole-Orbitrap Q-Exactive high-resolution mass spectrometer equipped with a heated electrospray interface (both from Thermo Fisher Scientific, Bremen, Germany). Aliquots (100 μL) of the pre-cooked chicken fillet extracts were transferred to chromatography vials, which were randomly placed into the thermostatted (4 °C) autosampler tray of the LC-HRMS. The pooled QC sample was run as every 5th sample throughout the whole LC-HRMS experiment. Separation was achieved by hydrophilic interaction chromatography (HILIC) using a zwitterionic SeQuant ZIC-pHILIC column (Merck, Darmstadt, Germany; 150 × 4.6 mm, 5 μm). The column was eluted using a mobile phase consisting of 20 mM ammonium carbonate (A, pH 8.3) and MeCN (B). Elution proceeded isocratically at a constant flow rate of 0.3 mL/min for 1 min with 80% B, followed by a linear gradient to 20% B in 29 min. Subsequently, the column was flushed with 8% B for 5 min and then returned to the starting conditions and equilibrated for 9 min. The mass spectrometer was run in positive and negative ion full-scan mode using fast polarity switching in the mass range *m/z* 58 to *m/z* 870. The mass resolution was set to 70,000 at *m/z* 200. The spray voltage was 2.8 and 3.2 kV (positive and negative mode, respectively), the ion transfer capillary temperature was 280 °C, and the sheath and auxiliary gas flow rates were 35 and 10 units, respectively. Xcalibur software (v2.3) was used for instrument control and LC-HRMS data acquisition.

Additional LC-HRMS/MS data were acquired in both positive and negative ionisation mode using data-dependent analysis (DDA) of the QC sample under the following conditions: Full-scan analyses were performed in three mass range segments (60–300 *m/z*, 300–600 *m/z*, and 600–900 *m/z*), and the top 5 most intense ions selected for fragmentation and product ions scanned with a mass resolution set to 17,500. Fragmentation was achieved by higher-energy collisional dissociation (HCD), including three different normalised collision energies (NCE 15%, 35%, and 65%).

2.4.4. Processing and quality control of metabolomics data

An overview of the experimental workflow, including the data processing, is given in Fig. 2. Raw data files were converted into the Analysis Base File format using the freely available Reifycs ABF converter (Reifycs Inc, Tokyo, Japan) and deconvoluted. Three different workflows of data processing were initially explored (**Supplementary Table 1**). After result evaluation, the data were subsequently processed in MS-DIAL (v4.38) (Tsugawa et al., 2015), applying specific parameters for both ionisation modes (**Supplementary Table 2**). The LC-HRMS/MS data were included to facilitate metabolite annotation by MS/MS spectra comparison to relevant features. Metabolites in the QC sample were identified using the AbsoluteIDQ HR p400 targeted metabolomics kit (Biocrates Life Sciences AG, Innsbruck, Austria) according to the vendor's recommendations. The resulting data matrix contained retention times (RT), *m/z*, and peak areas of blanks, QCs, and samples, normalised by the Locally Weighted Scatterplot Smoothing (LOWESS) function of the detected metabolic features. LOWESS normalises the drift of the MS signal intensities by using the intensities of the QCs interspersed in the analytical run. The QC values are smoothed by application of single-degree least-squares, and subsequently, the coefficients determined for the QCs are interpolated by cubic spline. Finally, the dataset is aligned to the spline result (Tsugawa et al., 2015).

The normalised dataset was processed using the R-based package MS-CleanR (Fraisier-Vannier et al., 2020), activating the following filters: blank signal subtraction (minimum blank ratio set to 0.8), background ion drift removal, and determination of relative standard deviation thresholds (RSD, set to 30) based on sample class and relative mass defect (RMD) window filtering (set by default to 50–3,000 ppm). As a second step, the data obtained for the two ionisation modes were merged and clustered according to the MS-DIAL Peak Character Estimation Algorithm, followed by parental signal extraction applying multi-level optimisation of the modularity algorithm. The maximum mass difference selected for feature relationships detection was established to 0.005 Da with a maximum RT difference of 0.025 min. The Pearson correlation links were considered only when the correlation coefficient was ≥ 0.9 and statistically significant ($\alpha = 0.05$). During the processing in MS-CleanR, molecular formula and a preliminary *in-silico* annotation using MS-Finder (v3.50) (Tsugawa et al., 2016) were assigned to the clusters considering a 5 ppm error for MS and 10 ppm error for MS/MS data.

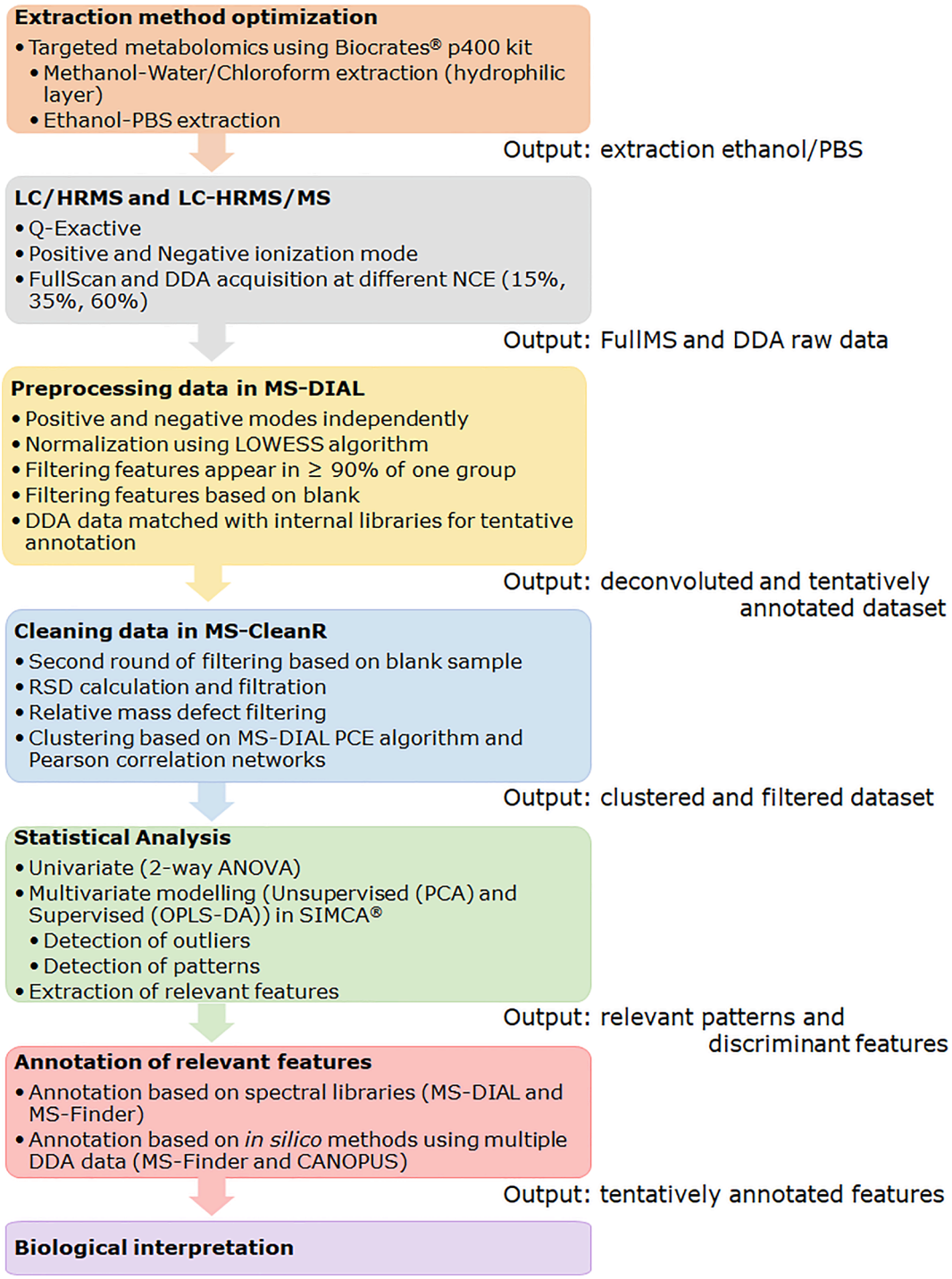

Fig. 2. Workflow of the metabolomics data processing.

2.5. Statistical Analyses.

2.5.1. Multivariate analysis

The processed dataset was log-transformed and Pareto-scaled in SIMCA® (v16; Sartorius Stedim Biotech, Umeå, Sweden) for multivariate analysis using unsupervised (principal component analysis, PCA) and supervised (orthogonal partial least-squares-discriminant analysis, OPLS-DA) models.

PCA was used to evaluate the instrumental variation (based on the QCs), detect potential outliers, and identify clustering patterns. Hotelling's T^2 test was used to statistically assess differences between groups and outlying samples of the ellipse region, defined as the 95% confidence interval of the modelled variation. OPLS-DA models were built to identify metabolite patterns or specific features discriminating between the different processing methods. In OPLS-DA modelling, it is possible to decompose data into "predictive" information related to the response to Y (in this study: the different processing methods of precooked chicken fillet) and "orthogonal" structured information that is uncorrelated to the response and could be associated with factors such as technical or biological variation, i.e., time in storage. The predictive and orthogonal components are characterised by respective types of variable importance in the projection (VIP) values, which help to interpret the most discriminant variables in the model. In this study, we included only the VIP values from the predictive component (VIPp). The default seven-

round cross-validation in the SIMCA® software package was applied for the discriminant analyses. Cross-validation of variance (CV-ANOVA) was performed to evaluate the reliability of the models, and p-values $\leq$ 0.05 were considered significant.

When two or more different OPLS-DA models contained the same reference group (i.e. HPP vs. VP, and MW vs. VP), we built Shared and Unique Structures (SUS)-plots using the p(corr) (a vector representing the correlation, hence the reliability, of the data in Y) values of each OPLS-DA model. This approach facilitated the extraction of relevant metabolites related to a specific model, allowing the identification of shared features between the two included models in either a similar or an inverse trend. A relatively strict cut-off of p(corr) $\geq |0.75|$ was applied for group-specific features and $\geq |0.5|$ for shared features.

2.5.2. Univariate analysis

For feature-wise analysis, a simple Welch's *t*-test was performed, comparing pairs of treatment classes individually. In this application, we used a false discovery rate cut-off of p-value < 0.05 to correct for multiple testing and false positives. Two-way ANOVA was applied to determine the effects of the SGS pre-treatment, VC-, HPP- or MW-processing, and period of storage as well as their respective interactions on the metabolome in the pre-cooked chicken fillet. In this case, we used the Bonferroni test with a cut-off $p < 0.05$ to correct for multiple testing and false positives.

2.6. Annotation

The annotation of metabolites that discriminated between processing methods started with interpreting feature cluster compositions and matching the information to the HRMS/MS data obtained for the QC sample by targeted analysis. Annotations were either attained automatically in MS-DIAL by matching the measured mass spectra to publicly available MS/MS spectrum repositories, comparing mass accuracies, RT, and isotope ratios (Tsugawa et al., 2015), or manually curated. Spectral similarity scores between measured features and reference metabolites were determined in MS-DIAL by utilising the combined values of dot-product and reverse dot-product, which are based on the relative sums of peak abundances and the matched fragments ratio. In the present study, we set the limit for the identification of metabolites to at least 850 (the maximum value of spectral similarity is 1000), and the best matches were selected. For the manually-curated annotation of the remaining features with unknown identity, we used the automated class assignment and ontology prediction tool CANOPUS (Dührkop et al., 2020) that is an integrative part of the SIRIUS software (v.4.4; online available) and does not require specific configuration settings for the systematic classification of metabolites from fragmentation spectra. The application of CANOPUS for the annotation of metabolite classes allowed us to set our findings in a biological context without the need to identify the exact nature of the features responsible for the observed variations in the metabolite profiles of the differently-processed pre-cooked chicken fillets. Using these applications, we were able to report the level of identification rigour for each relevant metabolite according to the proposed guidelines for metabolomics studies (Sumner et al., 2007):

3. Results and discussion

Consumers expect high-quality and innovative food products containing few additives that are safe to consume, sustainable, tasty, and can be stored for a substantial period of time. Novel processing technologies are developed to meet these demands by introducing gentle, nutrient-preserving conservation and sanitation methods. However, all treatments can influence the metabolite composition of processed food products, leading to differences in levels of, e.g. lipids, peptides, and carbohydrates (Kim et al., 2016; Li et al., 2021). Assessing the impact of new processing technologies on the food metabolome thus provides new knowledge regarding their safety and possible advantageous effects on quality parameters. Metabolomics is therefore increasingly used in food science due to the versatility of application purposes in, e.g. food safety, food authenticity, nutrition, and food processing. Metabolomic analysis can provide a broad overview of the chemical and biochemical changes in food and, consequently, make it possible to determine the impact of industrial processing on composition and quality. Notably, several pitfalls may hinder the exploitation of the full potential of food metabolomics and could lead to misleading interpretations. Especially, adequate and considerate statistical analyses of the acquired data are crucial to avoid the misidentification of discriminating metabolites, which could lead to inaccurate mapping of metabolic pathways or biological processes (Li et al., 2021). We have thus developed an analytical workflow including robust chemometric methods suitable for different food metabolomics applications. In the present study, this approach was used to investigate the effects of modern food processing methodologies such as SGS, VP, HPP, and MW on the metabolite composition of pre-cooked chicken fillets by untargeted metabolomic profiling with the aim to detect potential process-induced changes in food quality.

3.1. Optimisation of sample preparation and extraction

The food processing was conducted under conditions closely simulating industrial processing to increase their predictive value. Chicken fillets were obtained from a major producer of chicken products in Norway and processed in a food technology centre applying state-of-the-art cooking, chilling, and preservation equipment. The fillets were extracted using automated tissue homogenisation to reduce sample variation from preparation steps to a minimum, ensuring a sound starting point for the metabolomic analysis. Sample extraction was optimised in exploratory experiments showing that the EtOH-PBS extraction provided a broader metabolite coverage and produced generally higher concentrations of the metabolites included in the p400 Biocrates kit than the two-step MeOH-water/chloroform extraction (**Supplementary** Fig. 2). The one-step method was thus chosen for the extraction of all study samples. Fig. 2 presents a detailed overview of the procedures included in the metabolomics workflow.

3.2. Raw data processing and annotation

Raw data obtained by untargeted LC-HRMS analysis were initially processed using three different sequences of software tools (**Supplementary Table 1**). Workflow 1 and 2 used Compound Discoverer for pre-processing as the first step in the data processing pipeline, whereas in Workflow 3, MS-DIAL was used for this purpose. Comparison of the methods showed that models with similar characteristics were obtained using the three different workflows but that the numbers of relevant features were slightly varying. Most importantly, however, the rates of successful tentative metabolite annotations differed considerably, showing with 100% by at least CANOPUS class level the superiority of Workflow 3 due to improved data integration of the used software packages.

Consequently, all further data processing started with MS-DIAL (**Supplementary Table 2**), followed by data processing in MS-CleanR, together with MS-Finder, and CANOPUS. In MS-DIAL, only features present in at least 90% of the samples of one group (including QCs) were retained. We detected 3090 features (2241 in positive and 849 in negative ionisation mode, respectively) in the complete data set. Following quality assessment and clustering steps in MS-CleanR, 531 unique compounds were included in the statistical analyses (a detailed overview of all detected features can be provided on request). MS-CleanR assesses the quality of the data, detects ghost peaks, and merges connected features (i.e. different ions from both ionisation modes that are highly correlated and likely to arise from the same compound) into clusters. This step of the data processing generates "cleaner" datasets by reducing background noise and the inherent

collinearity typical in metabolomics datasets that could influence and compromise subsequent statistical analyses. In addition, the clustering process in MS-CleanR organised features together that were highly associated (i.e. adducts, in-source fragments, correlated features with opposite charge), increasing the confidence in the calculated molecular formulae, which is a prerequisite for correct compound annotation.

MS-Finder uses MS and MS/MS spectra of unknown compounds to determine molecular formulae and predicts structures, allowing comparison to databases and metabolite annotation. Our study showed the advantages of MS-Finder-supported *in-silico* classification of compounds, as used in our Workflows 2 and 3 (**Supplementary Table 1**) compared to the more traditional manual annotation in Workflow 1. The additional use of CANOPUS (Dührkop et al., 2020) further improved the yield of tentatively annotated features. CANOPUS provided the possibility to assign compound classes to otherwise unidentified features, for which no spectral reference data were available. Basing the CANOPUS computations on data generated in DDA mode during the HRMS/MS analysis, acquired by using different collision energies, provided better coverage of the MS/MS product ions. This was relevant in the annotation process as it allowed to query the different spectra separately and to determine the best matches.

It is noteworthy that MS-DIAL (Tsugawa et al., 2015) and MS-CleanR (Fraisier-Vannier et al., 2020) have several advantages over commercial software as they are cost-free, open-source, work with open-source format files (but also with vendor-formats in the most recent versions), process data relatively fast (as compared to other solutions used in our lab such as Compound Discoverer v3.1), and were developed to be integrated into continuous data processing workflows. Furthermore, their output can be easily imported into CANOPUS by only a few data reformatting steps.

3.3. Measures for increased stringency in statistical analyses

Evaluation of the output data of the MS-CleanR application by performing initial PCA, including visual inspection of the score plots in conjunction with the Hotelling's T^2 test, revealed that one sample in the SGS-MW group (day 1) had to be considered an outlier. It was therefore excluded from all further statistical analyses.

In the present study, we utilised established statistical tools to analyse metabolomics data while focusing on avoiding data over-interpretation. PCA plots comparing the differently-processed pre-cooked chicken fillets were generated (Fig. 3), and OPLS-DA models were calculated for the respective pairwise combinations (Table 1). Conclusions on their validity were not based solely on the determined scores (R^2X, R^2Y, Q^2), but also on CV-ANOVA p-values, which increases the validity of the results due to higher stringency (Wheelock & Wheelock, 2013). In a number of published metabolomics studies, the use of VIP projection values from OPLS-DA (or PLS-DA) has been shown to be purposeful for the extraction of discriminant variables. Comparably, we filtered our data with VIPp ≥ 1 as inclusion criterion, but in complex studies such as ours with many treatment groups, comparison between models can be impaired by changes in the VIP values of the selected variables for each iteration. Therefore, we based our selection criteria on the p(corr) values calculated for each relevant feature. Although there are no standardised cut-off values for the p(corr), the relatively high value set by us ($>|0.75|$) was favourable in the search for specific features discriminating between the treatment groups. The use of p(corr) values has been recommended as they are static and consider only the predictive weight in the model, making comparisons more feasible (Wheelock & Wheelock, 2013). Since p(corr) values are also suitable for comparisons across different models, we applied them in the statistical evaluation of three-dimensional SUS-plots, which facilitated the data interpretation and detection of significance in feature annotations.

3.4. Leads SGS pre-treatment to metabolome changes in differently-processed pre-cooked chicken fillets?

The comprehensive data set included six different treatment groups studied at two storage time points (Fig. 1). Consequently, numerous permutations of group comparisons were theoretically possible. We structured the approach by first studying the impact of SGS on the metabolite composition in SGS-treated vs. NSG pre-cooked chicken fillets subsequently processed by VP, MW, or HPP on day 1. The PCA score

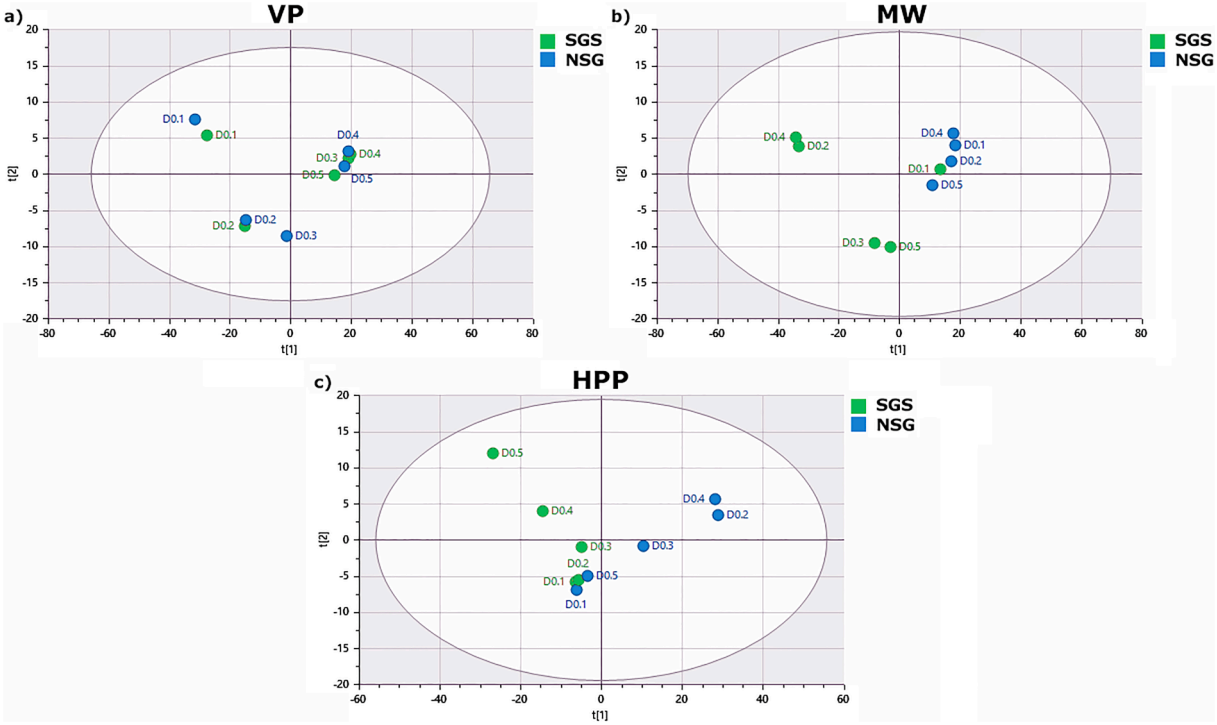

Fig. 3. PCA scores plot comparing VP-, HPP-, and MW-processed chicken fillets after NSG- and SGS-pre-treatment on day 1 of storage.

Table 1
Summary of OPLS-DA model characteristics for all pairwise comparisons of differently-processed chicken fillets (s. Fig. 1).

a) OPLS-DA Models comparing effects of SGS for HPP, MW and VP on day 1 of storage							
OPLS-DA Model	Processing	Day	LV	R^2X	R^2Y	Q^2	CV-ANOVA[a]
NSG vs. SGS	VP	1	n.m.[d]	–	–	–	–
NSG vs. SGS	MW	1	1 + 1 + 0	0.9	0.802	0.646	0.287
NSG vs. SGS	HPP	1	1 + 0 + 0	0.753	0.51	0.450	0.123
NSG vs. SGS	VP + MW + HPP	1	1 + 0 + 0	0.782	0.161	0.062	0.434
b) OPLS-DA Models comparing HPP and MW to VP w/w.o. SGS on day 1 of storage							
OPLS-DA Model	Processing	Day	LV	R^2X	R^2Y	Q^2	CV-ANOVA[a]
HPP vs. VP	NSG	1	n.m. [d]	–	–	–	–
MW vs. VP	NSG	1	n.m. [d]	–	–	–	–
HPP vs. VP	SGS	1	1 + 0 + 0	0.802	0.408	0.321	0.257
MW vs. VP	SGS	1	1 + 2 + 0	0.914	0.906	0.395	0.752
HPP vs. VP	NSG + SGS	1	n.m.[d]	0.772	0.094	−0.006	1
MW vs. VP	NSG + SGS	1	n.m.[d]	–	–	–	–
c) OPLS-DA Models comparing effects of SGS at different storage time points[b]							
OPLS-DA Model	Processing	Day	LV	R^2X	R^2Y	Q^2	CV-ANOVA[a]
NSG D1 vs. NSG D28	VP + MW + HPP	1; 28	1 + 1 + 0	0.771	0.932	0.861	3.03 E-10
SGS D1 vs. SGS D28	VP + MW + HPP	1; 28	1 + 2 + 0	0.807	0.971	0.933	7.63 E-13
d) OPLS-DA Models comparing effects of HPP, MW and VP at different storage time points[c]							
OPLS-DA Model	Processing	Day	LV	R^2X	R^2Y	Q^2	CV-ANOVA[a]
VP D1 vs. D28	NSG + SGS	1; 28	1 + 2 + 0	0.811	0.99	0.937	4.70 E-07
MW D1 vs. D28	NSG + SGS	1; 28	1 + 2 + 0	0.847	0.977	0.942	9.37 E-07
HPP D1 vs. D28	NSG + SGS	1; 28	1 + 1 + 0	0.738	0.942	0.855	3.83 E-06
e) OPLS-DA Models comparing effects of storage time for VP, MW and HPP w/w.o. SGS							
OPLS-DA Model	Processing	Day	LV	R^2X	R^2Y	Q^2	CV-ANOVA[a]
NSG D1 vs. D28	VP	1; 28	1 + 1 + 0	0.79	0.933	0.773	0.0719723
SGS D1 vs. D28	VP	1; 28	1 + 1 + 0	0.858	0.976	0.853	0.0257811
NSG D1 vs. D28	MW	1; 28	1 + 1 + 0	0.757	0.957	0.887	0.0354361
SGS D1 vs. D28	MW	1; 28	1 + 1 + 0	0.811	0.992	0.821	0.0413673
NSG D1 vs. D28	HPP	1; 28	1 + 2 + 0	0.839	0.96	0.848	0.0281981
SGS D1 vs. D28	HPP	1; 28	1 + 1 + 0	0.705	0.976	0.861	0.0226028

VP, vacuum packaging; HPP, high-pressure packaging; MW, microwave heating; SGS, soluble gas stabilization; NSG, no SGS pre-treatment; OPLS-DA, orthogonal partial least-square discriminant analysis; LV, latent variables; R^2X, total explained variance; R^2Y, goodness of fit; Q^2, predictive ability; CV-ANOVA, cross-validated ANOVA.

[a] CV-ANOVA p-values ≤ 0.05 were considered significant.
[b] significant metabolite features by S-plot in Table 2 and Fig. 4.
[c] significant metabolite features by SUS-plot in Table 3 and Fig. 5.
[d] no model could be built because the first predictive component was already not significant.

plots comparing SGS-VP vs. NSG-VP (Fig. 3**a**), SGS-MW vs. NSG-MW (Fig. 3**b**), and SGS-HPP vs. NSG-HPP (Fig. 3**c**) of the metabolite profiles revealed that there was considerable overlap between sample groups. The corresponding OPLS-DA models (Table 1**a**) were not significant (all CV-ANOVA p-values > 0.05), not showing any systematic pattern between the samples. Although a tendency for clustering in MW and HPP samples was perceptible in the PCA score plots, the within-group variation was too high and the sample numbers too low to reach a significant level in the OPLS-DA. More specifically, the SGS-treated samples spread considerably along the Y-axis in the OPLS-DA score plots indicating that the models could account for orthogonal variation. This was, however, not sufficient to create a valid predictive component. In a final attempt of defining the effect of SGS, we increased the sample numbers by merging the SGS and NSG samples of the different processing methods (Table 1**a**). This approach resulted, however, not in OPLS-DA models that could reveal systematic differences in the metabolite patterns between SGS-treated and -untreated samples on day 1.

3.5. Have different processing methods an effect on the metabolome of pre-cooked chicken fillets?

The potential impact of the different processing methods on the metabolite content in pre-cooked chicken fillets was investigated by pairwise comparisons of HPP vs. VP and MW vs. VP for both SGS and NSG samples on day 1 (Table 1**b**; Fig. 1). The VP samples were thus used as a common reference point in the corresponding OPLS-DA models, which did not indicate significant differences between the groups. The same was found when the SGS and NSG samples of each processing method were merged. Additionally-performed two-way ANOVA analysis investigating possible interactions of SGS pre-treatment and processing method did not show significant differences related to either SGS or processing method, nor did it reveal any interaction.

3.6. Can SGS pre-treatment mitigate time-dependent storage effects on the metabolome of pre-cooked chicken fillets?

A possible impact of the SGS pre-treatment on time-dependent changes in the metabolite contents in pre-cooked chicken fillets was investigated by comparing SGS- and NSG samples stored at 4 °C for one or 28 days. The data from the three processing methods were merged to increase sample numbers and focus on the SGS pre-treatment. Two highly significant OPLS-DA models were obtained for NSG-day 1 vs. NSG-day 28 and SGS-day 1 vs. SGS-day 28, respectively (Table 1**c**, **Supplementary** Fig. 3), showing a time-dependent effect of the storage period on the composition of the pre-cooked chicken fillets. When the p (corr) values of both OPLS-DA models were plotted against each other in a SUS-plot (**Supplementary Fig. 4**), it became evident that the observed changes were shared among groups and were therefore solely time-dependent. Features that changed exclusively as a result of the SGS pre-treatment were not identified. Forty-one features could be extracted from the SUS-plot as the most significant ($p(corr) > |0.5|$) for representing the time-dependency (Table 2**)**. The relative levels of 28 metabolites annotated in CANOPUS were increased after ended storage, while 13 were decreased. The absence of any SGS-related effect on the metabolite composition of the pre-cooked chicken fillets was confirmed using univariate statistical analysis by two-way ANOVA including all features. The analysis indicated a possible interaction of storage period

Table 2
Significant metabolite features extracted from SUS-plot combining the OPLS-DA models $NSG_{D1\ vs.\ D28}$ and $SGS_{D1\ vs.\ D28}$ (s. Table 1 and Fig. 4).

Code[a]	Clus. Size[b]	RT_{mean} [min]	m/z mean	Ion Form	Class CANOPUS	Level[c]	Id.[d] Level	Tentative Annotation	SGS[e] p (corr)	NSG[e] p (corr)
pos_2102	1	3.245	782.6556	$[M + Na]^+$	sphingolipids	class	3	–	0.8130	0.7759
pos_1670	2	3.347	370.2773	$[M + H\text{-}H_2O]^+$	carboxylic acid derivatives	subclass	3	*N*-palmitoyl methionine	−0.6883	−0.6691
pos_1718	1	3.358	386.3095	$[M + H]^+$	azole	class	3	–	−0.5924	−0.6635
pos_2127	1	3.359	820.5363	$[M + H]^+$	glycosyl-*N*-acylsphingosines	7	3	–	0.7906	0.6270
pos_1629	1	3.384	358.2774	$[M + H]^+$	α-amino acid amides	7	3	–	−0.6182	−0.5891
neg_660	1	3.459	319.227	$[M-H]^-$	hydroxyeicosatetraenoic acids	5	2	14R,15S-EpETrE	0.7442	0.6486
neg_621	2	3.490	295.228	$[M-H]^-$	linoleic acid derivatives	–	2	α -dimorphe-colic acid	0.6439	0.5809
neg_833	2	3.499	766.542	$[M-H]^-$	phosphatidylethanolamine	5	2	PE(18:0/20:4(5Z,8Z,11Z,14Z))	−0.5093	−0.5222
neg_827	2	3.536	746.518	$[M-H]^-$	amino acids	6	2	tetradecanoate	−0.5010	−0.5575
pos_1528	1	3.539	330.2631	$[M + H]^+$	acyl-carnitines	5	2	undenylcarnitine	0.8281	0.7715
neg_814	1	3.666	664.424	$[M-H]^-$	α -amino acid amides	7	2	1-pentadecanoyl-2-dodecanoyl-*glycero*-3-phosphoserine	0.8652	0.7971
pos_1348	1	3.739	286.2375	$[M + H]^+$	long-chain fatty acids	5	2	hexadecanoic acid, 16-amino-16-oxo-	0.9037	0.7162
neg_672	1	3.878	329.236	$[M-H]^-$	long-chain fatty acids	5	2	9-hydroxyocta-decanedioic acid	0.5999	0.7306
neg_259	2	3.901	171.103	$[M-H]^-$	medium-chain fatty acids	5	2	9-oxo-nonanoic acid	0.6557	0.6622
pos_1968	1	3.924	566.3803	$[M + H]^+$	phosphatidylcholines	5	2	1,2-didecanoyl PC	0.7882	0.6569
pos_1347	1	3.966	286.2012	$[M + H]^+$	acyl-carnitines	5	2	2-octenoyl-carnitine	0.8700	0.8421
neg_371	1	4.102	201.113	$[M-H]^-$	medium-chain hydroxy acids and derivatives	subclass	2	sebacic acid	0.8093	0.6550
neg_210	1	4.121	159.102	$[M-H]^-$	methyl-branched fatty acids	6	2	3-hydroxy-valproic acid	0.7350	0.6517
neg_260	1	4.298	171.103	$[M-H]^-$	medium-chain fatty acids	5	2	9-oxo-nonanoic acid	0.8222	0.8321
pos_1562	1	4.324	339.2032	$[M + H]^+$	hexoses	6	3	–	0.8589	0.8268
neg_773	2	4.421	494.292	$[M + C]^-$	α -amino acids and derivatives	6	3	4-{[4-(diethyl-amino)cyclohexa-2,5-dien-1-ylidene][4-(diethyl-amino) phenyl]methyl}-N,*N*-diethylaniline	0.8952	0.7899
pos_1610	1	4.474	353.2184	$[M + H]^+$	amino acid and derivatives	5	3	L-alanyl-L-leucyl-L-alanyl-L-proline	0.6193	0.5529
pos_782	1	4.482	210.1486	$[M + H]^+$	secondary carboxylic acid amides	6	3	–	0.9171	0.7650
pos_1857	1	4.594	461.3483	$[M + H]^+$	fatty acid esters	subclass	3	–	0.6172	0.5773
pos_707	1	6.343	200.1395	$[M + H]^+$	α -amino acids and derivatives	6	3	–	0.8361	0.8180
pos_1697	1	6.496	377.2542	$[M + H]^+$	dipeptides	6	3	–	0.6001	0.5439
neg_349	1	7.236	192.034	$[M-H]^-$	α -amino acids	7	3	γ -thiomethyl-glutamate or *N*-carboxy-L-methionine	−0.5080	−0.7198
neg_348	1	7.663	192.034	$[M-H]^-$	α -amino acids	7	3	γ -thiomethyl-glutamate or *N*-carboxy-L-methionine	−0.4998	−0.7122
neg_342	1	8.028	191.020	$[M-H]^-$	isocitrate	–	2	citric acid	0.6970	0.5574
pos_510	1	8.416	172.1080	$[M + H]^+$	guanidine	subclass	3	–	0.8788	0.9224
neg_145	1	8.594	142.051	$[M-H]^-$	α -amino acid and derivatives	6	2	5-OH-Me-cytidine	0.6245	0.5934
pos_207	1	8.669	127.0503	$[M + H]^+$	azole/imidazole	class	2	thymine	0.5837	0.6425
neg_347	1	9.343	192.034	$[M-H]^-$	amino acids	6	3	γ -thiomethyl-glutamate or *N*-carboxy-L-methionine	−0.5601	−0.7329
neg_147	1	9.497	142.051	$[M-H]^-$	amino acid and derivatives	5	3	6-oxopiperidine-2-carboxylic acid	0.7912	0.8106
pos_146	1	10.145	112.0507	$[M + H]^+$	pyrimidones	5	2	cytosine	−0.6228	−0.6209
neg_170	1	10.553	147.030	$[M-H]^-$	short-chain hydroxy acids and derivatives	7	2	D-2-hydroxy-glutaric acid	0.5949	0.5585
neg_522	6	11.734	242.079	$[M-H]^-$	pyrimidine nucleosides	class	2	cytidine	−0.6126	−0.7353
pos_1955	1	14.305	548.2128	$[M + H]^+$	γ -glutamyl peptides	6	3	–	−0.6592	−0.8164
pos_620	2	14.326	188.1033	$[M + H]^+$	*N*-acyl- α amino acids and derivatives	7	2	5-guanidino-3-methyl-2-oxopentanoic acid zwitterion	0.8461	0.7934
pos_1447	2	14.333	308.0906	$[M + H]^+$	γ -glutamyl peptides	6	2	glutathione	−0.6827	−0.8594
pos_1963	4	15.188	560.0801	$[M + H]^+$	purine ribonucleoside monophosphates	5	2	adenosine-5-diphosphoribose	−0.5619	−0.5339

[a] with ionization mode.
[b] cluster size.
[c] CANOPUS level: the classification considers the structure-based chemical taxonomy (ChemOnt) built on ClassyFire that uses only chemical structures and structural features for the automatic assignment of all known chemical compounds to a taxonomy consisting of > 4800 different categories. ChemOnt is organized as a tree, where the Kingdom is either Organic compounds or Inorganic compounds. Superclasses like Lipids and Lipid-like Molecules and Benzenoids are categories in a Kingdom. Pyrimidine nucleosides constitute a class, whereas guanidine is an example for a subclass. There can be up to 11 levels in the ontology.
[d] level of identification: level 1 – unambiguously identified metabolite; level 2 – putatively identified metabolite; level 3 – tentatively characterized metabolite class; level 4 – unidentified or unclassified metabolite, but differentiable.
[e] The direction of the change is indicated by the plus/minus sign in front of the number.

and SGS pre-treatment for only one feature corresponding to an ion with *m/z* 203.1397 (Bonferroni adjusted p-value = 0.035). It was tentatively annotated as the protonated molecule of the dipeptide alanyl-isoleucine.

3.7. Can different processing methods alter time-dependent storage effects on the metabolome of pre-cooked chicken fillets?

To study the impact of the three processing methods on the chicken metabolome during the storage period, we merged the data of SGS and NSG samples for each method. It was possible to build significant OPLS-DA models (CV-ANOVA p-value < 0.05) for, respectively, VP-day 1 vs. VP-day 28, MW-day 1 vs. MW-day 28, and HPP-day 1 vs. HPP-day 28 (Table 1**d**). Interestingly, when building a three-dimensional SUS-plot based on these three OPLS-DA models (**Supplementary Fig. 5**), we found that the majority of the observed changes in the metabolite compositions of the pre-cooked chicken fillets over time were similar for all three processing methods. We detected a relative decrease of 10 compounds and a relative increase of 28 compounds after 28 days of storage for VP, MW, and HPP, while 15 (7 increased, 8 decreased) features changed for at least two processing methods (**Supplementary Table 3**). Furthermore, the change of 10 compounds was observed for only one of the three processing methods. A two-way ANOVA, performed with the same data set, revealed that the interaction (processing method × SGS/NSG treatment) did not lead to significant differences. Thus, the observed changes in metabolite compositions were primarily dependent on storage time and were independent of the processing method (data not shown).

These findings were further investigated by generating OPLS-DA models for the pairwise comparison of samples from the storage days 1 and 28 belonging to the six treatment groups in this study (Table 1**e**, Fig. 1). It could be shown that neither the SGS pre-treatment nor the processing method did affect the metabolite composition, which was only dependent on the length of the storage period.

3.8. Importance of storage time for changes in the metabolome of pre-cooked chicken fillets – An indication of bacterial spoilage

Of all factors studied, the time in storage had the only significant impact on the metabolite profile in pre-cooked chicken fillets (Table 2, **Supplementary Table 3**). Changes in both directions, i.e., increasing and decreasing relative metabolite levels, could be observed (Fig. 4). Amino acids and their derivatives were the most affected of the denoted CANOPUS superclasses. Considering the diversity in this group, the deduction of underlying biological processes is challenging. A shift in the content of amino acid-related metabolites may imply the beginning denaturation of meat proteins. At the same time, the changes could be connected with starting bacterial growth. Several of the tentatively annotated features are related to the glutamine pathway: decreasing levels of γ-glutamyl-peptides, glutathione, and γ -thiomethyl-glutamate, a glutamic acid derivative occurring, e.g. in *E. coli* as a substrate of glutaminase, might indicate active microbial metabolism. Glutaminase catalyses the deamination of glutamine to glutamate, which is mainly responsible for causing the characteristic *umami* flavour, i.e. savoriness, in meat and fermented oriental food products such as soy sauce (Dermiki, Phanphensophon, Mottram, & Methven, 2013). However, L-glutaminase has also the capacity for γ -glutamyl transfer, possibly

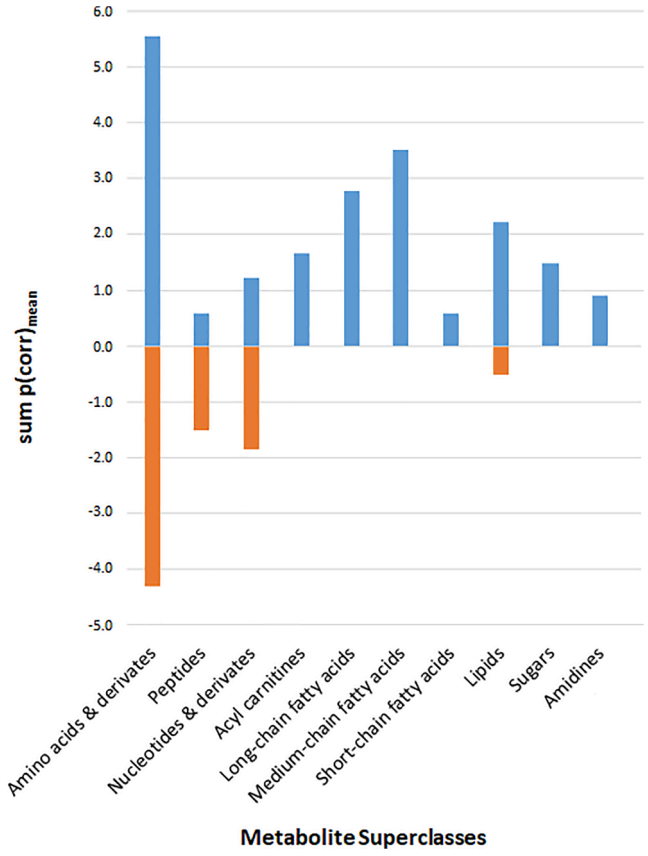

Fig. 4. Time-dependent changes (day 1 vs. day 28) in the metabolite profile of VP, HPP and MW-processed chicken fillets with or without SGS pre-treatment (Table 2). Tentatively annotated metabolites are divided in superclasses according to CANOPUS. The blue bars represent changes in positive direction, whereas the orange bars represent changes in negative direction. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

leading to a depletion of free glutamate and thereby diminishing the organoleptic properties and palatability of the product. Furthermore, γ -glutamyl-derived di- and tri-peptides, including glutathione, have been associated with the *kokumi* flavour (Yang, Bai, Zeng, & Cui, 2019), which has been described as a "taste enhancer" that magnifies and lengthens all the other five basic tastes. The observed decrease of these peptides (Table 2, Fig. 4) and of several nucleotides known for their contribution to *umami* proved that extended storage had an undesirable impact on the quality of the pre-cooked chicken fillets. Microbial activity was also suggested by the presence of amidine guanidine, which showed the highest relative level of all detected metabolites. In several bacteria, guanidine is produced from arginine and α-ketoglutarate and either used as a functional metabolite acting on guanidine-based riboswitches and transporters or as a nitrogen source in urea metabolism (Schneider et al., 2020). Interestingly, a significant formation of biogenic amines, decarboxylation products of free amino acids, such as putrescine and cadaverine (Wen et al., 2020) that are connected to meat

putrefaction, were not detected in the pre-cooked chicken fillets, showing the effectivity of the applied food processing methods to delay meat decay.

Several metabolites connected to the lipid metabolism, i.e. free fatty acids of different lengths, acyl-carnitines, and phospholipids, were significantly increased in the pre-cooked chicken fillets after 28 days of storage. This could mean that lipid deterioration processes had started. Lipid oxidation is an important cause for reduced meat quality, leading to undesirable changes in flavour, colour, and nutritive value (Love & Pearson, 1971). Both, intracellularly stored triglycerides and intracellular tissue phospholipids can be affected, the latter being especially labile due to their high content of unsaturated fatty acids. Changes in the oxidation state might thus lead to their release from the lipids and subsequent reaction with L-carnitine that is present in relatively high amounts in muscle tissue. Under physiological conditions, the formation of acyl-carnitines is necessary for transporting fatty acids across cell membranes into the mitochondria for subsequent β-oxidation (Reuter & Evans, 2012). Acylation of carnitine might still occur to a certain degree in stored meat, leading to considerably high levels as determined in the present study. This has, however, not been reported before, since comprehensive metabolomic analysis for food quality purposes is still rarely performed. Further studies should be initiated, focusing on the fate of different lipids during meat storage.

Alternatively, the observed rise in fatty acid and acylcarnitine levels could be associated with starting microbial infestation. The detected increase of several fatty acid esters (Table 2) could support this explanation because esters containing short-chain fatty acids such as acetic, butyric, isobutyric, propionic, or isovaleric acid are frequently found on spoiled meat as a result of bacterial metabolism (Casaburi, Piombino, Nychas, Villani, & Ercolini, 2015). Compared to the longer-chain fatty acids, fewer short-chain fatty acids were determined in the present study (Fig. 4); however, their detection by LC-MS is generally less efficient. The detection rate could be considerably improved using, e.g. gas chromatography (GC)-MS in additional experiments. Evidence for microbial activity on the stored pre-cooked chicken fillets was also indicated by the identification of phosphatidylethanolamines, which are used by the spoilage bacterium *B. thermosphacta* as a meat-derived energy source for the production of acetyl-CoA (Casaburi, De Filippis, Villani, & Ercolini, 2014).

During the storage period, the measurable levels of sugar metabolites increased significantly (Table 2, Fig. 4). The notable prevalence of hexoses strengthened the assumption of progressing bacterial growth since the extracellular polymers of bacteria contain D-glucose, D-galactose, and D-mannose, which were probably released by our sample preparation methods, including automated homogenisation and centrifugation (Brown & Lester, 1980).

3.9. Relevance of SGS, VP, MW, and HPP for the preservation of pre-cooked chicken fillets

SGS pre-treatment in combination with processing methods such as HPP (Argyri et al., 2018; Dang et al., 2021; Jackowska-Tracz & Tracz, 2015; Morales et al., 2009; Tananuwong, Chitsakun, & Tattiyakul, 2012) and MW (Apostolou et al., 2005; Morey et al., 2012; Zeinali et al., 2015) has the potential to decrease bacterial growth, thus preserving the physicochemical properties of pre-cooked chicken fillet and other meat products during storage (Dang et al., 2021; Rotabakk et al., 2006). In the present study, we did not observe that SGS pre-treatment caused significant changes in the metabolome of the pre-cooked chicken fillets or led to a reduction of bacterial growth during storage for 28 days. Thus, it appeared that SGS-treatment did not have a positive impact on the food quality and could be omitted.

Since the chicken fillets were pre-cooked in this study, the initial occurrence of bacteria was expected to be reduced, as we have found in a previous experiment (Dang et al., 2021). The anaerobic conditions that develop in VP-packaged meats can lead to changes in microbial compositions, favouring the growth of LAB, *Carnobacterium* spp. *and B. thermosphacta* (Baxter, 2000). These bacteria can shift to other substrates as major carbon sources such as amino acids, purine, and pyrimidine bases under aerobic or anaerobic conditions once that glucose is depleted. The deamination of amino acids or decomposition of nucleic acids can produce ammonia that is to some extent responsible for the off-odours of stored meat. Ketones such as diacetyl and acetoin have been reported as glucose metabolism byproducts in *B. thermosphacta*, *Carnobacterium* spp. and LAB (Casaburi et al., 2014; Pin, De Fernando, & Ordóñez, 2002). We were able to detect and tentatively annotate these compounds in the pre-cooked chicken fillet samples. Their respective levels did not change during storage, but their detection demonstrated the presence of metabolically active bacteria. Bacterial development in meat products depends on environmental conditions such as temperature, pH, and composition of the atmosphere. Packaging is, therefore, an effective method to regulate bacterial growth conditions. VP delays microbial growth during storage in combination with CO2-flushing, which enhances the antimicrobial effect (Baxter, 2000; Höll, Hilgarth, Geissler, Behr, & Vogel, 2020). It has, however, been reported that VP-packaging films are to some degree permeable for oxygen so that absolute anaerobic conditions cannot be achieved (Mangaraj, Goswami, & Mahajan, 2009). The greatest increase of bacterial counts in VP-packaged meat was observed between seven and 14 days of storage (Baxter, 2000). Considering the comparison of the different treatment groups in our study, VP was by far the most important measure for preserving the pre-cooked chicken fillets. Additional processing by MW or HPP did not result in recognisable changes in the metabolite profile during storage and implicitly did not improve resilience to bacteria.

HPP and MW apply physical stress during processing and have thus the potential to change metabolite profiles in treated products. The methods appear to have stronger effects on prokaryotic than eukaryotic cells, so that they are frequently used for sanitisation purposes. It has been shown that HPP reduces the viability of microorganisms by damaging the cell membrane and cell wall, compressing the vacuole and dissociating ribosomes (Garriga & Aymerich, 2009). At the same time, the technology was found to not notably alter the sensorial properties of cooked meat products. Supporting this evidence, we did not observe any significant impact of HPP at 600 MPa for 2 min or MW at 1 kW (3 × 15 s) on the metabolic signature of the pre-cooked chicken fillets in comparison to only VP-packaged samples, neither on day 1 nor day 28 of the storage period (Table 1). The observed changes in the metabolome were solely time-dependent and occurred regardless of the SGS-treatment and the processing method.

4. Conclusion

In conclusion, using extensive metabolomics with an optimised workflow for data processing and stringent statistical analysis, we were able to show that the shelf-life of pre-cooked chicken fillets depends mainly on effective VP-packaging. SGS pre-treatment prior packaging does not negatively affect the metabolic profile of the product, but does not have the potential to lengthen shelf-life either. The same was true for the modern food processing methods HPP and MW. While not changing the metabolite profile in the pre-cooked chicken fillets and therefore presumably not decreasing the sensory perception, these technologies did not provide any advantage concerning shelf-life within one month of storage.

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Oscar Daniel Rangel-Huerta: Conceptualization, Data curation, Formal analysis, Writing – original draft. **Silvio Uhlig:** Supervision, Writing – review & editing. **Lada Ivanova:** Methodology, Formal analysis. **Tem Thi Dang:** Conceptualization, Methodology. **Tone Mari Rode:** Conceptualization, Writing – review & editing, Supervision. **Estefanía Noriega Fernández:** Funding acquisition, Project administration. **Christiane Kruse Fæste:** Writing – original draft, Writing – review & editing, Project administration, Supervision.

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

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