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DOCTORAL THESIS

Optimising Analytical Methods for the Determination of Carbohydrates and their Derivates in Diverse Fermented Matrices

Dmitri Pismennõi

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Declaration:
Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for doctoral or equivalent academic degree.

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Analüütiliste meetodite optimeerimine süsivesikute ja nende derivaatide määramisekss erinevates fermenteeritud maatriksites

DMITRI PISMENNÕI

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Content

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Contribution to the papers in this thesis are:

I The author performed experiments related to the measurement of the selected group of analytes, prepared the tables and graphs for the manuscript, and helped with minor writing and editing of the manuscript.

II The author conducted most of the experiments, analysed the data and wrote the manuscript.

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IV The author conducted most of the experiments, analysed the data and wrote the manuscript.

Introduction

The growing population requires an increased food supply, making its sustainability questionable. Plant-based solutions to replace animal-derived products, such as milk and meat are gaining traction, offering a more sustainable way to meet dietary needs. The nutritional balance with plant-based products should be comparable to that with animal-based products, though the approach to achieving this is significantly different due to the individual natural properties of plant-based raw materials. As these materials are not always suitable in the "use-it-as-is" format, obtaining sustainable, healthy, balanced plant-based products presents a complex technological challenge. A potential solution is to ferment plant-based materials, which could significantly improve the properties of the final products and make them more desirable for the customer to procure.

The fermentation processes are well known and have been extensively studied for animal-based products, such as fermented dairy products: cheeses, kefirs, sour milk and yoghurts. On the other hand, the fermentation processes in plant-based raw materials are still poorly studied primarily due to the wide variety of these materials. Nuts, legumes, cereals and pseudocereals are all part of this group of materials. Their initial chemical, organoleptic and nutritional properties vary widely between species based on geographical, environmental, and seasonal factors. Therefore, their fermentation processes are highly dependent on the microorganism used and the availability of nutrients for microorganisms' growth. Bacteria have a lot of common pathways in utilising a wide variety of carbohydrates to proliferate and produce important metabolites and molecules responsible for flavour. The choice of the starting culture is highly dependent on the availability of carbohydrates in a selected plant-based raw material and the ability of a microorganism to digest said carbohydrates. Therefore, carbohydrate measurements play a significant role in optimising and describing fermentation processes in plant-based raw materials with suitable starter cultures. The diversity of carbohydrates found in nature presents a complex analytical challenge in measuring these sugars accurately and quickly, while obtaining valuable information about the composition of said analytes with a high degree of confidence.

This thesis aims to improve the methodology for the measurement of various carbohydrates in a wide range of plant-based raw materials to assess the consumption of carbohydrates with different starter cultures and their effects on final plant-based yoghurt alternatives and to explore the application of sugar derivatives in the lifecycles of common bacteria to provide improved input for metabolic modelling calculations.

Abbreviations

(*v/v*)	Volume percent
AmAc	Ammonium acetate
AmFor	Ammonium formate
BCW	Bacterial cell wall
BTH	*Bacteroides thetaiotaomicron*
DEA	Diethylamine
ECO	*Escherichia coli*
EPS	Exopolysaccharides
GluN	Glucosamine
GuHCl	Guanidine hydrochloride
HCl	Hydrochloric acid
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High-performance liquid chromatography
IEX	Ion exchange chromatography
IPA	Isopropanol
ISTD	Internal standard
LAB	Lactic acid bacteria
LC-MS	Liquid chromatography coupled to mass spectrometry
LLOQ	The lower limit of quantitation
LOD	Limit of detection
LOQ	Limit of quantitation
m/z	Mass-to-charge ratio
MeCN	Acetonitrile
MeOH	Methanol
MilliQ	Milli-Q® purified water (18.2 MΩ·cm)
MS	Mass spectrometry
Mur	Muramic acid
MWCO	Molecular weight cut-off
NAG	N-acetylglucosamine
NAM	N-acetylmuramic acid
PCA	Principal component analysis
PG	Peptidoglycan
PSDVB	Polystyrene divinylbenzene
SC	Starter culture
SD	Standard deviation
SIR	Selected ion recording
SPE	Solid phase extraction
STH	*Streptococcus salivarius* ssp. *thermophilus*
TIC	Total ion chromatogram

1 Literature overview

1.1 Fermentation of dairy-based yoghurts

For centuries, fermentation has been the standard technique for improving dairy products' sensory, nutritional and health properties [1]. Fermented dairy products are commonly produced using lactic acid bacteria (LAB). One of the most produced fermented dairy products is yoghurt. The most common approach to the production of yoghurts starts with mixing dairy material with starter cultures consisting of the *Streptococcus thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus* bacterial strains. The cooperative mechanism between these two strains induces a cascade of chemical changes in the starting material by growing on abundantly available dairy sugars and proteins (Figure 1).

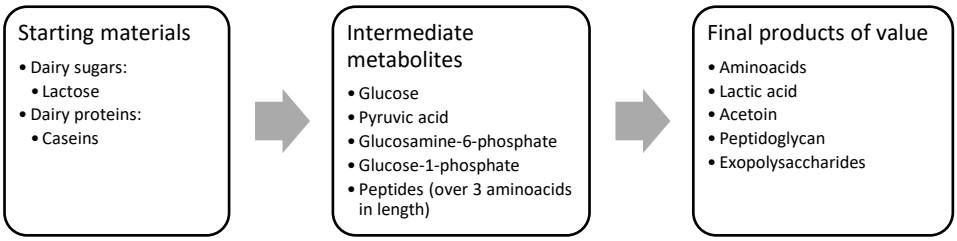


Figure 1. The general scheme of metabolism in lactic acid bacteria with selected examples of produced intermediate metabolites and end products in dairy starting materials

During fermentation, LABs efficiently utilise abundant dairy sugars, such as lactose, as fuel for their central metabolic processes, generating a diverse array of essential metabolites and final products of value. Dairy proteins and their proteolysis during the fermentation process are crucial due to the inability of selected LAB to synthesise amino acids to facilitate growth and produce important intermediate metabolites, which are integral to the bacteria [2]. In general, LAB possesses multiple transport systems to uptake different carbohydrate sources to facilitate the production of fermentation end products, e.g. L-lactic acid, acetoin and exopolysaccharides, which are responsible for the rheological, organoleptic, and chemical properties of fermented dairy products [2–4]. Due to the constant production of L-lactic acid during fermentation by LAB, the consumption of the starting material, for instance carbohydrates or proteins, and acidification rates should be monitored instrumentally to ensure the consistent quality of the final fermented product during the product development phase. The fermentation process usually stops when the fermented medium achieves a relatively low pH of 4.0 to 4.5 [5]. By prolonging fermentation and allowing LAB to consume alternative carbon sources, the accumulation of lactic acid could cause irreversible changes to the properties of a final product: excessively sour taste, undesired texture, pungent aroma, etc. Therefore, precisely monitoring changes by measuring pH, doing off-gas analysis, or detecting changes in sugar concentration between starting and final points during fermentation is essential to avoid unwanted effects on the final product's properties. Despite the health benefits of various fermented dairy products (*i.e.,* immunoactivity-promoting properties and balanced nutritional profile), the dairy industry causes environmental damage if farming practices are not kept sustainable.

Examples of such harmful effects are excessive excretion of manure into surface water, which promotes algal growth; methane production as a result of the dairy herd's lifecycle; and enormous land usage to accommodate dairy herds [6–8]. To alleviate or reduce environmental impact, alternative approaches based on the use of plant materials to obtain fermented products with similar or better health properties are rapidly gaining popularity in society [9,10] and challenging the food industry to develop new technological approaches as quickly as possible (i.e. within a decade).

1.2 Fermentation of dairy-alternative products

Recently, the market for alternative proteins has grown extensively in the EU, with alternative milk products having increasing sales [11,12]. While yoghurt alternatives make up only 9% of the alternative produce market, they contribute to over 9% of the total market, with a 10% growth rate, while dairy yoghurt sales have dropped by 4% over the same period [12]. This indicates a demand for alternative fermented non-dairy products, rapidly developing to fill this niche. The alternative non-dairy yoghurts mainly comprise different legumes, seeds, nuts, cereals and pseudocereals [13]. The wide variety of starting materials drastically affects the final qualities of fermented products, which should be reflected in customer satisfaction. The main problem with fermented non-animal-based products, such as milk, meat or yoghurts, are associated with strong astringent off-flavours in the starting material[14], and the presence of toxic substances, e.g., aflatoxins in peanuts [15]. Applying different bacterial strains, including LABs, could potentially solve those problems by reducing the concentrations of astringent or potentially hazardous compounds and improving the sensory parameters of end products.

However, applying bacterial cultures to raw, unprocessed plant materials to facilitate fermentation is not enough and is not as easy as with dairy products, as non-dairy starting material can be resistant to processing due to the natural protection mechanisms of the harvested materials. Therefore, raw plant materials are processed to aid fermentation performance and improve final product qualities. These processes can be subdivided into five large groups: disruption, extraction, formulation, fermentation and packaging [16]. Disruption processes are designed to break down natural protective layers of used raw natural materials of non-dairy origins. As a result of disruption processes, a lot of nutritionally beneficial compounds are released and made available for further processing. Still, undesirable or hazardous components of raw materials might also be present in the same medium. Thus, extraction processes are used to separate and gather as much as possible while removing components of unfavourable properties. The extraction processes may be mechanical, based on phase separation or particle size, and enzymes can be applied to digest larger molecules, such as starch, into a more processable form and improve the product's quality markers. During the formulation processing, extracted raw materials can be supplemented with additional nutritional components for further down-processing or to improve the final product's nutritional or sensory properties [13]. Applying all of those processing steps, the starting material can be used as a substrate for LAB to produce non-dairy fermented products more associated with traditional dairy alternatives. However, the processed starting material must still be compatible with the fermentative capabilities of LAB. Being one of the most studied microorganisms, LAB's main fermentation product is lactic acid, which is responsible for decreased pH in dairy products and thus is the primary factor in yoghurt's final quality attributes. Lactic acid is produced from glucose, which is made from sucrose or lactose,

depending on the substrate [2]. In a non-dairy starting material, sucrose or lactose might not be the dominant carbohydrate or even be present as many plant materials are prevalent in α-galactosides, *e.g.* raffinose or stachyose. Such complex carbohydrates require a particular enzyme, α-galactosidase, to digest those larger carbohydrates and not cause gastric problem from consuming plant-based products [17]. The utilisation of large carbohydrates by LAB strains can be bidirectional in terms of either the consumption or digestion of large oligosaccharides to facilitate the production of a substrate for fermentation or to secrete exopolysaccharides (EPS) into extracellular space. EPS are usually large molecules with molecular weights over 1 MDa and can be distinguished by their composition as either homoEPS or heteroEPS [18]. Due to the wide variety of EPS compositions synthesised by different LAB strains, the final properties of the product can be significantly influenced by the choice of EPS-producing strains and the choice of starting substrates. Thus, the importance of EPS lies in their protective and communication functions to the cell's potential antimicrobial, antioxidative and antitumour activity. Furthermore, EPS are vital for the food industry, as their routine application can significantly improve the organoleptic feeling of the final product by modifying the rheological properties of the final fermented product [19]. Overall, a wide variety of carbohydrates and their breakdown or biosynthesis products significantly affect any fermented products' organoleptic, chemical, and biological properties by either promoting or suppressing specific bacterial starter culture growth throughout the lifecycle.

1.3 Production and application of carbohydrate derivates during bacteria lifecycle

Considering the simplest metabolic model for any microorganism, the starting point for any process is the biotransformation of a glucose substrate to produce different molecules, which all play essential roles in the bacterial lifecycle [2]. One of the crucial parts of bacteria physiology is the bacterial cell wall (BCW), which shapes and protects bacteria from harmful external conditions. One of the main components for constructing BCW is peptidoglycan (PG), a polymeric structure consisting of carbohydrate moieties and interlinked amino acids [20].

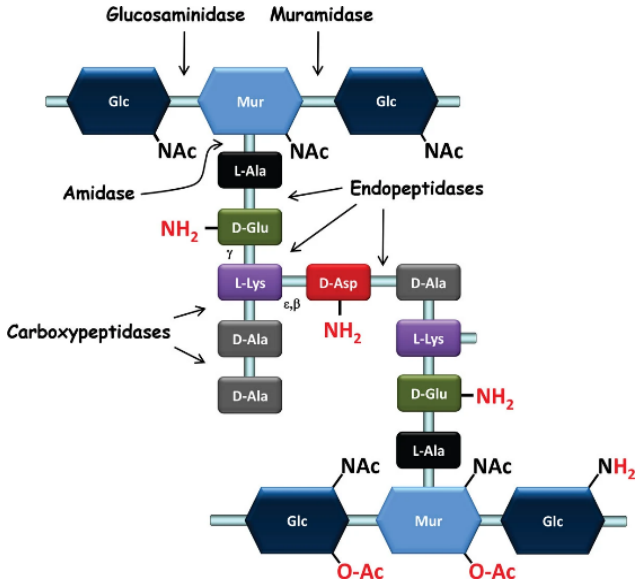


Figure 2. An example of a peptidoglycan structure is found in Lactobacillus Lactis, one of a LAB's representatives. Abbreviations used in the figure: Glc–glucosamine; Mur – muramic acid; NAc – N-acetylation; L-Ala – L-alanine; D-Glu – D-glutamic acid; L-Lys – L-lysine; D-Asp – D-aspartic acid; O-Ac – O-acetylation. Adapted from [21] under CC BY 4.0 license

Due to the complex nature of the PG structure and its significance to the lifecycle, all bacteria can synthesise PG from scratch in terms of starting the substrate. Glucose is, therefore, one of the most important substrates for any organism due to the ability of most microorganisms to biotransform it into any vital metabolite. The synthesis of PG starts with glucose uptake into the cell, attaching the phosphate group and inversing glucose-6-phosphate to fructose-6-phosphate via several enzyme-catalysed reactions. Afterwards, fructose-6-phosphate undergoes several more biotransformations until the polymeric carbohydrate-peptide chain is fully formed to be used afterwards during cell proliferation and division [22]. The thickness of PG can significantly influence the overall structural and functional properties of BCW, as well as being a host for any additional functionality anchored at the surface of BCW, such as teichoic acids, lipopolysaccharides and lipoproteins [20]. These functional groups provide bacteria with the storage of essential cations and protons for maintaining the pH gradient across cell walls [23] and morphological properties to allow cell division [24]. With recent developments in analytical methodologies and the rising popularity of metabolic computations, PG has regained its importance in basic understandings of how muropeptides can be used as signal molecules in bacterial interactions [25] or as targets for potential new antimicrobial compounds and, thus, a better understanding of hosts' immune response [26]. In addition, a recent study on cell size changes during bacterial growth showed the importance of knowing the structure and composition of BCW, which includes PG, to predict cell length or size based on growth rates [27]. However, our understanding of how BCW and, therefore, PG and all other constituents participate in communication between bacteria or any other host is quite limited. Improvements in this field require the application of specialised analytical techniques from several fields of instrumental analysis.

1.4 Analytical approaches to measure carbohydrates and their derivates

The analysis of carbohydrates in different matrices has a very long history due to the importance of carbohydrates in every aspect of the life cycle of any living organism. From paper partition separation in 1949 [28] to the present, when carbohydrate measurements are done virtually on any analytical instrument in the analytical laboratory, this wide variety of methods is beneficial because each can be finely tuned for more specific measurements of carbohydrates, providing advantages over others in different situations (Table 1).

Table 1. A non-exhaustive list of commonly used analytical techniques for measuring carbohydrates in various matrices.

Analytical technique	Class of carbohydrate	Reference
Paper partition chromatography	C5 and C6 carbohydrate	[28]
Ion exchange chromatography	Mono, di, trisaccharides from plant extract	[29]
	Mono, di, and trisaccharides from fermentation broths	[30]
	Mono and disaccharides from beverages and food	[31]
Capillary electrophoresis	Mono and disaccharides from plant extracts and fibres	[32]
	Mono and disaccharides from fermentation broth	[33]
Nuclear magnetic resonance spectroscopy	Carbohydrate profiling in non-fractioned beer	[34]
Gas chromatography	Monosaccharides from plant extract	[35]
	Monosaccharides from forest soil	[36]
High-performance liquid chromatography	Mono and disaccharides from milk	[37]
	C5 and C6 monosaccharides in urine	[38]
	Oligosaccharides from fermentation broths	[39]
	Mono and disaccharides in plant-based yoghurt alternatives	[40]
Fourier transform infrared spectroscopy	Mono and disaccharides in fruit juices	[41]
	Mono, di and polysaccharides in onion juices	[42]
Enzymatic assay	Sucrose in corn plants	[43]

This wide variety of methodologies used to analyse a vast assortment of different carbohydrates reveals the complexity of the measurement of carbohydrates. The main challenges in carbohydrate analysis are related to the lack of analyte-specific detection markers. They do not have any natural chromophores in the structure, limiting the use of specific ultraviolet or fluorescence detection; they are not readily volatile under gas chromatographic analysis, requiring a complex derivatisation process to improve volatility or introduce a suitable chromophore. These challenges are particularly expressed in the analysis of sugars with capillary electrophoresis, where either derivatisation or a specific sample preparation treatment is required [44–46]. Similar problems arise in the case of gas chromatographic analysis, where additional complexity in the form of multiple chromatographic peaks per single sugar appears, e.g., four peaks of glucose due to alpha- and beta isomers of both pyranose and furanose after derivatisation with trifluoracetic acid [47]. Spectroscopic techniques are also applicable with preference for the speed of analysis performance compared to any chromatographic or electrophoretic separation. Yet, they require comparatively expensive analytical instrumentation, such as high-resolution NMR [48] or performing analytical measurements and statistical modelling to quantify sugars with infrared spectroscopy [49]. On the other hand, liquid chromatography provides an efficient solution to measure carbohydrates in various matrices. Despite advances in instrumentation over the past 50 years, some methods are still based on stationary phases from before 1970 [50] in the form of Bio-Rad Aminex® HPX columns or equivalent columns from other manufacturers. The polystyrene divinylbenzene beads (PSDVB) used in this column type cannot separate different molecules by themselves; they must be doped with appropriate ionic ligands to facilitate separation by exploring ligand exchange or size exclusion mechanisms. While there are viable alternatives for measuring carbohydrate content in samples, this stationary phase has been dominant due to ease of operations. However, the main drawbacks of PSDVB-based columns are 1) they cannot separate multiple sugars with high enough resolution belonging to the same class, i.e. disaccharide resolution – Ca^{2+}-doped PSDBV column cannot resolve maltose from sucrose; 2) oligosaccharide resolution is also limited, requiring multiple differently doped PSDBV columns to resolve oligosaccharide with up to 10 monomeric units; 3) particles of PSDVB are usually 8 to 10 µm, which is suboptimal in terms of throughput or resolution due to lower number of theoretical plates per column length. Moreover, PSDVB-based stationary phases cannot withstand backpressures above 2000 psi or, in most cases, even 1000 psi, required for shorter run times due to the increased flow rate of a mobile phase, thus limiting the potential for high throughput analysis. The search for solutions to those challenges with PSDVB stationary columns resulted in the creation of alternative amino- or amide-based stationary phases, which belong to hydrophilic interaction chromatographic (HILIC) types of stationary phases. HILIC is characterised as an alternative to reversed phase chromatography and is the extension of normal phase chromatography employing a combination of commonly used aqueous mobile phases with stationary phases of high hydrophilicity [51]. Typical commercial examples of HILIC phases are Waters Corporations' BEH Amide, Resonac' Shodex Asahipak NH2P-50 4E, Phenomenex' Luna Omega Sugar, and comparable amino or amide-based columns with greater mechanical and chemical strength compared to ion exchange stationary phases provided by other manufacturers. Despite all of the recent advances in improving stationary phase resistance, the core problem with measuring various carbohydrates lies in analysing isomers due to their being almost completely identical in terms of physical properties.

Isomerism in carbohydrates is one of the most complex analytical challenges to overcome. Carbohydrates can be present in multiple isomeric forms, e.g. D- and L-isomers, α and β anomers, epimers, pyranose and furanose ring structures, and aldose-ketose isomerism. All of these isomers, which can be dynamically present at varying proportions in a solution, increase the difficulty of analysing carbohydrates in a complex mixture [52]. For instance, in the classic example of D-glucose, a composition of five different forms of D-glucose in aqueous solutions at 31°C is complicit with ratios of *38:62:0.5:0.5:0.002*, which corresponds to α-D-glucopyranose: β-D-glucopyranose: α-D-glucofuranose: β-D-glucofuranose: D-glucose(open chain) (Figure 3). Changing the temperature of the solution might significantly influence the final composition of a present carbohydrate, shifting the equilibrium towards one form or another. Additionally, the equilibrium can be influenced by the presence of a base or acid. However, these challenges can be overcome by applying specific derivatisation agents, using several chromatographic columns with different selectivity to characterise the sample's composition fully, or applying several instrumental methods to evaluate the sample comprehensively.

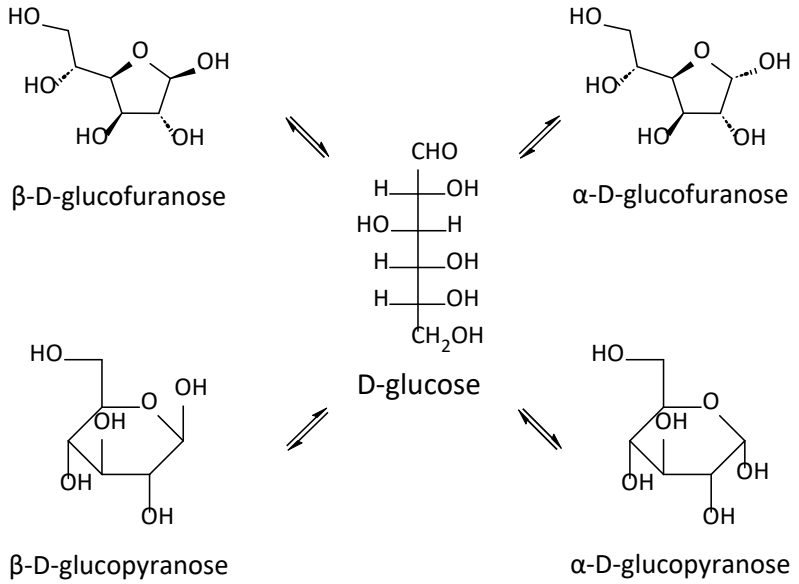


Figure 3. Dynamic distribution of D-glucose in water solutions at 31 °C [53]

The degree of complexity increases with polymeric carbohydrates, which can contain different monomeric substituents with varying positions of glycosidic bonds (Figure 4).

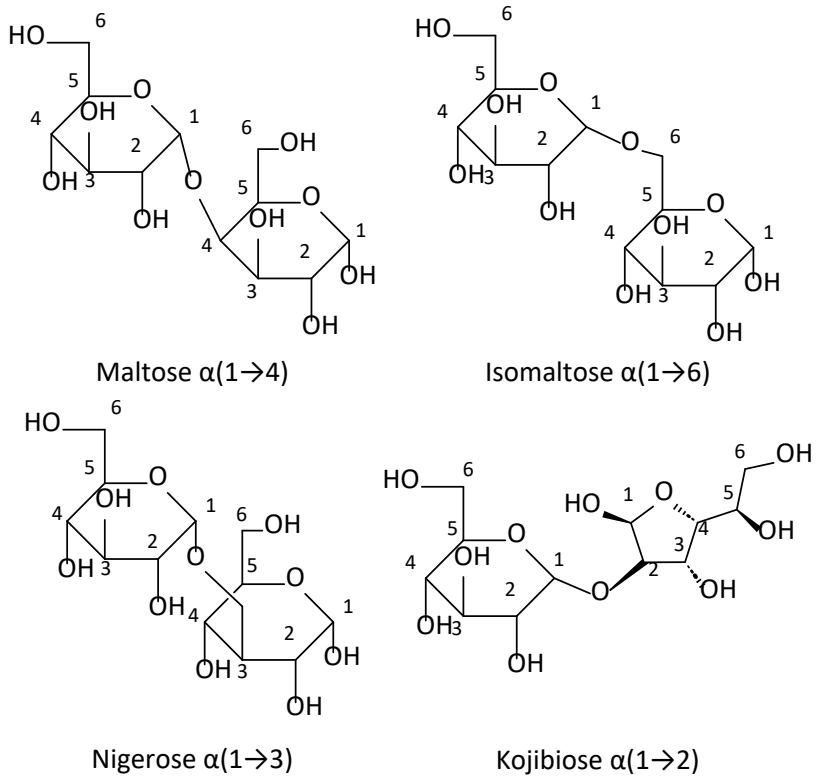


Figure 4. The examples of glycosidic bonds' locations in naturally occurring disaccharides

With advancements in complementary separation methods, such as cyclic ion mobility spectroscopy, it has become easier to separate large oligosaccharides or starting synthesis blocks made up of monosaccharides without complex sample preparation for evaluating their structures or anomeric compositions [54,55]. However, it should be noted that the separation is one part of a complex problem; the other part is how to quantify the separated sugars from various sources. In liquid chromatography, refractive index, evaporative light scattering, pulsed amperometric and mass spectrometric detection are the most common methods (Table 2). Additionally, ultraviolet detection is used whenever a specific chromophore is covalently attached to a carbohydrate via a derivatisation reaction [56].

Table 2. A general comparison between popular detection techniques used for sugar quantification. The "+" means good, "++" excellent, and "-" acceptable or poor. [57]

	Refractive index	Evaporative light scattering	Pulsed amperometry	Mass spectrometry
Sensitivity	+	++	++	++
Selectivity	-	-	-	+
Ease of operation	++	-	-	-
Gradient elution	-	+	+	+
Cost of operation (high marks – cheaper)	++	-	-	-

Despite even more detection techniques, such as nuclear magnetic resonance or optical rotation polarimetry, they still require a properly purified and isolated sugar to determine an analyte's absolute configuration.

In sum, these various approaches in separation or detection help define the composition and structural properties of various naturally and non-naturally occurring carbohydrates in the shortest possible time. However, the complexity of the sample matrix plays a significant role in choosing a suitable separation/detection technique. An incorrect or inefficient choice might lead to multiple days spent on sample preparation, analysis or data interpretation, depending on the complexity of the sample.

2 Aims of the study

The specific aims of the thesis are:

- The measurement of mono-, di- and trisaccharides during the development of oat-based fermented drinks, using a Bio-Rad Aminex© HPX-87C column together with refractive index detection,
- Improving the measurement of mono- and disaccharides with an application of novel HILIC stationary phases coupled with mass spectrometric detection in various fermented matrices,
- Determining the concentration of aminosugars through the HILIC-MS-based method in bacterial cell walls to improve metabolic modelling calculations.

3 Materials and methods

Below is a summary of the methodologies used in this work. Appendices 1 to 4 contain detailed explanations of the methodologies used and results.

3.1 The measurement of mono-, di- and trisaccharides in oat-based fermented drinks using IEX-RI (Study 1)

The fermentation of oat-based drinks with various LAB starter cultures was performed in-house at AS TFTAK (Tallinn, Estonia). The samples were provided after enzymatic treatment and at various sampling points during fermentation. The samples were cleaned with 3 kDa MWCO filters (Amicon Ultra 0.5, Merck KGaA, Darmstadt, Germany) spined at 14 000 *×g* for 20 minutes to trap all large interfering particles and molecules to obtain clean supernatant. The supernatant was diluted two times with MilliQ water, and 20 µl was injected into the HPLC system (Alliance 2695, Waters Corporation, Milford, MA, USA). A saccharide elution was performed using Bio-Rad Aminex© HPX-87C column (7.8x300 mm, 9µm, Bio-Rad Laboratories, Inc., CA, USA) heated in a column oven to 85 °C. The mobile phase was MilliQ water flowing at 0.6 ml/min. Eluted saccharides were detected with a refractive index detector (Waters 2414 RID, Waters Corporation, Milford, MA, USA). Due to the inability of IEX to separate isobaric compounds within the same group of carbohydrates, several marker compounds were used to represent a sum of appropriate compounds based on the length of saccharides: raffinose (trisaccharide marker), maltose (disaccharide marker) and glucose (monosaccharide marker). The standard compounds for the analysis were at least 99% pure and procured from Sigma-Aldrich (Darmstadt, Germany).

3.2 The measurement of mono- and disaccharides in the fermentation broth of *Streptococcus thermophilus* with HILIC-MS (Study 2)

A novel instrumental methodology based on an amino-based silica stationary phase was developed to improve the measurement of mono- and disaccharides in various fermentation matrices. Waters Acquity BEH HILIC (2.1x100 mm, 1.7 µm, Waters Corporation, Milford, MA, USA), Waters XBridge BEH Amide XP (3.0x150mm, 2.5 µm, Waters Corporation, Milford, MA, USA) and Phenomenex Luna Omega Sugar (2.1x150mm, 3 µm, Phenomenex Inc., Torrance, CA, USA) were screened for the best resolution between five selected sugars: fructose, glucose, galactose, sucrose and lactose. A Phenomenex Luna Omega Sugar column was chosen as it delivered the most consistent chromatographic performance. The analyte separation was achieved using a gradient elution of two mobile phases on the Waters Acquity UPLC© system (Waters Corporation, Milford, MA, USA). Mobile phase A was 99% MilliQ with 1% MeCN and 1 mg/L of GuHCl (*v/v*), and mobile phase B was 99% MeCN with 1% MilliQ and 1 mg/L of GuHCl (*v/v*). The detection of eluted saccharides was achieved using a mass spectrometer (Waters Quattro Premier XE, Waters Corporation, Milford, MA, USA) operated in SIR mode, looking for [M+Cl]- adducts of analytes. LC-MS measurements were done using two isotopically labelled standards, glucose-$^{13}C_6$ and lactose-$^{13}C_6$, to improve the accuracy and precision of the methodology. Isotopically labelled standards were procured from Sigma-Aldrich (Darmstadt, Germany) and Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA). Several sample clean-up procedures were tested: SPE with RP (Biotage Isolute® C18, 100 mg/1 mL, Biotage AB, Uppsala, Sweden),

NH_2 (Biotage Isolute® NH2, 100 mg/1 mL, Biotage AB, Uppsala, Sweden) and PLD (Biotage Isolute® PLD+, 50 mg/1 mL, Biotage AB, Uppsala, Sweden) stationary phases, MWCO filtration (Amicon Ultra 0.5, Merck KGaA, Darmstadt, Germany) and a simple dilute and filtrate approach with Millex-LCR filters (0.2 µm, Merck KGaA, Darmstadt, Germany). The methodology was applied to *Streptococcus thermophilus* fermentation broth to measure the change in carbohydrate concentration during the growth of bacteria. The fermentation was performed in-house at AS TFTAK (Tallinn, Estonia).

3.3 The analysis of mono- and disaccharides in plant-based yoghurt alternatives using HILIC-MS (Appendix 3)

A simplified cut-off filtration was applied to all samples to remove any large particles or biomolecules over the 3 kDa weight limit (Amicon Ultra 0.5, Merck KGaA, Darmstadt, Germany), which can interfere with the results of an analysis. Carbohydrate content and composition were described using a modified procedure from Publication 2 based on HILIC-MS methodology. Updated HILIC-MS methodology was done on Waters Acquity BEH Amide XP column (3.0 x 150 mm, 2.5 µm, Waters Corporation, Milford, MA, USA), and analytes of interest were detected with Waters Acquity QDa single quadrupole mass detector (Waters Corporation, Milford, MA, USA). The sugars were eluted using a gradient system consisting of two mobile phases. Mobile phase A was 80/20 MeCN/MilliQ (*v/v*), and mobile phase B was 90/5/5 MeCN/MilliQ/IPA (*v/v*). Both mobile phases additionally contained 0.5% of DEA (*v/v*) and 0.5 mg/L of GuHCl (*v/v*). The primary analytes of interest were fructose, glucose, sucrose, and maltose. The standard compounds were procured from Sigma-Aldrich (Darmstadt, Germany) and were at least 99% pure. The instrumental and sample preparation variations were compensated for with isotopically labelled internal standards – fructose-$^{13}C_6$, glucose-$^{13}C_6$, sucrose-$^{13}C_6$ and maltose-$^{13}C_{12}$. All isotopically labelled standards were procured from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA). Additional chemical and microbiological analyses were conducted within the analytical department at AS TFTAK (Tallinn, Estonia).

3.4 The measurement of aminosugars in bacterial cell walls of Gram-positive and Gram-negative bacteria with HILIC-MS (Appendix 4)

Streptococcus salivarius ssp. thermophilus DSM20259, *Escherichia coli* K12 MG1655 and *Bacteroides thetaiotaomicron* DSM 2079 were cultivated with optimal conditions for each strain in-house at AS TFTAK (Tallinn, Estonia). Fermentation broths were washed, concentrated and freeze-dried before commencing an analysis. Freeze-dried samples were subjected to chemical hydrolysis in 6N HCl with 1% phenol (*v/v*). The hydrolysis was performed in an Eppendorf Thermomixer® set at 100 °C and agitation set at 1000 rpm (Eppendorf AG, Hamburg, Germany). Hydrolysed samples were freed from acid by vacuum centrifugal evaporation and cleaned with a pass-through SPE cartridge (Biotage PLD+, 50 mg/1mL, Biotage AB, Uppsala, Sweden). A Waters Atlantis BEH Z-HILIC0 (2.1x150, 1.8 µm, Waters Corporation, Milford, MA, USA) column was employed to chromatographically separate analytes of interest on Waters Acquity H-Class Plus Bio chromatographic system. The eluted analytes were detected in SIR mode with Waters Acquity QDa single quadrupole mass detector (Waters Corporation, Milford, MA, USA). The primary analytes of interest were N-acetylmuramic acid, muramic acid, N-acetylglucosamine, and glucosamine. The standard compounds were at least 99% pure

and procured from Sigma-Aldrich (Darmstadt, Germany). A single isotopically labelled compound, glucosamine-$^{13}C_6$ (99 atom-% 13C, Omicron Biochemicals, Inc, South Bend, IN, USA), was used across all measurements to compensate for any interference.

3.5 Data acquisition, processing and analysis

Waters Empower 3 (Build 3471 FR5 SR4, Waters Corporation, Milford, MA, USA) was used for data acquisition, processing and analysis in **Study 1**, **Study 2** and **Study 4**. Waters MassLynx v4.1 (SCN805, Waters Corporation, Milford, MA) and Waters QuanLynx v4.1 (SCN805, Waters Corporation, Milford, MA) were used for data acquisition, processing and analysis in **Study 2**. Additionally, Microsoft Excel (Microsoft 365 Apps for enterprises, Microsoft Corp., Redmond, WA, USA) was used to perform additional data analysis in all studies.

4 Results and discussion

4.1 Carbohydrate dynamics of oat-based fermented drinks fermented with various LAB starter cultures (Study I)

This study was focused on exploring changes in carbohydrate concentrations and content during the fermentation of oat-based matrices. The fermentation was achieved using certified commercial starter cultures (SC) of LABs, which are specially blended to ferment plant-based raw materials, the composition of which is presented in Table 3.

Table 3. The bacterial composition of commercial vegan starter cultures (SC) was used in this study.

SC1	SC2	SC3	SC4
Streptococcus thermophilus	*Streptococcus thermophilus*	*Streptococcus thermophilus*	*Streptococcus thermophilus*
Lactobacillus bulgaricus	*Lactobacillus bulgaricus*	*Lactobacillus bulgaricus*	*Lactobacillus bulgaricus*
	Lactobacillus lactis	*Bifidobacterium lactis*	*Bifidobacterium lactis*
	Bifidobacterium lactis	*Lactobacillus acidophilus**	*Lactobacillus acidophilus**
	*Lactobacillus acidophilus**	*Lactobacillus plantarum**	*Lactobacillus paracasei**

* - LAB strains capable of starch and starch-like saccharides digestion**[58]**

It was observed that the compositions of SCs are very similar in terms of present microorganisms. However, the manufacturer did not disclose their proportions in SCs (Danisco® VEGE cultures from IFF, Inc.). Based on the knowledge provided by the manufacturer, it was known that these SCs are capable of fermenting plant-based matrices: soy, beans, cereals, etc. These capabilities allowed the production of oat-based fermented drinks. Like many plant-based raw materials, oats contain a lot of starch, which makes direct fermentation impossible due to the inability of most starter cultures to digest large chains of carbohydrates within reasonable fermentation time. Despite that, there are LAB strains, which contain α-amylase (*amyl*) genes, and thus they can grow on, e.g., starch as the sole carbon source, yet their number is somewhat limited. Some of the most popular microorganisms used in dairy and plant-based fermentation are *S. thermophilus* and *L. bulgaricus*, which both contain *amyl* in an inactivated state [58]. Therefore, the first step in producing any product from a plant-based raw material is to process complex carbohydrates into a more suitable medium for SC. An oat flake slurry was prepared from commercially available oat flakes, rinsed under cold tap water and blended using a household-grade blender. The oat slurry was subjected to enzymatic processing by applying several α-amylases, amyloglucosidases or their mixture. The enzymatic process was stopped with the inactivation of enzymes, with a rapid increase in temperature (up to 70 °C), and released carbohydrates were measured using the currently available methodology based on IEX-RI, employing a Bio-Rad Aminex® HPX87C column (Figure 5). Applying α-amylase and amyloglucosidase made it possible to break starch into more suitable feed components for SCs but presented another technological problem involving final prototype product. Applying mixtures of Fungamyl®+AMG® or BAN®+AMG® released an extremely high concentration of free

glucose, which can negatively affect SC fermentative abilities due to increased osmotic stress [59]. The Fungamyl®-based enzymatic treatment was chosen to hydrolyse starch in oat slurry as it has provided the best texture for the final oat fermented drink among professional assessors.

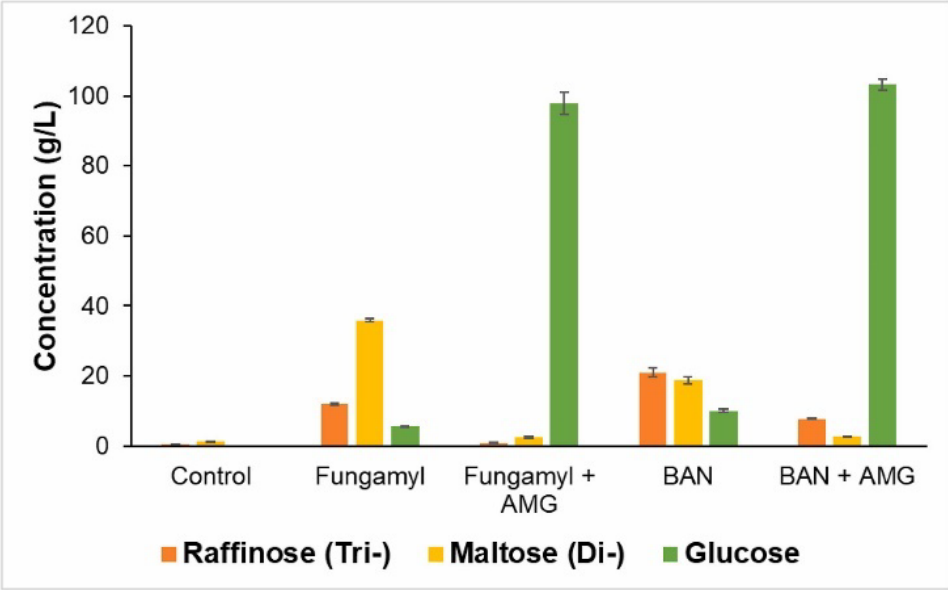


Figure 5. Carbohydrate concentration after the application of enzymes to soaked oat flakes. Di- and trisaccharides are present as the sum of respective saccharides based on the concentrations of marker compounds. AMG® (amyloglucosidase), BAN® (α-amylase) and Fungamyl® (fungal α-amylase) are commercially available enzymes from Novozyme (Bagsvaerd, Denmark). Adopted from [60] under CC BY 4.0

After enzymatic hydrolysis parameters were optimised, fermentation with four different SCs commenced. After enzyme inactivation, an oat slurry was used without additional treatment and spiked with different SCs. The final concentration of SC was about 10 bacterial activity units (proprietary measurement value of IFF Inc.) Based on the provided information by SCs' manufacturer, fermentation was expected to be completed in eight to ten hours, with the final pH of the product at 4.6. The fermentation was allowed to continue for the 24 hours at 40°C to understand better the underlying processes in the fermentation of plant-based materials. In 24 hours, the lowest pH value was achieved at 3.85 with SC2, negatively affecting the sensorial properties of the oat-based fermented drink. Based on measurements done over 24 hours, the optimal fermentation time for all SCs was up to 12 hours, which produced a variety of oat drinks with pH values between 3.82 (SC2) and 4.19 (SC1). These recorded pH values and fermentation dynamics were consistent with the values reported in the literature or the specifications provided by the manufacturer. Additionally, several important chemical parameters were measured throughout the fermentation process, e.g. short-chained organic acids, free amino acids, volatile compounds, and carbohydrates. The fermentation vessel sampling was performed at 3, 6, 9, 12 and 24 hours. The sugar measurements were performed using the same instrumental setup as with released carbohydrates after enzymatic treatment of the oat slurry (Figure 6). The choice of external standards was

based on a typical metabolic activity of LAB strains which do not prefer fructose as a carbon source during growth when a more favourable source is available, e.g., sucrose or glucose [2]. Consequently, all results are provided as a sum of compounds related to the same class of carbohydrate, e.g. the maltose measured concentration reflects the combined value of all potential disaccharides quantified in a sample.

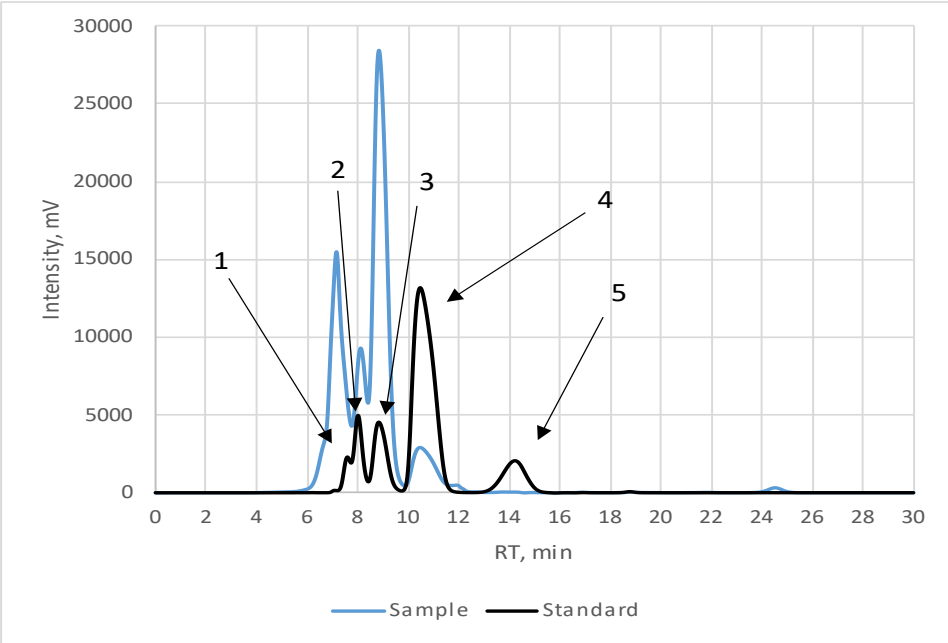


Figure 6. The typical chromatogram of external standards (black line) and the sample obtained after 24 hours of fermentation of the oat slurry with SC1 (blue line). The numbers on chromatograms respond to the set of external standards: 1 – stachyose(tetrasaccharide), 2 – raffinose (trisaccharide), 3 – maltose (disaccharide), 4 – glucose (monosaccharide) and 5 – fructose (monosaccharide)

Figure 7 provides an overview of carbohydrate concentration changes measured by the significant classes of carbohydrates: mono-, di- and trisaccharides. Overall, the concentration levels of disaccharides did not change significantly during the 24-hour-long fermentation, which might be attributed to the variety of disaccharides found in oats during various processing steps and the ability of SCs to consume said sugars to grow on. On the other hand, mono- and trisaccharides exhibited changes in their concentration levels during the fermentation process. These changes can be attributed to the affinity of LAB strains to consume monosaccharides as the most preferable carbon source. However, considering pH changes and the viability of SCs at lower pH values, the complete consumption of monosaccharides was not achieved over the 24-hour fermentation period. At the same time, the trisaccharide concentration changed, which might be due to the release and consumption of alternative longer polysaccharides in oat slurry. These long-chained oligosaccharides could have been digested and used as an alternative carbon source for growth by several LAB strains present in SC2 to SC4 (Table 3).

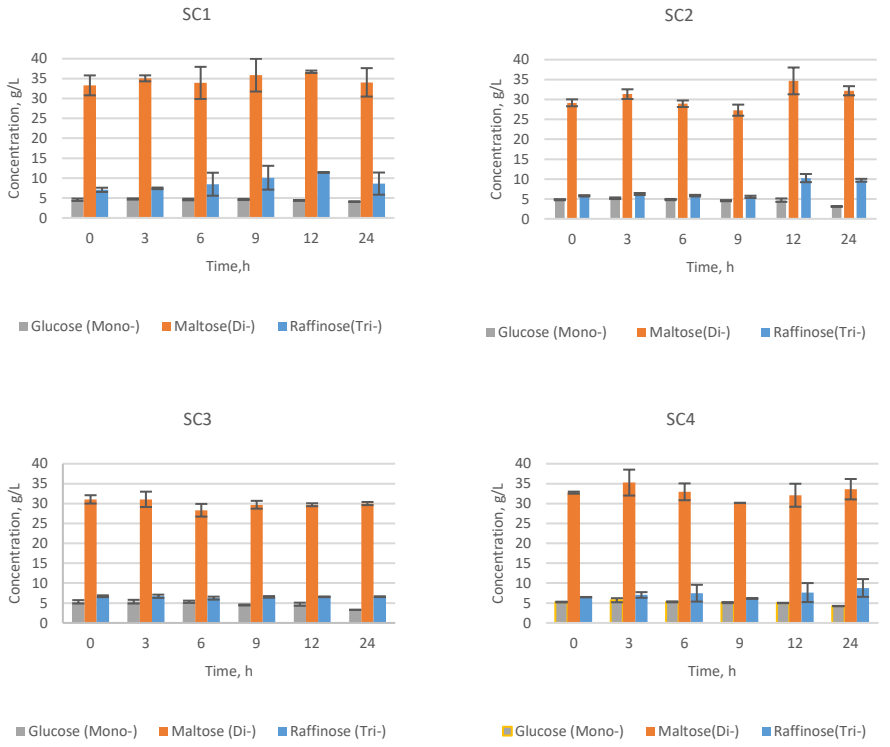


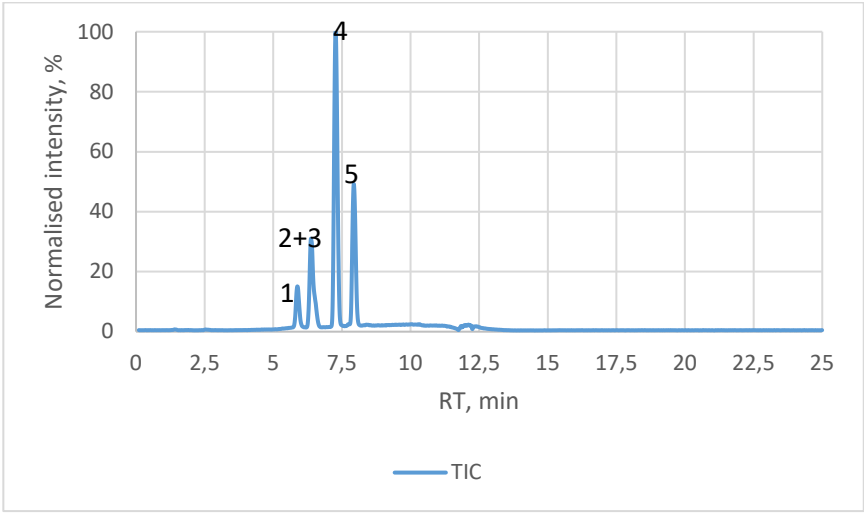
Figure 7. The carbohydrate concentration expressed as g/L during 24-hour sampling to determine the consumption of various sugars found in the oat slurry. The concentration of sugars is presented as the sum of all isobaric compounds belonging to the same order of carbohydrates. The data represents the mean values of three biological replicates ± SD (n=3)

Overall, the successful fermentation of oat-based drinks was achieved, highlighting the possibility of making different plant-based fermented alternatives. The oat-based fermented drinks achieved low pH values, around 3.8–4.2, and they could be used to develop yoghurt-like products, wherein typical pH values are around 4.0–4.4 [5]. However, a better analytical separation is required for a more precise explanation of the changes in various carbohydrate concentrations, especially commonly consumed mono- and disaccharides by bacteria, during the fermentation of plant-based materials. Thus, advancements in analytical methodologies should be utilised for the selective and reliable measurement of all potential mono- and disaccharides found in raw materials.

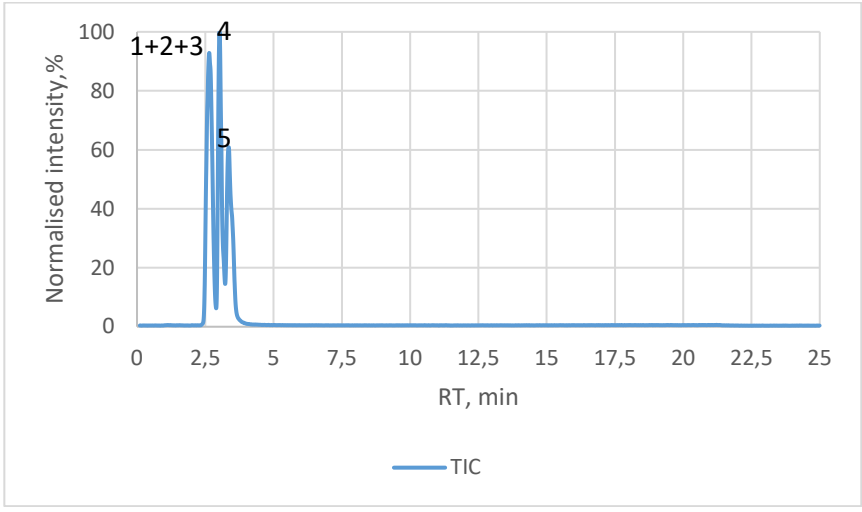
4.2 Improving the analytical methodology for measuring carbohydrates during *Streptococcus thermophilus* fermentation (Study II)

During experimental work in Publication I, it was suggested that the methodology for measuring saccharides with various lengths of monomers could be improved by performing a more selective and sensitive assay. Based on these requirements, it was proposed to develop a MS-based measurement to measure mono- and disaccharides in a fermentation medium harvested from *Streptococcus thermophilus* fermentation.

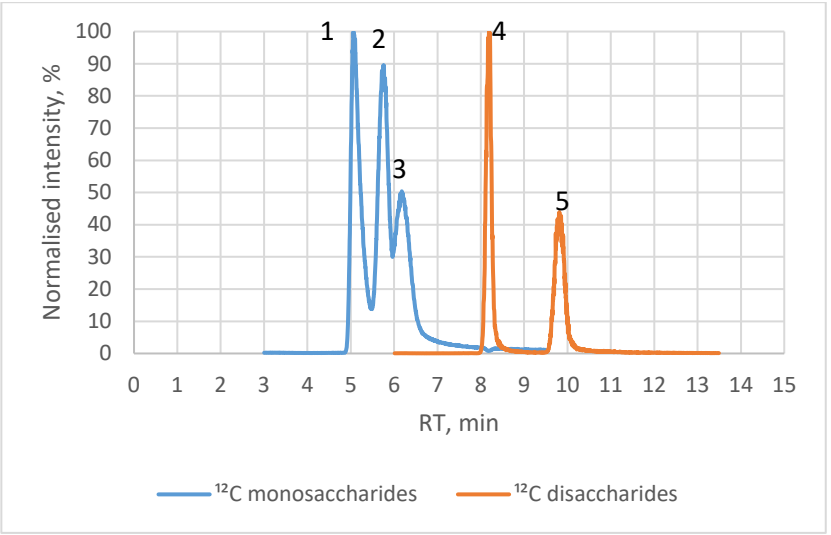
The choice of bacteria was based on a wide range of applications of *S. thermophilus* in the fermentation of both dairy and non-dairy, i.e., plant-based raw materials. The saccharide measurement with conventional stationary phases used in liquid chromatography, such as C18 and others, is problematic due to the fast isomerisation processes in the solutions. Several solutions could be used to reduce the number of available isomers at any given time. However, most of them require performing chemical derivatisation, which might be problematic based on the nature of the sample or the total concentration of all measurable carbohydrates. HILIC appears to be a prominent alternative to measure carbohydrates in the samples without performing derivatisationm thus alleviating the complexity of derivatisation. In this study, three different HILIC columns were chosen for separation of five different saccharides: fructose (1), glucose (2), galactose (3), sucrose (4) and lactose (5).



(a)



(b)



(c)

Figure 8. The screening results of three HILIC columns. The numbers represent detected saccharides. The panels show the separation of a mixture of mono- and disaccharides achieved on (a) Waters Acquity BEH Amide (3x150, 2.5 µm) (b) Waters Acquity BEH HILIC (2.1x100 mm, 1.7 µm) (c) Phenomenex Luna Omega Sugar (2.1x150 mm, 3 µm). The numbers on the chromatograms correspond to a set of external standards: 1 – fructose, 2 – glucose, 3 – galactose, 4 – sucrose and 5 – lactose. "TIC" stands for total ion chromatogram, "^{12}C monosaccharides" corresponds to monosaccharide [M+Cl]- adduct and "^{13}C disaccharides" corresponds to disaccharide [M+Cl]- adduct

Figure 8 shows the separation capabilities of commonly used HILIC columns for analysing carbohydrates based on amide-, bare silica- or amino-based stationary phases. Out of three tested columns, a bare silica type column (Waters Acquity BEH HILIC) had the worst performance regarding the separation capabilities between compounds in the monosaccharide group (Compounds 1 to 3). The amide-based column (Waters Acquity BEH Amide) was superior to the bare silica-type column in terms of fructose, glucose, and galactose separation. However, it failed to provide a resolution between glucose and galactose under current instrumental conditions. The hybrid amino/amide-based column (Phenomenex Luna Omega Sugar) provided the best-in-class separation between all studied monosaccharides and disaccharides. Therefore, Luna Omega Sugar was chosen as the primary column for analysing saccharides in the fermentation medium of *S. thermophilus*. Additional optimisations were performed, which included the optimisation of an analytical gradient, MS operating parameters, the addition of isotopically labelled internal standards and guanidine hydrochloride (GuHCl). Adding GuHCl improved the sensitivity and selectivity of measurements by providing a more specific adduct $[M+Cl]^-$[61]. The next step was to develop a suitable sample preparation protocol to remove excessive matrix components and allow a cleaner extract to be analysed. Various sample preparation techniques were tested and evaluated to determine their suitability for the intended purpose (Table 4).

Table 4. The sample preparation protocols, which were evaluated using fermentation broth of S. thermophilus.

Protocol Nr.	1	2	3	4	5	6
Step 1	Dilute 100 times with MilliQ					Dilute 50 times with MilliQ
Step 2	Centrifuge at 14000 rpm for 10 min					
Step 3	Filter through 0.2 µm filter	Dilute two times with MeCN: MilliQ mixture containing ^{13}C ISTD		Pass through 1 kDa MWCO filter	Pass through 3 kDa MWCO filter	Dilute four times with MeCN: MilliQ mixture containing ^{13}C ISTD
Step 4	Dilute two times with MeCN: MilliQ mixture containing ^{13}C ISTD	Pass through Isolute PLD+ cartridge	Pass through Isolute NH_2 cartridge	Dilute two times with MeCN: MilliQ mixture containing ^{13}C ISTD		Pass through Isolute NH_2 cartridge

Based on the results with the simulated matrix, which contained all five analysed carbohydrates in various ratios, protocol #1 was chosen as the optimal and suitable approach to afford clean samples. The additions of ISTD, glucose-$^{13}C_6$ and lactose-$^{13}C_6$ made it possible to compensate for variations during sample preparation and instrumental injection processes. The methodology was validated in terms of limits of detection (LOD) and quantitation (LOQ), linear range, linearity and the recovery of the two ISTDs used in all experiments (Table 5).

Table 5. The LOD, LOQ, linear range and linearity of the developed HILIC-MS method.

Analyte	Linear Range, mg/L	Linear Regression	R^2	LOD[1], mg/L	LOQ[2], mg/L	Recovery, %
Fructose	0.77–49.88	y = 1.3611x + 0.9873	0.9974	0.189	0.629	
Glucose	0.51–64.80	y = 0.6921x + 0.0765	0.9993	0.080	0.268	103.73 ± 1.69
Galactose	0.39–49.60	y = 0.3764x – 0.0112	0.9958	0.067	0.220	
Sucrose	0.93–59.75	y = 1.2610x + 0.9776	0.9935	0.232	0.704	111.04 ± 2.80
Lactose	0.38–49.10	y = 1.0851x – 0.0076	0.9996	0.048	0.159	

[1] – LOD = Blank mean value + 3.3*SD at lower limit of quantitation (LLOQ); [2]- LOQ = Blank mean value + 10*SD at LLOQ

This protocol was then applied to the fermentation broth of *S. thermophilus*. The protocol made possible the measurement of all the components of the starting fermentation medium on which *S. thermophilus* was grown (Figure 9).

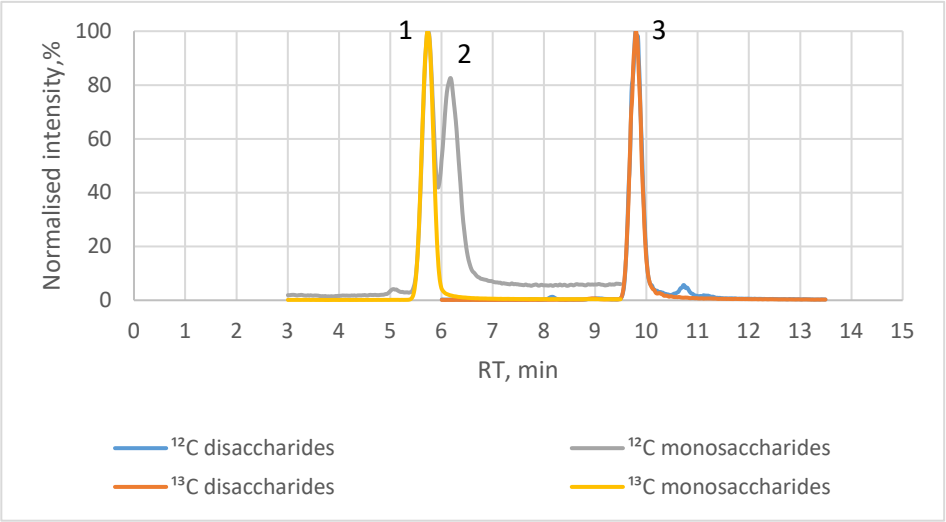


Figure 9. The chromatogram of the S. thermophilus fermentation sample during the growth on the lactose-based chemically defined medium. "^{12}C monosaccharides" corresponds to monosaccharide [M+Cl]- adduct, ^{12}C disaccharides" corresponds to disaccharide [M+Cl]- adduct, ^{13}C monosaccharides" corresponds to isotopically labelled monosaccharide [M+Cl]- adduct and ^{13}C disaccharides" corresponds to isotopically labelled disaccharide [M+Cl]- adduct. The numbers on the chromatogram correspond to this set of external or isotopically labelled standards: 1 – glucose, 2 – galactose and 3 – lactose

Based on the carbon source in the starting medium, it was observed that *S. thermophilus* used in these experiments was more likely to consume lactose as a primary carbon source during 24 hours of fermentation than sucrose (Figure 10). It was also seen that after 24 hours of fermentation, half of the starting lactose was found in the sampling points, indicating that fermentation might still be ongoing and could be prolonged to generate more biomass or increase the concentration of potentially beneficial intermediates.

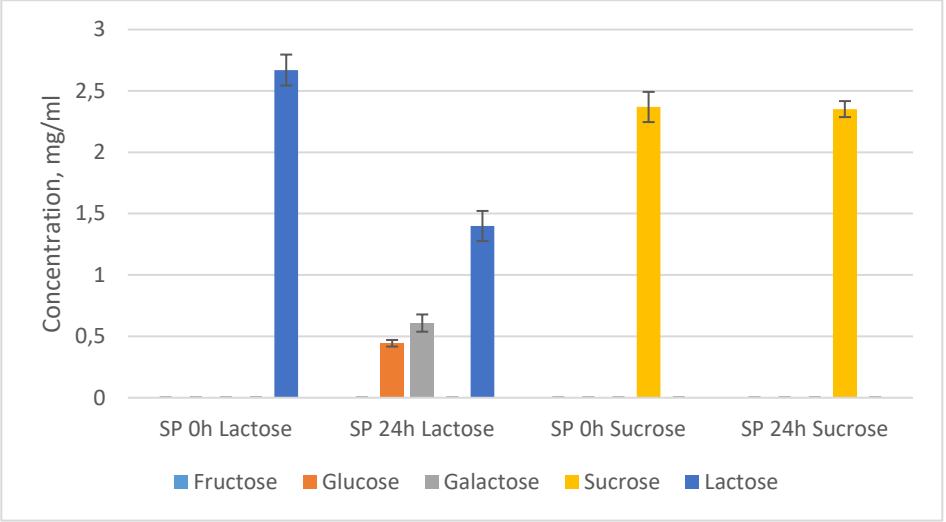


Figure 10. The carbohydrate measurements during the growth of S. thermophilus. "SP" stands for sampling point, and "Lactose" or "Sucrose" stands for the carbon source in the fermentation medium. The results are presented as mean value ±SD (n = 3)

These results show that the developed HILIC-MS methodology is capable of measuring structurally closely related saccharides, e.g. fructose, glucose, and galactose, in a single analytical run with little to no extensive sample preparation, providing a more than sufficient analytical measurement range to quantify major carbohydrates classes.

4.3 Screening of available carbohydrates in commercially available plant-based yoghurt alternatives (Study III)

Study II determined the optimal starting point to further fine-tune HILIC-MS methodology towards more diverse samples with more complex matrices as plant-based yoghurts alternatives. Therefore, it was used in a large cohort study of commercially available plant-based yoghurt alternatives. Twenty-five samples were procured from local markets in Estonia, Finland, and Germany. The selection criteria specified that products should not contain flavourings, a list of microbial cultures used for fermentation must be listed on a product label, and/or the application of fermentation must be mentioned in the product description. Overall, 2 lupin-, 10 soya-, 9 oat- and 4 coconut-based plant yoghurt alternatives were procured. In Publication II, a Phenomenex Luna Omega Sugar column was used as a primary column for separating sugar isomers, such as glucose-galactose. This column's chemistry is based on a hybrid amino/amido stationary phase, typical of HILIC columns. The column exhibited a substantial decrease in retention mechanisms after approximately 1000 injections, and it was observed that different batches of packing showed reduced performance in terms of glucose-galactose separation. Therefore, an alternative column was evaluated to provide the same selectivity but better resistance towards potential sample interferences. The new amide-based column was chosen from Waters Corporation – Waters XBridge BEH Amide XP, which proved to be more resistant due to the high resistance of the stationary phase to pressure and pH changes, as well as high temperature. The chromatographic separation of fructose (1), glucose (2), sucrose (3) and maltose (4) was achieved using a modified methodology from

[62] and [63]. The key differences were the modification of the chromatographic gradient to reduce the number of concurrently used mobile phases, thus reducing the consumption of organic solvents, and using a more rapid gradient ramp to accommodate faster sample turnaround while not sacrificing the measurements' selectivity. The mass spectrometric parameters remained almost the same, with only minor tuning performed due to a change in the measurement apparatus. The variability in sample preparation or instrumental response was corrected by using isotopically labelled internal standards – fructose-$^{13}C_6$, glucose-$^{13}C_6$, sucrose-$^{13}C_6$ and maltose-$^{13}C_{12}$. The method was validated in terms of LOD, LOQ, linear range, linearity and the recovery of the four ISTDs used throughout the measurements of standards and samples.

Table 6. The LOD, LOQ, linear range, linearity and recovery of the optimised HILIC-MS methods.

Analyte	Linear Range, mg/L	Linear Regression	R^2	LOD[1], mg/L	LOQ[2], mg/L	Recovery, %
Fructose	0.80–51.21	Y = -1.91e-01 X^2 + 4.70e-01 X + 4.25e-05	0.9999	0.065	0.196	103.88 ± 1.55
Glucose	0.78–49.93	Y = -7.69e-01 X^2 + 9.56e-01 X + 6.80e-05	0.9999	0.057	0.173	99.12 ± 3.44
Sucrose	0.83–53.27	Y = -1.71e-01 X^2 + 1.23e+00 X – 1.02e-04	0.9999	0.085	0.258	104.8 ± 3.46
Maltose	0.83–52.92	Y = -4.02e-01 X^2 + 1.20e+00 X – 1.54e-04	0.9999	0.128	0.389	92.26 ± 3.42

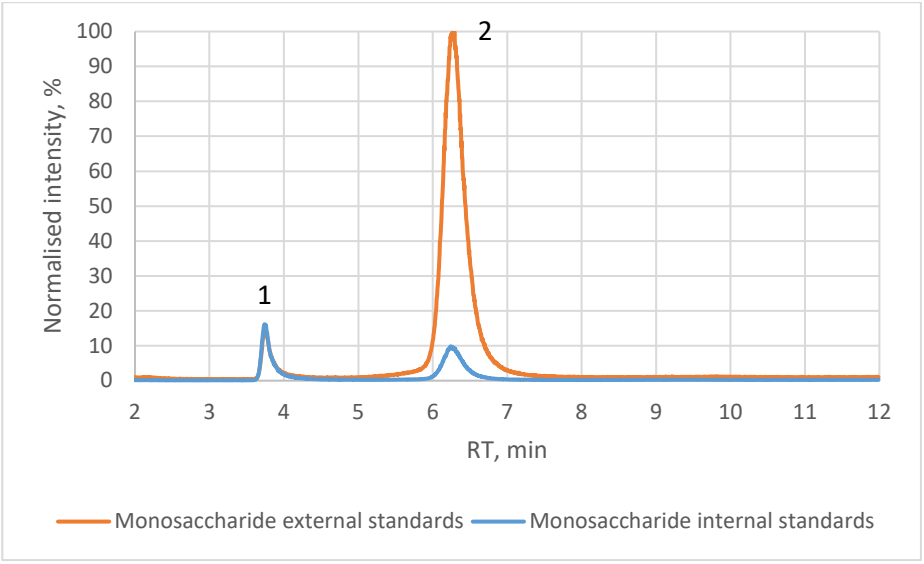
[1] – LOD = Blank mean value + 3.3*SD at lower limit of quantitation (LLOQ); [2]- LOQ = Blank mean value + 10*SD at LLOQ

The modified methodology was applied to all twenty-five samples of various origins: lupin, oat, coconut and soya (Table 7). The samples were prepared using a similar approach described in Publication II, using 3 kDa MWCO spin filters to clean them. The filtrates were diluted accordingly with MilliQ® water or a mixture of MilliQ® water and acetonitrile. In the final step, an ISTD mixture consisting of four components was added to each sample to afford the final dilution.

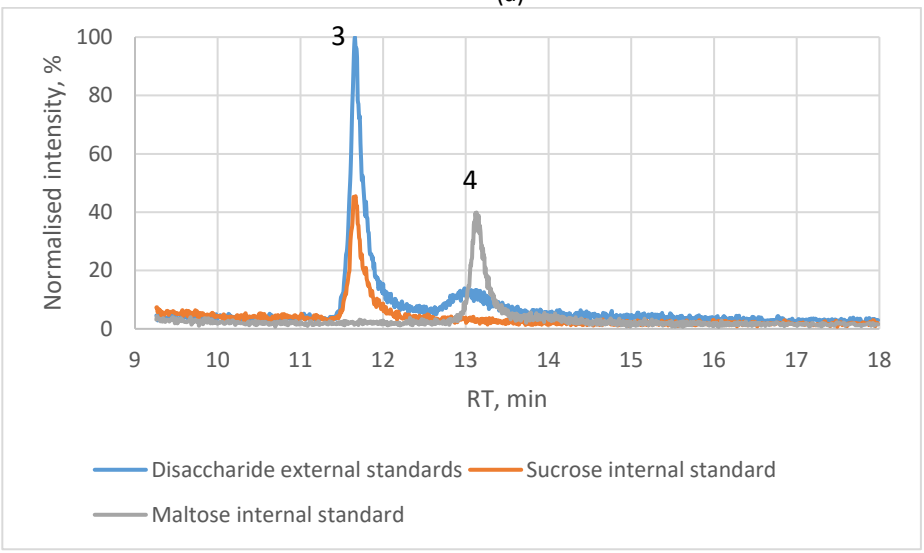
Table 7. The concentration of sugars in plant-based yoghurt alternatives with updated HILIC-MS methodology. The data are provided in g/100 g of product as the average value of three replicates with SD.

Sample name	Plant matrix	Fructose	Glucose	Maltose	Sucrose
VY 1	lupin	0.553 ± 0.059	0.494 ± 0.039	0.317 ± 0.013	0.028 ± 0.005
VY 2	lupin	4.83 ± 0.446	4.796 ± 0.435	0.316 ± 0.016	1.108 ± 0.089
VY 3	soya	0.095 ± 0.004	0.037 ± 0.002	0.001 ± 0	0.276 ± 0.016
VY 4	soya	0.032 ± 0.001	0.002 ± 0	0.001 ± 0.002	0.261 ± 0.039
VY 5	soya	0.009 ± 0	0.008 ± 0	0.001 ± 0.001	0.158 ± 0.005
VY 6	soya	0.026 ± 0.001	0.027 ± 0.001	0.001 ± 0	0.096 ± 0.004
VY 7	soya	0.062 ± 0.002	0.064 ± 0.004	0.001 ± 0.001	0.204 ± 0.018
VY 8	soya	0.062 ± 0.002	0.064 ± 0.004	0.002 ± 0.001	0.201 ± 0.007
VY 9	soya	0.031 ± 0	0.032 ± 0.002	0.036 ± 0.016	2.622 ± 0.092
VY 10	soya	0.015 ± 0.001	0.02 ± 0.003	0.018 ± 0.024	2.423 ± 0.025
VY 11	soya	0.011 ± 0.004	0.012 ± 0	0.001 ± 0.001	0.123 ± 0.003
VY 12	soya	0 ± 0	0 ± 0	0.019 ± 0.011	0.093 ± 0.008
VY 13	oat	3.897 ± 0.031	6.331 ± 0.158	1.636 ± 0.188	0.372 ± 0.014
VY 14	oat	0.029 ± 0.001	0.001 ± 0.002	0.028 ± 0.01	1.072 ± 0.068
VY 15	oat	0.031 ± 0.004	0.047 ± 0.001	3.345 ± 0.139	0.34 ± 0.004
VY 16	oat	0.033 ± 0.004	0.051 ± 0.003	3.722 ± 0.203	0.25 ± 0.007
VY 17	oat	0.008 ± 0.003	2.415 ± 0.151	0.984 ± 0.037	0.033 ± 0.004
VY 18	oat	0.024 ± 0.003	0.583 ± 0.016	0.01 ± 0.009	1.289 ± 0.043
VY 19	oat	0.029 ± 0.003	4.053 ± 0.11	0.141 ± 0.024	0.031 ± 0.003
VY 20	oat	0.017 ± 0.003	0.256 ± 0.025	0.675 ± 0.043	0.005 ± 0.009
VY 21	oat	0.018 ± 0.004	2.133 ± 0.088	1.28 ± 0.048	0.098 ± 0.009
VY 22	coconut	0.453 ± 0.018	0.316 ± 0.001	0.034 ± 0.051	1.801 ± 0.104
VY 23	coconut	0.001 ± 0.001	0 ± 0	0.012 ± 0.017	2.049 ± 0.148
VY 24	coconut	0.249 ± 0.007	3.419 ± 0.195	0 ± 0	0.489 ± 0.022
VY 25	coconut	0 ± 0	0 ± 0	0 ± 0	0.884 ± 0.072

The results of measurement showed that the methodology can successfully measure a wide range of concentration in complex plant-based samples with relatively low standard deviations between replicates. Additionally, due to the inherent sensitivity and selectivity of mass spectrometric detection, only 1 µl of sample extract had to be injected to achieve low detection limits (sub milligrams per litre). It was able to successfully separate all analytes of interest in the studied samples (Figure 11).



(a)



(b)

Figure 11. (a) The chromatogram of monosaccharides ("Monosaccharides external standards" corresponds to monosaccharide [M+Cl]- adduct and "Monosaccharides internal standards" corresponds to internal standard [M+Cl]- adduct) found in a coconut-based yoghurt alternative sample was analysed using the modified methodology. (b) The chromatogram of disaccharides ("Disaccharides external standards" corresponds to disaccharide [M+Cl]- adduct, "Sucrose internal standards" corresponds to sucrose internal standard [M+Cl]- adduct and "Maltose internal standards" corresponds to maltose internal standard [M+Cl]- adduct) found in a coconut-based yoghurt alternative sample was analysed with the modified method. The numbers on chromatograms correspond to this set of external or isotopically labelled standards: 1 – fructose, 2 – glucose, 3 – sucrose and 4 – maltose

Overall, applying a different HILIC stationary phase positively affected the outcomes of sugar measurements in a wide range of plant-based yoghurt alternatives, achieving the same throughput, sensitivity and repeatability yet being more resistant mechanically and chemically. The obtained carbohydrate concentration data was combined with several additional chemical and microbiological parameters: pH, short-chained fatty acid concentrations, total titratable acidity, and total and alive cell counts. The combined data were subjected to principal component analysis (PCA). Three major clusters were observed, and one outlier, VY13 (oat), did not belong to any major clusters. This can be explained by the fact that VY13 (oat) was a fermented plant-based drink, not a yoghurt alternative, were as the other 24 samples analysed in this study. Furthermore, it was observed that oat-, coconut- and lupin-based yoghurt alternatives are more closely related than soya-based alternatives. This might be attributed to the starch or other polysaccharides present, which might have been degraded while producing these plant-based yoghurt alternatives. Additionally, two samples, VY2 (lupin) and VY23 (coconut), formed their clusters due to extremely high concentrations of sugars and organic acids measured in these samples (Figure 12).

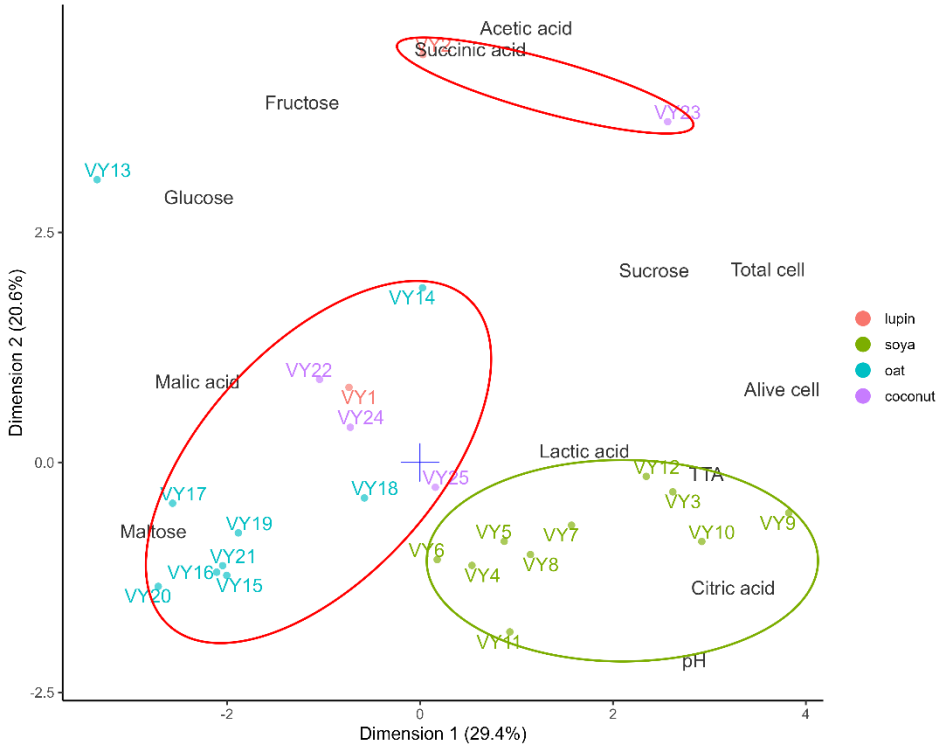


Figure 12. The PCA biplot of twenty-five plant-based yoghurt alternatives with colour marking depending on the origin of plant raw materials. The ordination was calculated and visualised in R 4.3.0 (The R Foundation for Statistical Computing, Vienna, Austria)

4.4 The quantitative approach to measuring bacterial cell wall monomers (Study IV)

The bacterial cell wall plays a vital role in the bacterial life cycle. It is used as the primary target in differentiating bacteria by the most common staining techniques, e.g. Gram or Chance. Applying these staining techniques could show the difference in the chemical composition of BCW, thus allowing for the qualitative differentiation of bacteria. The BCW is primarily composed of the outer membrane, which is a characteristic trait of Gram-negative bacteria, the peptidoglycan layer(-s), periplasmic space and cytoplasmic membranes. The thickness of the peptidoglycan layer is the most used differentiation parameter to describe the difference between BCW in various microorganisms. In Gram-staining, the thickness of the PG dictates whether crystal blue dye is retained or washed away after adding an alcohol solution. The crystal blue dye is retained in a thick PG layer. Thus, the stained microorganism is purple under the microscope or might be pink or red in the case of Gram-negative bacteria, which usually have a thinner PG layer. However, most staining methods lack a quantitative dimension, thus making it impossible to use vital information for metabolic modelling calculations. To resolve this issue and obtain the missing information on BCW content, the novel acidic hydrolysis-based HILIC-MS methodology has been developed and applied to several Gram-positive and Gram-negative bacteria. The target analytes for this methodology were chosen based on the standard structure of PG present in both Gram-negative and Gram-positive bacteria (Figure 2). N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) are present in all strands of PG across BCW, thus making them an excellent choice to target for quantification purposes. The amino acid bridges might also be used as additional analytes of interest. However, depending on the microorganism, variability in amino acids in the PG has been observed, making them inconsistent when used across various species without additional information [64]. At the beginning of method development, several chromatographic setups were evaluated to accurately measure NAM, NAG, muramic acid (Mur), and glucosamine (GluN). Table 8 provides an overview of the parameters used to optimise the measurement part of the methodology.

Table 8. The overview of experimental setups used to optimise the chromatographic performance of the measurements.

Chromatography column	Critical parameters of the column (Internal diameter, length, particle size)	Flow rate, µl/min	Mobile phase A	Mobile phase B
Waters Acquity UPLC BEH Phenyl	2.1 x 150 mm, 1.8 µm	300	0.1% Formic acid in MilliQ	MeOH
Waters Xbridge BEH Amide XP	3 x 150 mm, 2.5 µm	800	80/20/0.05 MeCN/MilliQ/ /DEA + 0.5 mg/L GuHCl	90/5/5/0.05 MeCN/MilliQ/IP A/DEA + 0.5 mg/L GuHCl
Phenomenex Luna Omega Sugar	2.1 x 150 mm, 3 µm	313	MilliQ + 0.5 mg/L GuHCl	99/1 MeCN/MilliQ + 0.5 mg/L GuHCl
Waters Atlantis Premier BEH C18 AX	2.1 x 100 mm, 1.7 µm	300	10 mM AmFor in MilliQ (pH = 3.75)	90/10 MeCN/10 mM AmFor in MilliQ (pH = 3.75)
Waters Atlantis Premier BEH Z-HILIC	2.1 x 150 mm, 1.7 µm	500	20 mM AmAc in MilliQ (pH = 4.75)	90/10 MeCN/20 mM AmAc in MilliQ (pH = 4.75)

Based on preliminary results, Waters Atlantis Premier BEH Z-HILIC was chosen as the optimal separation column of the tested ones. Figure 13 provides an overview of four separated analytes of interest with an optimised gradient elution program using LC-MS. Based on the previous studies (Publication II and III) concerning the measurement of carbohydrates, GuHCl was added to both mobile phases to improve the ionisation efficiency of studied analytes. NAG and GluN actively formed a more favourable adduct $[M+Cl]^-$ while NAM and Mur did not change their ionisation pattern and formed more classic adducts, such as $[M-H]^-$.

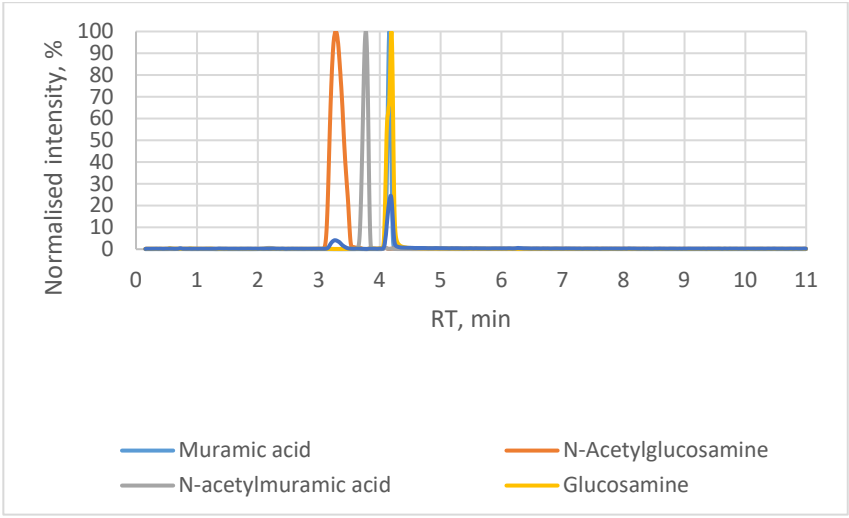


Figure 13. The optimised separation of four analytes of interest with a Waters Atlantis Premier BEH Z-HILIC column, including the isotopically-labelled internal standard – glucosamine-$^{13}C_6$. The measurement was performed using dedicated SIR channels

The cross-linked polymeric structure of PG presented a significant obstacle to quantifying both targets directly. The initial screening of how to digest PG into the smaller components was based on the enzymatic treatment with lysozyme, the enzyme specific to PG breakage. The lysozyme protocol was adapted from the vendor's instructions from the GenElute kit [65].

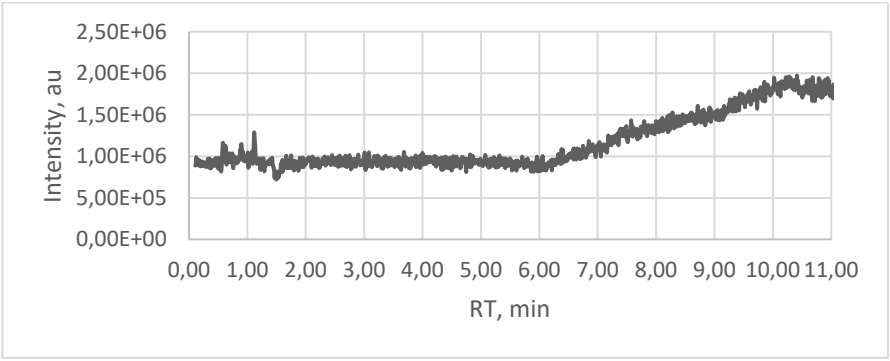


Figure 14. The TIC of S. thermophilus's wet biomass was subjected to the lysozyme treatment according to the vendor's instructions, which were obtained with optimised chromatographic conditions used in all experiments. The mass spectrometric measurement was performed under negative ESI conditions, scanning from 100 to 750 m/z at a 2 Hz scan rate

The experiments were performed with a Gram-positive bacterium, *Streptococcus salivarius ssp. Thermophilus* DSM20259, which possesses a thick PG layer. The wet biomass was subjected to the lysozyme protocol and measured with optimised chromatographic and mass-spectrometric parameters (Figure 14). The measurements were made in TIC to follow all possible ionisable molecules after applying the lysozyme solution to the sample. The recorded chromatogram showed a lack of produced chromatographic peaks. This indicated that the lysozyme application did not achieve its

goals of breaking the PG layer into the monomeric constituents; thus, the development shifted to the widely used technique: acidic hydrolysis with HCl and phenol. An example chromatogram obtained with this approach is shown in Figure 15.

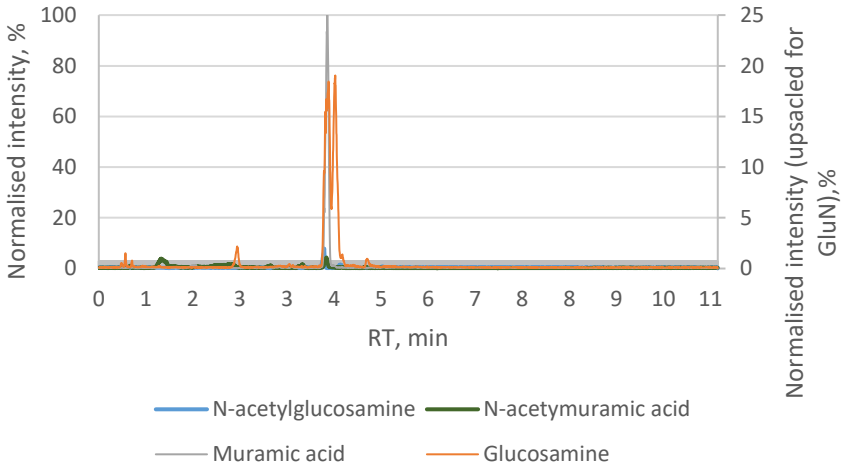


Figure 15. The SIR chromatogram of four analytes of interest was detected after S. thermophilus's acidic hydrolysis (t = 4 hours) using optimised chromatographic and mass-spectrometric parameters. The intensity of measured peaks was normalised to the highest detected peak (Mur) during the injection.

The sample preparation methodology was optimised regarding the sample amount used, the time spent on the hydrolysis and purification processes, and the improvement of the hydrolysis solution homogeneity. The method used one to two milligrams of dried biomass and 300 µl of 6M HCl with 1% phenol (*v/v*). The hydrolysis process was performed in classic Eppendorf tubes at 100 °C with agitation set at 1000 rpm in a thermally regulated mixer. The hydrolysate was freed from HCl and phenol with vacuum rotary evaporation, and the residue was redissolved in 50% aqueous MeCN (*v/v*) and purified using Biotage PLD+ columns. The stability of the analytes of interest was assessed with the parameters used for the hydrolysis of wet biomass, wherein the hydrolysis time was set to four hours (Table 9). The conditions used for hydrolysis showed that both NAG and NAM were hydrolysed into GluN and Mur, respectively. At the same time, both GluN and Mur showed exceptional resistance to these hydrolysis conditions and remained stable (recovery rates of 94–99%).

Table 9. The recovery values for pure standards (~1 mg/mL) after the acidic hydrolysis conditions used for the wet biomass of S. thermophilus.

Analyte	Recovery (%, n = 3)	SD (%, n = 3)
N-acetylmuramic acid	0	0
N-acetylglucosamine	0	0
Muramic acid	99.1	3.8
Glucosamine	94.2	1.9

The next step was to optimise the hydrolysis time for various bacteria, both Gram-positive and Gram-negative, as their PG layer thickness and outer membrane might present additional challenges. Three bacteria were chosen for these experiments:

Streptococcus salivarius ssp. Thermophilus DSM20259 (STH, Gram-positive), *Escherichia coli* K12 MG1655 (ECO, Gram-negative) and *Bacteroides thetaiotaomicron* DSM 2079 (BTH, Gram-negative). The optimisation of hydrolysis times for both types of bacteria was done with different sampling times based on the estimated thickness of the PG layer (Table 10).

Table 10. Optimisation of the acidic hydrolysis time of two Gram-negative bacteria. The results are presented as a percentage of analyte per 1 mg dry biomass. The analysis was performed in triplicates.

Sampling time, h	%Glucosamine (ECO) (n=3)	%Muramic acid (ECO) (n=3)	%Glucosamine (BTH) (n=3)	%Muramic acid (BTH) (n=3)
0.5	0.50 ± 0.02	0.47 ± 0.07	3.22 ± 0.23	0.49 ± 0.06
1	0.50 ± 0.02	0.51 ± 0.04	3.16 ± 0.03	0.52 ± 0.04
1.5	0.50 ± 0.08	0.52 ± 0.05	3.16 ± 0.16	0.51 ± 0.05
2	0.60 ± 0.06	0.59 ± 0.08	3.09 ± 0.33	0.55 ± 0.05
4	0.82 ± 0.10	0.88 ± 0.05	3.05 ± 0.02	0.57 ± 0.03

It was concluded that a complete hydrolysis of the PG layer could be achieved as fast as four hours. The recorded results correlated with previously published results for ECO [66,67]. For BTH, the concentration of GluN exceeded by multiple factors the stochiometric ratio of predicted GluN reported in the literature [66]. This unsatisfactory ratio's probable cause might have been the overproduction of UDP-N-acetylglucosamine, the essential metabolite to produce PG [22]. It was shown that the Escherichia coli K-12 strain NCM3722 can accumulate excess amounts of UDP-N-acetylglucosamine in its cytosol as a backup to the sudden changes in the growth environment [68]. This accumulation behaviour extended to the STH strain used in these experiments. During the optimisation of the hydrolysis time, it was observed that GluN concentrations slightly exceeded the stoichiometric ratio expected in the PG layers (Table 11). Furthermore, the overall concentration of detected monomers (8.2%) correlated with reported values for different LAB species of the Lactobacillales order (5.5–12.2 %) [69].

Table 11. Optimisation of time spent on the acidic hydrolysis of a Gram-positive bacterium. The results are presented as a mass percentage of analyte per 1 mg of dry biomass. The analysis was performed in triplicates.

Sampling time, hours	%Glucosamine (STH) (n=3)	%Muramic acid (STH) (n=3)
1.5	4.63 ± 0.12	3.47 ± 0.07
2	4.88 ± 0.03	3.51 ± 0.04
4	4.72 ± 0.31	3.52 ± 0.17
12	4.73 ± 0.27	3.53 ± 0.28
16	4.49 ± 0.42	3.38 ± 0.25

Overall, this methodology showed its potential to measure the monomeric constituents of the PG layer found in Gram-negative and Gram-positive bacteria. With appropriate modifications, it can be used in high-throughput screening due to the relative ease of handling samples.

5 Conclusions

This work aimed to develop and apply suitable analytical procedures for precise and selective monitoring of the saccharides during fermentation processes in plant-based products. The starting materials' diversity and richness in carbohydrate profiles showed the necessity of having quantitative yet selective analytical methods with the shortest turnaround time per sample.

- In **Study I**, fundamental research was performed into the fermentation of oats to produce plant-based milk alternatives. The IEX-RI method revealed a diverse range of carbohydrates in oats at various sampling points. However, the IEX-RI method also demonstrated limitations in separating carbohydrates of the same order, such as maltose from sucrose.
- **Study II** focused on developing the HILIC-MS method to quantify saccharides, which are hard to resolve with IEX-RI. The developed HILIC-MS method showed that it can separate isomers, including hard-to-resolve epimers. The influence of various sample preparation procedures on the measurement results and potential application in a high-throughput environment were studied with a simulated matrix and real-world samples obtained from S*treptococcus thermophilus* fermentation.
- In **Study III**, the carbohydrate profiling was performed using the HILIC-MS method, which was tailored to suit the aims of the measurements in the plant-based matrices. The study focused on mono- and disaccharides as they are essential feeding components for the proliferation of bacteria during fermentation. Four major carbohydrates – fructose, glucose, sucrose, and maltose – were consistently found throughout the study, demonstrating the method's robustness and precision despite the complexity of the studied matrices.
- In **Study IV**, we focussed more deeply and directly on the bacterial cell wall, which plays a significant role during the life cycle of bacteria. A newly developed acidic hydrolysis-based HILIC-MS methodology was able to measure the amino sugar component of BCW. The methodology has been validated on Gram-positive and Gram-negative bacteria, showcasing the applicability of the methodology for various bacteria regardless of Gram-staining. The results were in a good correlation with previously published studies and, with appropriate modifications, the methodology can be deployed in a high-throughput environment with proper laboratory automation.

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Abstract

Optimising analytical methods for the determination of carbohydrates in diverse fermented matrices

The rapidly growing world population may overwhelm conventional food production supply chains. Thus, alternative supply chains are being developed to feed the population sustainably. Plant-based fermented products are good alternatives to animal-based fermented products, such as meat and milk. However, the fermentation processes in plant raw materials have not been well studied compared to animal-based counterparts. The complexity of raw plant-based materials presents many technological problems, from the choice of starter cultures to the availability of feed components to such cultures in these materials. Correctly assessing bacteria growth requires focusing on the consumption of present sugars in plant-based matrices. To determine consumption, an analytical measurement of these carbohydrates must be performed. The aim of this thesis was to improve the measurement of carbohydrates in various fermented matrices and explore how bacteria use sugar derivates during their lifecycle. The initial step in improving the currently available instrumental methodologies was to establish the baseline with the measurement of consumed carbohydrates during the fermentation of oat flakes. The classic approach, applying an ion exchange-based stationary phase and refractive index detection (IEX-RI), was employed to measure mono-, di- and trisaccharide produced and consumed during the 24-hour fermentation of oats with four different starter cultures (SC). The results showed that, with the four SCs, the disaccharide concentration did not significantly change across all sampling points. In contrast, mono- and trisaccharide concentrations fluctuated at later sampling points, indicating the possible consumption or production of the carbohydrates. The main outcome in terms of analytical performance is that the classic approach of using IEX-RI can not effectively separate isobaric compounds belonging to the same order, e.g. maltose from sucrose. Therefore, a novel methodology based on hydrophilic interaction chromatography coupled with mass spectrometry (HILIC-MS) was developed and validated on fermentation broths from *Streptococcus thermophilus*. Five saccharides – fructose, glucose, galactose, sucrose and lactose – were successfully separated and quantitated using the optimal chromatographic and mass spectrometric conditions. Additionally, several sample preparation protocols were evaluated to afford the cleanest but still comprehensive sample. The HILIC-MS methodology was further finetuned and applied to a wide range of plant-based yoghurt alternatives made from lupin, oats, coconut, and soya. Four main sugars – fructose, glucose, sucrose, and maltose – were measured across all 25 samples with minor modifications to sample preparation protocols. The sugar measurement results were combined with additional chemical and microbiological analysis results to comprehensively describe the yoghurt alternative market. Furthermore, a HILIC-MS method was applied to the measurement of cell wall components, providing vital quantitative information about cell wall monomers: N-acetylglucosamine and N-acetylmuramic acid. The method was validated and applied on biomass produced from both Gram-positive and Gram-negative bacteria, highlighting the opportunity to perform this analysis in a high-throughput manner.

To conclude, these results show that the analytical complexity of carbohydrates and their derivates requires a specific approach in an instrumental setup. Correct quantification and distinguishing various sugars in a wide array of matrices is vital to producing a good plant-based product.

Lühikokkuvõte

Analüütiliste meetodite optimeerimine süsivesikute ja nende derivaatide määramisekss erinevates fermenteeritud maatriksites

Kiirelt kasvav maailma rahvastik võib koormata üle traditsioonilisi toidutootmise tarneahelaid. Elanikkonna jätkusuutliku toitmise nimel arendatakse alternatiivseid tootmis- ja tarneahelaid. Taimepõhised fermenteeritud tooted on hea alternatiiv traditsioonilistele lihast ja piimast valmistatud fermenteeritud toodetele. Võrreldes loomset päritolu toiduproduktidega ei ole taimse tooraine fermenteerimisprotsesse siiski niivõrd põhjalikult uuritud. Toorainete kompleksus tekitab palju tehnoloogilisi väljakutseid, alates juuretiste valimisest kuni toitainekomponentide kättesaadavuseni bakteridele nimetatud materjalides. Bakterite kasv sõltub taimepõhistes maatriksites olemasolevatest suhkrutest ja sellest, kas bakteri suudab selliseid suhkruid tarbida. Taimsete suhkrute mitmekesisus tekitab ka analüütilist probleemi ning bakterite kasvu ja suhkrute tarbimise kindlakstegemiseks tuleb erinevaid süsivesikuid analüütiliset määrata. Selle töö eesmärk oli parandada süsivesikute määramist erinevates kääritatud maatriksites ja uurida, kuidas kasutab bakter suhkruderivaate elutsükli jooksul. Esimene samm olemasolevate instrumentaalsete metoodikate parendamisel oli määrata kaerahelveste fermenteerimise ajal tarbitud süsivesikuid. Lähendes klassikalisel viisil, kasutati ioonivahetusel põhinevat statsionaarset faasi ja refraktomeetrilist detektorit (IEX-RI), et määrata tekkivaid ja tarbitavad mono-, di- ja trisahhariide 24 tundi kestnud kaerahelveste kääritamisel nelja erineva juuretisega. Tulemused näitasid, et kõigis proovivõtu punktides ei toimunud disahhariidide kontsentratsioonis olulist muutust. Samas mono- ja trisahhariidide kontsentratsioonid kõikusid hilisemates proovivõtu punktides, mis viitab nende süsivesikute võimalikule tarbimisele või tootmisele. Peamine tulemus analüütilisest seisukohast oli see, et klassikaline IEX-RI kasutamine ei suuda tõhusalt eraldada isobaarseid ühendeid, mis kuuluvad samasse perekonda, näiteks maltoosi lahutus sahharoosist. Seetõttu arendati välja ja valideeriti juba *Streptococcus thermophiluse* fermenteerimis-protsessi jälgmiseks uus metoodika, mis põhineb hüdrofiilsete interaktsioonide kromatograafia-massispektromeetrial (HILIC-MS). Viis sahhariidi: fruktoos, glükoos, galaktoos, sahharoos ja laktoos, eraldati ja mõõdeti edukalt, kasutades optimaalseid kromatograafilisi ja massispektromeetrilisi tingimusi. Lisaks hinnati mitmeid proovivalmistamise protokolle, et saada puhtaim ja samal ajal informatiivseim proov. HILIC-MS metoodikat arendati edasi ja rakendati laiale taimepõhise jogurti alternatiivi valikule, mis olid valmistatud lupiinist, kaerast, kookosest ja sojast. Kõigil 25 proovil mõõdeti nelja peamist suhkrut – fruktoosi, glükoosi, sahharoosi ja maltoosi. Põhjalikumaks turul olevate jogurti alternatiivide kirjeldakirjeldamiseks kombineeriti suhkrute määramise tulemusi täiendavate keemiliste ja mikrobioloogiliste analüüside tulemustega, HILIC-MS meetodit rakendati lisaks rakuseina komponentide mõõtmiseks, pakkudes olulist kvantitatiivset teavet rakuseina monomeeride – N-atsetüülglükosamiini ja N-atsetüülmuraamhappe kohta. Meetodit valideeriti ja rakendati nii Gram-positiivsetest kui ka Gram-negatiivsetest bakteritest toodetud biomassis, kinnitades analüüsi suurt läbilaskevõimet.

Kokkuvõttes näitavad saadud tulemused, et süsivesikute ja nende derivaatide analüütiline keerukus nõuab spetsiifilist lähenemisviisi instrumentaalsele seadistusele. Erinevate suhkrute kvantifitseerimine ja teineteisest eristamine erinevates maatriksites annab parema ülevaate toimuvatest protsessidest ja on hea taimepõhilise fermenteeritud produkti saamise oluliseks osaks.

Appendix 1

Publication I

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Research article

Starter culture growth dynamics and sensory properties of fermented oat drink

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ABSTRACT

In the present study, an oat drink, a plant-based alternative to dairy products, was developed by fermenting the oat base with different vegan starter cultures. The desired pH below 4.2 was achieved in 12 h, regardless of starter culture used. Metagenomic sequencing revealed that *S. thermophilus* was the dominating species, ranging from 38% to 99% of the total microbial consortia. At lower pH values, population of *L. acidophilus*, *L. plantarum* and *L. paracasei* continued to increase in fermented oat drinks. Lactic acid was produced between 1.6 and 2.8 g/L. The sensory panel showed that all fermented oat drinks had a sour odor and taste. The volatile compounds identified belonged to the ketone, alcohol, aldehyde, acids, and furan classes. The concentration of the most preferred volatile components, such as diacetyl and acetoin, increased during fermentation. However, sensory evaluation showed that all samples were associated with cereals and not dairy in terms of taste and odor. Rheological analysis showed the formation of weak gel-like structures in fermented oat drinks. Overall, fermentation improved flavor and texture of the product. This study provides a broad overview of the oat drink fermentation process from the perspectives of starter culture growth, microbial consortium dynamics, lactic acid bacteria metabolism, and sensory profile formation.

1. Introduction

Plant-based fermented drinks are the new reality towards a sustainable lifestyle. The world population will grow to 9.7 billion within five decades and there will not be enough animal-based food for all of us [1]. Plant-based foods also provide healthy and sustainable diet for mankind. To meet these needs, novel plant-based dairy alternatives that mimic animal-based foods must be developed.

Plant-based drinks, often called milk analogs, are gaining popularity every year. Cereals, legumes and nuts are the most preferred raw materials for making plant-based drinks [2,3]. Unfortunately, plant-based drinks often do not meet the requirements of traditional dairy products and require excessive fortification. Fermentation is a simple and natural way to improve the sensory, textural, and

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nutritional value of plant-based dairy alternatives. However, plant-based dairy alternatives are often not fermented, and both acid and live bacteria are added in the final stages of product preparation. Furthermore, if live cultures are used, very little is known about how traditional milk-derived bacteria behave and adapt to plant materials. Currently, we still lack information on culture growth kinetics, consortium composition, and flavor and aroma formation of plant-based fermented drinks. In order for bacteria to succeed, we need to understand how different starter cultures affect plant-based fermented drinks.

In our article, we decided to focus on oat drink because the consumption of oat drinks is a growing trend. Oats are healthy and less allergenic compared to soy or nut-based drinks. Oats grow in cool, moist conditions and in sandy loam to heavy clay soils with good drainage, which helps to cultivate oats in regions (e.g. Eastern Europe, Scandinavia, North America) where soy or nut cultivation could not be applicable due to weather and agricultural conditions [4].

Oats are rich in starch, protein, fiber (beta-glucans), antioxidants, vitamins, and healthy fats, with the higher protein and lipid content than other cereals [3,5]. The presence of starch turns the oat-based matrix into a gel. Enzymatic hydrolysis with α-amylase or sequential hydrolysis with α- and β-amylase to hydrolyze starch to glucose and shorter chain polysaccharides must be performed to maintain the fluidity of the beverage [3]. 80% of oat protein is globulins with an isoelectric point between 4 and 5. Fermented oat beverage often reaches this low pH concentrations, which causes protein aggregation and reduces the acceptance of the fermented product due to chalky and sandy mouthfeel [6]. However, nutritionally, oat proteins have a higher lysine content than wheat, which is the main limiting amino acid in cereals [7]. Oats also contain high amounts of unsaturated essential fatty acids, such as oleic acid (18:1) and linoleic acid (18:2), which have a significant impact on nutritional quality. Unfortunately, oats also contain a considerable amount of lipases, which can cause rancidity during oat processing [8].

Fermentation is one way to reduce the shortages of raw materials and improve the shelf-life, nutrition, and flavor of the product. Microorganisms involved in fermentation produce enzymes, vitamins such as folates, short chain fatty acids, amino acids, bacteriocins and exopolysaccharides, thereby improving the nutritional value, sensory properties, and shelf-life of the product. For example, riboflavin and folate are found in higher concentrations in fermented oat drink than in unfermented oat drink [9]. Short chain fatty acids produced by LAB increase the solubility of bioavailable calcium and enhance the synthesis of vitamins and bioactive peptides [10]. Fermentation also improves the appearance of plant-based dairy alternatives. Due to the production of organic acid and a decrease in pH, the effect on phenols has been shown to lighten the colour of the fermented product compared to unfermented oat beverage [11].

Lactic acid bacteria (LAB) are often used to positively manipulate plant-based dairy alternatives. To improve the aroma profile, *L. plantarum* NCIMB 8826 has been used in oat, wheat, barley, and malt matrices. Different volatile compounds such as esters, alcohols and aldehydes were produced during the growth, with each cereal broth having a unique volatile compound profile [12]. When traditional LAB strains were combined with *L. rhamnosus* LGG^R to ferment soy, oat, and coconut substrates, acetoin levels increased, and acetaldehyde decreased in the presence of LGG^R in all three bases. Oat samples additionally fermented with LGG^R demonstrated preferred notes, such as sourness, lemon, and fruity flavors. Gel firmness in coconut samples was improved in the presence of LGG^R, indicating that the addition of *L. rhamnosus* improves the textural and sensory perception of fermented plant-based dairy alternatives [13]. *L. plantarum* LP09 strain was used to ferment the oat flake beverage. Oat base fermentation increased polyphenols availability and antioxidant activity (25% and 70% higher, respectively). Additionally, sensory evaluation showed that the fermented oat flake drink has characteristics of a yogurt-like beverage, enhancing the overall intensity of odor and flavor compared to the unfermented control [11]. To conclude, plant-based beverages will play a major role in future diets, and the fermentation of these matrices by LAB needs to be thoroughly investigated.

In this study, we combined various analysis and analytical tools to understand how starter cultures affect the oat drink during fermentation. The novelty of the work lies on the complex overview, where the growth dynamics of the starter culture are monitored from chemical-, physical-, metabolic- and sensory aspects. For the first time, isothermal microcalorimetry and 16 S metagenomic analysis are combined to evaluate the starter culture properties and composition in a fermented oat drink. This knowledge provides a detailed overview of what novel food manufactures need to consider when formulating and developing plant-based dairy alternatives.

2. Materials and methods

2.1. Materials

2.1.1. Raw materials and chemicals

Veski Mati whole grain oat flakes were purchased from a local retailer. α-Amylase Fungamyl® 800 L (Fungamyl), α-amylase BAN® 480 L (BAN), and glucoamylase AMG® 300 L (AMG) were provided by Novozymes (Bagsvaerd, Denmark). For sugar analysis, D-(+)-Raffinose pentahydrate (Sigma-Aldrich, P/NR0250-25G), D-(+)-Maltose monohydrate (Sigma-Aldrich, P/N M2250-1 KG), D-(+)-Glucose (Sigma-Aldrich, P/N G7528-1 KG), were represented as marker compounds. For organic acid analysis, acetic acid (Honeywell, Fluka P/N 965,092), butyric acid (Sigma-Aldrich, P/N B1030500-500 mL), citric acid (Sigma-Aldrich, P/N 251,275-100G), formic acid (Supelco, P/N 5,330,020,050), isobutyric acid (Sigma-Aldrich, P/N l1754-500mL), isovaleric acid (Acros Organics, P/N AC156690100), lactic acid (Sigma-Aldrich, P/N L7022-10G), malic acid (Sigma-Aldrich,P/N M6413-25G), propionic acid (Sigma-Aldrich, P/N P1880-100G), succinic acid (Sigma-Aldrich, P/N 14,079-250G), valeric acid (Alfa-Aesar, P/N A16238. AP) were used as standards. For free amino acid analysis amino acid standard (Waters Corporation, WAT088122) + 3 additonal amino acids: L-asparagine (Serva, P/N 14,110), L-tryptophan (Serva, P/N 37,422) and L-glutamine (Sigma-Aldrich, G-3126) were used. For volatile compounds analysis in GC-MS, 4-methyl-2-pentanol (Sigma-Aldrich 109,916-25 mL) was used as internal standard.

2.1.2. Starter cultures

Commercial vegan starter cultures were used to ferment the oat drink. Vegan starter cultures states that the lactic acid bacteria in the culture mix are produced dairy free, on plant-based medium and are suitable for plant and/or vegetable-based products. The products fermented with vegan starter cultures are suitable for vegans. Starter cultures are abbreviated SC1 to SC4 throughout the article. SC1 contains *Streptococcus thermophilus* (*S. thermophilus*) and *Lactobacillus delbrueckii* spp. *Bulgaricus* (*L. bulgaricus*). SC2 contains *S. thermophilus*, *L. bulgaricus*, *Lactobacillus delbrueckii* spp. *Lactis* (*L. lactis*), *Bifidobacterium lactis* (*B. lactis*) and *Lactobacillus acidophilus* (*L. acidophilus*). SC3 contains *S. thermophilus*, *L. bulgaricus*, *B. lactis*, *L. acidophilus* and *Lactobacillus plantarum* (*L. plantarum*). SC4 contains *S. thermophilus*, *L. bulgaricus*, *B. lactis*, *L. acidophilus* and *Lactobacillus paracasei* (*L. paracasei*). The manufacturer did not provide information on the proportion of individual bacterial species in the starter cultures.

2.2. Fermented oat drink preparation

To prepare 1 L of oat drink, 160 g of oat flakes were weighed and washed for 10 s under cold running water. 840 mL of water was added to the flakes and homogenized (Polytron PT MR 2100, Kinematica, Switzerland) for 30 s. To increase the enzyme activity, $CaCl_2$ was added at a concentration of 0.01% (w/v oat base). Hydrolysis was performed with α-amylase (Fungamyl® 800 L or BAN 480 L) at a concentration of 0.88% (w/w oat flakes) and glucoamylase (AMG® 300 L) at a concentration of 0.4% (w/w oat flakes). The drink was hydrolyzed in a water bath (Julabo TW8, Julabo Labortechnik GmbH, Germany) for 60 min, ensuring +55 °C inside the liquid. After hydrolysis, the drink was strained through a muslin cloth and the solids were separated from liquid. The filtered drink was reheated to deactivate the enzymes in a water bath, where the internal temperature was maintained +85 °C for 15 min. The oat drink (enzymatically pretreated with Fungamyl) was cooled to +40 °C and divided into four aliquots. Each aliquot was inoculated with a commercial starter (SC1, SC2, SC3 and SC4). The freeze-dried culture was resuspended in sterile 0.85% saline and the inoculum concentration were 10 DCU. Fermentation was carried out for 24 h at +40 °C. For the various analysis, samples were collected and stored either at +4 °C (for sensory evaluation, NGS, rheological and titratable acid analysis) or −20 °C (for sugar, organic acid, free amino acid, and GC-MS analyses).

2.3. Isothermal microcalorimetry

Isothermal microcalorimetry can be used to monitor the heat flow produced by the microorganisms in real-time. This provides a continuous real-time signal proportional to the heat generated during bacterial growth and metabolism [14]. A TAM III 24-channel isothermal microcalorimeter (TA Instruments, New Castle, DE, USA) was used. Each 3 mL ampoule was filled with 2 mL of inoculated oat drink. The heat flow was monitored for 24 h at +40 °C. Data analysis was performed in Microsoft® Excel® (Version 2204 Build 16.0.15128.20240) by plotting power-time curves describing the heat release during the investigated process. The maximal specific growth rate μ_{max} (h^{-1}) was determined as the slope of heat production (Q) of the exponential growth phase over time (t). The maximum heat flow produced P_{max} (μW/mL), and the time maximal heat flow obtained t_{Pmax} (h) were obtained from the power-time curves. The value P_{max} characterizes the maximal heat rate production and reaching P_{max} can be considered as the end of the exponential growth phase. The heat release during the exponential growth phase Q_{exp} (J/mL) and Q_{tot} (J/mL) during the entire growth were determined from the area under the power-time curves.

2.4. pH measurement

iCinac (AMS Alliance, Rome, Italy) is a multi-channel system for real-time monitoring of pH, temperature, and redox potential. For analysis, 104 g of the sample was placed in an autoclaved 100 mL bottle with a hole in the cap for the sensor, placed in a +40 °C water bath (Julabo EH, Julabo Labortechnik GmbH, Germany) and connected to an electrode. Changes in pH were monitored for 24 h. Data analysis was performed in *Microsoft Excel* by plotting pH curves.

2.5. Starter culture consortium analysis

2.5.1. Microbial cell separation and genomic DNA extraction

To isolate bacterial cells from fermented plant residues, pellet from approximately 14 mL of fermented oat drink was resuspended in 10 mL of sterile 0.85% NaCl solution and centrifuged at 750×*g* (Hettich ROTANTA 460 R, fixed angle rotator) for 5 min at +6 °C. To pellet the microbial cells, the supernatant was transferred to a new 50 mL tube and centrifuged at 10,000×*g* for 15 min at +6 °C (Hettich ROTANTA 460 R, fixed angle rotator). The pellet was washed in 1 mL sterile 0.85% NaCl solution, divided into three aliquots (approximately 400 μL each) and centrifuged at 10,000×*g* for 10 min at +6 °C (Thermo scientific MicroCL 21 R). The supernatant was aspirated, and the pellet containing the microbial cells were stored at −20 °C until gDNA extraction.

Microbial cells precipitate from 400 μL aliquots (1/3 from all separated cells, approximately 100 mg of cells) were subjected to gDNA extraction according to the Quick-DNA™ Fungal/Bacterial Miniprep Kit protocol (ZR, Zymo Research, Irvine, CA, USA). The samples were thawed at room temperature for 10 min before the initiation of gDNA extraction. The quantity of the extracted gDNA was measured with a Qubit™ 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) using the dsDNA BR Assay Kit (Thermo Fisher Scientific).

2.5.2. 16 S library preparation, next generation sequencing and data processing

Amplicon libraries targeting the 16 S rRNA gene V4 hypervariable region by primer pair 515 F/806 R were prepared according to Illumina's dual indexing system. Multiplexed and normalized libraries were sequenced with iSeq100 Sequencing System (Illumina, San Diego, CA, USA) using iSeq 100 i1 Reagent and 2 × 150 cycles paired-end sequencing protocol. Previous activities were performed as published before by Kazantseva et al., [15]. The sequencing data was analyzed by an open-source BION-meta package (https://github.com/nielsl/mcdonald-et-al) according to the author's instructions [16,17].

2.6. Chemical, textural, and sensory analysis

2.6.1. Sugars and organic acid analysis

Enzyme-treated and fermented oat drink samples were centrifuged at 14,000×*g* for 20 min at room temperature (Hettich ROTANTA 460 R, fixed angle rotator). The supernatant was filtered through a 3 kDa molecular weight cut-off filter (Amicon® Ultra-0.5, Merck KGaA, Germany) and diluted with 2 parts of ultrapure water before analysis. Concentrations of sugars (D-(+)-Raffinose pentahydrate and D-(+)-Maltose monohydrate, represented as a marker compound), organic acids and D-(+)-Glucose were measured with a high-performance liquid chromatography (HPLC) system (Alliance 2695 system, Waters Corp., Milford, MA, USA), using a BioRad Aminex HPX-87C (for sugars) or BioRad Aminex HPX-87H (for organic acids) columns (7.8 × 300 mm, 9 μm particle size) (Bio-Rad Laboratories, Inc., CA, USA). A BioRad Micro-Guard Cation C guard column (4.6 × 30 mm, 9 μm particle size) with isocratic elution of ultrapure water at a flow rate of 0.6 mL/min at +85 °C was used for sugar analysis. H guard column (4.6 × 30 mm, 9 μm particle size) with isocratic elution of 5 mM H_2SO_4 at a flow rate of 0.6 mL/min at +35 °C was used for organic acid analysis. Waters 2414 refractive index detector was used for the detection and quantification of substances, which was paired with a Waters 2487 Dual Absorbance Detector for organic acid analysis.

2.6.1.1. Titratable acidity. Titratable acidity, reported as lactic acid, was measured with a DL22 Food and Beverage Analyzer (Mettler Toledo, Switzerland). 5 g of sample was mixed with 45 g of distilled water, mixed until homogeneous and titrated with 0.1 N NaOH. The results were calculated as % of lactic acid using Eq. (1).

$$\%\ lactic\ acid = \frac{mL \bullet N \bullet 90 \bullet 100}{V \bullet 1000} \tag{1}$$

Where: mL – NaOH usage for a sample in milliliters.

N – the normality of NaOH (0.1)
V – sample volume (5 mL)

2.6.2. Free amino acid analysis

Fermented oat drink samples were centrifuged at 14,000×*g* for 20 min at room temperature (Hettich ROTANTA 460 R, fixed angle rotator). The supernatant was filtered through a 3 kDa molecular weight cut-off filter (Amicon® Ultra-0.5, Merck KGaA, Germany) and diluted with 2 parts of ultrapure water before analysis. Prior to injection, free amino acids were derivatized with AccQ•Fluor Reagent (Waters Corp., MA, USA) according to the manufacturer's procedure. Analysis of free amino acids was performed on an ultra-performance liquid chromatography (UPLC) system (Acquity UPLC; Waters Corp., MA, USA), including a binary solvent manager, a sample manager, and a photodiode array detector (PDA), controlled by Waters Empower™ 3.0 software (Build 3471, Waters Corp., MA, USA). Separations were performed on Waters Acquity UPLC AccQ•Tag Ultra Column (2.1 × 100 mm, 1.7 μm particle size) operated at +55 °C. The injection volume was 1.5 μL, the amino acids were eluted at a flow rate of 0.3 mL/min, and absorbance was recorded at 260 nm. The running time was 25 min. Empower software (Waters Corp., MA, USA) was used for data processing.

2.6.3. Rheological analysis

Dynamic oscillatory measurements were carried out at +22 °C using a Physica Modular Compact Rheometer MCR 301 (Anton Paar GmbH, Graz, Austria) equipped with a Peltier temperature control unit *C*-PTD200 and a coaxial cylinder measuring system CC27 (outer and inner diameters 28.92 and 26.66 mm, respectively). Amplitude sweep was performed varying the strain from 0.01 to 100% at a constant frequency of 1 Hz. Frequency sweep was performed from 0.01 to 10 Hz at a strain value of 0.1%, staying within the linear viscoelastic (LVE) range. The storage (G′) and loss (G″) module were measured and plotted in double logarithmic scale against frequency and amplitude, respectively. The limit of the LVE range (γ_L), the yield stress (τ_y), and the storage modulus within the LVE range (G'_{LVE}) were determined from the amplitude sweep. The slope of G′ vs frequency (Δ log G'/Δ log f) was calculated from the double logarithmic plot of frequency sweep using Rheoplus/32 V^2.66 software (Anton Paar GmbH).

2.6.4. Volatile compound analysis

Identification and quantification of volatile compounds was performed using gas chromatograph system (2030; Shimadzu, Kyoto, Japan) equipped with mass spectrometer (8050NX Triple Quadrupole; Shimadzu, Kyoto, Japan). A ZB5-MS column (30 m length × 0.25 mm i. d. × 1.0 μm film thickness; J&W Scientific, Folsom, CA, USA) was used with helium as a carrier gas at linear velocity of 35 cm s^{-1}. The oven was programmed to ramp up from +40 °C at a rate of 7.5 °C/min to a final temperature of +280 °C with an additional holding time of 4 min (total run time 36 min). Mass spectra were obtained at an ionization energy of 70eV, detector voltage 1 kV and

with Q3 scan range of *m/z* 35–250. Non-targeted identification of volatile compounds was carried out using GCMS solution 4.52 software (Shimadzu, Japan) and retention indices (RI) calculated with n-alkanes. The identification of the compounds was verified by comparing experimental retention indices to NIST17 and FFNSC4 spectral libraries. Semi-quantitative evaluation using the internal standard (4-methyl-2-pentanol; 20 ppb) was performed to semi-quantify identified volatile compounds (in ISTD ppb-equivalents).

2.6.5. Sensory analysis

The sensory analysis was carried out by the sensory panel of Center of Food and Fermentation Technologies in a quiet room in accordance with ISO standard 8589:2007. The analysis was conducted by nine trained assessors (average age 32.8 ± 7.5) with previous experience in evaluating fermented plant-based dairy alternatives and fermented dairy products. All participants from a pool of highly trained evaluators in the sensory panel gave written consent to take part in the experiment. Participants were informed in advance of the purpose and the procedures of the study. Participants were assured of the confidentiality of their data. Taking part in the given study was voluntary and one could withdraw from the test at any time. Participants were in good health and had no known allergy to the components. Institutional approval for the research is not available due to Estonian requirements for human research.

A separate training session was carried out with selected samples prior the analysis, which familiarized assessors with the products and attributes for the assessment. The samples were kept refrigerated (+4 °C) until serving in 40 mL plastic cups coded with random three-digit numbers. Order of fermented drinks was followed by Williams Latin Square design. The appearance, odor, taste, and texture of samples were evaluated at 10-point scale ranging from 0 to 9 with anchor points (i.e., "0" - none; "1" - very weak; "5″ moderate; "9" - very strong). Panel members were encouraged to use water and crackers for palette cleansing. Samples were evaluated in three parallels, in total of two separate sessions. Unfermented oat drink was used as reference in both sessions. Assessors also had at least 2-h break between sessions to reduce sensory fatigue.

2.7. Statistics

Statistical analysis was performed in R version 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria). Package 'stats' 4.0.2 was used for calculating principal components, performing ANOVA and Tukey Honest Significant Differences test at 95% family-wise confidence level.

Principal component analysis (PCA) for volatile compounds was performed using "prcomp" R function and visualized with R package "ggplot2" version 3.3.0. Sample clusters on PCA biplot were shown by 95% confidence ellipses. Variables were centered and scaled. Data represents each sample in the form of mean of biological replicates (n = 3).

3. Results and discussion

3.1. Oat drink preparation

3.1.1. Enzymatic treatment

The pretreatment of oat drink with different amylases to hydrolyze starch and obtain sugars for both, the fermentation process and the overall flavor profile of the final product are shown in Fig. 1. The addition of AMG increased the glucose concentration almost 10-times compared to the treatments without AMG. In the BAN treatment, the concentration of trisaccharides (20 g/L) was the highest following disaccharides and glucose. Fungamyl alone increased the disaccharide concentration to 35.86 g/L after 1 h of incubation. Trisaccharides and glucose were in similar concentration. Fungamyl was chosen for the final production of the oat drink as it resulted in a suspension with a nice creamy texture (compared to other enzymes that resulted in an unstable texture), and a slightly sweet and not too sugary taste (data not shown). High glucose concentration was also not preferred due to osmotic stress, which can inhibit lactic acid bacteria fermentation [18].

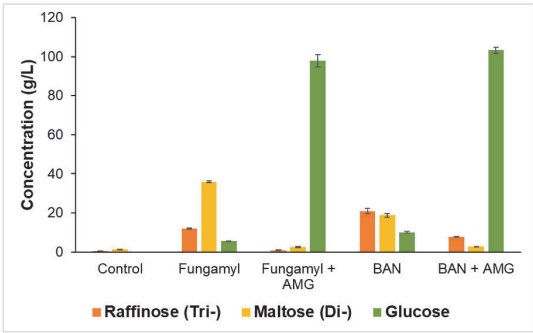

Fig. 1. Sugar concentration (g/L) after enzymatic treatment of oat drink. Tri- and disaccharides are indicated as sum, where either raffinose or maltose was used as markers, respectively. AMG, BAN and Fungamyl are abbreviations for corresponding enzymes (see Materials and methods). Data represents each sample in the form of mean of biological replicates ± SD (n = 3).

3.1.2. Duration of hydrolysis

The optimal time of enzymatic hydrolysis was determined at four timepoints and the results are shown in Fig. 2. The concentrations of trisaccharides, disaccharides and glucose were measured after 30, 60, 90 and 120 min of treatment with Fungamyl. Hexoses and disaccharides are the most favorable carbon source for lactic acid bacteria [19]. Since no significant increase in the concentration of tri- and disaccharides or glucose was observed after 60 min of hydrolysis, then 1 h enzyme treatment was considered sufficient for further experiments.

3.2. Fermentation of oat drink

3.2.1. Dynamics of fermentation process

Based on specification sheets for commercial starter cultures, plant-based dairy alternatives should be fermented for 8.5–10 h to reach a pH of 4.6. However, for this experiment, the process was followed for 24 h to observe the dynamics of a longer fermentation (Fig. 3). In 24 h, the most acidified oat drink with a pH of 3.85 was obtained in the SC2 starter culture. In this experiment, the optimal fermentation time was 12 h, as a pH < 4.6 would result in a more acidic taste pallet in the final product, comparable to kefir with a pH of 4.2. At this time point, the SC1 culture had the mildest acidity with a pH of 4.19, followed by the more acidic SC4 with a pH of 4.06, then SC3 with a pH of 3.91 and SC2 with a pH of 3.82. Luana et al. [11], showed that fermentation of oat beverage with *L. plantarum* LP09 achieved a pH of 4.2 in 8 h. The observation was in correlation with a current study where a pH of 4.2 was reached already in 7.3 h with SC3, which also contains *L. plantarum*. If the desired optimal pH were 4.6, which is more comparable with yoghurt, then SC3 and SC2 reached pH 4.6 in ~4.5 h, while SC1 and SC4 reached in ~6 h. The degree of acidification of the oat drink appeared faster than the specification sheets of the starter culture stated. However, products such as yoghurt have a shorter shelf-life at pH 4.6 than at pH 4.2 or lower. Karagül-Yüceer et al. [20], showed that pH affected the viability of contaminating bacteria. *E. coli* survival at pH 5 was 21 days, while at low pH (4.2) there was no survival at day 21. After 12 h of fermentation (samples collected to sensory evaluation, rheology, and GC-MS study) drinks with SC3 and SC2 were only 0.1 pH points apart, showing that these two cultures acidify quite similarly.

The lag-phase before fermentation varied from 1 to 1.5 h depending on the starter culture. A similar lag-phase was observed for heat power time curves. While SC3 and SC2 showed the fastest acidification, the maximal specific growth rate (μ_{max}) based on biomass heat production was highest for SC1 (Supplementary materials - Table S1) with 1.66 h^{-1}. The maximal specific growth rates of SC2 and SC3 were 1.55 h^{-1} and 1.53 h^{-1}, respectively. The lowest μ_{max} of 1.40 h^{-1} was observed for SC4. However, the time where exponential growth ended (P_{max}) was fastest with SC3 at 3.03 h (t_{Pmax}). The slowest grower, as already indicated by the maximal specific growth rate, was SC4, achieving the majority of the biomass in 4.4 h. Still, the maximal biomass (Q_{tot}) was produced by SC2 reaching the total heat production of 2.19 J/mL. SC2 also had the lowest pH, suggesting greater acid tolerance while maintaining metabolic activity at low pH. The next highest total heat production was obtained with SC3, showing 1.94 J/mL. Interestingly, the slowest grower, SC4, produced more biomass and remained metabolically active longer than SC1, which initially had the highest maximal specific growth rate but then rapidly slowed down. The maximal total heat production for SC4 and SC1 was 1.63 J/mL and 1.29 J/mL, respectively.

The heat curves show (Fig. 3) that all three starter cultures except SC1 had two growth phases. It may be that SC1 has only two species in the consortium, while the other starter cultures have five species in the consortium. The second slower growth phase may indicate several environmental changes. First, essential substrates were depleted, and the starter culture had to switch on another source of nutrients. However, this scenario is the least likely, while the carbon source was unlimited (Supplementary materials – Fig. S.1). A second and more probable scenario was that whatever limiting property appeared in the medium, then either the dominating culture had to switch metabolism or slowly growing species started to take the culture over. The most probable cause was a drop in pH between 5 and 10 h. The limiting effect of pH caused the termination of the exponential growth phase. The pH curves show an exponential acidification phase within two to 5 h after the pH reaches the inhibitory range and the dominating bacteria or the whole consortium needs to change their metabolism to adapt to the new conditions.

Fig. 2. Sugar concentration (g/L) by different duration of Fungamyl enzyme treatment. Tri- and disaccharides are indicated as sum, where either raffinose or maltose was used as marker, respectively. Data represents each sample in the form of mean of biological replicates ± SD (n = 3).

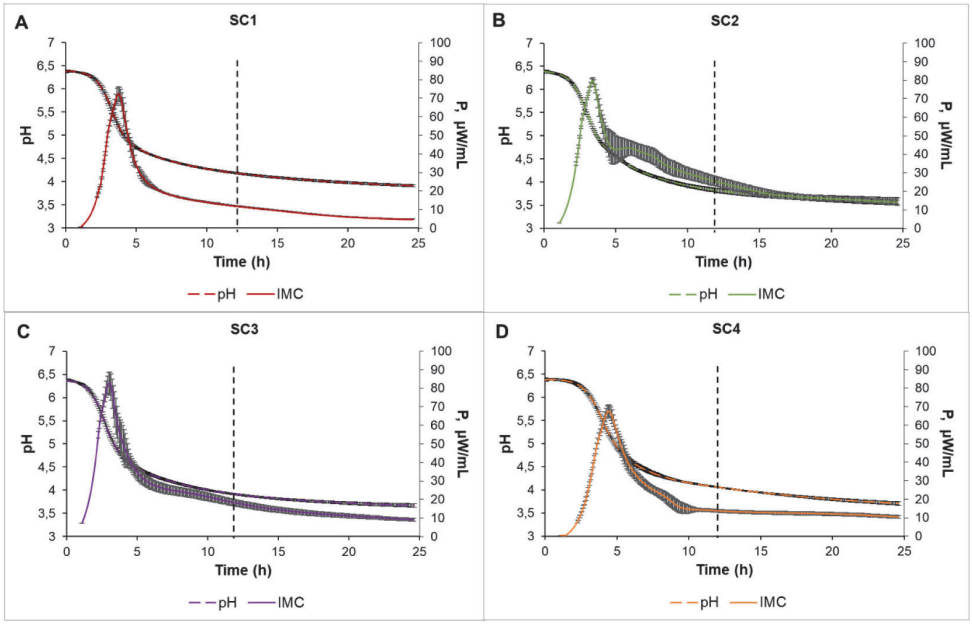

Fig. 3. Heat flow curves (marked as IMC) and pH curves (marked as pH) of each starter culture for 24 h. Heat flow and pH curve for SC1 is shown on graph A, for SC2 on graph B, for SC3 on graph C and for SC4 on graph D. Curves from IMC are power time curves (P) with µW/mL values. The vertical dotted black line indicates hour 12, where extra samples for sensory evaluation, rheology and GC-MS analysis were collected. Data represents each sample in the form of mean of biological replicates ± SD (n = 3).

3.2.2. Starter culture consortium composition

The acidification curves as well as the heat power curves showed the shift and changes in the metabolism of the starter cultures during fermentation. To further investigate dynamic shifts between starter cultures, 16 S rRNA sequencing was carried out every 3 h for up to 12 h, with the next and final sampling point after 24 h of fermentation. Fig. 4 shows the relative abundance of starter species from whole consortia. Before the oat drink was fermented, the native bacteria formed 2.6–13.9% of the total population. The native microbiota was gradually outcompeted by starter cultures and had decreased to 0.4% after 6-h of fermentation. The native microflora was dominated by *Bifidobacterium animalis*, *Lactobacillus gallinarum*, *Streptococcus salivarus*, *Streptococcus suis,* and *Pseudomonas azotoformans*, which can be found on the surface of cereals or in milk [21–23].

All four starter cultures show that *S. thermophilus* was the dominating species throughout the fermentation. The main reason for the dominance of *S. thermophilus* is that this species prevailed in starter culture right from the beginning – in SC1: 95.7%, SC2: 81.6%, SC3: 38% and in SC4: 60.3% of the total consortium.

The SC1 specification states the presence of two species: *S. thermophilus* and *L. bulgaricus*. The 0-h sampling point shows that *S. thermophilus* is the dominating species, while *L. bulgaricus* was less than 1%. *S. thermophilus* dominated throughout the fermentation, showing almost monocultural fermentation. Still, traces of *L. bulgaricus* were found throughout the fermentation. The rate of heat flow curves shows that SC1 was the only culture with a single growth phase, indicating that pH limitation decelerates the growth of *S. thermophilus*, but *L. bulgaricus* was not strong enough to take over the culture. The research by Adamberg et al. [24], showed that

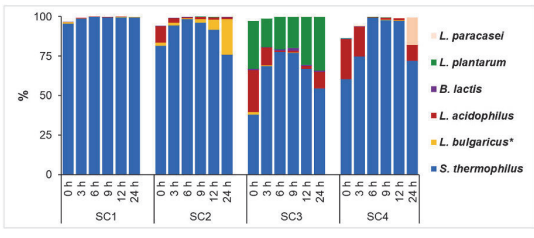

Fig. 4. Dynamics of starter culture consortium during fermentation process. Data is represented as bacterial proportion in percentages using 16 S rRNA gene next generation sequencing method. In SC2, *Lactobacillus delbrueckii* ssp. *Bulgaricus* (*L. bulgaricus*) and *Lactobacillus delbrueckii* ssp. *Lactis* (*L. lactis*), are marked as *L. bulgaricus**. Data represents each sample in the form of pooled biological replicates (n = 3).

S. thermophilus St20 was acid sensitive and unable to grow below pH 5.1, which is consistent with the present work.

SC2 has five different species in the culture, but amplicon sequencing does not distinguish between *L. delbrueckii* spp. *Bulgaricus* and *L. delbrueckii* spp. *Lactis* and identifies them as a single species (marked as *L. bulgaricus** in Fig. 4). At the beginning of fermentation, *S. thermophilus* was the dominating species, followed by *L. acidophilus* at 10.8%. The minority species were *L. bulgaricus/lactis* and *B. lactis*, with proportions of 1.8% and 0.2%, respectively. *B. lactis* was not detected at later timepoints, indicating that three other species took over the culture during the fermentation process. Interestingly, *L. bulgaricus/lactis*, which has only 1.8% abundance at the beginning, almost disappeared by 6th hour and after that, its proportion in the culture started to increase again, reaching 6.7% and 22.5% after 12 h and 24 h of fermentation, respectively. The same phenomenon occurs in cultures SC3 and SC4, where *S. thermophilus* reaches its maximal abundance by hour 6, after which its proportion begins to decrease, and other species start to take over the culture. At the end of the exponential growth phase, the heat flow increased slightly, creating peaks due to the secondary slow growth of the same three cultures (SC2, SC3, SC4), and after 9 h, the abundance of other populations in the starter consortium started to increase.

L. paracasei was initially not detected in the SC4 culture. It first appeared after 3 h of fermentation and was the second dominant species at 17.6% after 24 h. Generally, the species that dominated in the beginning of fermentation was the most abundant population until conditions were no longer favorable. In this starter culture comparison, *S. thermophilus* was the dominant species in all fermented oat drinks. However, in SC2 culture, after 24 h sampling point, *S. thermophilus* relative abundance is lower (75.8%) than at the starting point (81.6%). It seems that the starting conditions were suitable for the growth of *S. thermophilus*, but the limiting factor was the drop in pH, and the other starter cultures were able to slowly increase their proportion in the fermented oat drink. In this type of product development, *S. thermophilus* is the dominant species because the fermentation time is usually around 6–12 h or even less. Studies have shown that during the first hours of fermentation, *S. thermophilus* is the main lactose degrader, while in the later stages of yoghurt production, *L. bulgaricus* increases its abundance [25,26]. Compared to other LAB species, in addition to the dominant *S. thermophilus*, *L. plantarum* was constantly present in the SC3 consortium. This may be because *L. plantarum* originates from plant and is adapted to grow on plant material [27]. The dominance of the two species during fermentation indicates that these bacteria probably did not compete for the same carbon source. While *S. thermophilus* prefers to metabolize mono- and disaccharides, *L. plantarum* is capable of

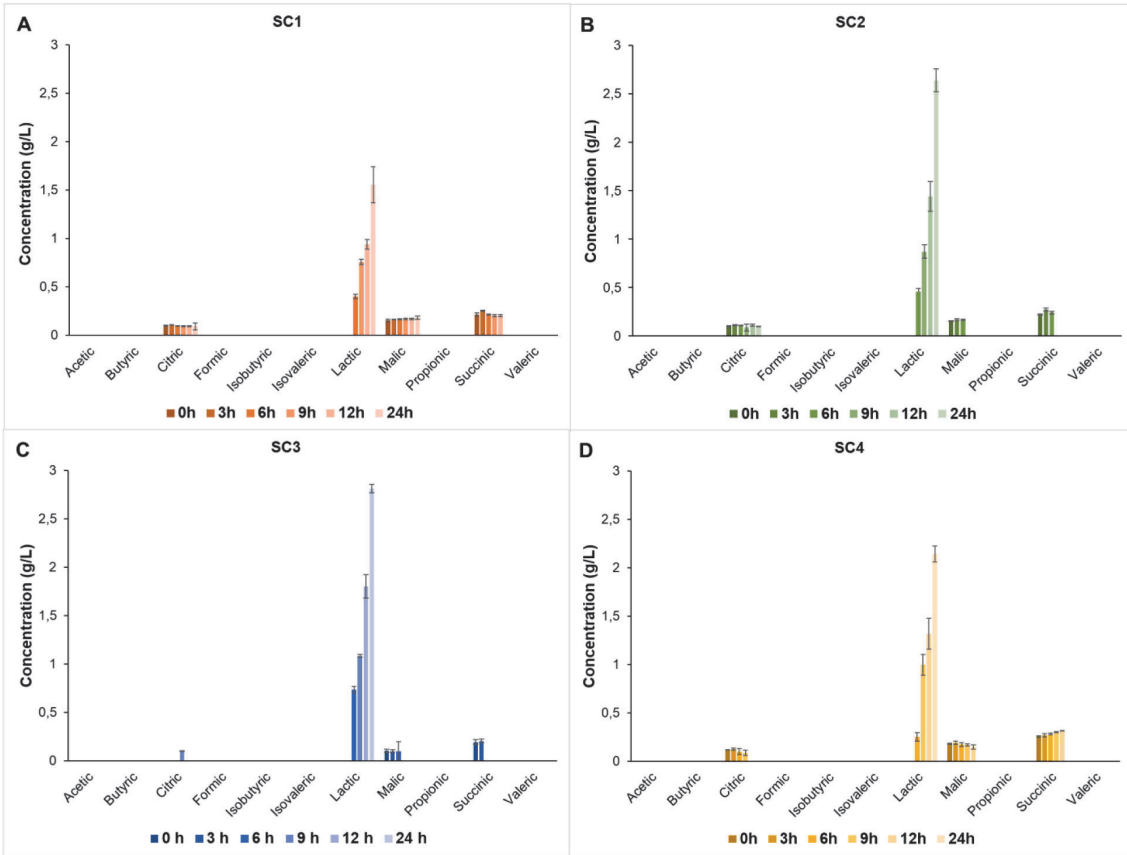

Fig. 5. Organic acid concentration (g/L) change during fermentation. Organic acid concentrations for SC1 is shown on graph A, for SC2 on graph B, for SC3 on graph C and for SC4 on graph D. Data represents each sample in the form of mean of biological replicates ± SD (n = 3).

fermenting different fractions from starch, both linear and branched subunits [28]. Luana et al. [11], showed that only *L. plantarum* strains were capable of fermenting oat beverages at +30 °C for 12 h up to a pH of 4.2, whereas drinks with *L. casei* and *L. paracasei* did not reach below a pH of 4.7. Moreover, in oat, barley, or malt-based substrates, *L. plantarum* was the only bacterium maintaining viability of 10^7 cfu/mL after 24 and 36 h of fermentation, compared to *L. acidophilus* and *L. reuteri* [29]. These observations indicate that *L. plantarum* should be considered as a valuable species for fermentation of plant material next to *S. thermophilus* with excellent acidification capability and high acid tolerance.

3.3. Chemical, textural, and sensory evaluation of fermented oat drink

3.3.1. Chemical parameters of fermented oat drink

Throughout the study (every 3 h up to 12 h and then after 24 h of fermentation) the concentration of sugars, organic acids and free amino acid was measured (Fig. 5, Supplementary materials – Fig. S.1, Fig. S.2). The initial sugar content was similar in all oat drinks with different starter cultures. The trisaccharide concentration was 6.5 g/L, the disaccharide concentration was 32 g/L, and the glucose concentration was 5 g/L. The results showed that after 24 h, glucose was the only measured sugar source consumed by the starter cultures. Interestingly, the concentration of tri- and disaccharide in some starter cultures even increased, indicating that even longer sugar polymers were probably present in the matrix. Thus, the bacteria likely consumed additional carbon sources besides glucose, increasing tri- and disaccharides residues in the medium. However, *S. thermophilus* cannot metabolize amylose and amylopectin [30, 31], preferring primarily monosaccharides. Still, amylolytic activity was observed in some LAB strains, resulting in the release of mono- and disaccharides [29].

The organic acid results from HPLC analysis show a homofermentative process for all starters (Fig. 5). The only acid produced during fermentation was lactic acid. Homolactic fermentation is a redox-neutral process where glucose is metabolized to lactic acid, thus ATP can be produced in glycolysis without accumulating the excess NADH in the cell. The highest concentration of lactic acid was reached at 24 h and from the highest to the lowest concentrations, SC3 produced the most at 2.81 g/L, followed by SC2 at 2.64 g/L, SC4 at 2.14 g/L and SC1 at 1.56 g/L. The uniqueness of SC3 is that it contains *L. plantarum*, a common lactic acid bacterium isolated from plants. The oat drink environment could be most suitable for this microorganism, and therefore lactic acid was produced in higher concentrations. Also, the metagenomic results showed that the appearance of *L. plantarum* increased after 24 h of fermentation. In all starter cultures, the lactic acid concentration increased more than 1.5 times with extra 12 h of fermentation.

None of the starter cultures produced ethanol during fermentation, indicating that the entire process was aerobic [32]. Acetic acid was also not measured with HPLC at different timepoints throughout the fermentation. However, under aerobic conditions, when lipoic acid is not available, *S. thermophilus* requires acetic acid to produce biomass [33]. It could be that the acetic acid was produced by other species (such as *L. paracasei*, *L. plantarum*) and was immediately consumed by *S. thermophilus*. Furthermore, *S. thermophilus* can also utilize lipoic acids, which are unique molecules from both plant and animal tissues that are also present in oats [34]. Lipoic acid is a co-factor of pyruvate dehydrogenase, which catalyzes the reaction from pyruvate to Acetyl-CoA, a key molecule in the breakdown of a carbon source and required for bacterial growth [35]. Some organic acids like citric, malic, and succinic acids were present in small quantities from the beginning, but all were consumed within 12–24 h in all batches. The dominating *S. thermophilus* was not able to utilize malic and succinic acids due to the lack of enzymes in the reductive branch of the TCA cycle. Thus, *S. thermophilus* did not grow during these hours, and probably other lactic acid bacteria consumed aforementioned organic acids. Unfortunately, citric acid was not measured properly in almost all SC3 timepoints, while in the sample this organic acid measurements were below the limit of qualification (LOQ). Still, citrate was depleted by the SC4 starter, containing *L. paracasei*. Analysis of the NCBI protein database reveals that the genomes of *L. plantarum* and *L. paracasei* contain genes encoding enzymes necessary for citric acid degradation.

Fluctuations in free amino acid concentration were measured throughout fermentation (Supplementary materials – Fig. S.2). The results indicate that the highest consumption occurred in 24 h and that free Asn, Asp and Glu concentrations decreased the most for all starter cultures. However, some free amino acids concentrations increased after 24 h of fermentation. An increase in Pro and Ser was detected in all starter cultures. The results are opposite found by Luana et al. [11], where Asp concentration increased and Ser concentration decreased in oat drink fermented with *L. plantarum*. The contradiction can be explained by the fact that Luana et al. [11], used a single *L. plantarum* culture, while the current study used a consortium. The increase in the concentration of some free amino acids was probably caused by the consumption of peptides, since it is energetically feasible to take in peptides of several amino acid

Table 1
Rheological parameters derived from amplitude and frequency sweeps conducted with control (unfermented oat drink) and fermented oat drink samples.

Starter	[a] G'_{LVE}, Pa	[b] τ_y, Pa	[c] Δ log G'/Δ log f, Pa/Hz
Control	0.59 ± 0.02^{a}	0.002 ± 0.000^{a}	0.11 ± 0.02^{ac}
SC1	1.09 ± 0.11^{b}	0.006 ± 0.005^{ab}	0.17 ± 0.04^{b}
SC2	0.83 ± 0.12^{ac}	0.003 ± 0.001^{a}	0.14 ± 0.02^{ab}
SC3	1.00 ± 0.16^{bc}	0.004 ± 0.000^{ab}	0.17 ± 0.03^{ab}
SC4	1.39 ± 0.15^{d}	0.011 ± 0.006^{b}	0.08 ± 0.03^{c}

Means with different letters are sign. Different from one another $p < 0.05$.
[a] G'_{LVE} – Storage modulus within the linear viscoelastic range.
[b] T_y – Yield stress.
[c] Δ log G'/Δ log f – Slope of storage modulus vs frequency.

long and excrete futile amino acids [36].

3.3.2. Rheological parameters of fermented oat drink

12 h fermented oat drink samples were collected for rheological analysis and amplitude and frequency sweeps were performed; these results are summarized in Table 1. Apart from the control, which was unfermented oat drink sample with G'_{LVE} of 0.59 ± 0.02 Pa and viscoelastic almost liquid-like behavior ($G' \leq G''$, data not shown), fermented oat drink samples had approximately two times higher G'_{LVE} and showed weak gel-like structures ($G' > G''$). Amplitude sweep analysis showed that among others fermentation with SC4 resulted in most gel-like characteristics. SC4 had the highest structural rigidity and gel strength, since it scored highest values among G'_{LVE} and τ_y, respectively. Frequency sweep curves also showed a weak gel-like character of all fermented oat drink samples over the whole frequency range indicating the presence of some network structure (data not shown). A lower slope of G′-curve at lower frequencies ($\Delta \log G'/\Delta \log f$) of the sample fermented with SC4 indicates the higher physical long-term stability and a lower tendency to sedimentation. This is in accordance with the information provided by the starter manufacturer, which described the SC4 with an ability to give a higher texture. SC4 consortium contains *L. paracasei* that, according to the NCBI protein database, has enzyme coding genes for EPS production. However, *L. plantarum* that was present in SC3 also produces EPS, but this batch did not have as high viscosity as SC4. The EPS produced by these two species were different and affected the structure of the final fermented product diversely [37,38]. The EPS production by starter culture is crucial for dairy alternative formulation, while this study also showed that unfermented samples had poor texture, were layered and were the first ones to lose their structure. The rheological analysis pairs with sensory analysis results, which showed that SC4 had more viscous mouthfeel.

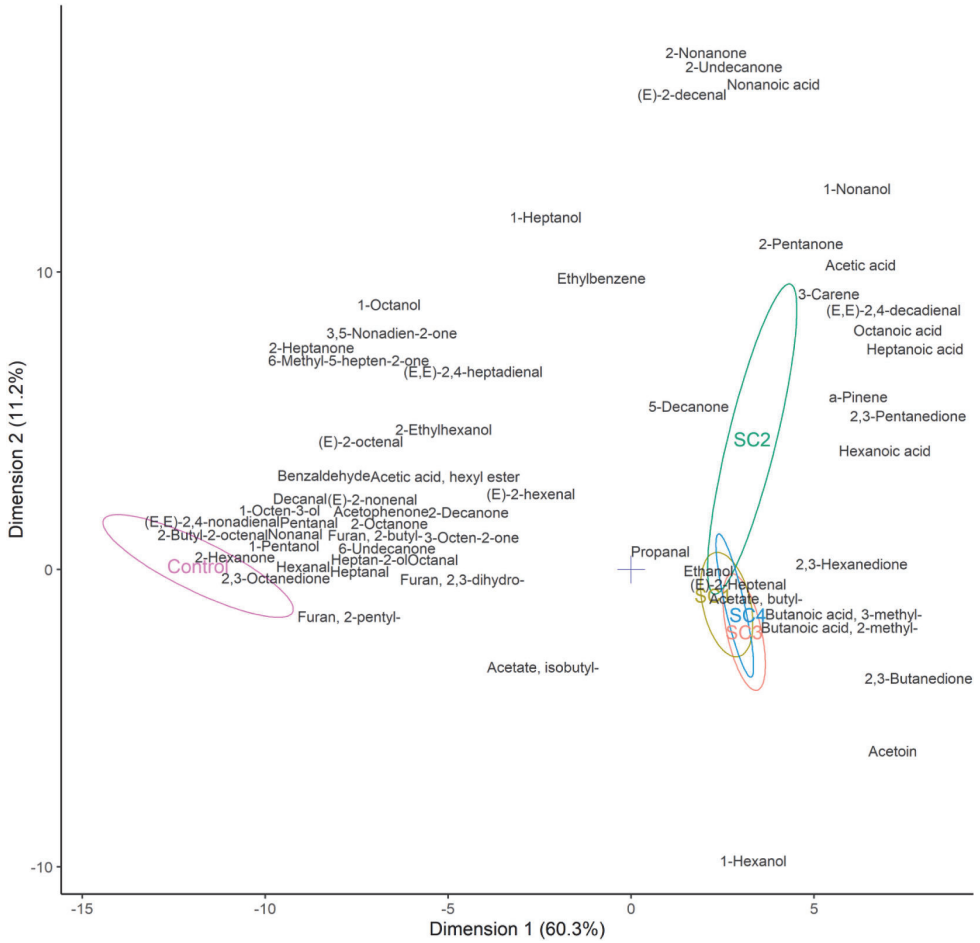

Fig. 6. PCA biplot of oat drink (control) and oat drinks fermented with different starter cultures (SC1-4), showing correlations with volatile compounds extracted by HS-SPME. Sample clusters are shown by 95% confidence ellipses. Variables are centered and scaled. Data represents each sample in the form of mean of biological replicates (n = 3).

3.3.3. Volatile compounds of fermented oat drink

More than 60 volatile compounds were identified after analyzing the oat drink bases fermented with four different starter cultures. The correlation between volatile compounds in unfermented and fermented oat drink is shown in Fig. 6. Most of the volatile compounds identified belonged to the aldehyde, ketone, acid, furan, and alcohol classes.

Aldehydes, such as pentanal, hexanal, heptanal, octanal, nonanal, decanal and benzaldehyde give sweetness and green notes to non-inoculated drinks in both odor and taste perception. For example, the unfermented drink had a hexanal (grass) concentration of 645 parts per billion (ppb), while the fermented samples had less than 10 ppb (data not shown). Hexanal was reported to be the most significant volatile in dry non-thermal treated oats. It is the result of oxidation of linoleic acid and is also the main volatile component in rancid oat groats [39]. Furthermore, nonanal (fatty, citrus) and decanal (fatty, orange) were only present in trace amounts in the fermented samples but were abundant in the unfermented samples**.** Nonanal is a product of degradation of oleic acid and is derived from fresh, untreated oat groats [39].

Meanwhile, ketones were more prominent in the fermented samples. Concentrations of creamy ketones like diacetyl (2,3-butanedione), acetylproprionyl (2,3-pentanedione), acetylbutyryl (2,3-hexanedione) and acetoin (2-butanone, 3-hydroxy-) were high in all inoculated samples but only traces were present in unfermented samples. The above-mentioned ketones give fermented samples a sweet, round, more balanced flavor. Diacetyl concentrations increased during fermentation, being highest for SC1 and SC4 at 226 ± 2.6 and 223 ± 5.9 ppb, respectively (data not shown). Diacetyl production is inconsistent with the results of [29], who stated that *L. plantarum, L. acidophilus* and *L. reuteri*, could only produce diacetyl in malt-based media, but not in oat and barley media. It could be that other lactic acid bacteria, such as *S. thermophilus*, were required for diacetyl production [40]. SC3 (consortium combination additionally includes *L. plantarum*) and SC4 (consortium additionally includes *L. paracasei*) inoculated beverages had two-fold higher acetoin concentrations compared to the other two starters (data not shown). Furthermore, the concentrations of some unwanted ketones were reduced by fermentation. 2-Heptanone (blue cheese), 2,3-octanedione (herbal), 2-octanone (overripe, moldy) and acetophenone (almond, musty) were present in the inoculated samples at lower concentrations than in the blanks, with one exception where 2-nonanone (green, herbal) concentration was higher in SC2 inoculated samples.

Acids, namely acetic acid (vinegar), 3-methyl- and 2-methylbutanoic acids (cheesy) and hexanoic acid (goat cheese) increased in all fermented samples. According to the GC-MS results, acetic acid has the highest relative intensity of the identified acids. The highest production of acetic acid was detected in drinks inoculated with SC2 and SC3. Sensorially, acetic acid gives a sharp acid flavor, while lactic acid gives a mild sour flavor. Acetic acid in SC2 was 21 ± 1.1 ppb, in SC3 14 ± 1.7 ppb, in SC4 9 ± 0.1 ppb and in SC1 6 ± 0.3 ppb (data not shown). Acetic acid concentrations correlated with the results of sensory analysis of acidic flavor intensity in the decreasing order: SC2>SC3>SC4>SC1. However, no acetic acid was detected in the HPLC analysis. The explanation is that *S. thermophilus* (present in all four starter cultures) consumed significant amounts of acetic acid and the sensitive GC-MS analysis was able to detect traces in the fermented oat drink. Furthermore, the concentrations of hexanoic (cheesy, goat), octanoic (blue cheese) and nonanoic (cheese, dairy) acid increased in all fermented samples. This is in accordance with the results of Salmeron et al. [12], who found that octanoic and nonanoic acids were characteristic of oat drink fermented with *L. plantarum* NCIMB 8826, but were not released during fermentation of wheat, barley, and malt drink.

Concentrations of 2-Butylfuran (wet hay) and 2-Pentylfuran (beany, earthy) were higher in blanks, but decreased five-fold in all fermented samples. 2-Pentylfuran comes from fresh oat groats [39]. Amongst the detected alcohols, green and herbal notes were prevailing. 1-Hexanol (floral, green) was present in high concentrations in all samples. In the fermented samples, 1-Pentanol (green) was 3-fold and 1-Octen-3-ol (mushroom, earthy) was ten-fold lower. The results are consistent with previous studies showing 1-pentanol in fresh oat groats and 1-hexanol and 1-octen-3-ol in hydrated groats [39].

3.3.4. Sensory properties of fermented oat drink

In this experiment, the optimal time for fermentation to reach a kefir-like product was 12 h, and samples were collected for sensory evaluation at this time point. All fermented oat drink samples had an overall similar odor, taste, and textural properties that differed from the unfermented sample. After fermentation, an increase in sourness of odor and taste was observed (Fig. 7). The sourness of odor

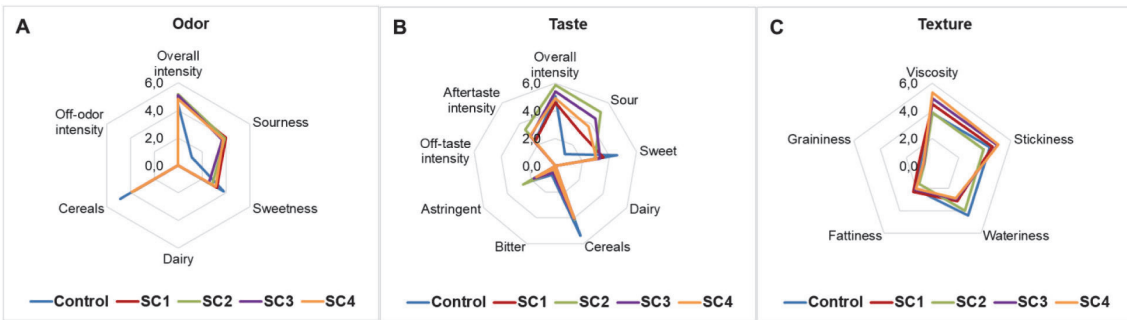

Fig. 7. Sensory profile of oat drinks (control and fermented with different starter cultures for 12 h) by odor (A), taste (B), and texture (C). Mean values of assessors are indicated (n = 9).

increased from 1.1 to 4.0 points and of taste increased from 1.1 to 5.1 points. Simultaneously with the sourness increase, the sweetness decreased slightly. Since the total sugar concentration measured by HPLC was not affected by fermentation, the decrease in sweetness perception may be due to the increase in sourness. Although diacetyl and acetoin concentrations increased during fermentation, all samples were associated with cereals but not dairy products in both odor and taste. Luana et al. [11], showed that fermentation with *L. plantarum* increased mainly sour taste and reduced artificial and earthy notes in fermented oat drinks. None of the samples had off-odors or -flavors. Among the fermented samples, SC2 was the highest and SC1 was the least sour. This is in accordance with the organic acid production and the specifications provided by the manufacture. The fermented drinks had low values in bitterness, while unfermented sample was the most bitter with a value of 0.7. A higher astringency and aftertaste intensity were observed for SC2, which might be related to the higher sourness of the sample.

The viscosity of the fermented samples was higher than of unfermented drink, except for SC2. SC4 was evaluated as the most viscous (Fig. 7). The sensory analysis is consistent with the results of the rheological measurements, which showed that SC4 had the most gel-like structure, and with the information provided by the starter manufacturer, which described SC4 to provide a high texture. Fermented oat drinks had higher stickiness and lower wateriness than the unfermented drink. The samples had low scores in fattiness and there was no graininess. This result is on contrary to the data of Luana et al. [11], who found a change in viscosity in the opposite direction during oat base fermentation. The reduction in thickness during fermentation could be related to the viscosity of the oat base, while 25% of oat flour was used compared to our 16%.

Perceived sourness was compared with the results of titratable acidity (Supplementary materials – Fig. S3). The results were in correlation and consistent with the iCinac and organic acid analysis results. Of the fermented oat beverages, SC2 was the most sour and had the highest acid content, followed by SC3 and SC4, with SC1 being the least sour. Titratable acidity was 0.51, 0.45, 0.38 and 0.3% of lactic acid for SC2, SC3, SC4 and SC1, respectively.

4. Conclusion

The global demand for plant-based dairy alternatives is constantly increasing. However, little is known about the fermentation processes of plant-based drinks. The main objective of the present study was to understand and demonstrate the changes in pH, consortium population, metabolism, chemical composition, structural and sensory properties during oat drink fermentation. The oat drinks were fermented with four different vegan starter cultures containing various lactic acid bacteria. Combining isothermal microcalorimetry with 16 S metagenomic analysis showed that a kefir-like product with pH < 4.2 was obtained after 12 h and that *S. thermophilus* was the dominant species throughout the fermentation. In the later stages of fermentation *L. acidophilus, L. plantarum* and *L. paracasei* slowly increased in the fermented oat drink, but never reached the dominant concentration in the beverage. The prevalence of *S. thermophilus* was also indicated by organic acid production, with lactic acid being the most produced metabolite. Concentrations of the most favored volatile compounds such as diacetyl and acetoin increased during fermentation. However, during sensory evaluation, all samples were associated with cereal and not dairy in terms of odor and taste during sensory evaluation. Fermented oat drinks had approximately twice the G'_{LVE} and had a weak gel-like structures ($G'>G''$) compared to the unfermented oat drink base. Rheological analysis combined with sensory analysis revealed a more viscous mouthfeel for oat drinks fermented with SC4. This comprehensive study shows the growth dynamics of various vegan starter cultures and changes in the composition of bacterial consortium, which in turn affected the sensory and textural formation of the fermented oat drink and helps food manufactures to choose suitable starter cultures for the plant-based dairy alternative production.

Author contribution statement

Mary-Liis Kütt: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Kaisa Orgusaar, Irina Stulova: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Reimo Priidik, Indrek Morell: Analyzed and interpreted the data; Wrote the paper.

Dmitri Pismennõi, Helen Vaikma, Aili Kallastu, Aleksandra Zhogoleva, Tiina Kriščiunaite: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Data availability statement

Data included in article/supp. Material/referenced in article.

Declaration of interest's statement

The authors declare no competing interests.

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Ethical statement

All participants from a pool of highly trained evaluators in the sensory panel gave written consent to take part in the experiment. Participants were informed in advance of the purpose and the procedures of the study. Participants were assured of the confidentiality of their data. Taking part in the given study was voluntary and one could withdraw from the test at any time. Participants were in good health and had no known allergy to the components. Institutional approval for the research is not available due to Estonian requirements for human research. Ethical statement is also included at the end of the manuscript.

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.heliyon.2023.e15627.

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Appendix 2

Publication II

Pismennõi, D., Kiritsenko, V., Marhivka, J., Kütt, M.-L., & Vilu, R. (2021). Development and Optimisation of HILIC-LC-MS Method for Determination of Carbohydrates in Fermentation Samples. Molecules, 26(12), Article 12.
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molecules

MDPI

Article

Development and Optimisation of HILIC-LC-MS Method for Determination of Carbohydrates in Fermentation Samples

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Abstract: Saccharides are the most common carbon source for *Streptococcus thermophilus*, which is a widely used bacterium in the production of fermented dairy products. The performance of the strain is influenced by the consumption of different saccharides during fermentation. Therefore, a precise measurement of the concentrations of saccharides in the fermentation media is essential. An 18-min long method with limits of quantitation in the range of 0.159–0.704 mg/L and with ^{13}C labelled internal standards employing hydrophilic interaction chromatography coupled to mass spectrometric detection-(HILIC-LC-MS) allowed for simultaneous quantification of five saccharides: fructose, glucose, galactose, sucrose, and lactose in the fermentation samples. The method included a four-step sample preparation protocol, which could be easily applied to high-throughput analysis. The developed method was validated and applied to the fermentation samples produced by *Streptococcus thermophilus*.

Keywords: saccharides; HILIC-LC-MS; *Streptococcus thermophilus*; fermentation

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1. Introduction

Streptococcus thermophilus is a gram-positive facultative anaerobic bacterium mostly known for its role in the production of fermented dairy products. As a part of the lactic acid bacteria (LAB) group, it is a widely studied and well-known microorganism. As production of lactic acid by *S. thermophilus* is dependent on carbohydrate utilisation, the choice of available carbohydrate is usually dictated by strain ability to digest certain disaccharides—mainly sucrose and lactose [1]. Bacterial consumption of monosaccharides—fructose, glucose. and galactose, was found to be suppressed in most common strains. Nevertheless, the mutant strains, able to consume monosaccharides, were created to study the alternative ways for lactic acid production by utilisation of low-molecular carbohydrates [2–4].

Carbohydrates are a vast class of chemical compounds with a similar structure comprised of either furanose or pyranose skeleton core. Carbohydrate analysis employing chromatography has a long history starting from paper partition chromatography of selected monosaccharides in 1949 [5]. The advances in the chromatographic field have helped to achieve better separation and selectivity [6,7]. The main path to analyse carbohydrates was to use either gas chromatography with derivatisation of saccharides or liquid chromatography employing ion-exchange resins [8]. The development of novel stationary phases for both gas and liquid chromatography increased the number of applications where carbohydrate analysis could be performed from samples obtained from various sources, i.e., raw nutritional materials, animals, bacteria, humans, and so forth [9–12]. At the same time, developments in ion chromatography and electrophoresis allowed to measure carbohydrates in similar matrices creating the alternative ways for the measurements

of saccharides [13–16]. Nowadays, the most commonly used method is to measure carbohydrates to use ion-exchange resin with a refractive index (RI) detector, as carbohydrates do not have any chromophores. The use of this combination is shown in Association of Official Analytical Chemists (AOAC) or International Organization for Standardization (ISO) methods [17]. The main disadvantage with RI-based detection is a relatively low selectivity, sensitivity, and elution program restriction compared to more advanced detection techniques such as evaporative light scattering detector (ELSD) [18,19], charged aerosol detection (CAD) [20] or mass spectrometric (MS) detector [21–23]. Out of advanced methods, MS-based detection offers the most selectivity as active mass filtration could clean up a substantial portion of noise interference originated from the sample matrix [24]. Therefore, the liquid chromatography coupled to mass spectrometer (LC-MS) system became a prominent choice to perform carbohydrate analysis with little to no extensive sample preparation [25,26].

The aim of this work was to develop and validate a rapid and sensitive method for the quantitative determination of five saccharides: fructose, glucose, galactose, sucrose, and lactose, by employing a rapid, selective, and sensitive methodology based on hydrophilic interaction chromatography coupled to mass spectrometric detection (HILIC-LC-MS) and isotopically labelled glucose and lactose as internal standards. The method development included optimisation of sample preparation, validation and application of the method towards the determination of carbohydrates metabolised by *S. thermophilus* in fermentation broth samples.

2. Results and Discussion

2.1. Chromatographic and Mass Spectrometric Optimisation

The initial screening involved testing of the performance of several columns with hydrophilic interaction (HILIC) stationary phase. The testing of Waters BEH HILIC and BEH Amide revealed that even though the columns are clearly capable of separation between mono- and disaccharides, the inter-class separation of closely matched carbohydrates is impossible (Figure 1).

During method scouting, it became evident that BEH HILIC column could not achieve an acceptable separation of 3 monosaccharides of interest. Employing Waters BEH Amide column showed the separation between fructose and glucose-galactose pair close to the baseline. The separation of the glucose-galactose pair requires more resolving power as the epimer separation is proven to be complex. The acceptable separation between epimers was reached using Phenomenex Luna Omega Sugar column, which allowed a repeatable and precise determination of closely eluting glucose and galactose (Figure 2).

To improve the sensitivity of measured analytes, the decision to enrich the mobile phases with guanidine hydrochloride solution was made to facilitate the formation of $[M + Cl]^-$ adduct instead of $[M\text{-}H]^-$ ion. The addition of chloride ion to saccharide molecules provided better ionisation, cleaner spectra at the baseline level (Table S1). It thus decreased the amount of sample injected on the column to achieve a satisfactory chromatographic and mass spectrometric result [27,28]. The variations in flow rate were also studied as the mock-up method was transferred from the column with a larger inner diameter, making it incompatible with the current column choice. Therefore, several different flow rates were evaluated to achieve optimal separation between all analytes: 300, 313 and 350 µL/min (Figure S1–S3). The flow rate 313 µL/min resulted in the most optimal separation among targeted carbohydrates. The column temperature was also studied, and two temperatures were tested: 25 and 35 °C. The higher temperature was ruled out as unfavoured due to more unsatisfactory performance in terms of chromatographic separations, which was in accordance with reports in the literature [29].

2.2. Sample Preparation Optimisation and Measurement of Fermentation Samples

Several variants of sample preparation were proposed during initial consideration for adequate sample preparation for maximum elimination of matrix components (Table 1).

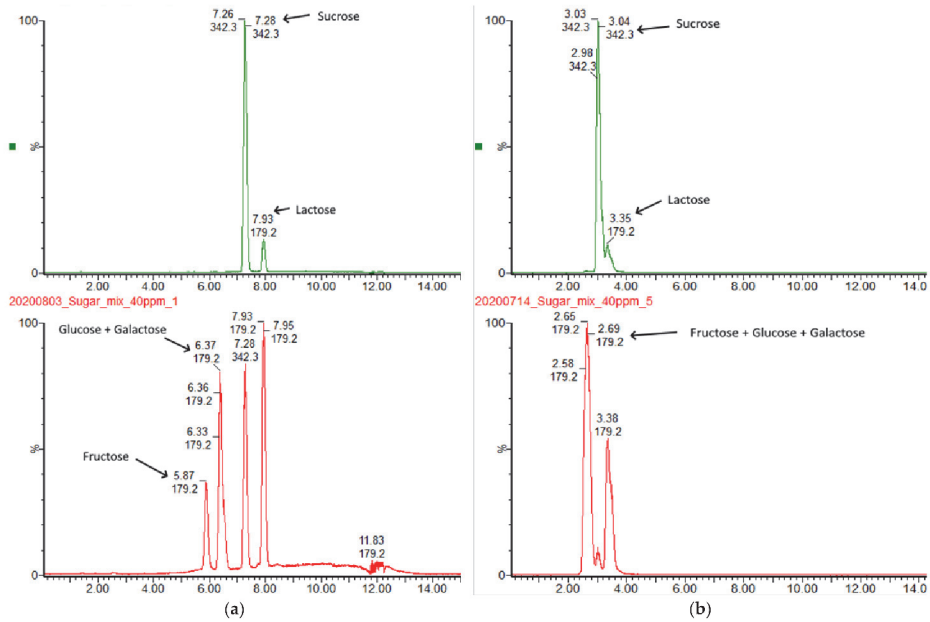

Figure 1. (**a**) Separation of mono- and disaccharides on Waters BEH Amide column with mobile phases containing 0.1% NH_4OH; The elution was achieved using gradient elution at 170 µL/min flow rate. Solvents were: A—MilliQ + 0.1% NH_4OH, B—MeCN + 0.1% NH_4OH. Gradient program was: 0.0–10 min linear ramp 0.1–60% A, 10.01–25.00 hold at 0.1% A. The detection was performed in Single-Ion-Reaction (SIR) mode (**b**) Separation of mono- and disaccharides on Waters BEH HILIC column with mobile phases containing neat solvents. The elution was achieved using gradient elution at 170 µL/min flow rate. Solvents were: A—MilliQ, B—MeCN. Gradient program was: 0.0–20 min linear ramp 0.1–40% A, 20.01–30.00 hold at 0.1% A. The detection was performed in Single-Ion-Reaction (SIR) mode at a concentration level of 10 µg/mL for all saccharides.

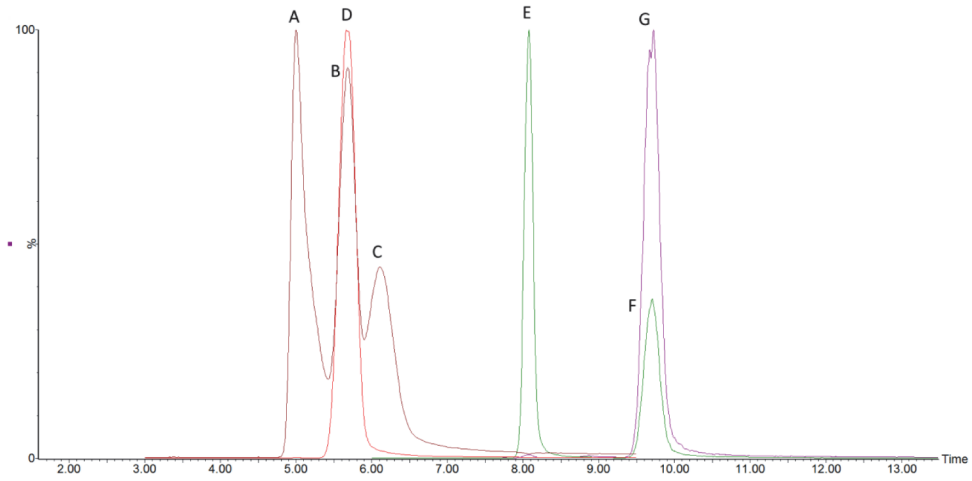

Figure 2. The representative chromatogram of separated mono- and disaccharides with internal ^{13}C-labeled standards on the Luna Omega Sugar column. Peaks are labelled as: A—fructose, B—glucose, C—galactose, D—glucose-$^{13}C_6$, E—sucrose, F—lactose and G—lactose-$^{13}C_6$. Peak heights are normalised.

Table 1. Comparison of sample preparation protocols.

Protocol Nr.	1	2	3	4	5	6
Step 1	Dilute 100 times with MilliQ					Dilute 50 times with MilliQ
Step 2	Centrifuge at 14000 rpm for 10 min					
Step 3	Filter through 0.2 µm filter	Dilute 2 times with MeCN: MilliQ mixture containing ^{13}C ISTD		Pass through 1 kDa MWCO filter	Pass through 3 kDa MWCO filter	Dilute 4 times with MeCN: MilliQ mixture containing ^{13}C ISTD
Step 4	Dilute 2 times with MeCN: MilliQ mixture containing ^{13}C ISTD	Pass through Isolute PLD+ cartridge	Pass through Isolute NH_2 cartridge	Dilute 2 times with MeCN: MilliQ mixture containing C13 ISTD		Pass through Isolute NH_2 cartridge

As the protocols were being tested firstly with a simulated matrix composed of chemically defined medium (CDM) [30] and external standards, it was found that variants 2, 3, 4 and 6 did not produce expected results as analytes were detected in lower amount compared to other protocols or no analytes of interest were found during the measurement (Table S2). Protocol nr 1 and 5 were chosen for additional evaluation as their performance with simulated matrix was found to be acceptable. The optimised protocols nr. 1 and 5 were applied towards harvested fermentation broth. It was found that protocol nr 1 produced a higher number of impurities which negatively affected MS performance by leaving more residue on the source cone compared to protocol nr 5, which included the usage of molecular weight cut-off filters (Figure 3).

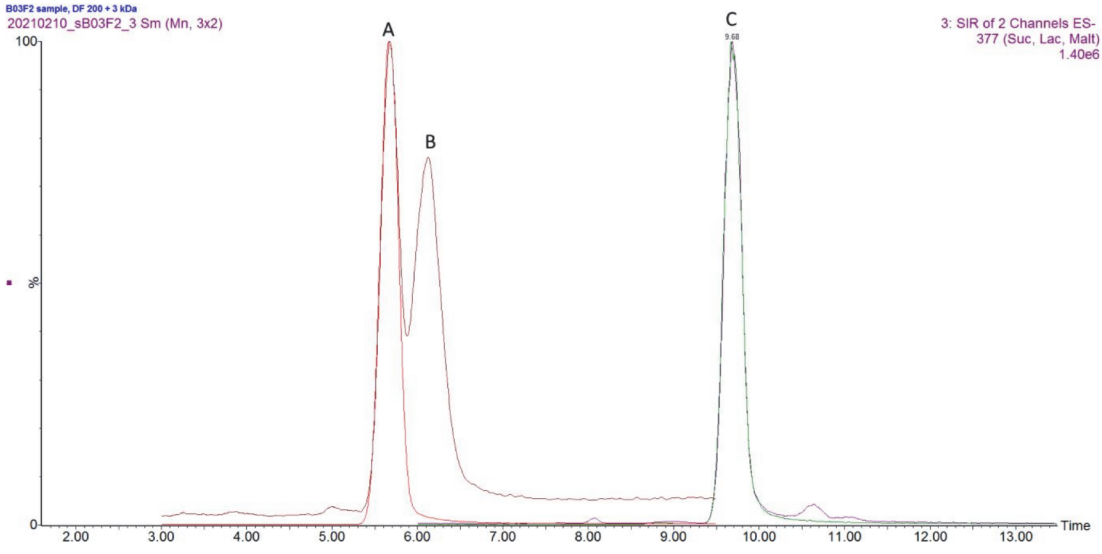

Figure 3. The overlaid chromatogram of the fermented sample subjected to extraction protocol nr. 5. Peaks are labelled as follows: A—Glucose and Glucose-$^{13}C_6$, B—Galactose, C—Lactose and Lactose-$^{13}C_6$.

After sample preparation protocol optimisation was completed and sample preparation protocol nr 5 was chosen as the primary option to perform sample preparation. Six samples obtained during the fermentation process at different time points were analysed for carbohydrate content (Table 2).

Table 2. Description of the harvested fermentation samples produced by *S. thermophilus*.

Sample Name	Sampling Time Point, h	Carbohydrate Source
BO3F1_1	24	Sucrose
B3F1_2	24	Sucrose
BO3F2_1	24	Lactose
B3F2_2	24	Lactose
B3F1H0	0	Lactose
B3F2H0	0	Sucrose

The samples were injected in triplicates. (Figure 4).

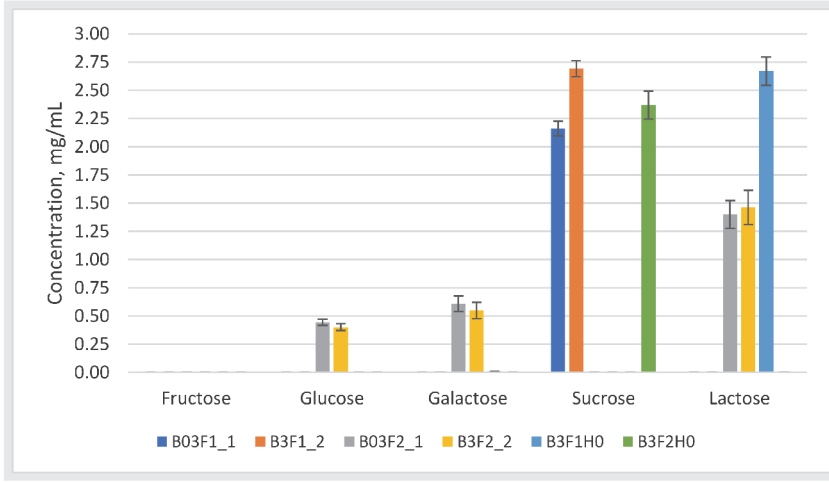

Figure 4. Quantified carbohydrates (mg/mL) in the measured samples. The bars represent the concentration levels of the found saccharides in the fermentation samples. The sample naming follows B stands for batch fermentation; number 3 or 03 states the number of experiments, F1 or F2 states the reactor number and _1 or_2 states the number of parallel. H0 denotes 0 h sampling point.

It was found that neither of the samples contained fructose at levels exceeding the limit of quantification. Other samples contained either sucrose or lactose at a higher concentration due to its presence in CDM. It was shown in samples B03F2_1 and B3F2_2 that the bacterium was able to produce monosaccharides by breaking down larger saccharide. In other samples, no by-products of disaccharide breakdown were detected, which could indicate that bacteria would utilise either sucrose or lactose as a carbon source for the production of other molecules such as organic acids. The results were subjected to carbon balance calculations based solely on the saccharide content in the fermented and blank samples. The calculations showed that measured saccharide content is correlatable with calculations e.g., carbon balance calculation errors were 25.6 $\pm$ 16.1% (n = 6) on average between fermented samples. The obtained values were found to be acceptable as calculations did not take into the account presence of other organic materials commonly present in the fermentation broth.

2.3. Validation Results

When the development and optimisation of methodology were finished, validation was performed to evaluate the method linear range, limits of detection and quantifications, recoveries and the stability of prepared samples. First of all, linear range and linearity were evaluated via the repeated measurements of standard solutions consisting of 8 individual points obtained from stock's serial dilution (Table 3).

Table 3. The linear range, regression equation, limits of detection. and quantification of five saccharides.

Analyte	Linear Range, µg/ml	Linear Regression	R^2	LoD [1], mg/L	LoQ [2], mg/L
Fructose	0.77–49.88	y = 1.3611x + 0.9873	0.9974	0.189	0.629
Glucose	0.51–64.80	y = 0.6921x + 0.0765	0.9993	0.080	0.268
Galactose	0.39–49.60	y = 0.3764x − 0.0112	0.9958	0.067	0.220
Sucrose	0.93–59.75	y = 1.2610x + 0.9776	0.9935	0.232	0.704
Lactose	0.38–49.10	y = 1.0851x − 0.0076	0.9996	0.048	0.159

[1] LoD = Blank mean value + 3.3*standard deviation at LLOQ; [2] LoQ = Blank mean value + 10* standard deviation at LLOQ.

After linearity was found to be acceptable (R2 $\geq$ 0.99 or higher) for all sugars in this study, the repeatability of the method was studied. Repeatability of retention times and peak areas were studied first with six replicate injections of standard solution (Table 4). The repeatability of the method was studied across four independent days to confirm the stability of the retentions time and peak areas of the analytes.

Table 4. Repeatability of retention times and peak areas of measured carbohydrates.

Analyte	Mass of Measured Ion, m/z	Retention Time, min	Retention Time RSD, %		Peak Area RSD %	
			Inter-Day, % (n = 6)	Intra-Day, % (n = 4)	Inter-Day, % (n = 6),	Intra-Day, % (n = 4)
Fructose	215	5.06	0.26	1.27	2.90	3.75
Glucose	215	5.75	0.42	1.59	2.69	4.01
Galactose	215	6.18	0.50	1.50	3.62	3.28
Sucrose	377	8.16	0.23	1.32	3.46	2.96
Lactose	377	9.81	0.19	1.60	4.70	2.40
Glucose—$^{13}C_6$	221	5.70	0.39	0.59	2.13	1.48
Lactose—$^{13}C_6$	383	9.73	0.44	0.50	4.63	2.23

Recovery of the sample preparation was determined by analysing fermentation samples, whereas ^{13}C-internal standards were spiked prior to sample preparation steps and compared to sample preparation procedure where ^{13}C-internal standards were added in the last stage of the procedure. Recovery was calculated according to Equation (1):

$$\text{Recovery} = \text{Peak area in a spiked sample}/\text{Peak area in a non-spiked sample} * 100 \quad (1)$$

The recovery experiments consisted of injections of 2 samples in triplicate. The recorded recovery values were 103.73 $\pm$ 1.69% and 111.04 $\pm$ 2.80% for ^{13}C-labeled glucose and ^{13}C-labeled lactose, respectively. The obtained values are in the $\pm$20% range. Furthermore, we have investigated the stability of the prepared standard solutions in a ready-to-use form stored at +4 and −20 °C. The prepared standards were stable at +4 degrees for one week whereas peak area of analytes has not changed by more than 5%. The analytes stored at −20 degrees for 1 month showed stable retention factors or all measured compound except for sucrose, which response factor after 1 month of storage at −20 °C had changed by 15%.

3. Materials and Methods

3.1. Reagents and Chemicals

Standards of mono- and disaccharides: D-fructose, D-glucose, D-galactose, D-(+)-sucrose, D-lactose monohydrate, and ammonia solution (25%, LC-MS LiChropur™ grade) were obtained from Sigma-Aldrich (Darmstadt, Germany). Glucose-$^{13}C_6$ (Glu-$^{13}C_6$, U-$^{13}C_6$, 99%, chemical purity 98%) and lactose monohydrate (Lac-$^{13}C_6$, UL-$^{13}C_6$glc, 98%+) were procured from Cambridge Isotope Laboratories Inc. (Tewksbury, MA, USA). Ultrapure

water (18.2 MΩ.cm) was prepared with MilliQ® Direct-Q® UV (Merck KGaA, Darmstadt, Germany). Acetonitrile (MeCN; LiChrosolv, HPLC gradient grade), and guanidine hydrochloride (GuHCl; ≥99%) were acquired from Sigma-Aldrich (Darmstadt, Germany). Biotage Isolute®PLD+, C18 and NH_2 were procured from Biotage Sweden AB (Uppsala, Sweden). Amicon Ultra-0.5 centrifugal filter unit (3 kDa) and Millex-LCR filters (Pore size 0.2 μm, Filter Dimension 13 mm) were obtained from Merck KGaA (Darmstadt, Germany) and Microsep Advance Centrifugal Devices with Omega Membrane 1K filter unit was purchased from Pall Corporation (Port Washington, NY, USA).

3.2. Preparation of Standard Solutions

The stock solution of each individual saccharide was prepared in MilliQ® water and stored at −20 °C. Solutions of isotopically labelled standards were dissolved in aqueous MeCN (50%, *v/v*) and stored at −50 °C. Working solutions for the determination of analytes were prepared firstly by diluting the stock solution with 100% MeCN, and after each working solution was prepared in aqueous MeCN (50%, *v/v*) water. Calibration curves were built for fructose (0.39–49.875 ppm), glucose (0.506–64.800 ppm), galactose (0.388–49.600 ppm), sucrose (0.467–59.750 ppm) and lactose (0.384–49.100 ppm). Glucose-$^{13}C_6$ and lactose-$^{13}C_6$ were added prior to injection to the autosampler vial, and their concentration in the vial was set at 15.925 and 12.825 ppm, respectively. The calculations of calibration curves used response factors, which were calculated according to Equation (2).

$$\text{Response Factor (RF)} = \text{Area of analytes} \times (\text{Concentration of internal standard}/\text{Area of internal standard}) \quad (2)$$

Calibration curves were built using eight-point measurements of serially diluted standards. The regression was found by fitting a point to a linear equation.

3.3. Liquid Chromatography

Samples were analysed using a Waters UPLC® system (Waters Corporation, Milford, MA, USA) coupled with a Waters Quattro Premier XE Mass Spectrometer equipped with ZSpray™ Source and controlled by Waters MassLynx™ 4.1 (V4.1 SCN805, Waters Corporation, Milford, MA). Mobile phases were as follows: (A) 99% MilliQ® + 1% MeCN + 1 mg/L of GuHCl and (B) 99% MeCN + 1% MilliQ® + 1 mg/L of GuHCl. Weak needle wash was composed of 10% MilliQ® in MeCN (*v/v*), and strong wash needle consisted of 10% MeCN in MilliQ® (*v/v*). Seal wash was aqueous MeCN (50%, *v/v*). Samples were stored at an autosampler which held temperature at 8 °C. The injection volume was 2 μL. Several columns were tested: Waters Acquity UPLC® BEH HILIC (2.1 × 100 mm, 1.7 μm, Waters Corporation, Milford, MA), Waters XBridge® BEH Amide XP (3.0 × 150 mm, 2.5 μm, Waters Corporation, Milford, MA), Phenomenex Luna Omega Sugar column (2.1 × 150 mm, 100 Å, 3 μm, Phenomenex Inc., Torrance, CA, USA). To prevent harm to any analytical column, ACQUITY UPLC Column in-line filter unit (Waters Corporation, Milford, MA) with installed 0.2 μm stainless steel filter was used in all experiments with all tested columns. The column temperature was held at 25 degrees of Celsius for the duration of all experiments. The gradient was as follows: 0–10 min linear gradient 10–25% A, 10–12 min hold at 25% A, 12.01–14 min hold at 35% A, 14.01–18 min hold at 10% A. Flow rate was set at 313 μL/min.

3.4. Mass Spectrometry

The analytes were ionised under negative electrospray ionisation conditions with optimised source conditions. The source temperature was set at 120 °C, high-purity nitrogen was fed into the source at 50 L/h (cone) and 800 L/h (desolvation) and heated to 350 °C. The capillary voltage was set at −2.3 kV, cone voltage at 25 V and extractor voltage 3 V. The values for efficient ionisation were found by infusing a standard solution of individual saccharide (*ca* 25 ppm) in 50% aqueous MeCN (*v/v*) at the combined flow from UPLC and integrated syringe pump at 250 μL/min. For measurement of analytes, single ion monitoring (SIR) experiments were chosen as saccharides possess no valuable fragments for ubiquitous identification, therefore, making it unnecessary to perform multiple reaction

monitoring (MRM) type of experiments. The SIR channels were based on molecular ion with added chloride as $[M+Cl]^-$. Data acquisition was performed in Waters MassLynx™ V4.1 (SCN805, Waters Corporation, Milford, MA, USA). Data analysis was performed in Waters QuanLynx™ V4.1 (SCN805, Waters Corporation, Milford, MA, USA) and Microsoft Excel (Microsoft 365 Apps for enterprise).

3.5. Bioreactor Experiments, Bacteria Growth Description

Streptococcus thermophilus was inoculated into fresh M17 medium (lactose as carbon source) at the rate of $1e^7$ cfu/mL (1%) and cultivated until OD_{600} reached a value of 0.8. Then the culture was inoculated into a bioreactor containing 300 mL of CDM achieving 100-fold dilution. CDM contained either sucrose or lactose as a carbon source. Cultivation was conducted in anaerobic conditions maintained by the constant flow of N_2 into the medium flask and N_2:CO_2 mixture (80:20, *v/v*) into the reactor vessel at 150 and 300 mL/min, respectively.

Culture outgrowth was monitored by the rate of medium acidification and using the turbidimetric sensor. After 7 h of batch growth, the stability of culture was achieved, and flowthrough was initiated with a dilution rate of 0.25 h^{-1}. The flow was maintained for 20 h, ensuring culture stabilisation. At the chemostat point, the culture samples were taken for subsequent analysis. HPLC samples were centrifuged at 14,000 rpm for 10 min. Supernatants were frozen and stored at −20 °C.

3.6. Sample Preparation

Frozen samples were fully thawed at room temperature until a clear solution was obtained. Thawed samples were serially diluted 100-fold before further steps. Diluted samples (1000 µL) were firstly centrifuged at 14,000 rpm for 10 min to remove any remaining solid residue. The supernatant (500 µL) was then transferred to a 3 kDa molecular weight cut-off (MWCO) filter (Amicon® Ultra-0.5, Merck KGaA, Germany). The MWCO filter was then centrifuged at 14,000 rpm for 20 min. The supernatant obtained was diluted with a 50% aqueous MeCN (*v/v*) mixture containing Glu-$^{13}C_6$ and Lac-$^{13}C_6$ 2-fold before analysis.

3.7. Method Validation

The developed method was assessed for linearity (as a correlation coefficient of R^2 of calibration curve), the limit of detection and quantification (as the standard deviation of the measured sample at the lowest calibration points multiplied by 3 or 10, respectively), recovery (as spiked sample vs. un-spiked) and matrix effect [31].

4. Conclusions

In summary, we have developed the HILIC-LC-MS method for the rapid and simultaneous determination of five saccharides in just 18 min without employing complex sample preparations steps. The methodology can be applied to the simplest instrumentation consisting of liquid chromatograph and single quadrupole mass detector. The addition of a common mobile phase additive such as guanidine hydrochloride is a viable option to increase signal insensitivities while reducing the baseline noise. The mass spectrometric detection helps with the selectivity of the methodology as mass spectrometer could filter out matrix interfering components thus providing cleaner and unambiguous spectra. The simultaneous measurement of fructose, glucose, galactose, sucrose, and lactose could be done in a fraction of time and less consumed solvent compared to classical methods used elsewhere [10,11,16,32]. The method developed here suits for both to identify and characterise the metabolism of various starter cultures (like here with *Streptococcus thermophilus*) as well as to detect the sugar profile of different food matrices. Furthermore, the monosaccharide quantities could be used in a calculation of the carbon balance in a single cell model (SCM) to assess the productivity of the strain during fermentation. The applicability of the method for quantification of the larger oligosaccharide chains together with smaller

saccharides, for example, to analyse their consumption by gut microbiota, could be further determined in the future.

Supplementary Materials: Figure S1: The chromatogram of 5 saccharides subjected to the optimised gradient elution program with flow rate of 300 µL/min, Figure S2: The chromatogram of 5 saccharides subjected to the optimised gradient elution program with flow rate of 313 µL/min, Figure S3: The chromatogram of 5 saccharides subjected to the optimised gradient elution program with flow rate of 350 µL/min, Table S1: The peak areas of analytes while using neat solvents or solvents with added guanidine hydrochloride, Table S2: The peak areas of five saccharides in this study subjected to different extraction protocol in order to perform the sample preparation optimisation.

Author Contributions: Conceptualisation, D.P., V.K. and R.V.; methodology, D.P.; software, V.K.; validation, D.P. and V.K.; formal analysis, D.P. and V.K.; investigation, D.P., V.K. and J.M.; resources, V.K. and J.M.; data curation, D.P. and V.K.; writing—original draft preparation, D.P.; writing—review and editing, J.M., M.-L.K., R.V.; visualisation, D.P.; supervision, R.V.; project administration, D.P.; funding acquisition, M.-L.K. and R.V. All authors have read and agreed to the published version of the manuscript.

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Sample Availability: Not available.

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Appendix 3

Publication III

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Microbiological, chemical, and sensorial characterisation of commercially available plant-based yoghurt alternatives

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ABSTRACT

Consumer demand for plant-based dairy alternatives has increased rapidly during the past few years and the market has been saturated with a wide variety of alternative products. The general aim of this study was to broaden the understanding of the composition and characteristics of currently commercially available plant-based yoghurt alternatives focusing especially on the content of live bacteria. The bacterial composition, including the content of live bacteria in yoghurt alternatives, was evaluated using metagenetic sequencing of the 16S rRNA gene amplicons in combination with the novel PMAxx treatment approach. The content of organic acids, sugars, and volatiles was measured, and descriptive sensory analysis was carried out to comprehensively describe the products. While the main ingredient (soya, oat, coconut, or lupin) determined the general characteristics of the product, significant differences were observed in both chemical and microbiological composition and sensorial attributes even among the yoghurt alternatives made from the same plant ingredient.

1. Introduction

Yoghurt – a fermented dairy product, is a staple in a healthy diet. Traditionally, yoghurt is produced by fermenting milk with a starter culture composed of lactic acid bacteria, commonly *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp *bulgaricus* (*Leroy and De Vuyst 2004*) During the past decade, a growing number of consumers have become interested in plant-based alternatives to replace dairy products in their diets (McCarthy et al. 2017; European Commission 2018; Paul et al. 2020; Ignaszewski 2022). Food producers have met that demand and brought a wide range of vegan yoghurt alternatives to the market. Vegan yoghurt alternatives are plant-based emulsions, which aim to resemble traditional dairy yoghurt in terms of flavor, aroma, and texture. The main ingredients of yoghurt alternatives include plant extracts, oils, starches, fibres, stabilisers, and flavorings. These products are made from a variety of plants, the most popular being soya, oat, almond, and coconut. While yoghurt is a fermented milk product, plant-based alternatives can be produced either by fermentation or by combining and processing ingredients using different physical and chemical methods to resemble yoghurt and meet the consumers' expectations (Tangyu et al. 2019; Montemurro et al. 2021; Harper et al. 2022). In addition to the main plant ingredient, vegan yoghurt alternatives can contain oils, starches, fibres, stabilisers, and natural flavorings. Often the products are also enriched with vitamins and minerals, B12 and calcium being the most widely used (Paul et al. 2020).

Flavor and mouthfeel are considered the most significant criteria for high consumer acceptance, yet the nutritional value is becoming increasingly important as one of the reasons for transferring from animal-based foods to plant-based alternatives and as the potential health benefits (Paul et al. 2020; Zandona et al. 2021). Switching from yoghurt to plant-based alternatives may lead to a reduction in the consumption of saturated fatty acids and cholesterol, but also limit the intake of high-quality proteins, vitamins, and minerals (Mäkinen et al. 2016; Clegg et al. 2021). If the vegan yoghurt alternatives are chemically acidified instead of being fermented with starter cultures, consumers are also deprived of the potentially beneficial bacteria and the metabolites produced by those microorganisms in the fermented products (Chandan et al. 2008).

While many of the products list lactic acid bacteria and/or bifidobacteria among the ingredients, the number of living microorganisms in the end product is generally unknown to the consumer. Some products list both bacterial cultures as well as organic acids on the label, which can raise the question of whether the product has been fermented or chemically acidified. Indeed, the production of plant-based yoghurt al-

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ternatives is not standardised and there is no universal understanding of how these products should be produced. Little published information is available about the microbial composition of vegan yoghurt alternatives. This study aimed to identify the microbial communities and determine the proportion of live bacteria together with the evaluation of chemical composition and volatiles' profile of commercially sourced plant-based yoghurt alternatives to better understand the current situation in the product category.

2. Materials and methods

2.1. Samples

Twenty-five samples of plant-based yoghurt alternatives were sourced from retail stores in Estonia, Finland, and Germany. The criteria for sample selection were that the products should preferably not contain added flavorings, must list microbial cultures as one of the ingredients and/or refer to fermentation. The list of the studied plant-based yoghurt alternatives is shown in Table 1. As several manufacturers did not have unflavored versions of the products available, some flavored samples were also included to obtain a better overview of the discrepancies between plant-based yoghurts from different producers.

2.2. DNA extraction

Microbial cells from commercial yoghurt alternatives were isolated aseptically. For that, 20 mL or g of each product was diluted in 30 mL of sterile 0.85% NaCl, 0.05% Tween 20 solution (Sigma), vortexed thoroughly and centrifuged at 500 × g for 15 min at 6 °C (Hettich ROTANTA 460R, fixed angle rotator). To collect microbial cells, the supernatant was transferred to a new 50 mL tube and centrifuged at 5000 × g for 15 min at 6 °C. The cells were washed in 2 mL of sterile 0.85 % NaCl solution, transferred to a 2 mL tube and centrifuged at 5000 × g for 15 min at 6 °C (Thermo Scientific MicroCL 21R). The final pellet was resuspended in 800 µL of NaCl solution and divided into two 400 µL aliquots. For viable cell discrimination, one aliquot was treated with PMAxx reagent (Biotium, Fremont, CA, USA). The PMAxx-cells suspension was incubated in dark for 10 min on a shaker. Then the samples were exposed to blue light by PMA-Lite™ LED Photolysis device (Biotium) for 20 min with intermittent inversion. Untreated aliquots for total cell number estimation and PMAxx treated aliquots were centrifuged at 5000 × g for 15 min at 6 °C, the supernatant was aspirated, and the pellets were kept at -20 °C until gDNA extraction.

Before DNA extraction, cells pellets were re-suspended in 150 - 195 µL 1 x PBS, and $3 \times 10^4 - 1 \times 10^8$ cells of ZymoBIOMICS™ Spike-in Control I or Spike-in Control II (High Microbial Load, Low Microbial Load respectively (Zymo Research, Irvine, CA, USA)) were added to the samples. Microbial cells were subjected to gDNA extraction according to the Quick-DNA™ Fungal/Bacterial Miniprep Kit protocol (ZR, Zymo Research, Irvine, CA, USA). The quantity of the extracted gDNA was measured by Qubit™ 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) using dsDNA HS Assay Kit (Thermo Fisher Scientific).

2.3. Metagenomic analysis of 16S rRNA gene amplicons

Amplicon libraries targeting the 16S rRNA gene V4 hypervariable region by primer pair 515F/806R were prepared according to Illumina's dual indexing system. Multiplexed and normalized libraries were sequenced on MiSeq Sequencing System (Illumina, San Diego, CA, USA) using MiSeq v2 Reagent Kit and 2 × 150 cycles paired-end sequencing protocol. Previous activities and sequencing data analysis were performed as published before (Kazantseva et al. 2021).

2.4. Sensory analysis

Sensory analysis was conducted in the Center of Food and Fermentation Technologies (Tallinn, Estonia) in isolated panel booths in a sensory room (in accordance with ISO 8589:2007). The expert sensory panel consisted of nine highly trained assessors (in accordance with ISO 8586:2012), who had previous experience in assessing plant-based dairy alternatives. Panellists were aged between 22 and 43 years old, with an average age of 33 years. Participants gave written consent to take part in the sensory analysis. Participation in the given study was voluntary and panellists could withdraw from the test at any time. Selected panellists were informed in advance of the purpose and the procedures of the study. All participants were in good health and had no known allergies to the components.

Descriptive sensory analysis was carried out over a two-day period involving two replicates per sample. Samples were presented in transparent 40 mL plastic cups coded with three-digit random numbers. Before serving at room temperature (20 °C), samples were stored refrigerated (4 °C). During the assessment, the order of the samples was different for each assessor and followed Williams' Latin square design to avoid the effect of presentatio (Macfie et al. 1989) The first set of replicate samples were served so that samples from similar raw materials were included to allow comparability for this particular group and to avoid the convergence effect of very distinctive samples (Kemp et al. 2018). In the second set of replicates, however, different raw materials were combined to ensure comparability between different product types.

The evaluation was completed in seven sessions consisting of 6-8 samples in each. Each session lasted about 30 min. After each session, there was at least 1-hour break to avoid sensory fatigue. During the sessions, assessors were encouraged to clean the palate with unsalted water crackers (Pladis LTD, London, UK) and spring water (Saku Läte OÜ, Estonia, Tallinn, Estonia). Samples were evaluated on a 10-point structured line scale with word anchors (0 "none", 1 "very weak", 5 "moderate", 9 "very strong"). The attribute list was developed based on previous literature and previous experience. A full list of sensory terms and descriptions for evaluating plant-based yoghurt alternatives is shown in Appendix 1.

The sensory analysis focused on odour and taste properties. For both modalities, the following attributes were evaluated: overall intensity, raw material, sour, sweet, fruity, floral, cheesy, dairy, and additive. For taste, there were additional characteristics assessed such as saltiness, bitterness, and astringency. For each sample, the odour was first assessed by sniffing, after which the sample could be assessed by taste. There was also a possibility to add additional comments to a voluntary text box under each modality.

Sensory data were collected by using RedJade (RedJade Sensory Solutions LLC, Martinez, CA, USA). Sensory results were considered in subsequent analyses if at least half of the assessors scored an attribute higher than 0. Ordinations for Principle Component Analysis (PCA) were calculated and visualized in R 4.1.2 (The R Foundation for Statistical Computing, Vienna, Austria) using "prcomp" function. Data for ordinations were mean-centred and scaled by the standard deviation. Partial least squares discriminant analysis (PLS-DA) was done using R library "mixOmics" 6.19.1.

2.5. Chemical analyses

2.5.1. pH, titratable acidity, and concentration of organic acids

The pH of the yoghurt alternatives was measured with pH meter S20 Seven Easy equipped with InLab 413 electrode (Mettler-Toledo GmbH, Greifensee, Switzerland). Total titratable acidity (TTA), reported as lactic acid, was measured with DL22 Food and Beverage Analyzer (Mettler Toledo, Switzerland) according to the manufacturer's instructions. 5 g of sample was mixed with 45 g of distilled water, stirred until homogeneous and titrated with 0.1 N NaOH. The results were calculated as to % of lactic acid.

Before the chromatographic analysis of the organic acids, 1 g of sample in duplicate was centrifuged (21000 × g, 5 min, 20 °C). 500 µL of sample supernatant was ultra-filtered (14000 × g, 30 min, 20 °C) using Amicon Ultra-3K centrifugal filter device, cut-off 3 kDa (Milli-

Table 1
Studied plant-based yoghurt alternatives.

Sample code	Product name	Main ingredient	Added bacteria	Added acidity regulators	Added flavoring
VY1	Lughurt Plain from Made with Luve	lupin	*S. thermophilus, L. bulgaricus*	-	flavoring
VY2	Lughurt Plain from Made with Luve (blueberry)	lupin	*S. thermophilus, L. bulgaricus*	lemon juice concentrate	flavoring, blueberries (3.6 %), black currants (2.7 %)
VY3	Sojade (So Soja, Natural)	soya	selected live cultures of which *Bifidus* and *Acidophilus*	-	-
VY4	Sojade (So Soja, alternative zu quark)	soya	ND	-	-
VY5	ProVamel. Soya. Coconut	soya	*S. thermophilus, L. bulgaricus*	citric acid, malic acid	natural coconut flavoring
VY6	ProVamel. Soya. Ohne Zucker	soya	*S. thermophilus, L. bulgaricus*	citric acid	-
VY7	ProVamel. Soya. Skyr Style	soya	*S. thermophilus, L. bulgaricus*	citric acid	-
VY8	ProVamel. Soya. Quarkalternative	soya	*S. thermophilus, L. bulgaricus*	citric acid	-
VY9	Alpro High Protein	soya	*S. thermophilus, L. bulgaricus*	Na-citrate, citric acid	natural flavoring
VY10	Alpro Natural	soya	*S. thermophilus, L. bulgaricus*	Na-citrate, citric acid	flavoring
VY11	Alpro Natur ohne zucker	soya	*S. thermophilus, L. bulgaricus*	Na-citrate, citric acid	natural flavoring
VY12	Rainbow Hapendatud mahesojatoode	soya	ND	-	-
VY13	BonSoya juuretisega kaerajook (õuna-kirsi)	oat	ND	-	apple-cherry concentrate (12%)
VY14	Valio Oddlygood Mieto maustamaton	oat	selected live cultures of which *Bifidus* and *Acidophilus*	malic acid	flavoring, coconut beverage (29%)
VY15	Oatly Havregurt Turkisk	oat	ND	malic acid, lactic acid	-
VY16	Oatly Havregurt Natureli (Hapendatud maitsestamata kaeravahepala)	oat	ND	malic acid, lactic acid	-
VY17	Yosa Greek Style Natural	oat	ND	-	-
VY18	Fazer Aito Kauravälipala Havrebaserad gurt	oat	ND	-	-
VY19	Planti Yog Oat Maiustamaton Naturell	oat	ND	-	-
VY20	Juustoportti Friendly Viking's O'gurt Natural	oat	ND	malic acid, lactic acid	-
VY21	Benecol Maustamaton kauragurtti	oat	*S. thermophilus, L. bulgaricus*	-	-
VY22	The Coconut Collaborative Natural Coconut Yog	coconut	*S. thermophilus, L. bulgaricus, L. acidophilus, B. lactis*	-	-
VY23	Cocodeli vegan natural	coconut	ND	-	-
VY24	Koko Plain smooth&mellow	coconut	*S. thermophilus, L. bulgaricus*	-	natural flavoring
VY25	Harvest Moon	coconut	*S. thermophilus, L. bulgaricus, L. acidophilus, B. lactis*	-	-

pore, USA). The filters were previously rinsed three times with MilliQ water to remove glycerol residues. Appropriate dilutions (2-15 times) were made in duplicate with Milli-Q water. The concentrations of organic acids were determined by high-performance liquid chromatography (HPLC, Alliance 2795 system, Waters, Milford, MA, USA), using a BioRad HPX-87H column (Hercules, CA, USA) with isocratic elution of 0.005 M H_2SO_4 at a flow rate of 0.6 mL/min. Refractive index (RI) (model 2414; Waters, USA) and UV (210 nm; model 2487; Waters, USA) detectors were used for the quantification of the substances.

2.5.2. Sugars

The measurement of sugars was based on (Pismennõi et al. 2021) article with modifications. The chromatography was performed using Waters BEH Amide XP column (3.0 × 150 mm, 2.5 μm) from Wa-

ters Corporation (Milford, MA, USA). The sugars were eluted with a binary gradient consisting of mobile phase A (90%/5%/5% v/v Acetonitrile/Ultrapure water/Isopropyl alcohol) and mobile phase B (78%/20%/2% v/v Acetonitrile/Ultrapure water/Isopropyl alcohol), both containing additionally 0.05 % (v/v) diethylamine and 0.5 mg/L guanidine hydrochloride. The column was heated to 90 ˚C. The injection volume was set to 1 µl and the run time was set to 25 min. The data were acquired and processed with Empower 3.0 software (Waters Corporation, Milford, MA, USA). The samples were centrifuged at 14000 × g upon arrival and supernatant was used for subsequent analysis. The supernatant was firstly diluted with ultrapure water, then diluted with 50 % aqueous acetonitrile (v/v) containing C13-isotopically labelled standards.

2.5.3. Volatile compounds

Extraction of volatiles was carried out using solid-phase microextraction (SPME). 0.5 g of each was weighed into a 10 mL sample vial. The vials were pre-incubated at 50 ˚C for 5 min. SPME fibre (30/50 μm DVB/Car/PDMS Stableflex, length 1 cm) was used to adsorb/absorb the volatile compounds from the headspace (HS) for 30 min. The absorbed/absorbed volatile compounds were subsequently desorbed into a GC injection port for 5 min.

Identification and quantification of volatile compounds was performed using a gas chromatograph system (Nexis GC-2030; Shimadzu, Kyoto, Japan) equipped with a mass spectrometer (GCMS-TQ8050NX; Shimadzu, Kyoto, Japan). A ZB5-MS column (30 m length × 0.25 mm i.d. × 1.0 μm film thickness; Phenomenex, Torrance, CA, USA) was used with helium as a carrier gas at a linear velocity of 35 cm s^{-1}. The oven was programmed to ramp up from 40 ˚C at a rate of 5 ˚C/min to a temperature of 190 ˚C and at a rate of 25 ˚C/min from 190 ˚C to 280 ˚C with an additional holding time of 4 min (total run time 36 min).

Mass spectra were obtained at ionization energy of 70 eV with a mass-to-charge ratio scan range of 35 to 350. For each sample, three analytical replicates were made.

Non-targeted identification of volatile compounds was carried out using GCMSsolution software (Shimadzu, Japan) and retention indices (RI). Experimental retention indices were calculated using the retention times of the eluting compounds normalized to the retention times of adjacent n-alkanes. The identification of the compounds was verified by comparing experimental retention indices to NIST17 and FFNSC libraries. Semi-quantitative approach against an internal standard (4-methyl-2-pentanol; 400 ppb) was used to quantify identified volatile compounds (in IS ppb-eq.).

3. Results

3.1. Bacterial composition

Fig. 1 represents the results of the metagenetic analysis in the form of proportions of total and alive dominating bacteria and their estimated cell numbers. The bacterial composition of commercial yoghurt alternatives was evaluated by an amplicon-based 16S rRNA gene next-generation sequencing approach. To identify the number of viable bacteria, PMAxx-pretreatment of cells and spike-in addition were applied. Untreated cells were considered as a total bacterial load of the sample and included in the analysis as separate data.

For better comparison, the products were grouped according to the starter cultures mentioned on the labels as shown in Table 1 and Fig. 1. Thus, five distinctive groups were formed: 1) contained *Streptococcus salivarius subsp thermophilus* and *L. delbrueckii subsp bulgaricus*; 2) *S. thermophilus, L. bulgaricus, Lactobacillus acidophilus, Bifidobacterium animalis subsp lactis;* 3) prepared with *L. acidophilus* and Bifidobacteria; 4) mentioned bacterial culture usage for fermentation; 5) using an acidic base for the production of the yoghurt alternatives.

The most representative was group number one, where all plant bases such as lupin (VY1, VY2), soya (VY5-VY11), oat (VY21), and coconut (VY24) were represented. All eleven products belonging to the group showed the presence of both the abovementioned bacterial cultures. Based on the analysis of total bacterial DNA, samples with lupin as the main ingredient included significant proportions of *Lacticaseibacillus rhamnosus*, and 1-11% of *L. acidophilus* and *L. crispatus,* neither of which was stated on the product label. The latter two, however, were not detected among alive bacterial consortia of the respective samples. The dominating bacteria for all other plant bases was *S. thermophilus,* which represented around 99% of the detected bacteria in all vegan yoghurt alternatives in this group. The detected number of bacterial cells from 1 g of the products ranged from 7.56×10^7 for oat products to about 2×10^9 for soya products. However, not all detected bacteria were alive. The lowest viability was observed for sample VY1 (30.9%), while in VY5 all detected bacteria were alive.

The second group was represented by only two products (VY22 and VY25). Both were coconut-based, but one did not contain *B. lactis*, and the level of *L. acidophilus* in the other was very low (0.004%). Moreover, these species were not detected among the live bacteria, but several environmental species, such as *Acinetobacter johnsonii, Aquabacterium parvum*, and *Pseudomonas aeruginosa,* were observed for VY22. Instead, these products contained *L. rhamnosus* not marked on the product label. Also, the number of alive bacteria and their viability percentage for VY22 were very low (6.17×10^2 cells/g and 12%, respectively).

The third group of products consisted of two yoghurt alternatives as well. These were soya (VY3) and oat-based (VY14) and included mainly *S. thermophilus* and only traces of declared *L. acidophilus* and *B. lactis.* The products contained *L. delbrueckii* and all these mentioned bacteria were alive, with the estimated number of 2.08×10^9 and 1.48×10^7 cells per gram of product for VY3 and VY14, respectively. Though, the proportion of viable bacteria for oat-based VY14 was only 6.5%.

The fourth group labelled as “culture” consisted of seven fermented products, where four were oat-based (VY13, VY17, VY18, and VY19), two were made from soya (VY4 and VY12), and one was a coconut-based yoghurt alternative (VY23). As for all previously described groups, the main species these products contained was *S. thermophilus.* All products differed by their bacterial composition and included up to three additional lactic acid bacteria (LAB) species. However, only three species of alive bacteria were detected in the coconut yoghurt alternative VY23 (*S. thermophilus, L. delbrueckii,* and *L. rhamnosus),* while all other products contained one or no additional bacteria in combination with *S. thermophilus.* Despite the estimated number of alive bacteria for this group being in the range of 1.52×10^7-1.07×10^9, the ratio of alive to total bacteria differed from 3.8% to 45.6%.

The fifth and last “acidic base” group was represented by three oat products (VY15, VY16, and VY20). Their common features were a low number of detected bacteria (2.63×10^4 to 1.65×10^6) and a minimal proportion of alive bacteria in the samples (0.4%-2.9%). All of them contained *S. thermophilus,* but for VY20, the potentially pathogenic *P. aeruginosa* was identified as a dominant species among alive bacteria. Moreover, this product had *Corynebacterium* spp and *Staphylococcus aureus* in their composition, which were not on the list of viable species. *P. aeruginosa* was also detected in VY15 and VY16 (31.9% and 8.7%, respectively), together with other environmental strains not involved in LAB fermentation.

Overall, the minimum amount of alive bacteria in analyzed products was at least 2.2×10^2 cells per gram for oat-based VY20, with the maximum detected number of 2.08×10^9 for VY3 soya product.

The next step of our evaluation was to understand the common bacteriological features or dissimilarities between the products. Non-metric multidimensional scaling (NMDS) analysis, seen in Fig. 2 for total and alive consortia at the species level was performed.

Most products for total bacterial consortia clustered together, excluding several outsiders such as VY1, VY2, VY17, VY19, VY20, VY23, and VY25 as presented in Fig. 2a. All of them, in combination with the dominant *S. thermophilus* species, contained *L. rhamnosus* or non-LAB bacteria, distinguishing them from other groups. Clustering of alive

Sample	TOTAL, %												Estimated cells number per g of product	ALIVE, %										Estimated cells number per g of product	Alive/Total cells ratio, %
	Streptococcus thermophilus	*Lactobacillus bulgaricus*	*Lactobacillus acidophilus*	*Bifidobacterium lactis*	*Lacticaseibacillus rhamnosus*	*Lactobacillus crispatus*	*Lactobacillus gallinarum*	*Corynebacterium unclassified*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*			*Streptococcus thermophilus*	*Lactobacillus bulgaricus*	*Lactobacillus acidophilus*	*Bifidobacterium lactis*	*Lacticaseibacillus rhamnosus*	*Acinetobacter johnsonii*	*Aquabacterium parvum*	*Pseudomonas aeruginosa*			
Streptococcus thermophilus, Lactobacillus bulgaricus (L. delbrueckii subsp bulgaricus)																								
VY1	22,5	0,01	4,5		71,1						7.51×10^8		10,3	0,004		1,7	85,7					2.32×10^8	30,9	
VY2	17,8	0,01	1,0		68,1	10,9					1.87×10^9		7,9	0,004			88,1					9.08×10^8	48,4	
VY5	98,7	1,17									8.72×10^8		98,8	1,14								1.02×10^9	100,0	
VY6	98,2	1,63									4.27×10^8		93,7	1,48								2.74×10^8	78,1	
VY7	99,5	0,31									1.71×10^9		99,1	0,69								9.08×10^8	53,2	
VY8	99,5	0,29									1.03×10^9		98,7	1,16								7.53×10^8	72,9	
VY9	99,7	0,16									1.96×10^9		99,6	0,22								8.97×10^8	45,7	
VY10	99,7	0,17									1.22×10^9		99,9	0,06								1.04×10^9	85,4	
VY11	99,8	0,07									2.13×10^8		99,7	0,12								5.69×10^7	36,4	
VY21	99,7	0,14									7.56×10^7		99,7	0,01								4.08×10^7	50,0	
VY24	99,4	0,49									1.12×10^9		99,6	0,29								3.89×10^8	35,0	
Streptococcus thermophilus, Lactobacillus bulgaricus, Lactobacillus acidophilus, Bifidobacterium lactis (B. animalis subsp lactis)																								
VY22	80,1	0,25	1,97	0,25							5.45×10^4		25,0	1,4			1,4	5,1	5,1	24,0		6.17×10^2	12,0	
VY25	76,1	0,08	0,004		22,7						1.44×10^9		69,7	0,07			29,9					4.38×10^8	30,8	
Selected live cultures of which *Bifidus* and *Acidophilus*																								
VY3	99,3		0,08	0,41							2.63×10^9		99,4	0,004	0,009	0,53						2.08×10^9	79,2	
VY14	75,8	7,4	14,2	2,5							5.87×10^8		72,6	10,8	14,3	2,1						1.48×10^7	6,5	
Culture																								
VY4	90,4		1,3	4,4							9.08×10^8		91,3			8,3						2.43×10^8	26,7	
VY12	99,5										2.36×10^9		99,8									1.07×10^9	45,6	
VY13	94,1	1,9	2,9								7.77×10^6		95,6				3,7					1.52×10^7	8,2	
VY17	32,9				61,3	3,2					9.64×10^7		16,7				80,7					1.82×10^7	18,9	
VY18	99,9										1.55×10^9		99,9									5.92×10^7	3,8	
VY19	61,2		1,1				34,7				2.80×10^8		91,3		7,8							1.42×10^8	37,3	
VY23	55,0	22,1			14,0	1,6					2.24×10^9		50,4	8,2			32,2					9.78×10^8	43,7	
Acidic base																								
VY15	92,7	6,9									4.53×10^5		39,5	1,8	1,3			4,5	1,2	31,9		$*1.62 \times 10^3$	0,4	
VY16	98,5	1,3									1.65×10^6		60,0				1,3	5,3		8,7		1.05×10^4	2,9	
VY20	28,5							25,5	2,6	12,0	2.63×10^4		7,0					7,9	3,0	36,0		2.27×10^8	1,0	

Fig. 1. Cluster groups of analyzed yoghurt-alternatives according to the package label. Proportions of total and alive dominating bacteria and their estimated cell numbers identified by 16S rRNA sequencing are shown. Samples are indicated by different colours: brown corresponds to coconut, blue – lupin, red – oat, and green – to soya bases. All data are normalised to DNA input except for the sample marked as *, in which case a normalisation was not possible due to the low library concentration.

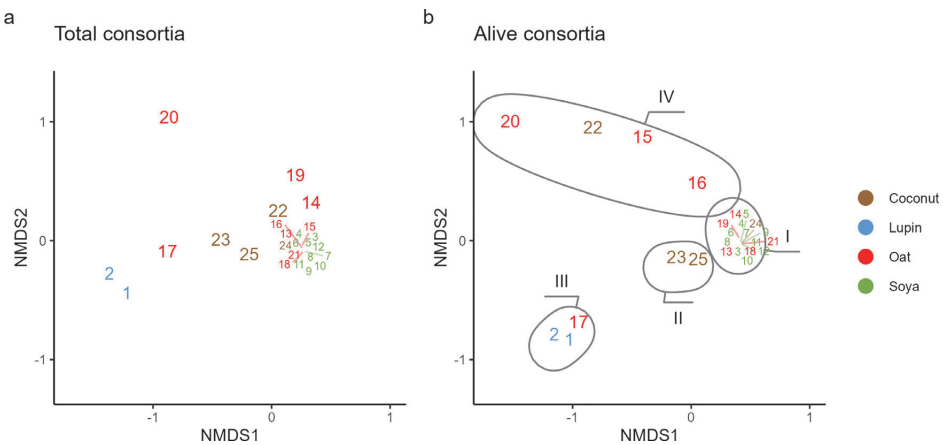

Fig. 2. Non-metric Multi-dimensional Scaling (NMDS) plots represent total (a) and alive (b) bacteria consortia of yoghurt alternatives clustered by identified species groups. Four distinctive clusters grouped by similar alive bacteria species consortia are marked.

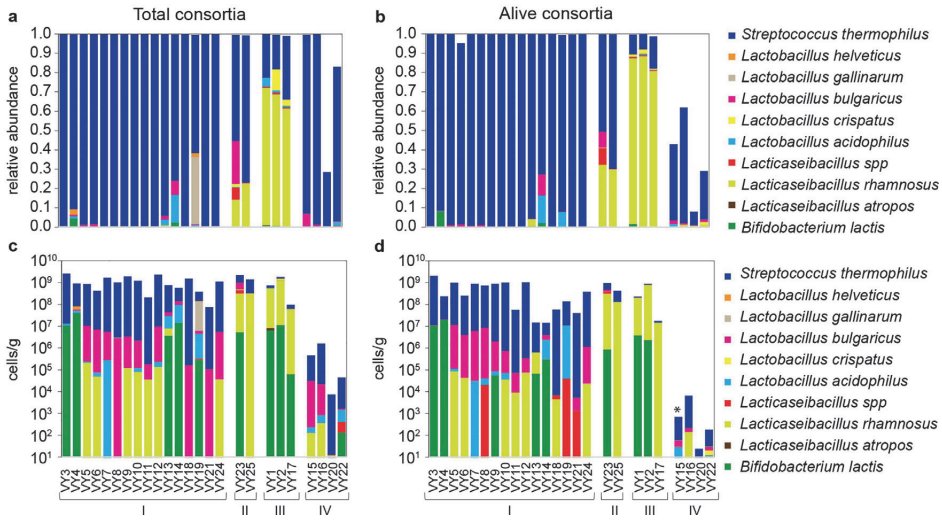

Fig. 3. 16S rRNA amplicon sequencing data for total (a, c) and alive (b, d) bacterial consortia of yoghurt-alternatives represented as relative abundances (a, b) and estimated cell number per g of the product (c, d) values. Asterix * indicates the sample which was not possible to normalise in the library preparation stage due to the low DNA concentration and therefore should not be compared with all other samples.

bacteria was more discriminatory with four typical groups as seen in Fig. 2b. Most of the plant-based yoghurt alternatives formed one big cluster (I). Two coconut products VY23 and VY25 (cluster II) were distinctive by their increased level of *S. thermophilus*, a significant amount of *L. rhamnosus*, and the presence of *L. delbrueckii*. The third cluster (III) formed by VY1, VY2, and VY17 products differed by a dominant bacterial strain, which was *L. delbrueckii* (80.7-88.1%) but not *S. thermophilus* (7.9-16.7%). VY15, VY16, VY20, and VY22 are grouped due to the presence of environmental bacteria in their composition in addition to typical LAB cultures (cluster IV). Three of the four products here were oat-based.

The complete taxonomical distribution of the relative abundances of bacterial species (a, b) and estimated cell number per 1 gram of product (c, d) for total (a, c) and alive (b, d) consortia is represented in Fig. 3. As seen from the figure, LAB composed almost 100% of all identified bacteria for most of the analyzed plant-based yoghurt alternatives. However, oat-based VY15, VY16, VY20, and the coconut product VY22 that clustered separately had additional environmental bacterial species. This difference is even more profound in the case of alive consortia, where LAB represented less than half of the detected bacteria.

3.2. Chemical analyses

3.2.1. pH and TTA

Table 2 summarises the pH and TTA measurements of the analyzed yoghurt alternatives. pH was in the range of 3.6-4.8 having the lowest values in lupin-based samples (pH 3.6-3.7) and the highest values in soya-based samples (pH 4.5-4.8). The pH values varied the most among the oat-based sample group (pH 3.8-4.4). TTA was higher in soya- and lupin-based samples compared to products based on oat and coconut. There is no straightforward dependence between the results of pH and TTA. The higher concentrations of lactic and/or citric acid were also determined in soya and lupin-based products. The highest variability in TTA values was also observed within oat-based samples with the highest values in the samples VY13, VY15 and VY16 where the acids have been added as an acidity regulator or are included in the flavoring. All the rest oat samples had quite low TTA values compared to all the samples analyzed.

3.2.2. Organic acids

Concentrations of selected organic acids measured in the samples are shown in Table 2. Several yoghurt alternatives contained added acids according to the list of ingredients marked on the products (see Table 1). Lactic acid was the most prominent acid found in the yoghurt alternatives, detected in the range of 3.5-5.8 g/L. A significantly higher amount of lactic acid was measured in samples (VY15, VY16) where the acid was added to the product in addition to the amount of acid that may have been produced during the fermentation process. The concentration of citric acid was higher if both, Na-citrate as well as citric acid were added to the product compared to citric acid being the only acidity regulator. Some samples based on soya (VY 3, VY4, VY12) contained citric acid, even though it was not listed as an ingredient. The three aforementioned products contained a relatively high number of living bacteria, which could indicate the acid was produced during fermentation. Acetic and succinic acids were determined in several samples having higher concentrations in samples based on lupin (VY1, VY2), soya (VY14), and coconut (VY22, VY23). A significant amount of malic acid was detected in sample VY13 due to added flavoring (apple-cherry concentrate) and in sample VY22 as it was based on coconut. Malic acid may have been derived from the coconut base. A slightly lower amount of malic acid was detected in the samples VY15 and VY16 where it was added as the acidity regulator together with lactic acid. In all other samples, only trace amounts of malic acid were detected. Both malic and lactic acids were listed among the ingredients of oat-based sample VY20 but were detected in very low amounts – 0.17 g/L and 0.31 g/L, respectively.

A partial least squares discriminant analysis (PLS-DA) was conducted on acid content and microbial cells grouped based on the presence of acidity regulators. The data is presented in Appendix 2. Oat samples where acids were added (a), were measured as often having a higher concentration of citric acids. Although, figure in the Appendix 2 indicates that according to product labelling, malic acid and lactic acid were added. Lactic acid was found at a prominent level in some samples (VY15 and 16). For soya products (b), citric, malic, formic, and succinic acid were often found in samples with added acidity regulators, which is partly supported by indications of added citric acid. Oat and soya samples without acidity regulators generally had higher cell numbers, which means that these samples may have been naturally fermented and additional acid was not added.

Table 2
Concentration (g/L) of sugars (fructose, glucose, maltose, sucrose) and organic acids (acetic, citric, lactic, succinic, malic), pH and Total Titratable Acidity (TTA) in plant-based yoghurt alternatives.

Sample code	g/L									pH	TTA, %
	Fructose	Glucose	Maltose	Sucrose	Acetic acid	Citric acid	Lactic acid	Succinic acid	Malic acid		
VY1	5.53	4.94	3.17	0.28	0.11	0.05	5.21	0.05	ND	3.71	0.53
VY2	48.30	47.96	3.16	11.08	0.14	1.72	4.14	0.09	ND	3.63	0.65
VY3	0.95	0.37	0.01	2.76	0.05	1.36	4.09	0.02	0.06	4.64	0.50
VY4	0.32	0.02	0.01	2.61	0.05	0.76	4.98	0.02	0.07	4.76	0.51
VY5	0.09	0.08	0.01	1.58	0.05	1.52	3.79	0.03	0.21	4.57	0.51
VY6	0.26	0.27	0.01	0.96	0.05	1.86	4.00	0.03	0.09	4.47	0.55
VY7	0.62	0.64	0.01	2.04	0.05	1.89	3.93	0.02	0.07	4.48	0.72
VY8	0.62	0.64	0.02	2.01	0.04	1.92	3.99	0.02	0.08	4.49	0.73
VY9	0.31	0.32	0.36	26.22	0.03	6.50	4.53	0.02	0.07	4.51	0.96
VY10	0.15	0.20	0.18	24.23	0.03	5.39	4.18	0.01	ND	4.57	0.71
VY11	0.11	0.12	0.01	1.23	0.03	5.18	3.76	0.02	0.09	4.64	0.71
VY12	0.00	0.00	0.19	0.93	0.07	2.41	5.84	0.05	0.12	4.6	0.64
VY13	38.97	63.31	16.36	3.72	0.06	ND	1.39	0.11	2.06	4.05	0.46
VY14	0.29	0.01	0.28	10.72	0.16	0.14	4.28	0.17	0.25	4.43	0.33
VY15	0.31	0.47	33.45	3.40	0.02	0.08	6.93	ND	1.51	4.02	0.66
VY16	0.33	0.51	37.22	2.50	0.04	0.05	7.77	ND	1.26	4.15	0.47
VY17	0.08	24.15	9.84	0.33	0.05	0.03	1.88	0.01	ND	3.82	0.24
VY18	0.24	5.83	0.10	12.89	0.05	0.03	1.25	0.01	0.08	4.39	0.16
VY19	0.29	40.53	1.41	0.31	0.02	0.02	2.04	0.01	0.09	4.41	0.22
VY20	0.17	2.56	6.75	0.05	0.01	0.04	0.31	0.00	0.17	3.84	0.14
VY21	0.18	21.33	12.80	0.98	0.02	0.07	2.32	0.02	0.14	4.37	0.27
VY22	4.53	3.16	0.34	18.01	0.09	0.23	3.48	0.09	2.53	4.53	0.40
VY23	0.01	0.00	0.12	20.49	0.22	0.44	6.19	0.20	ND	4.32	0.50
VY24	2.49	34.19	0.00	4.89	0.08	0.09	3.63	ND	0.56	4.17	0.49
VY25	0.00	0.00	0.00	8.84	0.06	0.27	4.10	0.02	ND	4.26	0.30

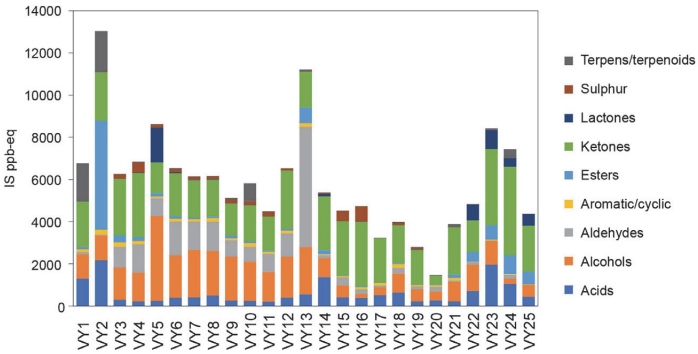

Fig. 4. Volatiles in yoghurt alternatives are summed and grouped by chemical classes.

3.2.3. Sugars

The content of glucose, fructose, sucrose, and maltose was measured in all products and the results are summarised in Table 2. Fructose was detected only in flavored samples – VY2 (lupin-based product with blueberry and black currants) and VY13 (oat-based product with apple-cherry flavoring). The highest amount of glucose was also observed in the mentioned samples. The highest amount of sucrose was detected in soya-based samples VY9 and VY10 which most probably originated from flavoring added to the products. The most diverse sugar profile was seen in oat-based samples where glucose, maltose, and sucrose were presented in different ratios. Oat-based samples were most likely enzymatically treated to improve the texture by degrading the starch and releasing mono- and disaccharides. The coconut-based samples contained sucrose in higher amounts but also glucose and fructose to some extent.

3.2.4. Volatile compounds

The results for volatiles' measurements are presented in Fig. 4. In total 192 volatile compounds were detected across all the samples. The total list of molecules together with their relative concentrations can be found in Appendix 3. Lupin and coconut samples had higher total acid contents compared to soya and oat-based products. The exception was the oat-based sample VY14 with also high acetic acid content. The dominating volatile acid in most of the samples was acetic acid followed by butanoic and hexanoic acids. Lupin and soya contained a higher number of different alcohols compared to oat and coconut products, which can be seen in Fig. 4. 1-hexanol and (Z)-3-hexen-1-ol which have "green" and "leafy" notes were the most dominating alcohols detected in the analyzed samples. The presence of different aldehydes in the highest quantities characterized soy-based products, where hexanal (grassy) was the most dominating compound. The oat-based sample VY13 differed from the other samples by high concentrations of furfural (caramel) and benzaldehyde (bitter almond) most probably coming from the used flavorings. Esters were mainly found in higher concentrations in coconut-based samples. There were some exceptions like lupin-based sample VY2 and oat-based sample VY13 which had also high ester content which could be related to the fruit-based ingredients. Ketones varied inside each raw material group. The main ketones present were with "creamy" and "dairy" notes like 2,3-butandione, 2,3-pentanedione, and acetoin which are typical fermentation products or could originate from added flavorings rather than from plant-based raw materials. Lactones which are with "creamy" and "coconut" notes were present in coconut-based samples as expected. Soya-based sample VY5 was also high in lactones,

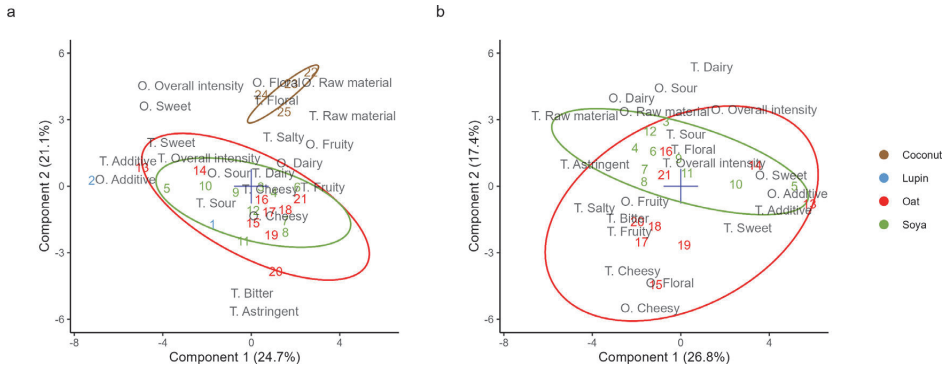

Fig. 5. PCA is based on sensory results of all samples by a) all main ingredients and by b) oat and soy samples. Sample clusters are shown by 95% confidence ellipses. Titles in parentheses show the variance explained by the component. Abbreviations: „ O" for odour and „T" for taste.

specifically in gamma-octalactone due to the addition of coconut flavoring. Also, the oat-based sample VY14 contained more lactones explained by added coconut beverage. The content of terpenes was quite low in most samples. The most dominating compounds were generally p-cymene (woody) and D-limonene (citrus) present in considerably higher concentrations in lupin-based samples VY1 and VY2 and in coconut-based sample VY24. Soya-based sample VY10 differed from all the other samples by the high concentration of menthol (peppermint).

3.3. Sensory analysis

All the results of the sensory analyses are presented by their average concentrations in Appendix 4 and descriptions for different attributes in Appendix 1. In Fig. 5a Principal Component Analysis (PCA) of sensory results showed that coconut was the most distinctive group from all the samples by their intense raw material odour and taste (in this case, coconut) and low bitterness and astringency. As there were only four coconut-based samples and two lupin-based samples, no clear conclusions can be made about these groups. Further sensory data analysis focuses only on soya and oat samples in order to determine, whether there are specific clusters within these groups. As shown in Fig. 5b, soya samples tended to have higher sourness, dairyness, and raw material odour/taste. It can be observed that samples with higher sourness were also often perceived as more dairy-like. There was some variation within the oat group, as some samples were perceived with sweeter off-flavors, while others were more salty, astringent, and bitter. A few samples had also some cheesiness (VY15 and 17), which is associated more with fermented, fruity, floral notes rather than clear dairy flavor.

In Fig. 5 it can be seen that some samples were assessed to have additive notes linked to their flavor additives (e.g. fruit flavoring in Table 1). Additionally, PLS-DA was conducted to further see the effect of added flavorings on the sensory profile. This is shown in Appendix 5. The two oat samples (a) with added flavorings were generally sweeter with additive notes, while other samples without added flavorings tended to have stronger raw material notes in taste and smell, some even with high bitterness, astringency, and saltiness. Soya samples (b) with added flavors were generally less varied in terms of odour and taste, but with added flavorings, sweeter and sour notes were perceived. It appears, that the soya and oat products successfully used flavorings to disguise flavors characteristic of plants, such as the raw material flavor, but also bitterness and astringency.

4. Discussion

As consumer interest in a plant-based lifestyle increases, the market is continuously enriched with yoghurt alternatives made from a variety of ingredients using innovative technologies. Since there is significant heterogeneity among the consumer preference for plant-based alternatives and the availability of raw materials differs regionally (Cardello et al. 2022; Ignaszewski 2022), it is a challenge for the producers to select suitable main ingredients – cereals, pulses, nuts, or seeds, and develop products that can attract a wider audience in a highly competitive product category. The characteristics of yoghurt alternatives vary significantly and are dependent on several factors. Though some raw materials (e.g., coconut) have distinct characteristics, which determine the flavor of the final product, then for others, multiple factors (e.g., starter cultures, processing parameters, added flavorings) influence the properties even more than the main ingredient resulting in high diversity in the yoghurt-alternatives product category.

The objective of the current study was to assess the diversity of commercially available plant-based yoghurt alternatives and to obtain a better understanding of their microbial composition, especially the content of live bacteria, and how that is linked to the chemical composition and sensorial properties of the products.

The main observation of the study was that there is a significantly greater heterogeneity in the plant-based yoghurt category compared to traditional dairy yoghurts (Aktar 2022). The main ingredient (soya, oat, lupin, and coconut) to a great extent determined the sensorial and chemical characteristics of the yoghurt. According to the literature, fermentation and/or addition of organic acids can help to hide plant attributes (Emkani et al. 2022; Laaksonen et al. 2021), though it appears the effect is insufficient and selecting suitable flavor additives can give better results.

Furthermore, there were significant differences observed in both chemical and microbiological composition and sensorial attributes among the yoghurt alternatives made from the same plant base. The highest diversity was observed in the case of oat-based products. It means, there are several variables (variety of crops, protein powder properties, processing parameters, fermentation parameters, additives, etc.) that can influence the characteristics of the final product either enhancing or diminishing the off-aromas and -tastes of the main ingredients. This makes the development of plant-based dairy alternatives to meet consumers' expectations a significant challenge.

The key role of fermentation is for the starter bacteria to produce organic acids and aroma compounds that give yoghurt its characteristic odor and taste (Chandan et al. 2008). In dairy yoghurts, starter bacteria lower the pH by producing large quantities of lactic acid from lactose, which is the main fermentable substrate in milk. Depending on the plant base used for the production of yoghurt alternatives, the range of available substrates can vary (Grasso et al. 2020). If enzymatic treatment is applied to degrade starch in the base, it will result in a high concentration of fermentable sugars, such as glucose and maltose (van der Maarel

et al. 2002). As only the composition of the end products was evaluated in this work, the initial fermentable sugar content remains unknown. In all studied samples, lactic acid was the most prominent organic acid detected. If Na-citrate was used as an acidity regulator, a significant amount of citric acid was also observed.

Though fermentation has shown a positive effect on reducing different key molecules in plants causing "beany" and "green" aroma notes (Harper et al. 2022), the characteristic raw material taste was still recognisable in most of the analyzed samples. Around 100 volatile molecules have been identified in typical dairy yoghurt, while acetaldehyde, diacetyl, acetoin, acetone, and 2-butanone have been brought out as the main contributors to the typical flavor of the product (Cheng 2010). These molecules were also present in all the analyzed samples. However, the number of different volatiles determined in analyzed samples is mostly considerably higher compared to dairy yoghurt, which brings out the challenges in plant-based developments. The presence of different molecules that have "green", "fatty and "beany" notes are mainly from aldehydes, furans, ketones, and alcohols (Singh 2021; Vaikma et al. 2021), which have been identified also in the current study, are limiting the acceptability of plant-based products in general. These raw material-specific notes are difficult to hide, and the selection of a neutral base is possible as the first step toward a successful product. On the other hand, the study found that some soya and oat products successfully used flavorings that masked plant-specific nuances (e.g., raw material intensity, bitterness, astringency). Therefore, the selection of processing steps and parameters as well as a smart selection of additional ingredients are crucial for developing successful products.

Compared to traditional yoghurts, the approach to producing plant-based yoghurt alternatives can vary remarkably (there is no gold standard) resulting in a high diversity among the products in this category. The sensory and chemical composition, as described before, along with the microbial profile can differ significantly. The microbial composition of the products along with the content of live lactic acid bacteria should be one of the key indicators to consider when evaluating such products. Based on the results of this study, the classical yoghurt starter culture *S. thermophilus* (Chandan et al. 2008; Iyer et al. 2010) was the most prominent species found in yoghurt alternatives indicating its versatility and adaptability to ferment various raw materials. Although *S. thermophilus* exhibited a high relative abundance among both total as well as live consortia, its cell numbers were often overshadowed by species belonging to the lactobacillus genus originating from both the starter as well as other sources (possibly raw materials and ingredients, as well as the production environment). The content of live bacteria detected in the yoghurt alternatives varied notably, indicating possible differences in the preparation approach of the products. Additionally, of the 25 products evaluated 8 contained significant numbers of live bifidobacteria, which have been associated with widely described health benefits (Picard et al. 2005; O'Callaghan and van Sinderen 2016; Hidalgo-Cantabrana et al. 2017).

In order to evaluate the microbiological composition of plant-based yoghurt alternatives, an enhanced metagenetic sequencing approach was used to assess the proportion of live bacteria among the total bacterial consortium. The developed method is based on the pre-treatment of cells by PMAxx reagent to discriminate between total and alive bacteria in the consortia. Moreover, the definite number of bacterial spike-in control cells was added to the cells isolated from the products, which went through the whole 16S NGS pipeline starting from DNA isolation, library preparation up to bioinformatic analyses and was the basis for the calculation of the number of bacteria in the samples. These combined approaches helped to characterize the products more rigorously, differentiate between whole and viable bacteria consortia, and get estimated cell values. This methodology possesses several advantages compared to the classical microbiological method of plating, as it does not rely on a specific medium or optimal temperature and gives a better overview of bacteria in the products - not all bacteria are cultivable under the selected conditions and might be excluded from the analysis.

Despite the number of bacterial species in the products obtained by this methodology being an estimate, all samples can be reliably compared to one another, and some joint conclusions can be drawn. Oat-based yoghurt alternatives were shown to contain a smaller number of viable bacteria; coconut and lupin products mostly formed distinctive clusters based on the composition of viable bacteria. This demonstrates that different technologies can be applied in the preparation of plant-based yoghurt alternatives depending on the ingredients and expectations for the final product.

As to the authors' knowledge, this is the first study in which a comprehensive analysis of commercially available plant-based yoghurt alternatives was carried out focusing on the evaluation of the microbial and chemical composition as well as sensorial attributes. Although our findings provide new insight into the current state of the yoghurt alternatives market, the main limitations of this study were the insufficient number of plant-based yoghurt samples made from a wider range of main ingredients and sourced from different markets. More in-depth analyses for evaluating the nutritional aspects of the products are still being conducted.

Ethical statement

An internal scientific research committee of the Center of Food and Fermentation Technologies (Tallinn, Estonia) approved the design of the sensory study. Participants in the sensory test were selected from a pool of highly trained evaluators who volunteered to perform the sensory assessment. The selected panelists had previous experience in evaluating non-dairy analogues and provided written consent. The participants were informed in advance of the purpose and the procedures of the study. Participation was voluntary and one could withdraw from the test at any time. Panelists were in good health and had no known allergy to the components.

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Appendix 1. Sensory terms and descriptions for evaluating plant-based yoghurt alternatives

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.

Data Availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fufo.2022.100212.

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Appendix 4

Publication IV

Pismennõi, D., Kattel, A., Belouah, I., Nahku, R., Vilu, R., & Kobrin, E.-G. (2023). The Quantitative Measurement of Peptidoglycan Components Obtained from Acidic Hydrolysis in Gram-Positive and Gram-Negative Bacteria via Hydrophilic Interaction Liquid Chromatography Coupled with Mass Spectrometry. Microorganisms, 11(9), 2134. https://doi.org/10.3390/microorganisms11092134

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Article

The Quantitative Measurement of Peptidoglycan Components Obtained from Acidic Hydrolysis in Gram-Positive and Gram-Negative Bacteria via Hydrophilic Interaction Liquid Chromatography Coupled with Mass Spectrometry

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Abstract: The high throughput in genome sequencing and metabolic model (MM) reconstruction has democratised bioinformatics approaches such as flux balance analysis. Fluxes' prediction accuracy greatly relates to the deepness of the MM curation for a specific organism starting from the cell composition. One component is the cell wall, which is a functional barrier (cell shape, exchanges) with the environment. The bacterial cell wall (BCW), including its thickness, structure, and composition, has been extensively studied in *Escherichia coli* but poorly described for other organisms. The peptidoglycan (PG) layer composing the BCW is usually thinner in Gram− bacteria than in Gram+ bacteria. In both bacteria groups, PG is a polymeric mesh-like structure of amino acids and sugars, including N-acetylglucosamine, N-acetylmuramic acid, and amino acids. In this study, we propose a high-throughput method to characterise and quantify PG in Gram-positive and Gram-negative bacteria using acidic hydrolysis and hydrophilic interaction liquid chromatography coupled with mass spectrometry (HILIC-MS). The method showed a relatively short time frame (11 min analytical run), low inter- and intraday variability (3.2% and 4%, respectively), and high sensitivity and selectivity (limits of quantification in the sub mg/L range). The method was successfully applied on two Gram-negative bacteria (*Escherichia coli* K12 MG1655, *Bacteroides thetaiotaomicron* DSM 2079) and one Gram-positive bacterium (*Streptococcus salivarius* ssp. *thermophilus* DSM20259). The PG concentration ranged from 1.6% *w*/*w* to 14% *w*/*w* of the dry cell weight. The results were in good correlation with previously published results. With further development, the PG concentration provided by this newly developed method could reinforce the curation of MM.

Keywords: peptidoglycan; HILIC-MS; hydrolysis; n-acetylmuramic acid; n-acetylglucosamine; muramic acid; glucosamine; biomass composition analysis

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1. Introduction

Peptidoglycan (PG) is a component of the bacterial cell wall involved in both the cell shape [1] and the resistance to osmotic stress. The PG layer is a mesh-like structure constantly synthesised, remodelled, and repaired to adjust to changes in the cell environment and physiology (division, sporulation). This mesh is composed of alternating units of N-acetylglucosamine (NAG) and N-acetylmuramic acids (NAM), connected with β-1-4 bonds and adjoined in long strands. The PG synthesis starts in the cytoplasm with the formation of glucosamine-6-phosphate from fructose-6-phosphate. After six more enzymatic reactions, an NAG residue is linked to an NAM, which bears a peptide stem consisting of five amino acids. This monomeric disaccharide peptide unit is called lipid II. During the

polymerisation of PG, several units of lipid II are linked to each other via a short peptide bridge. The length of the PG strands, the composition of the disaccharide peptide units [2], and the thickness of the mesh [3] vary between strains [4]. Based on the PG KEGG pathway map (map00550), the synthesis of lipid II could cost up to seven ATP molecules, one UTP molecule, and one NAD(P)+ molecule with glucose as a carbon source [5]. In addition, one acetyl-Coa molecule, one phosphoenolpyruvate molecule, and one undecaprenyl phosphate molecule are mobilised. Most ATP is consumed during the extension of the peptide stem with one ATP molecule per added amino acid. The PG layer is thus an energy and amino acids sink that should be further characterised, especially for its application in in silico metabolic modelling analysis. In the absence of specific information, the biomass composition is usually derived from the closest well-known organism, such as *E. coli* or *B. subtilis*. Improving the specificity and stoichiometry of the biomass reaction within models would certainly reinforce the accuracy of the predicted fluxes [6,7]. However, to keep up with the rapid pace of in silico approaches, the methods for biomass characterisation must be constantly improved.

The analysis of PG is performed in two principal ways: a qualitative description of PG in a selected strain of bacteria [8–11] or a quantitative measurement of selected biomarkers, e.g., muramic acid (Mur) [12,13] or meso-diaminopimelic acid (mDAP) [14]. The typical approach to obtain quantitative results from measurements of selected biomarkers from PG is to perform acidic hydrolysis of biomass to release those biomarkers in their free unbound form, making them detectable via various analytical techniques [15]. Liquid chromatography coupled with mass spectrometry is the most well-known technique for unambiguously determining peptidoglycan layer components or their structure. Depending on the type of the instrument, quantitative and qualitative data could be obtained through various sample preparation methodologies reported in the literature [13,16]. Due to the nature of NAM and NAG, they are usually derivatised to improve their volatility for gas chromatographs with mass spectrom·etery analysis [17] or reduced via strong bases to eliminate anomeric centres [8]. However, the high complexity of sample preparation methods raises the risk of introducing potential contaminants and increasing the sample turnover time accordingly. Therefore, the present work aims to introduce a simple, rapid, and reliable methodology for quantitative measurements of N-acetylmuramic acid, N-acetylglucosamine, and products of their acidic hydrolysis via hydrophilic interaction liquid chromatography coupled with mass spectrometry.

2. Materials and Methods

2.1. Chemicals and Materials

The standards for the components of the peptidoglycan layer, including N-acetylmuramic acid (NAM), N-acetylglucosamine (NAG), muramic acid (Mur), and glucosamine hydrochloride (GlcN), were obtained from Sigma-Aldrich (Darmstadt, Germany). Glucosamine-$^{13}C_6$ hydrochloride (GlcN-C13, UL-$^{13}C_6$, 99% ^{13}C enrichment) was procured from Omicron Biochemicals, Inc. (South Bend, IN, USA). Ammonium acetate (AmAc, HiPerSolv CHROMANORM® for LC-MS), ammonium formate (AmFor, HiPerSolv CHROMANORM® for LC-MS), and acetic acid (AA, HiPerSolv CHROMANORM® for LC-MS) were purchased from VWR International GmbH (Wien, Austria). Acetonitrile (MeCN; LiChrosolv, HPLC gradient grade), isopropanol (IPA; LiChrosolv, HPLC gradient grade), formic acid (FA, LC-MS grade), diethylamine (DEA, ≤99% purity), phenol (ACS reagent, 99.0–100.5% purity), hydrochloric acid (HCl, 36.5–38% purity), guanidine chloride (GuHCl, ≤99% purity), and ammonium hydroxide (25%, LC-MS LiChropur™ grade) were procured from Honeywell (Charlotte, NC, USA). Ultrapure water (≤18.2 MΩ·cm) was produced in-house with MilliQ® HX7040SD equipped with MilliQ LC-PAK (Merck KGaA, Darmstadt, Germany). Biotage Isolute® PLD+ columns were bought from Biotage Sweden AB (Uppsala, Sweden).

2.2. Preparation of Standard Solutions

The stock solutions of individual peptidoglycan layers' components were prepared firstly in ultrapure water and stored at −20 °C. The solution of the isotopically labelled standard was prepared in ultrapure water and stored at −50 °C. The working solutions of both non-labelled and labelled standards were prepared via dilution in 100% MeCN and then serially diluted with 50% aqueous MeCN (*v*/*v*). Calibration curves were built for N-acetylmuramic acid (0.66–42.50 mg/L), N-acetylglucosamine (1.58–50.50 mg/L), muramic acid (0.63–40.85 mg/L), and glucosamine (0.80–52.25 mg/L). The internal standard was added to the autosampler vial before the injection, and its concentration was fixed at 6.81 mg/L. The calibration curves were built using the response factor calculated according to Equation (1).

$$\text{Response Factor} = \text{Area of analytes} \times (\text{Concentration of internal standard}/\text{Area of internal standard}) \quad (1)$$

Calibration curves were built using seven-point measurements of serially diluted standard solutions. The regression was found by fitting points to a quadratic equation.

2.3. Liquid Chromatography

The standards and samples were analysed using a Waters Acquity H-Class Plus Bio UPLC® system (Waters Corporation, Milford, MA, USA) coupled with a Waters Acquity QDa detector (Waters Corporation, Milford, MA, USA) controlled by Waters Empower 3 (Build 3471 FR5 SR4, Waters Corporation, Milford, MA, USA). The optimal mobile phases (MPs) were (MPA) 20 mM ammonium acetate in ultrapure water with adjusted pH = 4.75 ± 0.02 and (MPB) 90% MeCN + 10% 20 mM ammonium acetate in ultrapure water with adjusted pH = 4.75 ± 0.02. The wash solvent was 10% ultrapure water in MeCN (*v*/*v*), and the purge solvent composition was 10% MeCN in ultrapure water (*v*/*v*). The seal wash was 20% MeCN in ultrapure water (*v*/*v*). The standards and samples were stored in the autosampler compartment cooled to 8 °C. The injection volume was set to 3 µL. Several columns were tested: the Waters XBridge® BEH Amide XP (3.0 × 150 mm, 2.5 µm, Waters Corporation, Milford, MA, USA), Waters Atlantis Premier BEH C18 AX (2.1 × 100 mm, 1.7 µm, Waters Corporation, Milford, MA, USA), Phenomenex Luna Omega Sugar column (2.1 × 150 mm, 100 Å, 3 µm, Phenomenex Inc., Torrance, CA, USA), Waters Acquity UPLC® BEH Phenyl (2.1 × 100 mm, 1.7 µm, Waters Corporation, Milford, MA, USA), and Waters Atlantis Premier BEH Z-HILIC (2.1 × 150 mm, 1.7 µm, Waters Corporation, Milford, MA, USA). An ACQUITY UPLC Column in-line filter unit (Waters Corporation, Milford, MA, USA) with an installed 0.2 µm stainless steel filter was used in all experiments with all tested columns. The optimal column temperature was 60 °C for the duration of an analytical run. The optimised gradient was as follows: 0–1 min for 5% A, 1–3 min for linear gradient 5–55%, 3.01–5 min for linear gradient 55–60%, 5.01–7 min for a hold at 60% A, and 7.01–11 min for a hold at 5% A. The optimal flow rate for those experiments was found to be 500 µL/min.

2.4. Mass Spectrometry

The analytes were ionised under electrospray ionisation conditions with optimised source conditions. The source temperature was set at 120 °C, and high-purity nitrogen was fed into the source at 1200 L/h (desolvation) and heated to 600 °C. The optimisation was conducted by injecting a high concentration standard with varying parameters, such as the capillary voltage, cone voltage, and gas temperature, with the optimised gradient parameters described earlier. The capillary voltage was set at −0.8 kV, and the optimal cone voltage for all compounds was found at −5 V. Firstly, the measurements were conducted in the scan mode to find the appropriate adducts for each compound of interest. During these experiments, appropriate single-ion recording channels were selected for optimal detection and analyte identification. The chosen adducts and selected ion recording (SIR) channels corresponding to m/z can be found in Table 1:

Table 1. The adducts and *m*/*z* values for analytes of interest.

No.	Analyte	Adduct	*m*/*z*
1	N-acetylmuramic acid	$[M-H]^-$	292.1
2	N-acetylglucosamine	$[M+Cl]^-$	256.1
3	Muramic acid	$[M-H]^-$	250.1
4	Glucosamine	$[M+Cl]^-$	214.1
5	Glucosamine-$^{13}C_6$	$[M+Cl]^-$	220.1

Data were acquired and analysed in Waters Empower 3 (Build 3471 FR5 SR4, Waters Corporation, Milford, MA, USA). Additional data analysis was performed in Microsoft Excel (Microsoft 365 Apps for enterprise, Microsoft Corp., Redmond, WA, USA) and GraphPad Prism (v 9.0.0, GraphPad Software, LLC, Boston, MA, USA).

2.5. Bacterial Growth Description

All strains were cultivated at 37 °C, but the gas environment and media were modified according to species. *Escherichia coli* K12 MG1655 (ECO) was cultured aerobically on the shaker, set at 110 rpm (Ecotron, Infors HT, Switzerland). For ECO, modified LB-Luria broth was used, which contained (per litre) 10 g of casitone (Tryptone Plus, Fluka Analytical), 5 g of yeast extract (NuCell 545), 0.5 g of NaCl, 2.93 g of K_2HPO_4, and 4.65 g of KH_2PO_4.

Streptococcus salivarius ssp. *thermophilus* DSM 20259 (STH) was cultivated aerobically in the incubator in M-17 broth (Sigma-Aldrich). *Bacteroides thetaiotaomicron* DSM 2079 (BTH) was cultured in the thermostat inside the anaerobic chamber (COY box, Coy Laboratory Products Inc., Grass Lake, MI, USA) with an atmosphere of 2.5 ± 0.5% H_2, 10% CO_2, and balanced N_2. The medium composition was as follows (per litre): 5 g of D-glucose, 2.5 g of casitone (Tryptone Plus, Fluka Analytical), 2.5 g of yeast extract (NuCell 545), 1 g of L-cysteine HCl, 2.93 g of K_2HPO_4, 4.65 g of KH_2PO_4, 0.9 g of $(NH_4)_2SO_4$, 0.9 g of NaCl, 0.28 g of KOH, 1 g of $NaHCO_3$, 10 mg of hemin, 10 μg of biotin, 10 μg of cobalamin, 30 μg of para-aminobenzoic acid, 150 μg of pyridoxamine, 50 μg of folic acid, 0.09 g of $MgSO_4 \cdot 7\ H_2O$, and 0.09 g of $CaCl_2 \cdot 2\ H_2O$.

2.6. Sample Preparation

After growth, all cultures were washed with a saline solution to eliminate media components interfering with the analytical measurements and concentrated 20 times to reach higher biomass densities. After concentrating, cultures were washed again with ultrapure water for further purification. A washed bacterial culture was then aliquoted into smaller portions and frozen at −50 °C until analysis. Analysed aliquots were thawed at room temperature. The thawed bacterial culture was transferred to a 10 mL volumetric flask and filled with ultrapure water up to the mark. The dissolved bacterial culture was then freeze-dried in a 2 mL Eppendorf vial. The dried bacterial culture was then subjected to acidic hydrolysis. The hydrolysis was performed with a mixture containing 6N hydrochloric acid (HCl) with 1% phenol (*v*/*v*) in an Eppendorf Thermomixer® C thermomixer (Eppendorf AG, Hamburg, Germany) with a temperature set at 100 °C and stirring at 1000 rpm. The hydrolysis was carried out for 4 h. As samples contained a large quantity of HCl and phenol, the mixture of HCl and phenol was removed with vacuum centrifugal evaporation. Dried hydrolysed bacterial cultures were resuspended in 50% aqueous MeCN (*v*/*v*) before the clean-up with Biotage PLD+ cartridges. Samples were loaded on Biotage PLD+ cartridges and eluted in vacuo to ensure clean extraction. The extracts were diluted accordingly with 50% aqueous MeCN (*v*/*v*), and, as the last step, the isotopically labelled internal standard was added to the autosampler vial.

2.7. Method Validation

The developed method was assessed for linearity (as a correlation coefficient of R^2 of the calibration curve), the limit of detection and quantification (as the standard deviation

of the measured sample at the lowest calibration points multiplied by 3 or 10, respectively), and the recovery of analytes during the sample preparation steps [18].

3. Results

3.1. Optimisation of Chromatographic and Mass Spectrometric Parameters

Our first experiments were performed by injecting only NAM and NAG as primary components of any PG layer in Gram-positive and Gram-negative bacteria. Five different columns were selected and tested based on the nature of the analytes of interest. The chromatogram obtained for each of the five columns is reported in Figure 1. The performances of the columns were evaluated based on the capability to separate the different PG monomers: (1) NAG, (2) NAM, (3) Mur, and (4) GlcN.

During the development of a liquid chromatographic method, it was observed that most of the tested columns could not either retain the compounds of interest or present peak splitting for analytes of interest due to the formation of anomers. Waters Atlantis Premier BEH Z-HILIC was the most efficient out of five columns in terms of the separation power and retention of analytes on the column. A high column temperature and fast flow of mobile phases were applied to improve peak shapes and reduce anomers' formation during the analytical run. The chromatograms obtained with the final optimised method are provided in Figure 2.

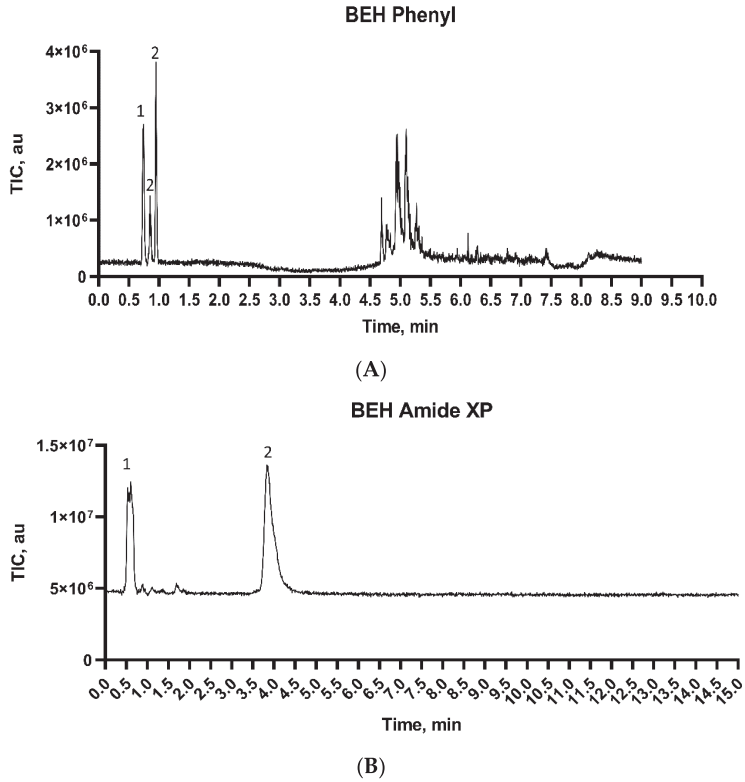
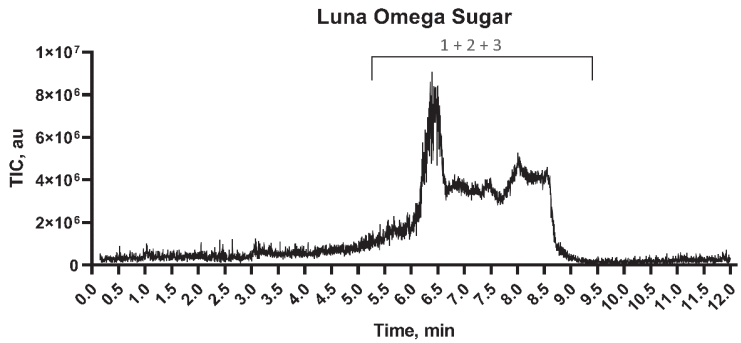
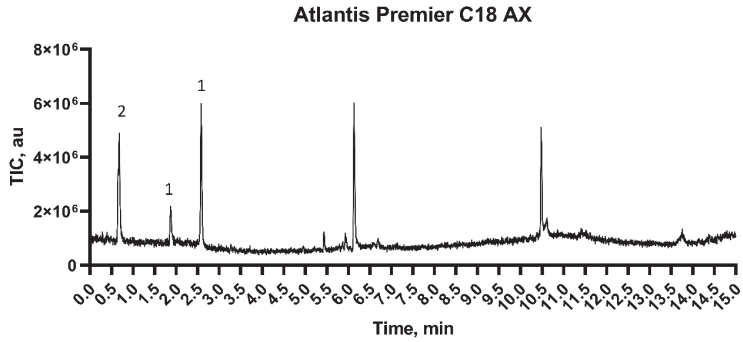


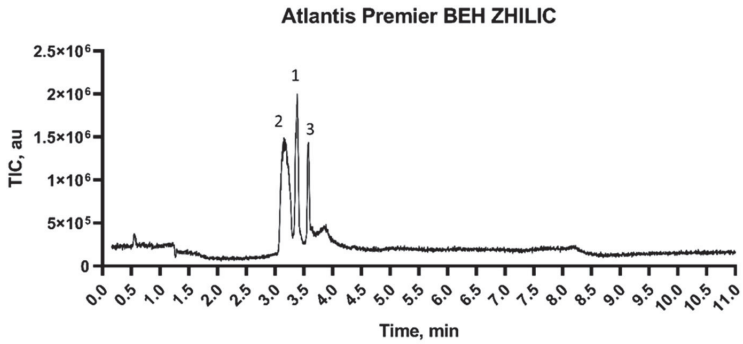
Figure 1. *Cont.*



(**C**)



(**D**)



(**E**)

Figure 1. (**A**) Waters Acquity BEH Phenyl column (2.1 × 100 mm, 1.7 µm). Mobile phases were (MPA) 0.1% FA in ultrapure water and (MPB) MeOH. Gradient elution was used, and the flow rate was 300 µL/min. (**B**) Waters XBridge BEH Amide XP (3.0 × 150 mm, 2.5 µm). Mobile phases were (MPA) 80/20/0.05 MeCN/ultrapure water/DEA + 0.5 mg/L GuHCl and (MPB) (**A**) 90/5/5/0.05 MeCN/ultrapure water/isopropanol/DEA + 0.5 mg/L GuHCl. The gradient elution program was used, and the flow rate was 800 µL/min. The methodology was adapted from reference [19]. (**C**) Phenomenex Luna Omega Sugar (2.1 × 150 mm, 3 µm). The mobile phases were (MPA) 100 ultrapure water + 0.5 mg/L GuHCl and (MPB) 99/1 MeCN/ultrapure water + 0.5 mg/L GuHCl. The gradient elution program was used, and the flow rate was 313 µL/min. The methodology was adapted from reference [20]. (**D**) Waters Atlantis Premier BEH C18 AX (2.1 × 100 mm, 1.7 µm). The

mobile phases were (MPA) 10 mM of AmFor in ultrapure water with pH = 3.75 and (MPB) 90/10 MeCN/10 mM AmFor in ultrapure water with pH = 3.75. The gradient elution program was used, and the flow was set to 300 µL/min. (**E**) Waters Atlantis Premier BEH Z-HILIC (2.1 × 150 mm, 1.7 µm). The mobile phases were (MPA) 20 mM of AmAc in ultrapure water with pH = 4.75 and (MPB) 90/10 MeCN/20 mM AmAc in ultrapure water with pH = 4.75. The gradient elution program was used, and the flow rate was set to 500 µL/min.

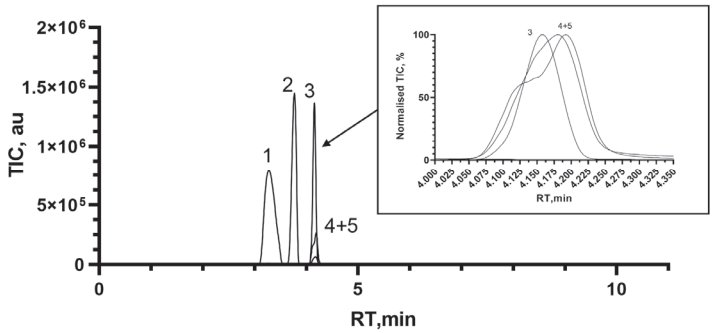


Figure 2. The optimised separation of NAG (1), NAM (2), Mur (3), GlcN (4), and GlcN-$^{13}C_6$ (5) obtained on Waters Atlantis Premier BEH Z-HILIC column in SIR experiments. The zoomed part is shown in normalised TIC levels.

Due to the presence of a trace amount of chloride ions in the mobile phases, a chloride adduct formation for NAG and GlcN was observed, which allowed for more precise and cleaner spectra for those analytes [21]. However, NAM and Mur's adduct formation had not been driven by the chloride ions in the mobile phases and was measured as deprotonated adducts. The reproducibility of adduct formation was closely monitored, and the repeatability of recorded response factors was very high. The gas flow and capillary voltages were set according to the manufacturer's recommendations for operation at the high flow of mobile phases.

3.2. Methodology Validation

The optimised LC-MS methodology was validated, and the results are presented in Table 2.

Table 2. Regression equations, correlation coefficients (R^2), limits of detection (LoD), and quantification (LoQ) for analytes of interest.

Analyte	Regression Equation	R^2	LoD, mg/L	LoQ, mg/L
N-acetylmuramic acid	$Y = -2.45 \times 10^{-2}\,X^2 + 4.83 \times 10^{0}\,X - 3.10 \times 10^{0}$	0.9947	0.116	0.386
N-acetylglucosamine	$Y = -5.83 \times 10^{-2}X^2 + 6.77 \times 10^{0}\,X + 3.46 \times 10^{0}$	0.9973	0.345	1.149
Muramic acid	$Y = 1.78 \times 10^{-2}\,X^2 + 1.76 \times 10^{0}\,X - 1.16 \times 10^{0}$	0.9933	0.048	0.159
Glucosamine	$Y = -7.96 \times 10^{-3}\,X^2 + 9.18 \times 10^{-1}\,X - 1.33 \times 10^{-1}$	0.9999	0.015	0.050

Additionally, the methodology was controlled for the repeatability of retention times (RTs) and peak areas in standards over several independent experiments by injecting a standard mixture (Table 3).

Table 3. The inter- and intraday repeatability of RT and peak area expressed as relative standard deviation (RSD), %.

Analyte	RT		Peak Area	
	Interday RSD, % (n = 3)	Intraday RSD, % (n = 4)	Interday RSD, % (n = 3)	Intraday RSD, % (n = 4)
N-acetylmuramic acid	0.11	0.15	1.82	3.63
N-acetylglucosamine	0.12	0.20	3.19	5.00
Muramic acid	0.09	0.14	1.54	2.62
Glucosamine	0.08	0.12	0.20	1.03
Glucosamine-$^{13}C_6$ [a]	0.08	0.13	8.68	9.6

[a] Internal standard for quantification.

3.3. Optimisation of Sample Preparation Procedures and Measurements of Bacterial Biomass

Based on the literature on breaking down the bacterial wall with enzymes [22], the first experiments were conducted with a STH bacterial culture, which should possess a thick PG layer. The wet biomass (WBM) was aliquoted in several Eppendorf tubes and subjected to hydrolysis via a lysozyme solution according to a protocol published by Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) [23]. The first results provided by this approach showed that the PG layer could not be hydrolysed into individual components, and, overall, no PG components were detected (Figure A1).

The current method was further optimised by improving the cell wall hydrolysis based on procedures described and adapted in the literature [24–26]. Reported methods tend to require very harsh and complex conditions, such as an oxygen-free environment, a high load of HCl acid to sample amount, and significant processing times due to the manual labour involved in preparing fully enclosed glass vessels containing both sample and hydrolysis reagents. The scaled-down methodology was tested to minimise processing times and decrease sample turnaround times. The amount of dried sample was reduced to *ca* 1–2 mg of dried biomass (DBM) and mixed with 300 µL of 6 M HCl containing 1% phenol (*v*/*v*). Furthermore, the samples were agitated for 4 h at 1000 rpm to improve the hydrolysis, and the temperature was reduced to 100 °C from the commonly employed 110–115 °C.

Additionally, the maintenance of the complete integrity of the standards after the acidic hydrolysis was used for WBM was evaluated. The hydrolysis was performed using an aliquot of a standard stock solution (*ca* 1 mg/mL). HCl with 1% phenol was evaporated in vacuo, and the residue was redissolved in 50% aqueous MeCN (*v*/*v*). This mixture was passed through a Biotage PLD+ cartridge to remove impurities from sample matrices. The filtrate was diluted with an internal isotopically labelled standard and injected on LC-MS as described in Section 2.3 of the Section 2. The recovery values for analytes were calculated and are presented in Table 4.

Table 4. The recovery values for pure standards after acidic hydrolysis under conditions used for WBM.

Analyte	Recovery (%, n = 3)	SD (%, n = 3)
N-acetylmuramic acid	0	0
N-acetylglucosamine	0	0
Muramic acid	99.1	3.8
Glucosamine	94.2	1.9

The hydrolysis reaction time was screened based on the Gram-staining of selected bacteria. In the case of Gram-negative bacteria, hydrolysis was conducted for four hours, sampling at 0.5, 1, 1.5, 2, and 4 h. For Gram-positive bacteria, the hydrolysis reaction time was prolonged to 16 h with sampling at 1.5, 2, 4, 12, and 16 h. All experiments were performed in triplicate, and an example chromatogram is shown in the Appendix A Section

(Figures A2–A5). The percentage of analyte mass concentration per 1 mg of dry cell weight (*w*/*w*) is reported in Tables 5 and 6.

Table 5. The results of acidic hydrolysis performed with DBM of ECO and BTH in triplicate (Gram-negative bacteria).

Sampling Time, h	%Glucosamine (ECO)	%Muramic Acid (ECO)	%Glucosamine (BTH)	%Muramic Acid (BTH)
0.5	0.50 ± 0.02	0.47 ± 0.07	3.22 ± 0.23	0.49 ± 0.06
1	0.50 ± 0.02	0.51 ± 0.04	3.16 ± 0.03	0.52 ± 0.04
1.5	0.50 ± 0.08	0.52 ± 0.05	3.16 ± 0.16	0.51 ± 0.05
2	0.60 ± 0.06	0.59 ± 0.08	3.09 ± 0.33	0.55 ± 0.05
4	0.82 ± 0.10	0.88 ± 0.05	3.05 ± 0.02	0.57 ± 0.03

Table 6. The results of acidic hydrolysis performed with DBM of STH in triplicate (Gram-positive bacteria).

Sampling Time, h	%Glucosamine	%Muramic Acid
1.5	4.63 ± 0.12	3.47 ± 0.07
2	4.88 ± 0.03	3.51 ± 0.04
4	4.72 ± 0.31	3.52 ± 0.17
12	4.73 ± 0.27	3.53 ± 0.28
16	4.49 ± 0.42	3.38 ± 0.25

For Gram-negative bacteria, the release of PG components has increased with prolonged hydrolysis times, reaching its maximum concentrations by the 4th hour. However, for Gram-positive bacteria, longer hydrolysis times resulted in a slight decrease in quantified PG components with more significant experimental errors between replicates. Under optimised hydrolysis conditions, the recovery of PG's hydrolysis products was 94 to 99% (Table 4), whereby GlcN showed a slight sign of degradation under used conditions. For Gram-positive bacteria, the hydrolysis time might be reduced to 2 h while maintaining high yields of PG components with low experimental errors. This behaviour could be attributed to structural differences between Gram-positive and Gram-negative bacteria in terms of an outer layer of the cell wall. In Gram-negative bacteria, the outer layers are the lipopolysaccharide layer and outer membrane, which might reduce the hydrolysis reaction rate.

4. Discussion

Acidic hydrolysis is a very viable and proven methodological approach to breaking down the rigid structures of the bacterial cell independent of the thickness of the cell wall.

The results showed that, for widely studied microorganisms such as *Escherichia coli* K12 (ECO), the PG layer components' concentration was in the range of 1.7% of the sum of amino sugars components per 1 mg of dry cell weight (Table 5). This value is in good agreement with previously published results on *Escherichia coli* B/r [27], which shows methodology robustness towards Gram-negative bacteria. Additionally, the PG concentration in BTH was at levels exceeding 3.6% (Table 5). According to a previously published study, the concentration of PG in BTH was calculated within the range of 1.1% to 1.3% [28]. As the PG layer comprises stoichiometrically equal amounts of both NAG and NAM, it was hypothesised that BTH could hold extra amounts of UDP-NAG as an intermediate of the cell wall synthesis. Additionally, it has been reported that, in *Escherichia coli* K-12 strain NCM3722, UDP-NAG is stored in the cytosol for synthesising the cell wall in case of changes in the growth environment, which could also be the case with BTH [29]. If PG components for BTH are calculated based on the stoichiometry principle, the sum of PG components for BTH would be in a proposed range of 1.14% of PG components per 1 mg of dry cell weight, which is in good agreement with calculated values of PG concentration

from the literature. As for Gram-positive bacteria, STH showed a significantly higher concentration of PG components than Gram-negative ones (Table 6). There was no reported value for PG % in cell walls of STH, but for other Gram-positive bacteria, such as from the *Lactobacillales* order, the values for hexosamines, e.g., NAG and NAM, are in the range of 5.2–12.2% [30]. This range indicates that STH's recorded sum of amino sugars of 8.2% is in good agreement with other similar Gram-positive bacteria, which possess a thick peptidoglycan layer. The greater concentration of NAG in STH compared to NAM could be attributed to the presence of UDP-NAG in the cytosol, as discussed previously.

5. Conclusions

With the developed HILIC-MS methodology described in this study, four different PG components could be accurately and repeatably quantified from the cell walls of both Gram-positive and Gram-negative bacteria. Although requiring sophisticated equipment, the HILIC-MS methodology allows an accurate quantification from a small amount of biomass and small working volume. The turnaround time could be significantly reduced from hours to minutes by employing different heat sources, such as microwave-based irradiation, at the hydrolysis step. The HILIC-MS methodology can also be adapted for 96-well plates for a higher throughput. In addition, the composition in PG of more prokaryotes should be determined in further studies. Nonetheless, the results obtained combined with metabolome and proteome data constitute a valuable input for the MM.

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Appendix A

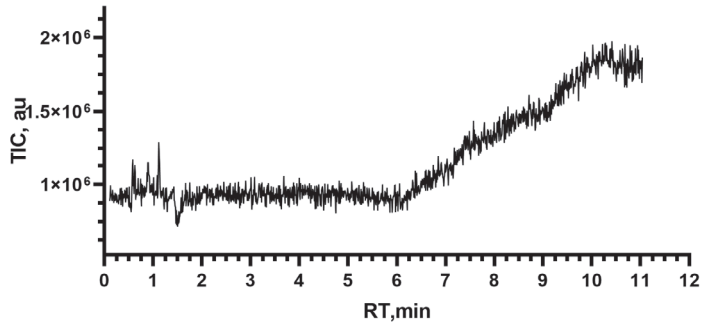

Figure A1. The LC-MS chromatogram obtained after lysozyme application on STH WBM.

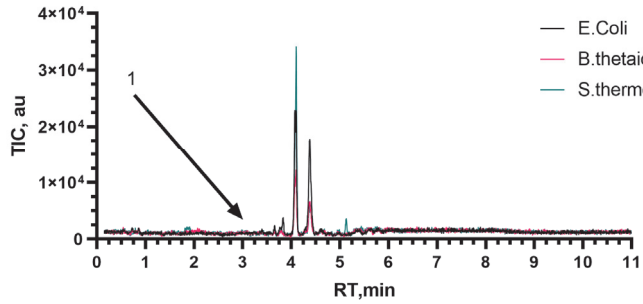

Figure A2. The LC-MS chromatogram of NAG (1) obtained during 4 h of acidic hydrolysis.

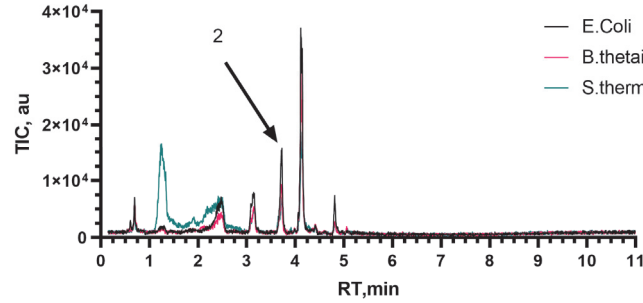

Figure A3. The LC-MS chromatogram of NAM (2) obtained during 4 h of acidic hydrolysis.

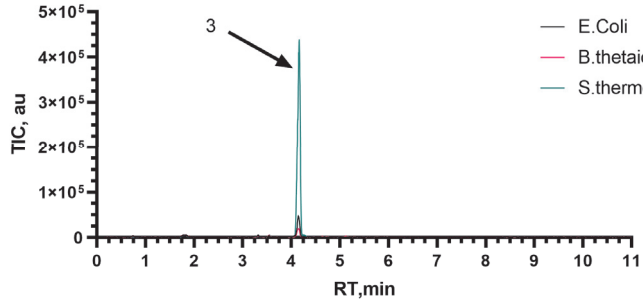

Figure A4. The LC-MS chromatogram of GlcN (3) obtained during 4 h of acidic hydrolysis.

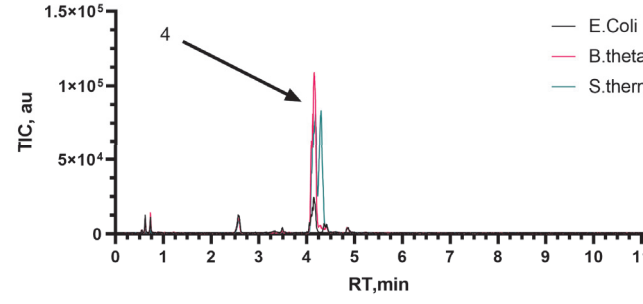

Figure A5. The LC-MS chromatogram of Mur (4) obtained during 4 h of acidic hydrolysis.

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