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Mass Spectrometric Analysis of Bioactive Metabolites from Lactobacilli

by

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This work is dedicated to my parents, Monica and Milton Black, for their endless support, guidance and love.

Abstract

Lactobacilli are commonly used in food fermentations. Preservation and changes in food quality due to fermentation arise because of the growth, metabolism and enzymatic activity of these organisms. Enzymatic pathways of lactobacilli can also be exploited for the production of bioactive compounds. In this work, *Lactobacillus* spp. were used to enzymatically produce hydroxy fatty acids with anti-fungal activities and oligosaccharides with anti-adhesion properties. These bioactive compounds were found to be present as mixtures of geometric and positional isomers. In order to characterize individual isomers with minimal preparatory steps, liquid chromatography/tandem mass spectrometry (LC-MS/MS) methods were developed.

Lactobacillus hammesii and *Lactobacillus plantarum* converted linoleic acid into a racemic mixture of anti-fungal 10-hydroxy-*cis*-12-octadecenoic and 10-hydroxy-*trans*-12-octadecenoic acid by means of hydratase enzymes. When produced in sourdough bread 10-hydroxy-12-octadecenoic acid and anti-fungal 13-hydroxy-*cis*-9,*trans*-11-octadecadienoic acid, the latter which is enzymatically formed by flour lipoxygenase in the presence of reducing agents, increased the mould-free storage-life of the bread. Results from LC-MS/MS methods allowed for conversion pathway elucidation of linoleic acid to conjugated linoleic acid by lactobacilli.

Lactobacillus spp. containing galactosidase enzymes were used to form composite oligosaccharides with anti-adhesive properties. Using LC-MS/MS analysis, several novel oligosaccharides formed by β- and α-galactosidase were

identified. In particular, Galβ-(1→4)-GlcNAc was formed, which is the core structure in human milk oligosaccharides and acts as a competative inhibitor to enteropathogenic *Escherichia coli*.

The LC-MS/MS methods developed in this work proved useful in investigating structure-function relationships of anti-fungal lipids and anti-adhesive oliogsaccharides. This research can be further applied to increase the variety of bioactive compounds identified for food protection and health promotion.

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6-PG	6-phosphogluconate
α/β-Gal	Alpha/beta-galactosidase
APPI	Atmospheric pressure photo ionization
CCE	Crude cell extract
CI	Chemical ionization
CID	Collison induced dissociation
CLA	Conjugated linoleic acid
CFU	Colony forming units
COS	Chito-oligosaccharide
DMOX	4, 4-Dimethyloxazoline
EI	Electron impact ionization
ELSD	Evaporative light scattering detector
EMP	Embden-Meyerhof-Parnas
ESI	Electrospray ionization
FAME	Fatty acid methyl ester
GC	Gas chromatography
GOS	Galacto-oligosaccharide
HeOS	Hetero-oligosaccharide
HMOS	Human milk oligosaccharide

HPAEC-PAD	High performance anion exchange chromatography with pulsed amperometric detection
HPLC	High performance liquid chromatography
HSCCC	High-speed counter-current chromatography
IR	Infrared spectroscopy
LAB	Lactic acid bacteria
LC	Liquid chromatography
MIC	Minimum inhibitory concentration
mMRS	modified deMan-Rogosa-Sharpe
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
m/z	Mass to charge ratio
NMR	Nuclear magnetic resonance
qTOF	Quadrupole time-of-flight
UV	Ultraviolet spectroscopy
XIC	Extracted ion chromatogram

CHAPTER 1

1. Introduction

1.1. The genus *Lactobacillus*

The first pure culture of *Lactobacillus* species was isolated in 1901 by using an enrichment culture technique developed by Martinus Beijerinck (Beijerinck, 1907; Overmann, 2006). A little over a century later, the genus *Lactobacillus*, one of the largest of the order *Lactobacillales*, comprises over 150 species (Salvetti *et al*., 2012), with approximately six species identified annually (Hammes & Hertel, 2009). Generally, *Lactobacillus* are Gram-positive, rod shaped, non-spore forming, aero-tolerant or anaerobic, with a G+C DNA content range of 32–55% mol %. Species within the genus *Lactobacillus* exhibit a variety of phenotypical, biochemical and physiological traits. These traits lead to species specific nutritional requirements and metabolism; however, all lactobacilli produce lactic acid as the major end product of carbohydrate metabolism (Axelsson, 2004). It is from the production of lactic acid that the genus *Lactobacillus* is considered to be lactic acid bacteria (LAB), which is a group that consists of other taxonomically related genera (Axelsson, 2004). Orla-Jensen (1919) first classified LAB according to their physiological and morphological characters. LAB are divided according to the enzymes employed for metabolism: obligately homofermentative, facultatively heterofermentative or obligately heterofermentative (Hammes and Vogel, 1995). Homofermentative species metabolize hexoses using the Embden-Meyerhof-Parnas (EMP) pathway almost exclusively to lactic acid. These lactobacilli employ fructose-1,6-biphoshate-

aldolase to ferment hexoses, but lack phosphoketolase to ferment either pentoses or gluconate. Facultative heterofermentative lactobacilli exhibit both aldolase and phosphoketolase enzymes. Hexoses and pentoses are utilized by the EMP and 6-phosphogluconate (6-PG) pathway, respectively. In the presence of hexose, enzymes of the 6-PG pathway are repressed and saccharides are metabolized through the EMP pathway. Obligate heterofermentative lactobacilli metabolize pentoses and hexoses exclusively through the 6-PG pathway. Lactic acid, carbon dioxide, ethanol and acetic acid are end products of the 6-PG pathway.

Morphological, physiological and biochemical characteristics are not reliable for taxonomic identification of lactobacilli (Hammes & Hertel, 2009). *Lactobacillus* species are more accurately classified by sequencing 16S rRNA genes. Little association remains between traditional metabolic-based classification and phylogenic relatedness. As a result, sequencing techniques have allowed for a multitude of new *Lactobacillus* species to be identified in the last two decades (Hammes & Hertel, 2009). By analyzing 16S rRNA genes of bacteria, phylogenic relationships have been proposed to associate *Lactobacillus* into seven main groups: *L. buchneri* group, *L. casei* group, *L. delbrueckii* group, *L. plantarum* group, *L. reuteri* group, *L. sakei* group and *L. salivarius* group (Hammes & Hertel, 2006). However, the relationships between the groups are ambiguous. As an example, the genus *Pediococcus* is closely related to the *Lactobacillus* genus, and species were often misclassified as *Pediococcus*-like before being properly reclassified to *Lactobacillus* species (Leisner *et al*., 2000;

Haakensen *et al*., 2011). Due to their interrelatedness, *Pediococcus* species are intermixed with the *Lactobacillus* group (Hammes & Hertel, 2006). As new species of *Lactobacillus* and *Pediococcus* are identified, the phylogenic groupings do not become clearer; instead, additional groupings are proposed. Salvetti *et al.* (2012) recently reviewed the phylogenic analyses based on the 16S rRNA gene sequence for the *Lactobacillus* genus and divided the genus into 15 main groups instead of the previous seven. Within these groups, species are often heterologous in terms of their phenotypic properties, confirming that genotypic data, specifically 16S rRNA based sequencing, is more reliable for current taxonomic classification.

The adaptation of lactobacilli to specific ecological niches and resulting biosynthetic pathways is reflected by the size of the genome. Reconstruction of genes from the common ancestor of *Lactobacillales*, indicated that lactobacilli reduced the genome size during their evolution (Makarova *et al*., 2006). Thus it was postulated that during the transition to more nutrient-rich media, lactobacilli lost genes for biosynthesis of nutrients. It is interesting to observe that species of *Lactobacillus* also vary in their genome size, which appears to relate to how narrow or broad their respective biosynthetic capabilities are. *L. sanfranciscensis* TMW 1.1304 has a small genome size of 1.29 Mbp; however, it is highly adapted to sourdough fermentations and rapidly propagates to outcompete other microorganisms (Vogel *et al*., 2011). In comparison, to *L. plantarum* WCFS1, has a larger genome size of 3.31 Mbp. *L. plantarum* is encountered in various

ecological niches and has the capability to adapt to environments as represented by a relatively large amount of regulatory and transport genes (Kleerebezem *et al*., 2003). In terms of food fermentations, characterization of the bacterial genome is important to determine genes encoding enzymes that are important in flavor and texture forming pathways, in order to predict or control the overall quality of the food product.

1.2. Lactobacilli in foods

For more than 10,000 years, lactobacilli have been employed in food fermentations and thus have been part of the human diet. Second to drying, food fermentations are the world's oldest methods of preservation (Prajapati & Nair, 2003). Rapid anaerobic growth by *Lactobacillus* species together with the production of acids, antibacterial, and antifungal compounds inhibit the growth of other organisms in food fermentations (Molin, 2003). Because of their history of safe use, many *Lactobacillus* species were awarded GRAS status (generally recognized as safe) by the U.S. Food and Drug Administration (Bourdichon *et al*., 2012). According to Health Canada (2013), in order to demonstrate a history of safe use, evidence must be supplied that the organism does not infer harm, over several generations and a variety of genetically different human populations. The organism must also not harbor antimicrobial resistance and if genetically modified, any differences between novel and conventional strains must be assessed. Currently, lactobacilli are widely exploited for their health benefits as

probiotics and also serve as vaccine carriers (Barrangou *et al*., 2011; Mohamadzadeh *et al*., 2009; Kajikawa *et al*., 2012).

Food preservation by fermentation with lactobacilli both alters the organoleptic properties of the food and improves its nutritional quality (Poutanen *et al*., 2009; Steele *et al*., 2012; Costello *et al*., 2013). The adaptation of lactobacilli to utilize enzymes that regulate carbohydrate, peptide/amino acid and lipid metabolism has led them to achieve a dominant role in food fermentations. Lactobacilli are utilized to perform the main conversions in dairy, meat, vegetable products, sourdough, wine and coffee fermentations (Hammes & Hertel, 2006).

1.2.1. Carbohydrates

The ability of lactobacilli to metabolize carbohydrates during fermentations, resulting in the acidification of foods, is their most characteristic feature. By understanding the enzymatic pathways of bacteria and which substrates are available in the food matrix, the quality of fermented end products can be better predicted. Lactobacilli contain enzymes that metabolize carbohydrates by the 6-PG pathway, the EMP pathway or both. Heterofermentative *Lactobacillus* species utilize hexoses and pentoses from foods via the 6-PG pathway. Disaccharides such as maltose, sucrose and lactose are transported directly into the cell by specific permeases, transporters or the phosphoenol pyruvate phosphotransferase system (PEP-PTS) (Kandler, 1983; Gänzle *et al*., 2007). Once within the cell, glycosyl hydrolases or phosphorylases act to lyse disaccharides (Gänzle *et al*., 2012). Generally, hexose metabolism by the 6-PG pathway yields lactate, ethanol

and carbon dioxide as the major products (Axelsson, 2004). In the presence of electron acceptors, acetyl-phosphate from hexose metabolism is further converted to acetate. Examples of electron acceptors in foods are fructose, oxidized glutathione and short chain aldehydes (Stolz *et al*., 1996; Vermeulen *et al*., 2006b; Vermeulen *et al*., 2007a). Homofermentative *Lactobacillus* strains undergo the EMP pathway for carbohydrate metabolism in which lactic acid is formed; additionally, by this pathway the conversion of flavor forming aldehydes is reduced (Vermeulen *et al*., 2007a).

The metabolism of organic acids is linked to carbohydrate metabolism due to the production of pyruvate, an intermediate in hexose metabolism. Citrate, if available in the food matrix, is utilized by lactobacilli to produce acetate, diacetyl, acetoin, 2, 3-butanediol and carbon dioxide (Axelsson, 2004). Homofermentative formation of diacetyl imparts a "butter" flavor, and depending on the food type this can be a desired or an undesired trait (Masschelein, 1986). Citrate metabolism of lactobacilli has been well characterized in dairy, wine and sourdough fermentations (Hugenholz, 1993; Liu, 2002; Gänzle *et al*., 2007). Malate is another organic acid that can be utilized by lactobacilli to produce lactate and carbon dioxide; this reaction is considered a carbohydrate secondary fermentation (Axelsson, 2004). In wine and cider, beverages are de-acidified and this reaction is known as the malo-lactic fermentation (Wibowo *et al*., 1985; Jarvis *et al*., 1995).

1.2.2. Proteins

Similar to carbohydrate metabolism, peptide metabolism by lactobacilli produce flavor compounds and flavor precursors important in food fermentations. Lactobacilli require an exogenous nitrogen source to be present in the media for metabolism and growth (Hammes & Hertel, 2009). Extracellular proteinases are necessary for protein degradation, as only oligopeptide, peptide or amino acid forms are utilized (Savijoki *et al*., 2006). Flavor compounds produced by protein metabolism in foods are plentiful; however, flavor compounds may or may not be beneficial depending on the type of food. For example, in cheese, fermentation of branched chain amino acids by *Lactobacillus* species results in the formation of aldehydes, alcohols and fatty acids. These aldehydes include 3-methyl butanal and 2-methyl butanal, which are described as malty and fruity, are desired for cheese flavor but are a defect in fermented milk products (Kieronczyk, 2001; Marilley & Casey, 2004). For amino acid metabolism, the main enzymatic pathways of lactobacilli are transamination, decarboxylation and the lysing of sulfur-containing amino acids (McSweeney & Sousa, 2000; Gänzle *et al*., 2007; Hammes & Hertel, 2009). Specific examples of sulphur compounds converted by lactobacilli in dairy, wine and meats are: methanethiol, methional, dimethyl disulphide, and dimethyl trisulphide, and these compounds impart garlic, boiled potato-like and cooked cabbage flavors (Pripis-Nicolau *et al*. 2004; Martínez-Cuesta *et al*., 2013). Flavor compounds resulting in the transamination and decarboxylation of amino acids are 3-methyl butanol, 2-methyl butanol, 2-methyl

propanol, 2-methylbutyric and isobutyric acids which confer alcoholic, fruity, sweaty, rancid, fecal, and putrid flavors (Nakae & Elliott, 1965a; Nakae & Elliott, 1965b; Kieronczyk, 2001; Marilley & Casey, 2004).

In sourdough, glutamine is liberated from gluten and by glutaminase is converted to glutamate by *L. sanfranciscensis* producing bread with more savory flavor (Vermeulen *et al*., 2007b). Additionally, in sourdough, ornithine is derived from lactobacilli from the conversion of arginine by the arginine-deiminase pathway (De Angelis *et al*., 2002; Gänzle *et al*., 2007). Ornithine is a precursor to the compound 2-acetyl-1-pyrroline which is converted during baking and contributes to a roasty flavor in bread (Thiele *et al*., 2002).

1.2.3. Lipids

Lipid metabolism of *Lactobacillus* species also results in compounds that contribute to the flavor of foods; however, the pathway mechanisms for lipid utilization have been far less studied than that of carbohydrate and protein metabolism by lactobacilli. In foods, lipids occur as triglycerides, containing fatty acids at *sn*-1, -2, and -3 bound by esters to a glycerol backbone. A few *Lactobacillus* species such as *L. fermentum*, *L. plantarum* and *L. casei* produce lipases or esterases to cleave triglycerides releasing free fatty acids (Gobbetti *et al*., 1996; Gobbetti *et al*., 1997; Lee & Lee, 1990). Free fatty acids contribute to the flavor profile of the food at different thresholds depending on the chain length and degree of unsaturation and have been described as having a fatty, soapy and/or bitter flavor (Schieffman & Dackis, 1975). Hydroxy fatty acids are

produced by lipoxygenase activity, or by hydratase enzymes from specific lactobacilli reacting with unsaturated fatty acids (Sjögren *et al*., 2003; Kishimoto *et al*., 2003; Yang *et al*., 2013). Heating hydroxy fatty acids in the presence of water forms lactones (Eriksen, 1976), which are flavor compounds described as imparting coconut, peachy or green butter notes (Hatchwell, 1996). Lactobacilli do not utilize lipids for metabolism as they do carbohydrates; however, the presence of fatty acids is required for growth of the organism, as fatty acids are incorporated into the cellular membrane (Hammes & Hertel, 2009). Generally, oxidative reactions converting unsaturated fatty acids into aldehydes are responsible for undesirable lipid flavor formations in foods. If heterofermentative lactobacilli are present, these aldehydes may be transformed by alcohol dehydrogenase into alcohols with higher flavor thresholds (Vermeulen *et al*., 2007a). The reduction of aldehydes to alcohols is advantageous for heterofermentative lactobacilli, as it releases additional energy for growth; however, aldehydes are key aroma compounds of wine and sourdough bread (Grosch & Schieberle, 1997; Culleré *et al*., 2007).

1.3. Lactobacilli and biotransformation

Functional compounds are compounds that possess bioactive or healthful properties and are produced by lactobacilli in traditional food fermentations; however, until more recently, the demand for these products was low. Interest has accumulated due to the acceptance of these compounds as “natural”, considering

the substrates to be converted originate from foods and are not chemically synthesized (Canadian Food Inspection Agency, 2012). With governmental legislation limiting preservatives and consumer demand for foods that are high in quality, natural, safe, minimally processed and have a prolonged storage-life, innovative substitutes are required to achieve these necessities.

Currently, the most widely studied compounds produced by lactobacilli with bioactivity are bacteriocins. Bacteriocins are ribosomally synthesized proteins with antimicrobial activity. Since bacteriocins are degraded by the proteases of the mammalian gastrointestinal tract, they are considered safe for human consumption (Cleveland *et al*., 2001). Although bacteriocins will not be reviewed here, since they are not enzymatically produced, there are select reviews to which the reader is directed for more information (Drider *et al*., 2006; Vignolo *et al*., 2012).

Bioactive compounds produced in enzymatic conversions during lactobacilli fermentation, differ due to the types of substrates and species that are present. Genomic sequencing of *Lactobacillus* species has led to the prediction of metabolic pathways, and therefore, biochemical transformation (Mayo *et al*., 2008). However, the link between genome sequences of lactobacilli that code for specific enzymes and the conversion substrates to those with bioactive function must be first investigated. A non-exhaustive list of bioactive compounds produced through conversion of substrates by lactobacilli has been compiled in **Table 1-1**. These compounds are considered bioactive as they promote safety of food

products or the health of the consumer beyond basic nutrition. Bioactive compounds can either be formed directly in the food or added after purification and isolation steps to create a functional food (Hsieh & Ofori, 2010).

Within each example given within **Table 1-1**, precursor substrates converted by lactobacilli are not substantially active compared to the products. Products differ from substrates in terms of structure, and this change in structure allows for functionality of the products. Relationships between function and structure can be assessed by assaying for activity and structurally characterizing products formed by *Lactobacillus* species. Sometimes only slight differences occur between substrates and products with activity, thereby requiring sophisticated tools for their analyses.

Table 1-1. Overview of select bioactive compounds enzymatically converted by lactobacilli

Category	Compound name	Precursor	Bioactivity	Strain Example	Reference
Protein	VPP & IPP tripeptides	Casein or gluten	antihypertensive	*L. helveticus; L. reuteri*	Ueno *et al*., 2004; Hu *et al*., 2011
	β-Lactoglobulin-derived peptides	*β*-Lactoglobulin	immunomodulatory effects	*L. paracasei*	Prioult *et al*., 2004
	IKHQGLPQE, VLNENLLR & SDIPNPIGSENSEK	Casein	antibacterial	*L.acidophilus*	Hayes *et al*., 2006
	GABA (γ-aminobutyric acid)	Glutamate	antihypertensive and antitumorigenic	*L. brevis*	Higuchi *et al*., 1997; Zhang *et al*., 2012
	Phenyllactic acid	Phenylalanine	antibacterial; antifungal	*L. plantarum*	Lavermicocca *et al*., 2000
Carbohydrate	Inulin	Sucrose	prebiotic; texture	*L. johnsonii; L. reuteri; L.acidophilus*	Anwar *et al*., 2008; Tieking *et al*., 2005
	Exopolysaccharides	Glucose, galactose, and phosphate	cholesterol reduction, prebiotic, and immune modulation	*L. delbrueckii* subsp. *bulgaricus*	Uemura *et al*., 1998
		Lactose or glucose		*L. rhamnosus*	Van Calstern *et al*., 2002

Table 1-1. Continued: Overview of select bioactive compounds enzymatically converted by lactobacilli

Category	Compound name	Precursor	Bioactivity	Strain Example	Reference
Carbohydrate	Galacto-oligosaccharides	Lactose	prebiotic	*L. reuteri*	Splechtna *et a*l., 2006
	Hetero-oligosaccharides	Lactose and mannose, fucose or GlcNAc	prebiotic, competitive inhibitors to pathogenic bacteria	*L. fermentum; L. plantarum; L. delbrueckii* subsp. *bulgaricus*	Schwab *et al*., 2011
	Reuterin (3-hydroxypropionaldehyde**)**	Glycerol	antibacterial; antifungal	*L. reuteri*	Axelsson *et al*., 1989
Lipids	3-hydroxydecanoic acid, 3-hydroxy- *cis* -5-dodecenoic acid, 3-hydroxydodecanoic acid and 3-hydroxytetradecanoic acid	Unsaturated fatty acids	antifungal	*L. plantarum*	Sjögren *et al*., 2003
	Conjugated linoleic acid	Linoleic acid (*cis*-9-*cis*-12-octadecenoic acid)	affects immune response, insulin sensitivity, and body fat composition	*L. acidophilus*	Ogawa *et al*. 2001
				L. plantarum	Kishino *et al*., 2002
Phenolic	Aglycone	Isoflavone glucosides	antioxidation, antimutagenic, reduction of post-menopause symptoms	*L. acidophilus; L. paracasei*	Wei *et al*., 2007
				L. rhamnosus	Marazza *et al*., 2009
				L. fermentum; L. plantarum; L. rhamnosus	Di Cagno *et al*., 2010

Table 1-1. Continued: Overview of select bioactive compounds enzymatically converted by lactobacilli

Category	Compound name	Precursor	Bioactivity	Strain Example	Reference
Phenolic	Equol	Daidzein (4´,7-dihydroxyisoflavone)	antioxidant, antiandrogenic, lowers plasma total cholesterol, anti-inflammatory	*L. rhamnosus*	Tamura *et al*., 2011
				L. delbrueckii subs. *bulgaricus*	Li & Jing, 2010
				L. fermentum; L. plantarum; L. rhamnosus	Di Cagno *et al*., 2010
Other	Hydrogen peroxide	Glucose metabolism	antimicrobial, flavor	*L. crispatus* *L. jensenii*	Vallor *et al*., 2001
	Diacetyl			*L. casei*	Branen & Keenan, 1971; Lanciotti *et al*., 2003
				L. rhamnosus	Jyoti *et al*., 2003
	Organic acids (lactate & acetate)			All lactobacilli sp.	Axelsson, 2004

1.4. Analysis of bio-active metabolites from lactobacilli

Bioactive compounds produced by lactobacilli are often structurally similar to corresponding substrates or co-products that have little or no biological activity. In order to fully identify bioactive compounds or groups of compounds that possess activity, analytical techniques with high structural specificity are required. For example, a challenging structural identification might involve isomeric compounds, such as an isomeric amino acid sequence, carbohydrates differing only by linkage type, or the location of functional group along a lipid hydro-carbon chain. Many such isomeric structures exist, for example conjugated linoleic acid (CLA) is a term used to describe a fatty acid mixture of positional and geometric 18:2 isomers containing conjugated double bonds; however, only specific CLA structures possess biological activity (McCrorie *et al*., 2011). Glucans, such as reuteran, α-(1→4)-, α-(1→6)-linked, act as molecular decoys for enterotoxigenic *Escherichia coli* to potentially inhibit mammalian infection; while α-(1→6)-linked dextran has no activity (Wang *et al*., 2010).

Spectroscopic and spectrometric analytical techniques are widely used for the structural analysis of biological compounds. The most common types of spectroscopy employed in structural studies of organic molecules are infrared (IR), ultraviolet (UV) and nuclear magnetic resonance (NMR) techniques, while mass spectrometry (MS) is used for biological spectrometric structural analysis. An IR spectrum allows for the detection of functional groups in compounds by absorbance at specific infrared wavelengths that are unique to the molecular

group in question, but other analytical methods must be used to determine the locale of the functional groups within the molecule (Solomons & Fryhle, 2004). UV detection indicates the presence of compounds containing chromophores that absorb specific UV range wavelengths due to electronic transition; however, no characteristic spectra are obtained for structural identification and many compounds have absorbance at similar wavelengths (Almiro da Paixão, 2009). Both IR and UV techniques are most informative for pure samples, and can be used in conjunction with chromatography (Khan *et al*., 2012). Alternatively, NMR can be used to produce characteristic spectra for compound structural identification by measuring the absorption of a magnetic field and electromagnetic energy by nuclei with unpaired protons or neutrons; however, pure samples are required and often two-dimensional experiments are performed to structurally characterize biological compounds (Byrne, 2007).

Mass spectrometry (MS) separates ionized compounds in the gas phase according to their mass to charge ratio (*m/z*). Chromatography, either gas (GC) or liquid (LC), can be coupled with MS to provide the extra element of compound separation so that pure compound peaks can be analyzed. GC separates analytes based on volatility and polarity, and the eluting analytes are ionized by chemical ionization (CI) or electron impact ionization (EI) before MS analysis. The first step toward identifying the eluting compounds from the GC is by fragmentation using a fixed energy of 70 eV with EI. Under these conditions, EI mass spectra are reproducible and can be searched against vast collections of EI spectra of

known compounds (eg. National Institute of Standards and Technology/Environmental Protection Agency/National Institute of Health Mass Spectral Library and Wiley Registry of Mass Spectral Data). However, GC/MS is limited to the separation of low molecular weight analytes that are both thermally stable and volatile, and often samples must be derivatized prior to analysis in order to achieve these criteria (Poole, 2012). Sample derivatization can be time consuming and prolonged run times may be necessary for isomer separation (Black & Curtis, 2013; Stolyhwo & Rutkowska, 2013). However, where resolution is still not readily achievable, more sophisticated two-dimensional GC systems offer the possibility of enhanced isomer separation through the use of two columns in sequence that differ in polarity (Villegas *et al*., 2010).

In contrast to the ionization techniques used for GC/MS, those used in LC/MS are more suitable for nonvolatile and thermally labile compounds, as an estimated 85% of compounds in nature fall into this category (Dass, 2007). LC/MS systems also have a selection of compatible ionization sources operating at atmospheric pressure. Atmospheric pressure ionization is compatible with direct analysis of solutions and hence, can be easily coupled with LC. Two atmospheric pressure sources, electrospray ionization (ESI), which generally ionizes more polar and higher mass compounds, and atmospheric pressure photo ionization (APPI), which generally ionizes less polar and lower mass compounds, can be used as complimentary sources to ionize compounds over a wide range of polarity (de Hoffmann & Stroobant, 2007; Marchi *et al*., 2009). Additionally, ESI

leads to minimal in-source fragmentation of analytes producing a greater abundance of molecular ions for analysis (**Figure 1-1**). Tandem mass spectrometry (MS/MS or MS^n) is applied to structurally identify compounds or to quantify compounds using multiple-reaction-monitoring. MS/MS is performed by selecting specific precursor ions and fragmenting them through collisional activation at a selected collision energy with a target gas to produce product ions. The product ions are separated by *m/z* in another region of the instrument, and the resulting product spectrum allows for information on precursor ion structure to be obtained (de Hoffmann & Stroobant, 2007). Due to the advancement of modern LC/MS systems, rapid identification of compound classes and individual compounds from biological samples is widely used in lipidomics and metabolomics (Roux *et al*., 2011). Quantification of analytes is also possible with the use of external calibration curves or isotope labeled compounds (Giavalisco *et al*., 2009). Derivatization of analytes for LC/MS analysis is usually unnecessary and often not recommended due to possible artifact formation and the introduction of variance, such as degradation of the compounds and introduction of contaminants (Xu *et al*., 2010). However, derivatization may be applied in some cases to support ionization or enhance fragmentation (Xu *et al*., 2011).

LC/MS is one of the most important analytical techniques, as this robust system fulfills the need for rapid, sensitive and selective analytical measurements (Holčapek *et al*., 2012). For these reasons, LC/MS is suitable for the separation, structural identification and quantification of bioactive components in

complicated mixtures (Di Stepfano *et al*., 2012). For the purpose of analyzing bioactive metabolites from lactobacilli, LC/MS has the capability for structural and quantitative analysis alone; however, it can be paired with orthogonal analytical techniques for method validation.

1.5. Hypothesis

Currently, LC/MS is the method of choice for the analysis of a wide range of biologically active compounds. However, many lipids and carbohydrates are still routinely analyzed using GC/MS (Dass, 2007; Christie & Han, 2010). These GC/MS methods for structural characterization of lipids and carbohydrates usually involve derivatization procedures and often extended run times to obtain the required compound resolution (Dass, 2007; Christie & Han, 2010; Xu *et al*., 2010, Wittenburg *et al*., 2013; Yang *et al*., 2013). In this thesis it is hypothesized that carbohydrate and lipid bioactive metabolites from lactobacilli can be structurally characterized by LC/MS without a considerable amount of sample preparation. In order to test this hypothesis, experiments were conducted to meet the following objectives: (1) to determine that lactobacilli convert fatty acid and saccharides into active compounds, (2) to use a LC/ quadrupole time-of-flight MS system to structurally identify fatty acid and oligosaccharide active compounds in complicated mixtures with little or no derivatization and (3) to determine structure-function relationships between identification and activity of fatty acid and oligosaccharide compounds.

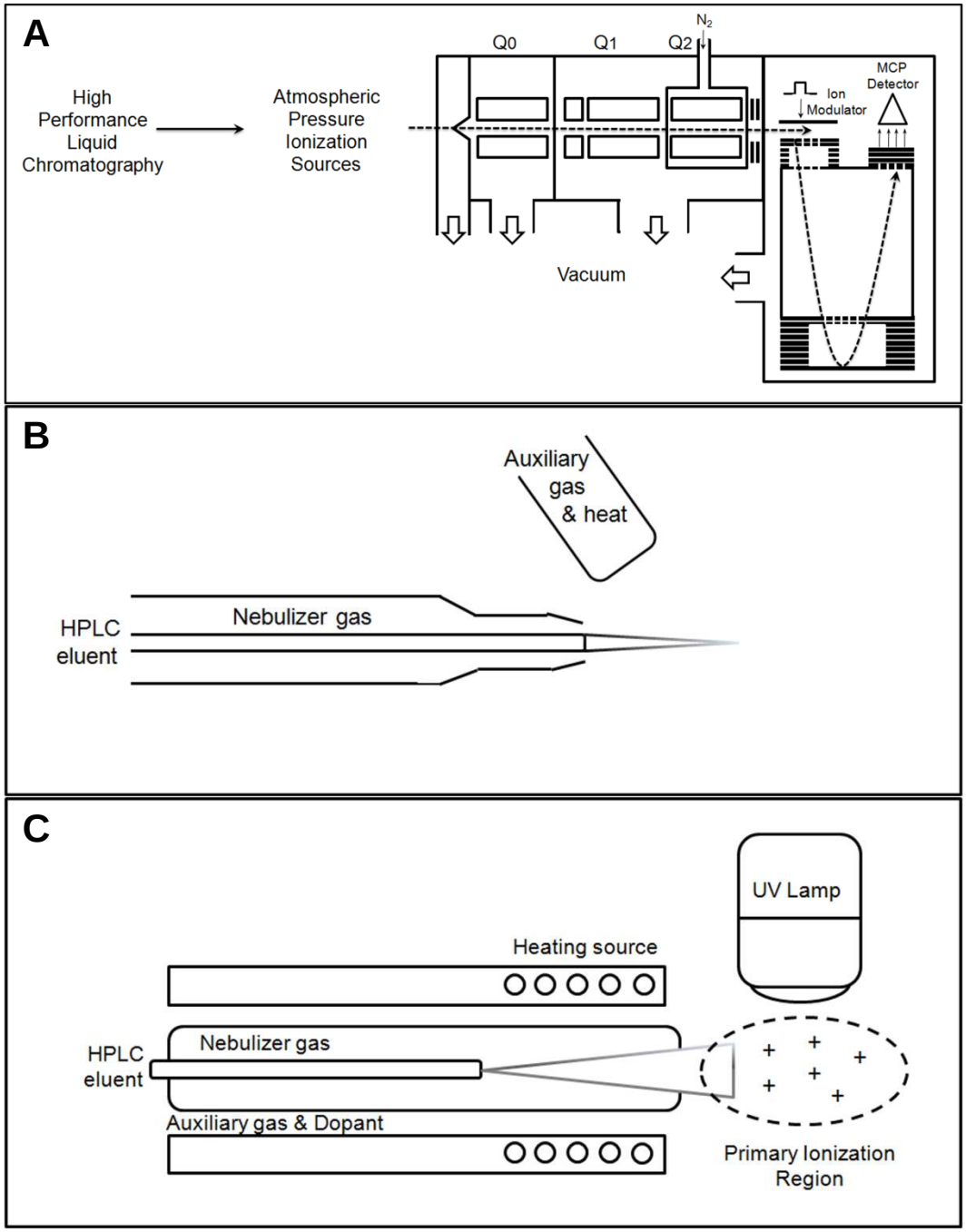

Figure 1-1. HPLC coupled with an Applied Biosysems/Sciex mass spectrometry system from to characterize analytes in complicated matrixes. (A) QStar Elite, a quadrupole time-of-flight hybrid mass spectrometer system; (B) Turbospray ESI source; (C) Photospray APPI source.

1.6. References

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CHAPTER 2

2. Antifungal hydroxy fatty acids produced during sourdough fermentation[1]

2.1. Introduction

Sourdough bread has an extended mold-free storage-life compared to conventionally leavened products (Salovaara, 2004; Smith *et al*., 2004) and the presence of metabolites from specific strains of lactobacilli contributes to the prolonged storage-life of sourdough bread (Coda *et al*., 2008; Coda *et al*., 2011; Ryan *et al*., 2011). While the fermentation microbiota of traditional sourdough is controlled by the fermentation conditions and the choice of raw materials, the industrial production of sourdough often relies on single strains of lactobacilli with defined metabolic properties (Brandt, 2007; Corsetti, 2013). To date, cyclic dipeptides, phenyllactic acid, acetic and propionic acids, and short-chain hydroxy-fatty acids have been identified as antifungal metabolites of sourdough lactobacilli (Lavermicocca *et al*., 2000; Schnürer, 2005; Zhang *et al*., 2010). However, these compounds are either not produced in effective quantities in sourdough fermentations, or adversely affect the quality of the product when produced in active concentrations. Cyclic dipeptides, such as 2,5-diketopiperazines, are produced in quantities 1000-fold below the MIC against molds, and are accompanied by bitter or metallic flavors if present in higher quantities (Ryan *et al*., 2009a). Similarly, the amount of phenyllactic acid produced in sourdough is

[1] A version of this chapter has been published. Black, B. A., Zannini, E., Curtis, J. M. & Gänzle, M. G. (2013). Applied and Environmental Microbiology. 79, 1866-1873.

Asterisks (*) indicate contributions by E. Zannini

1000 times less than the required amount for activity (Lavermicocca *et al*., 2000; Valerio *et al*., 2004; Ryan *et al*., 2009b). Cooperative metabolism in sourdough of *Lactobacillus buchneri* and *Lactobacillus diolivorans* produced acetic and propionic acids in concentrations of 4 and 3 g L^{-1} respectively, in sourdough (Zhang *et al*., 2010). Acetic and propionic acid formation during sourdough fermentation contributed to mold inhibition in bread (Zhang *et al*., 2010), however, their concentrations remain below the MIC for mold inhibition at 7.2 g L^{-1} and 4.4 g L^{-1}, respectively, and increased concentrations adversely affect sensory properties of bread.

Pseudomonas aeruginosa transformed linoleic acid to a mixture of mono-, di- and tri-hydroxy fatty acids with antifungal activity against wide range of crop fungal pathogens (Hou, 2008; Martin-Arjol *et al*., 2010). However, *P. aeruginosa* is not suitable for use in food fermentations. Lactic acid bacteria also convert linoleic acid to hydroxy fatty acids (Kishimoto *et al*., 2003; Volkov *et al*., 2010); however, this conversion was not demonstrated in food fermentations and it remains unknown whether hydroxy fatty acids produced by lactobacilli have antifungal activity. Hence, the aim of this study was to determine whether lactobacilli convert linoleic acid to metabolites with antifungal activity, to assess whether this conversion can be achieved in sourdough fermentation, and to determine whether conversion of linoleic acid in sourdough delays fungal spoilage of bread. The screening of lactobacilli focused on sourdough isolates that

were previously shown to convert linoleic and oleic acids to hydroxylated metabolites (Shahzadi, 2011).

2.2. Materials and methods

2.2.1. Chemicals and standards

cis-9-*cis*-12-Octadecadienoic (linoleic) acid, 12-hydroxy-*cis*-9-octadecenoic (ricinoleic) acid, 12-hydroxy octadecanoic acid, octadecanoic (stearic) acid, *cis*-9-octadecenoic (oleic) acid and distearin with > 99% purity, were purchased from Nu-Chek Prep, Inc. (Elysian, MN). 9,10-Dihydroxystearic acid (> 90%) was supplied by Pfaltz and Bauer (Waterbury, CT). Cysteine-HCl (≥ 98%), trizma hydrochloride (> 99%), lipoxidase from *Glycine max* (soybean) type I-B (≥ 50,000 units mg^{-1}) were purchased from Sigma-Aldrich, (St. Louis, MO). Fisher Scientific (Ottawa, Canada) supplied microbiological media, HPLC grade chloroform, methanol and acetic acid. Solvents were of analytical grade unless specified otherwise.

2.2.2. Strains and growth conditions

Lactobacillus sanfranciscensis ATCC27651, *Lactobacillus reuteri* LTH2584, *Lactobacillus pontis* LTH2587, *Lactobacillus hammesii* DSM16381, *Lactobacillus plantarum* TMW1460 and TMW1701 were cultivated on modified DeMan-Rogosa-Sharpe (mMRS) (de Man *et al*., 1960) containing 10 g L^{-1} maltose, 5 g L^{-1} fructose and 5 g L^{-1} glucose with a pH of 6.2. Lactobacilli were incubated under microaerophilic conditions (1% O_2, balance N_2) for 24 h at 37 °C

(*L. reuteri*) or 30 °C (all other strains). *Mucor plumbeus* FUA5003, *Aspergillus niger* FUA5001 or *Penicillium roqueforti* FUA5005 (Zhang *et al*., 2010) were used as target organisms for antifungal assays. Fungal cultures were grown on malt extract agar medium at 25 °C for 72 h and spores were harvested from plates using a 0.9% NaCl (w/v), 0.1% (w/v) Tween-80 solution and scraping with a Drigalski spatula. Mycelia debris was removed by filtering the solution through sterilized cotton gauze. The spore count was standardized to 10^4 or 10^2 spores mL^{-1} with a haemocytometer (Fein-Optik, Jena, Germany)

2.2.3. Screening of antifungal activity*

mMRS broth was used to screen the ability of lactobacilli to convert linoleic acid into antifungal compounds. Lactobacilli strains were inoculated into 15 mL of mMRS and incubated for 24 h. The cells were washed twice with 0.85% NaCl and resuspended in 10 mL of 0.85% NaCl. For each strain, 5% (v/v) 10^9 cfu mL^{-1} washed cells were inoculated into 20 mL mMRS broth. Additionally, linoleic acid at 0, 2 or 4 g L^{-1} was added. Inoculated broth was incubated with shaking at 120 rpm for 8 days. After 1, 4 and 8 days of incubation, 1 mL culture supernatant was withdrawn, centrifuged (4000 × g for 10 min) and sterilized by filtration (0.45 µm, MCE; Milipore Corp., Bedford, MA). Organic extracts of culture supernatants were obtained by extraction with chloroform 2:1 (v/v). The organic phase was collected by centrifugation (4000 × g for 5 min) and the upper layer was again extracted in the same manner. Both culture supernatants and organic extracts were dried under nitrogen gas and tested for antifungal activity.

Sourdough was prepared by mixing 10 g white wheat flour and 10 mL tap water, and inoculation with 5% (v/v) 10^9 cfu mL^{-1} lactobacilli suspended in saline. Linoleic acid (0, 2, or 4 g kg^{-1}) was added as a substrate and dough was incubated at 30 °C, or 37 °C for 8 d. At days 1, 4 and 8, 2 g samples of sourdough were removed for extraction and analysis. All sourdough fermentations were routinely characterized with regards to cell counts and pH to verify growth of lactobacilli, and to ensure the identity of fermentation microbiota with the inoculum. Aqueous extracts were obtained by centrifugation (4000 × g for 10 min). To obtain organic extracts, 2 mL of water and 1.5 mL isopropanol were added to 2 g sourdough. The pH of the mixture was adjusted to 2.5 ± 0.05 using 5 M HCl and adding 2 mL of water. The organic phase was extracted by mixing 2 mL isopropanol, 2 mL water and saturating with NaCl to obtain phase separation. Solids were removed by centrifugation, and the organic phase was collected. Both aqueous and organic extracts were dried under nitrogen gas and were reserved for further analysis.

2.2.4. Antifungal activity assay

The assay used to determine MIC values were performed as serial two-fold dilutions using a microtiter plate well method described by Magnusson and Schnürer, 2001. Microtiter plates were inoculated with mMRS broth containing 10^4 spores mL^{-1} of *A. niger*, *M. plumbeus* or *P. roqueforti* and incubated at 25 °C. The MIC was determined as the lowest concentration of sample inhibition growth. Organic solvents in the samples were removed by evaporation under a laminar

flow hood prior to the addition of fungal spores. Triplicate independant experiments were performed.

2.2.5. Combined liquid chromatography/atmospheric pressure photo ionization – mass spectrometry

Un-derivatized organic extracts were analyzed by LC/APPI-MS. Separations were conducted on an Agilent 1200 series LC system (Agilent Technologies, Palo Alto, CA) at 25 °C using a YMC PVA-Sil column (150 mm × 2.0 mm i.d., 5 µm; Waters Ltd., Mississauga, Canada). Lipid samples dissolved in chloroform were eluted using an injection volume of 5 µL and a gradient of (A) hexane with 0.2% acetic acid and (B) isopropanol with 0.2% acetic acid at a flow rate of 0.2 mL min^{-1}. The gradient was as follows: 0 min 99% A; 20 min 70% A; 20.1 min 99% A; for a total run time of 27 min, including the time to equilibrate the column. Negative ion APPI-MS was performed on a QStar® Elite hybrid orthogonal Q-TOF mass spectrometer coupled to a PhotoSpray® source with Analyst® QS 2.0 software (Applied Biosystems/MDS Sciex, Concord, Canada). The source and mass spectrometer conditions were: nebulizer gas 70 (arbitrary units), auxillary gas 20, curtain gas 25, ionspray voltage -1300 V, source temperature 400 °C, declustering potential (DP) -35 V, focusing potential -130 V and DP2 -13 V with a scanning mass range of *m/z* 50-700.

2.2.6. Identification and quantitation of antifungal compounds

For isolation of antifungal compounds, mMRS was fermented with *L. hammesii* or *L. sanfranciscensis* and 4 g L^{-1} linoleic acid for 4 days. Cultures were

extracted twice with two volumes chloroform/methanol 85:15 (v/v). The organic phase was then dried under vacuum at 30 °C and was stored at -20 °C under nitrogen gas. Up to 25 mg of extracted sample was loaded onto a conditioned Sep-Pak 500 mg silica cartridge (Waters Ltd., Mississauga, Canada), washed with 20 mL of chloroform, and hydroxy fatty acids were eluted with 10 mL 50% isopropyl alcohol in chloroform (v/v). The hydroxy fatty acid fraction was dried under nitrogen and dissolved in chloroform at 30 mg mL^{-1}. For further fractionation according to hydroxyl group number, semi-preparative high performance liquid chromatography was performed on an Agilent 1200 series LC system (Agilent Technologies, Palo Alto, CA). An injection volume of 100 µL was loaded onto a Zorbax Rx-SIL semi-prep column (9.4 mm × 250 mm i.d., 5 µm, Agilent Technologies). Separations were performed at 23 ºC at a flow of 3 mL min^{-1} with a gradient analogous to the analytical column aforementioned. Separations were monitored by diode array detection (DAD) at 210 nm, and confirmed by splitting the post-column flow to an evaporative light scattering detector (ELSD) at 60 ºC with 3.5 standard L min^{-1} nitrogen gas. Fractions were collected in 0.1 min time-slices, analyzed by mass spectrometry for purity, and assessed for their antifungal activity by MIC assays in triplicate independant experiments.

For the further fractionation of C18:1 mono-hydroxy fatty acids, a Supelcosil LC-18-DB column (10mm × 250 mm i.d., 5 µm; Sigma-Aldrich, Oakville, Canada) was employed with a 3 mL min^{-1} flow rate and a gradient of 50%

acetonitrile and 50% water at 0 min, increasing to 100% acetonitrile at 35 min. A total of 20 µL was injected of a 1 mg mL^{-1} fatty acid extract in chloroform/methanol. Fractions were collected from 13 – 15.5 min and LC/APPI-MS confirmed the absence of other compounds prior to assessment for antifungal activity.

For relative quantification, peak areas percentages of hydroxy fatty acids were compared to the peak area of the same hydroxy fatty acid in extracts from cultures of *L. hammesii* supplemented with linoleic acid. mMRS and sourdough were fermented for 4 days with *L. hammesii* or *L. sanfranciscensis*, or were chemically acidified with 4:1 lactic/acetic acid (v/v) to pH 3.5. Linoleic acid was added at either 0 or 4 g L^{-1}. mMRS was extracted directly with methanol/chloroform as described above. Wheat doughs were extracted using the Bligh and Dyer method (Bligh & Dyer, 1959). Briefly, the wheat dough was lyophilized and to the dried sample 1:2:0.8 chloroform/methanol/water (v/v/v) was added. The mixture was homogenized at 10, 000 rpm for 1 min and left at ambient temperature for 1 h. One part each chloroform and water was added for phase separation, the solution was mixed again and the lower phase was collected. Each broth and dough lipid extract was dried under nitrogen and reconstituted in chloroform/methanol 85:15 (v/v) to 1 mg mL^{-1} with 5 µg mL^{-1} distearin added as an internal standard. All samples were then analyzed by LC/APPI-MS and the Analyst® software was used to determine peak areas. Each peak was normalized using the response of the distearin internal standard. For optimization of

lactobacilli metabolites over time, the same fermentations were prepared and sampled at 24 h intervals over 8 days. All relative quantifications were performed in triplicate independent experiments with a minimum of three technical repeats.

For absolute quantification of the antifungal fatty acid in sourdough starter, dough and bread, 200 mg lyophilized samples were extracted by the Bligh and Dyer method (Bligh & Dyer, 1959). The lyophilized samples were first spiked with 150 µg of ricinoleic acid standard to measure extraction recovery. Each extraction was adjusted to a volume of 5 mL with chloroform after 25 µg of distearin internal standard was added. An external standard of ricinoleic acid was used to construct a calibration curve, with the assumption that ionization efficiency was similar to the unknown mono-hydroxy C18:1 product. All samples from triplicate independent experiments were analyzed in duplicate by LC/APPI-MS.

2.2.7. Enzymatic production of coriolic acid

To test the activity of different components of C18 fatty acids, 13-hydroxy-*cis*-9-*trans*-11-octadecadienoic (coriolic) acid was produced in a one-step method (Shahzadi, 2011). Linoleic acid was added at to a concentration of 3 mM to a 0.1 M Trizma® hydrochloride buffer (pH 9.0) containing cysteine in a 4:1 molar ratio to linoleic acid. After the addition of 0.16 g L^{-1} lipoxygenase, the reaction was carried out under a gentle stream of oxygen at room temperature for 5 min and then transferred to an incubator at 25 °C with shaking speed of 150 rpm for 25 min. At the end of incubation period, the buffer was adjusted to pH 2 with 1 N

HCl and extracted three times with chloroform containing 15% (v/v) methanol. Coriolic acid was purified from unreduced peroxide fatty acids by semi-preparative silica chromatography as outlined above. LC/APPI-MS confirmed the identity and the preparation and the absence of contaminants.

2.2.8. Sourdough fermentation and bread preparation

L hammesii and *L. sanfranciscensis* were used to prepare sourdough bread. A volume of 1% (v/v) overnight cell culture was used to inoculate mMRS broth and was incubated for 24 h at 30 ºC. Cells were washed twice and suspended in sterile tap water to a concentration of 10^9 CFU mL^{-1}. Sourdough was prepared by mixing white wheat flour, sterile tap water and culture in a ratio of 2:1:1 (w/w/w), and 4 g kg^{-1} linoleic acid to homogeneity. The dough was fermented at 30 ºC for 2 days. Samples were taken after 0, 1 and 2 d for analysis of cell counts, pH-values, and the concentration of organic acids and ethanol. The identity of the fermentation microbiota and the inoculum was verified by observation of a uniform and matching colony morphology metabolite profiles. During sourdough fermentation, cell counts for *L. hammesii* and *L. sanfranciscensis* reached 9 log CFU mL^{-1} after 24 h and remained constant after 48 h (**Appendix 1**, **Figure 1A-1** for methods). The pH values of sourdough after 24 h of fermentation were 3.3 ± 0.1 (*L. sanfranciscensis*) and 3.4 ± 0.1 (*L. hammesii*). The pH values remained consistant after 24 h of fermentation; *L. hammesii* and *L. sanfranciscensis* produced 64.9 ± 6.1 and 70.6 ± 2.4 mmol lactate (kg sourdough)$^{-1}$, respectively, and 11.1 ± 1.6 and 12.5 ± 2.3 mmol acetate (kg sourdough)$^{-1}$, respectively.

Dough was prepared with bread formulations shown in **Table 2-1**.

Table 2-1. Bread formulation.

Ingredients	Recipe (g)				
	Non-acidified	Chemically acidified	Fermented	Propionate	Coriolic acid
Flour	200	200	180	200	200
Water	130	130	110	130	130
Salt	4	4	4	4	4
Yeast	4	4	4	4	4
Acid mix[a)]	0	1.3	0	0	0
Sourdough[b)]	0	0	40	0	0
Calcium propionate	0	0	0	0.8	0
Coriolic acid	0	0	0	0	0.3

[a)] Mixture of lactic and acetic acid (4:1 v/v) to yield a dough pH of 3.9 ± 0.5.
[b)] Fermented by either *L. hammesii* or *L. sanfranciscensis* supplemented with 4 g kg^{-1} linoleic acid.

Bread was prepared with 20% addition of sourdough; non-acidified dough, chemically acidified dough and dough supplemented with 0.4% (w/w) calcium propionate or 0.15% (w/w) coriolic acid were prepared as references. Dough was mixed for 8 min (Kitchen Aid, K45SS, Hobart, OH) and proofed for 25 min at 30 ºC and 85% relative humidity in a proofer (Cres-Cor, 12711, Cleveland, OH). After the first proof, the dough was molded, placed into tins (202 × 102 × 55 mm) and proofed under the same conditions for an additional 105 min. Dough was baked in a convection oven (Bakers Pride Canada, X-300L, Lachine, Canada) at 180 ºC for 25 min. The loaves were cooled to room temperature on racks for 120

min, where samples were taken for antifungal testing, pH determination and quantification of antifungal compounds by LC/APPI-MS.

Determination of bread pH was measured by homogenizing a 10-fold dilution of bread crumb in deionized water. Growth of mold on bread was measured by slicing the bread in 25 mm thick uniform slices under sterile conditions and placing into sealed sterile plastic bags with filter tips inserted to allow the exchange of oxygen. Bread slices were inoculated with a spore suspension containing 10^2 spores mL^{-1} in a 0.9% NaCl (w/v), 0.1% (w/v) Tween-80 solution. The spore suspension was sprayed five times in each corner of the bread slice and once in the middle delivering 89.1 ± 3.1 µL spore suspension for *P. roqueforti* and 90.3 ± 3.3 µL *A. niger* spore suspension with each spray. Additional samples were sliced in an open baking area to allow environmental contamination without inoculation. Slices were incubated for 15 days at 20 ºC and monitored every 12 h. The time to visible mycelial growth was reported as mold-free shelf life.

The effect of sourdough fermentation was determined in triplicate independent experiments (triplicate sourdough fermentation and baking). Statistical analysis was conducted using SAS 9.3 with Tukey's pairwise multiple comparison test. Significant differences were reported at a confidence level of $p \leq 0.05$.

2.3. Results

2.3.1. Selection of sourdough lactobacilli with antifungal activity*

Seven strains of lactobacilli that are known to convert linoleic acid (Shahzadi, 2011) were screened for antifungal activity to identify lactobacilli that specifically convert linoleic acid to antifungal metabolites. Inhibitory activity of culture supernatant or organic extracts from cultures in mMRS or sourdough was investigated against *A. niger* and *M. plumbeus*. **Table 2-2** shows the activity of culture supernatants after 4 days fermentation with *A. niger* as indicator strain. Comparable results were obtained with *M. plumbeus* (**Table 2-3**).

Table 2-2. MIC of aqueous extracts from cultures in mMRS and sourdough with differing levels of linoleic acid. Samples were extracted at 4 days of fermentation and tested for activity using *Aspergillus niger* as an indicator. MIC analysis was performed after 4 days of indicator growth and data are shown as means ± standard deviation of triplicate independent experiments.

Starter culture	**MIC ($mL \cdot L^{-1}$) mMRS Broth**			**MIC ($mL \cdot L^{-1}$) Sourdough**		
	Linoleic acid concentration					
	0 $g \cdot L^{-1}$	**2 $g \cdot L^{-1}$**	**4 $g \cdot L^{-1}$**	**0 $g \cdot L^{-1}$**	**2 $g \cdot L^{-1}$**	**4 $g \cdot L^{-1}$**
L. sanfranciscensis ATCC 27051	83±0	70±17	83±0	35±10	17±5	42±0
L. plantarum TMW 1460	70±20	9±2	42±0	28±10	42±0	17±6
L. plantarum TMW 1701	56±20	10±0	70±24	83±0	83±0	35±12
L. reuteri LTH 2584	42±0	9±2	56±24	17±5	21±0	14±6
L. pontis LTH 2587	70±20	83±0	70±24	42±0	42±0	21±0
L. hammesii DSM 16381	14±5	17±5	3±2	28±10	21±0	10±0

Table 2-3. MIC of aqueous extracts from cultures in mMRS and sourdough with differing levels of linoleic acid. Samples were extracted at 4 days of fermentation and tested for activity using *Mucor plumbeus* as an indicator. MIC analysis was performed after 4 days of indicator growth and data are shown as means ± standard deviation of triplicate independent experiments.

Starter culture	**MIC ($mL \cdot L^{-1}$) mMRS Broth**			**MIC ($mL \cdot L^{-1}$) Sourdough**		
	Linoleic acid concentration					
	0 $g \cdot L^{-1}$	**2 $g \cdot L^{-1}$**	**4 $g \cdot L^{-1}$**	**0 $g \cdot L^{-1}$**	**2 $g \cdot L^{-1}$**	**4 $g \cdot L^{-1}$**
L. sanfranciscensis ATCC 27051	83±0	17±5	83±0	17±5	17±5	17±6
L. plantarum TMW 1460	9±2	7±2	69±24	42±0	21±0	10±0
L. plantarum TMW 1701	69±20	7±2	83±0	35±10	28±10	5±0
L. reuteri LTH 2584	41±0	21±0	42±0	14±5	17±5	14±6
L. pontis LTH 2587	56±20	17±5	35±12	42±0	14±5	21±0
L. hammesii DSM 16381	21±0	10±0	10±0	17±5	10±0	4±2

L. plantarum TMW1460, *L. reuteri* LTH2584 and *L. hammesii* DSM16381 exhibited the strongest activity in both mMRS broth and sourdough medium. However, *L. hammesii* was the only strain where higher concentrations of linoleic acid lead to a stronger antifungal effect. Organic extracts exhibited higher activity compared to the culture supernatants (**Table 2-4**).

Table 2-4. MIC of organic extracts from cultures in mMRS and sourdough with differing levels of linoleic acid. Samples were extracted at 4 days of fermentation and tested for activity using *Aspergillus niger* as an indicator. MIC analysis was performed after 4 days of indicator growth and data are shown as means ± standard deviation of triplicate independent experiments.

Starter culture	**MIC ($mL \cdot L^{-1}$) mMRS Broth**			**MIC ($mL \cdot L^{-1}$) Sourdough**		
	Linoleic acid concentration					
	0 $g \cdot L^{-1}$	**2 $g \cdot L^{-1}$**	**4 $g \cdot L^{-1}$**	**0 $g \cdot L^{-1}$**	**2 $g \cdot L^{-1}$**	**4 $g \cdot L^{-1}$**
L. sanfranciscensis ATCC 27051	2±0	2±0	6±2	1±0	5±2	6±2
L. plantarum TMW 1460	2±0	8±0	8±0	2±0	2±0	3±1
L. plantarum TMW 1701	6±2	2±0	3±1	4±0	1±0	8±0
L. reuteri LTH 2584	1±0	1±0	3±1	5±2	8±0	3±1
L. pontis LTH 2587	8±0	1±0	8±0	2±0	2±0	2±0
L. hammesii DSM 16381	4±1	2±0	1±0	6±2	5±2	1±0

The strongest activity of the organic extract from mMRS broth was from *L. reuteri*, *L. pontis* and *L. hammesii*; the strongest activity in sourdough extracts was observed with *L. sanfranciscensis*, *L. plantarum* and *L. hammesii*. Again, *L. hammesii* exhibited a strong effect in either media and addition of linoleic acid increased the antifungal effect against *A. niger* (**Table 2-4**) and *M. plumbeus* (**Table 2-5**).

Table 2-5. MIC of organic extracts from cultures in mMRS and sourdough with differing levels of linoleic acid. Samples were extracted at 4 days of fermentation and tested for activity using *Mucor plumbeus* as an indicator. MIC analysis was performed after 4 days of indicator growth and data are shown as means ± standard deviation of triplicate independent experiments.

Starter culture	**MIC ($mL \cdot L^{-1}$) mMRS Broth**			**MIC ($mL \cdot L^{-1}$) Sourdough**		
	Linoleic acid concentration					
	0 $g \cdot L^{-1}$	**2 $g \cdot L^{-1}$**	**4 $g \cdot L^{-1}$**	**0 $g \cdot L^{-1}$**	**2 $g \cdot L^{-1}$**	**4 $g \cdot L^{-1}$**
L. sanfranciscensis ATCC 27051	1.6±0.4	1.6±0.4	6±2	0.6±0.2	0.6±0.2	8±0
L. plantarum TMW 1460	1±0	1±0	8±0	8±0	1.6±0.4	6±3
L. plantarum TMW 1701	1.2±0.4	1.2±0.4	0.6±0.3	2±0	2±0	8±0
L. reuteri LTH 2584	8±0	2±0	6±2	6±2	5±2	1.6±0.5
L. pontis LTH 2587	2±0	1.2±0.4	4±0	1±0	2±0.4	6±2
L. hammesii DSM 16381	8±0	8±0	1.6±0.5	8±0	1.2±0.4	0.3±0.1

Overall, results indicate that *L. hammesii* converts linoleic acid to a hydrophobic compound with antifungal activity.

2.3.2. Preliminary characterization of antifungal compounds

Antifungal compounds were fractionated from organic extract of *L. hammesii*. Corresponding extracts from *L. sanfranciscensis* were also fractionated for comparison. The organic extract from both strains in mMRS medium was shown by normal phase LC/APPI-MS analysis to be a mixture of carbon 18 fatty acid isomers with from 0 to 3 hydroxyl groups and 0 to 3 double bonds (**Figure 2-1**).

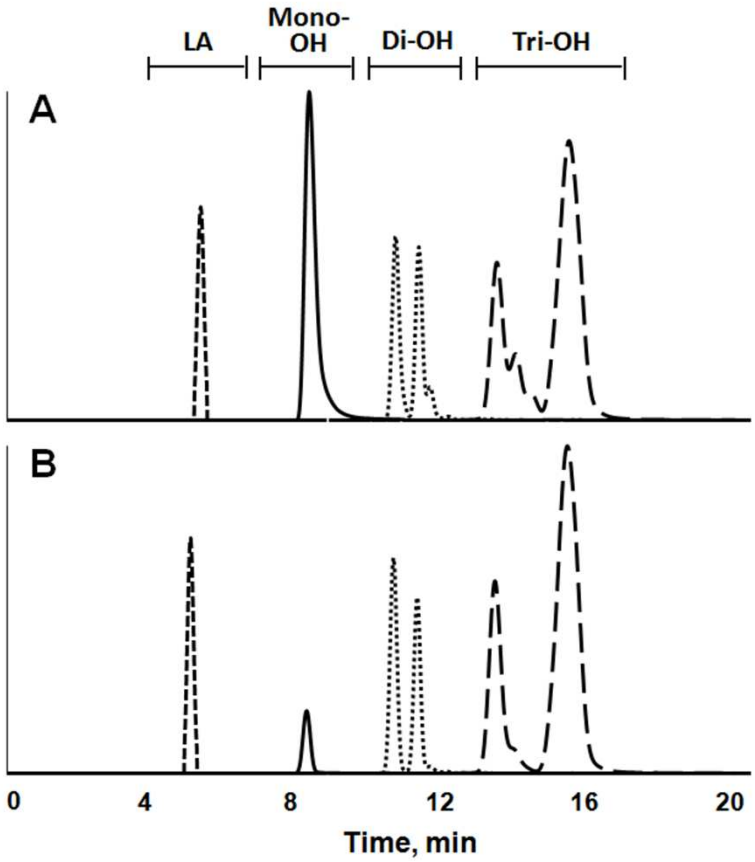
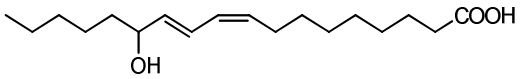
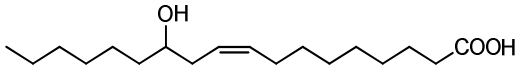
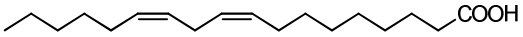
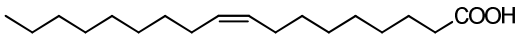
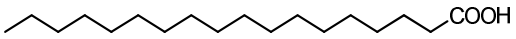
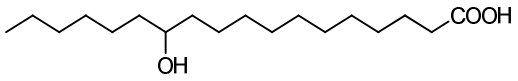
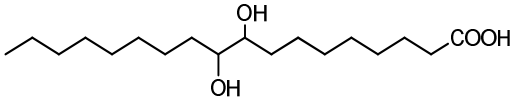


Figure 2-1. LC/APPI-MS extracted ion chromatogram (XIC) overlay of organic extract of sourdough fermented with *L. hammesii* in presence of 4 g L^{-1} linoleic acid (**Panel A)** and extract of sourdough fermented with *L. sanfranciscensis* in presence of 4 g L^{-1} linoleic acid (**Panel B)**. Shown are the $[M-H]^-$ ions of *m/z* 279 corresponding to linoleic acid (LA); *m/z* 293 – 299 corresponding to saturated, mono-, di- and tri-unsaturated mono-hydroxy C18 fatty acids (solid line); *m/z* 309 – 315 corresponding to saturated, mono-, di- and tri-unsaturated di-hydroxy C18 fatty acids (dotted line); and *m/z* 325 – 331 corresponding to saturated, mono-, di- and tri-unsaturated tri-hydroxy C18 fatty acids (dashed line). Separations were performed on a Waters YMC silica column.

Detection with ELSD revealed that mono-hydroxy fatty acids in extracts from cultures of *L. hammesii* accounted for greater than 90% of the hydroxylated lipid product peak area in the chromatogram (**Appendix 1**, **Figure 1A-2**). The peak area for mono-hydroxy fatty acids in extracts from *L. hammesii* was 6.5 times larger compared to extracts from *L. sanfranciscensis;* di- and tri-hydroxy fatty acids were at or below the limit of detection for LC/ELSD for both strains (**Appendix 1**, **Figure 1A-2**). Fatty acids were fractionated by hydroxyl group number and tested for antifungal activity. Mono-hydroxy fatty acids from either *L. hammesii* or *L. sanfranciscensis* exhibited antifungal activity (**Table 2-6**). The MIC of di-hydroxy and tri-hydroxy fatty acids was greater than 20 g L^{-1}. LC/MS analysis of the mono-hydroxy fraction from *L. hammesii* indicated that it consisted almost exclusively of a single compound. The main compound in the mono-hydroxy fraction of *L. hammesii* produced an $[M-H]^-$ ion with *m/z* 297.2403, indicating a mono-hydroxy C18:1 fatty acid with the composition $C_{18}H_{33}O_3$. Contrary to this, the same fraction from *L. sanfranciscensis* consisted of many mono-hydroxy fatty acids and isomers.

Table 2-6. MIC of fatty acids isolated from culture supernatants of *L. hammesii* and *L. sanfranciscensis*, reference compounds, and enzymatically produced coriolic acid. MIC analysis was performed after 3 days of growth with *Aspergillus niger* or 5 days growth with *Penicillium roqueforti*, as an indicator strain; data are shown as means ± standard deviation of triplicate independent experiments. The MIC of di- and tri-hydroxy-fatty acids from *L. hammesii* and *L. sanfranciscensis* was higher than 20 g L^{-1}.

Compound	MIC (g L^{-1})		Structure
	A. niger	*P. roqueforti*	
Mono-OH FA *L. hammesii*	0.7 ± 0.2	0.1 ± 0.06	Mono-hydroxy, mono-unsaturated C18 fatty acid
Mono-OH FA *L. sanfranciscensis*	5.9 ± 0.0		Mixture of saturated and unsaturated mono-hydroxy C18 fatty acids
Coriolic acid	0.7 ± 0.2	0.1 ± 0.08	
Ricinoleic acid	2.4 ± 0.0		
Linoleic acid	4.0 ± 0.0		
Oleic acid	> 20		
Stearic acid	> 20		
12-OH-stearic acid	> 20		
9, 10 di-OH-stearic acid	> 20		

Because mono-hydroxy fatty acids from *L. hammesii* or *L. sanfranciscensis* differed in their antifungal activity, the influence of fatty acid structure on

antifungal activity was examined. The mono-hydroxy fraction from *L. hammesii* and coriolic acid were the most active with a MIC of 0.7 and 0.1 g L^{-1} using *A. niger* and *P. roquefortii* as indicator strains, respectively. The MIC of ricinoleic acid against *A. niger* was 2.4 g L^{-1}. Oleic and stearic acids and saturated hydroxy fatty acids exhibited no antifungal activity (**Table 2-6**). The C18:1 mono-hydroxy fatty acid from *L. hammesii* was purified by reverse phase chromatography to attribute antifungal activity to a single compound. The purified compound inhibited *A. niger* with a MIC of 0.7 ± 0.2 g L^{-1} and *P. roqueforti* with a MIC of 0.1 ± 0.06 g L^{-1}.

2.3.3. Quantification of conversion products

In sourdough, hydroxy fatty acids may be produced by enzymatic or chemical oxidation in addition to microbial metabolism. Particularly the oxidation of linoleic acid by lipoxygenase, followed by chemical reduction to coriolic acid may contribute to the pool of hydroxy fatty acids (Shahzadi, 2011). To distinguish between chemical, enzymatic and microbial conversions, a quantification of fatty acids in organic extracts from mMRS and sourdough was performed. Since authentic standards were not available, hydroxy fatty acids were quantified relative to the concentration of the same compounds in culture supernatants of *L. hammesii*. Relative quantification was performed in extracts from cultures of *L. hammesii* or *L. sanfranciscensis* in mMRS and sourdough, and chemically acidified controls (**Table 2-7**). Peak areas for each fatty acid were expressed as a

percentage of the peak area of the same fatty acid in the supernatant of *L. hammesii* grown mMRS with 4 g L^{-1} linoleic acid.

Table 2-7. Relative quantitation of C18 hydroxy fatty acids in mMRS and sourdough by LC/MS. Hydroxy fatty acid concentrations are expressed relative to the concentration of the same compound in the *L. hammesii* mMRS + LA sample. Data are shown as means ± standard deviation of triplicate independent experiments. LA, addition of 4 g L^{-1} linoleic acid.

No. of hydroxyl groups[a)]	**Mono-OH C18 fatty acids**		**Di-OH C18 fatty acids**	
No. double bonds[b)] **Strain / matrix**	**0**	**1**	**0**	**1**
***L. hammesii* / mMRS + LA**[c)]	**100±12**	**100±3**	**100±11**	**100±4**
L. hammesii / mMRS	258±43	1±0	249±24	9±1
L. sanfranciscensis / mMRS + LA	190±31	30±3	126±28	28±2
L. sanfranciscensis / mMRS	341±51	0±0	203±28	7±3
L. hammesii / dough + LA	31±6	29±1	58±11	176±8
L. hammesii / dough	15±2	15±1	35±13	20±7
L. sanfranciscensis / dough + LA	8±2	3±0	36±10	123±8
L. sanfranciscensis / dough	7±2	4±1	10±5	9±0
Chemically acidified / mMRS + LA	5±1	1±0	16±1	21±5
Chemically acidified / dough + LA	2±1	0±0	12±1	17±0

[a)] Number of hydroxy groups on C18 fatty acids. [b)] Number of double bonds on C18 fatty acids. [c)] The concentration of fatty acids extracted from *L. hammesii* grown in mMRS in presence of linoleic acid was used as a reference (100%).

Mono-hydroxy octadecenoic acid concentrations in fermentations with *L. hammesii* including linoleic acid were 20-fold higher compared to controls containing no linoleic acid, or chemically acidified controls without bacterial metabolism (**Table 2-7**), demonstrating that it is a microbial metabolite from linoleic acid. The relative concentration of the mono-hydroxy C18:1 antifungal metabolite was higher in mMRS than in sourdough and more abundant in fermentations with *L. hammesii* compared to *L. sanfranciscensis*. Absolute quantification of the mono-hydroxy C18:1 from *L. hammesii* revealed that it is produced in sourdough to a concentration of 0.73 ± 0.03 g kg^{-1}, a level that is equivalent or higher than the MIC.

Culture supernatants from mMRS without the addition of linoleic acid show a high amount of mono- and di-hydroxy saturated fatty acids (**Table 2-7**). These products thus likely result from metabolism of other fatty acids, i.e. the hydration of oleic acid (Volkov *et al.*, 2010), a component of Tween 80. Sourdough fermentations with added linoleic acid generated a large amount of di-hydroxy octadecenoic acid, suggesting that flour-derived enzymes or microbial conversion of fatty acids present in dough play a role in their formation. The relative concentration of mono-hydroxy fatty acids with two or three double bonds and di-hydroxy fatty acids with two or three double bonds were high in the chemically acidified controls, and their absolute concentration was low (**Appendix 1**, **Table 1A-1**). This result indicates that these compounds result from chemical or enzymatic oxidation rather than microbial metabolism.

2.3.4. Concentration of hydroxy fatty acids in dough and bread

Initially, the fermentation time to achieve high concentrations of C18 hydroxy fatty acids was optimized. Sourdough with linoleic acid was fermented with *L. hammesii* and *L. sanfranciscensis* for up to 8 d and samples were taken every 24 h for analysis with LC/APPI-MS. The peak area for the antifungal C18:1 hydroxy fatty acid peaked by 2 d of fermentation for *L. hammesii* and *L. sanfranciscensis* and remained at a constant level throughout subsequent incubation (**Appendix 1**, **Figure 1A-3**). Therefore, sourdoughs for use in bread making were fermented for 2 d. Bread dough prepared with *L. hammesii* sourdough contained 0.13 ± 0.02 g kg^{-1} mono-hydroxy C18:1 after proofing. After baking, 0.11 ± 0.02 g kg^{-1} remained, corresponding to a loss of 14%. Coriolic acid was also present in the bread fermented with *L. hammesii* at a concentration of 0. 13 ± 0.03 g kg^{-1}, confirming its formation from linoleic acid, enzymes and reducing agents in the wheat flour. Bread supplemented with 1.5 g kg^{-1} coriolic acid contained 1.2 ± 0.09 g kg^{-1} coriolic acid after proofing and 1.1 ± 0.02 g kg^{-1} coriolic acid after heating, corresponding to a baking loss of 12%. The pH value of bread was 5.3 ± 0.1 for non-acidified control and bread supplemented with calcium propionate or coriolic acid. Chemically acidified bread and sourdough bread fermented with *L. sanfranciscensis* or *L. hammesii* had pH values of 4.1 ± 0.1, 4.3 ± 0.1 and 4.4 ± 0.1, respectively.

2.3.5. Effect of sourdough fermentation and coriolic acid on fungal spoilage of bread

The effect of sourdough fermentation on fungal spoilage was evaluated by challenge with two different fungal strains and after environmental contamination. Sourdough bread was compared to bread prepared in a straight dough process without additives, and to bread with 0.4% calcium propionate or 0.15% coriolic acid. Chemical acidification or addition of sourdough fermented with *L. sanfranciscensis* had no effect on growth of *A. niger* or *P. roqueforti* compared to the control. Environmental contaminants, however, were inhibited by inclusion of sourdough fermented with *L. sanfranciscensis* (**Figure 2-2**). Bread prepared with *L. hammesii* sourdough inhibited growth of all molds relative to the control, and delayed growth of environmental contaminants when compared to *L. sanfranciscensis* sourdough or the chemically acidified control. Coriolic acid supplemented bread exhibited inhibitory effects against all molds when compared to the control, and was as effective as 0.4% calcium propionate for bread inoculated with *P. roqueforti* and environmental contaminants.

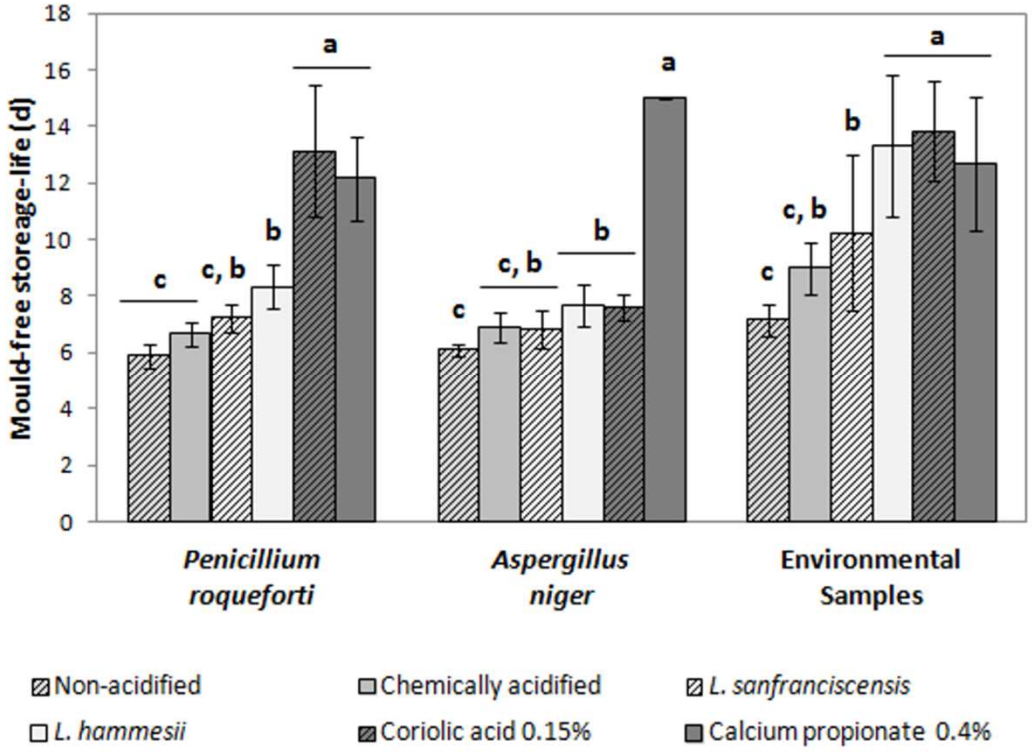


Figure 2-2. Mold free shelf life of bread - non-acidified control, chemically acidified bread, sourdough bread fermented with *L. sanfranciscensis* or *L. hammesii*, and bread supplemented with 0.15% coriolic acid or 0.4% calcium propionate. Sourdough was supplemented with 4 g kg^{-1} linoleic acid and fermented with *L. hammesii* or *L. sanfranciscensis*. Bread slices were inoculated with *A. niger*, *P. roquefortii*, or contaminated by environmental fungal spores during handling, and stored until visible mold growth, or for 15 days. Data are shown as means ± standard deviation of triplicate independent experiments. Values for bread inoculated with the same mold that do not share a common superscript are significantly different ($p < 0.05$).

2.4. Discussion

This study demonstrated that *L. hammesii* DSM16381, an isolate from sourdough (Valcheva *et al*., 2005), converts linoleic acid to a mono-hydroxy octadecenoic acid with antifungal activity. Thus, hydroxy fatty acids produced by food fermenting lactic acid bacteria (Ogawa *et al*., 2001; Kishimoto *et al*., 2003; Volkov *et al*., 2010; Shahzadi, 2011) exhibit antifungal activity. Moreover, linoleic acid metabolism by lactic acid bacteria was previously not observed in food fermentations (Kishimoto *et al*., 2003; Volkov *et al*., 2010). *L. hammesii* produced higher quantities of the mono- hydroxy C18:1 fatty acid than *L. sanfranciscensis*, and demonstrated higher antifungal activity than other lactobacilli. Moreover, coriolic acid exhibits antifungal activity and its activity is comparable to the hydroxy fatty acid produced by *L. hammesii.* Sourdough bread prepared with *L. hammesii* delayed fungal spoilage of bread and coriolic acid delayed fungal spoilage of bread for up to 15 days.

Lactobacilli hydrate linoleic acid to 13-hydroxy-9-octadecenoic acid or 10-hydroxy-12-octadecenoic acid (Ogawa *et al*., 2001; Kishimoto *et al*., 2003). The hydratase of lactic acid bacteria converting linoleic and oleic acids to hydroxy fatty acids was recently characterized (Volkov *et al*., 2010; Yang *et al*., 2013). Hydratases of lactobacilli produce predominantly 10-hydroxy-octadecenoic acid (Yang *et al*., 2013). In *Lactobacillus acidophilus*, the proportion of hydroxy-fatty acids in the cytoplasmic membrane increased at a higher growth temperature (Fernández Murga *et al*., 1999), suggesting a role of hydroxy fatty acids in

membrane homeostasis. Correspondingly, over-expression of the hydratase in lactic acid bacteria increased their heat resistance (Rosberg-Cody *et al*., 2011). Cells change the fatty acid composition of the plasma membrane in response to altered environmental conditions to maintain a liquid-crystalline state (Quinn, 1981; Annous *et al*., 1997). Hydroxy-C18:1-fatty acids decreased the phase transition temperature of the membrane, stabilizing the liquid–crystalline state (Jenske *et al*., 2008). Under direct comparison, the same unsaturated fatty acid had much less impact on membrane properties (Jenske *et al*., 2008).

The antifungal activity of hydroxy fatty acids (Hou, 2008) is likely also linked to their interaction with membranes. Partitioning of hydroxy fatty acids into fungal membrane has been proposed to increase membrane permeability (Sjögren *et al*., 2003; Pohl *et al*., 2008; Pohl *et al*., 2011). Our results demonstrate that antifungal activity is highly dependent on fatty acid structure. Unsaturated mono-hydroxy fatty acids exhibited antifungal activity but saturated hydroxy fatty acids or unsaturated fatty acids were not active. This suggests that at least one double bond and one hydroxyl group along a C18 aliphatic chain are required for antifungal activity. Remarkably, the mono-hydroxy C18:1 fatty acid produced by *L. hammesii* had higher activity than the mixture of mono-hydroxy fatty acids extracted from *L. sanfranciscensis*. Moreover, the 13-hydroxy-*cis*-9-*trans*-11-octadecadienoic (coriolic) acid had higher antifungal activity than 12-hydroxy-*cis*-9-octadecenoic (ricinoleic) acid. The *trans* configuration in coriolic acid has no effect on antifungal activity (Kobayashi *et al*., 1987; Avis, 2007), indicating

that the exact positioning and configuration of hydroxyl groups and double bonds also affects antifungal activity.

The antifungal activity of metabolites from lactic acid bacteria in bread has, to date, not been attributed to a single compound, but rather to their synergistic activity with substrate- or yeast-derived compounds (Coda *et al*., 2011; Ryan *et al*., 2011; Lavermicocca *et al*., 2000; Zhang *et al*, 2010). This study demonstrated that enzymatic and microbial activities generate antifungal hydroxy fatty acids from linoleic acid. Coriolic acid, the product of enzymatic conversion of linoleic acid, has antifungal activity that is equivalent to the linoleic acid metabolite from *L. hammesii*. Conversion of linoleic acid to coriolic acid depends on lipoxygenase activity to generate fatty acid peroxides and thiols to reduce fatty acid peroxides to hydroxy fatty acids (Shahzadi, 2011); both lipoxygenase activity and low-molecular weight thiols are present in wheat (sour)dough (Vermeulen *et al*., 2006; Belitz *et al*., 2009). Chemical and enzymatic oxidation generates additional hydroxy fatty acids in wheat dough, although identification of all of the isomeric structures has not yet been achieved. Microbial conversion of linoleic acid during growth of *L. hammesii* and *L. sanfranciscensis* produced C18:1 mono hydroxy fatty acids. Mono-hydroxy-C18:1-fatty acids produced at the dough stage were relatively stable with a baking loss of less than 15% after baking. The concentration of the antifungal hydroxy C18:1 fatty acid from *L. hammesii* in bread was at or below the MIC; nevertheless, bread prepared with *L. hammesii* sourdough delayed growth of *A. niger*, *P. roquefortii*, and environmental

contaminants. The comparison to bread prepared with *L. sanfranciscensis* sourdough indicates that microbial conversion of linoleic acid contributes to the antifungal activity of sourdough. The antifungal activity of 0.15% coriolic acid – a concentration exceeding the MIC against *A. niger* and *P. roquefortii* two to tenfold - was comparable to the preservative effect of 0.4% calcium propionate. The bitter taste threshold level for di- and tri-hydroxy fatty acids was 4 and 2 g L^{-1}, respectively (Baur *et al*., 1977); whereas, mono-hydroxy fatty acids have a higher taste threshold (Biermann *et al*., 1980). Mono-hydroxy fatty acids thus delay or prevent fungal spoilage of bread without adverse impact on the sensory properties of bread.

In conclusion, *L. hammesii* converts linoleic acid to a mono-hydroxy octadecenoic acid with antifungal activity. This conversion was observed in sourdough fermentations supplemented with linoleic acid but generation of hydroxy fatty acids in sourdough also occurred through enzymatic or chemical oxidation. Mono-hydroxy octadecenoic acid in combination with substrate derived coriolic acid inhibited of mold growth on sourdough bread. The use of coriolic acid and antifungal metabolites from linoleic acid as natural antifungals is not limited to food preservation. Antifungal metabolites from lactobacilli may complement or substitute chemically derived fungicides for use in seed treatment and crop protection (Hernández *et al*., 2012).

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3. Antifungal lipids produced by lactobacilli and their structural identification by liquid chromatography/atmospheric pressure photo ionization – tandem mass spectrometry[2]

3.1. Introduction

Lactobacillus species are widely used in food fermentations (Hammes & Hertel, 2009). Lactobacilli convert linoleic acid into hydroxy fatty acids and conjugated linoleic acid (CLA) (Ogawa *et al*., 2001; Kishimoto *et al*., 2003; Kishino *et al*., 2009; Andrade *et al*., 2012). The first step of this conversion, the hydration of linoleic acid to 13-hydroxy-9-octadecenoic acid or 10-hydroxy-12-octadecenoic acid, is catalyzed by hydratases (Kishimoto *et al*., 2003; Volkov *et al*., 2010; Yang *et al*., 2013). The subsequent synthesis of conjugated fatty acids from hydroxy fatty acids involves enzymes that have not yet been characterized in purified form (Kishino *et al*., 2002; Kishino *et al*., 2009). However, CLA formation by lactobacilli has been observed in buffer fermentations but not in growing cultures (Ogawa *et al*., 2001; Kishino *et al*., 2002; Xu *et al*., 2005; Kishino *et al*., 2009). Overall, information on the physiological role of linoleic acid conversion in lactobacilli remains scarce (O'Flaherty & Klaenhammer, 2010).

[2] A version of this chapter has been published. Black, B. A., Sun, C., Zhao, Y., Gänzle, M. G. & Curtis, J. M. (2013). Journal of Agricultural and Food Chemistry, 61, 5338-5346; Sun, C., Black, B. A., Zhao, Y., Gänzle, M. G. & Curtis, J. M. (2013). Analytical Chemistry, 85, 7345-7352.

Asterisks (*) indicate contributions by C. Sun

CHAPTER 3

The biological activities of conjugated linoleic acids and hydroxy fatty acids are highly related to lipid structure. The active isomers of CLA include *cis*-9, *trans*-11 and *trans*-10, *cis*-12; both of these isomers elicit different biological and physiological effects including changes in immune response, insulin sensitivity, and body fat composition (Pariza *et al*., 2001; Risérus *et al*., 2002; Song *et al*., 2005; Gaullier *et al*., 2005). However, conflicting results have arisen in human studies as to the healthful effects of CLA (McCrorie *et al*., 2011). Only the *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers have been studied, while others remain untested or elicit concern (Kramer *et al*., 1998).

Hydroxy fatty acids produced by lactobacilli exhibit antifungal activity (Black *et al*., 2013). Similar to the biological activity of CLA, only specific hydroxy-fatty acids display antifungal action (Black *et al*., 2013). For example, the antifungal activity of 13-hydroxy-*cis*-9-*trans*-11-octadecadienoic (coriolic) acid produced by the conversion of lipoxygenases, as well as a hydroxy fatty acid metabolite produced by *Lactobacillus hammesii*, were substantially higher than that of 12-hydroxy-*cis*-9-octadecenoic (ricinoleic) acid. Furthermore, saturated hydroxy fatty acids have been shown to be inactive (Black *et al*., 2013). This difference in activity appears to relate to the location of a hydroxyl group and the unsaturation(s) along the 18 carbon chain.

Since subtle structural differences of linoleic acid metabolites from lactobacilli strongly affect their biological activity, it is necessary to fully elucidate these fatty acid metabolite structures. This can be challenging since

many isomeric forms may be present and the most abundant forms are not necessarily the most active. Combined results from infrared spectroscopy (IR), nuclear magnetic resonance (NMR) and mass spectrometry (MS) have been used for the analysis of lipid metabolites from lactobacilli (Ogawa *et al*., 2001). However, relatively large amounts and/or purified samples are required for complete identification. Analysis of hydroxy fatty acids and CLA isomers by gas chromatography coupled to mass spectrometry (GC/MS) requires sample derivatization, long run times and ultimately geometric isomers are still difficult to differentiate (Volkov *et al*., 2010; Yang *et al*., 2013). Alternatively, liquid chromatography-mass spectrometry (LC/MS) can be used for the analysis of fatty acids with the advantage that purification and derivatization is not always necessary (Lima & Abdalla, 2002). In addition, the use of atmospheric pressure photo ionization (APPI) sources, available for many LC/MS systems, has been shown to have high ionization efficiency for non-polar compounds such as lipids, but with less matrix effect compared to electrospray ionization (ESI) (Robb & Blades, 2008).

The aim of this study was to develop methods for the isolation and structure elucidation of antifungal compounds produced by lactobacilli. The preparative purification of antifungal mono-hydroxy fatty acids was achieved using high-speed counter-current chromatography (HSCCC), an alternative to semi-preparative HPLC for the fractionation of complex extracts (Ito, 2005). A normal phase LC/APPI-MS/MS method was developed for the analysis of the antifungal

hydroxy fatty acids and applied to elucidate the structure of these compounds produced by select strains of lactobacilli. In this way, it was hoped to investigate the pathway of conversion from linoleic acid into hydroxy fatty acids and other metabolites by lactobacilli.

3.2. Materials and methods

3.2.1. Chemicals and standards

Cis-9-*cis*-12-octadecadienoic (linoleic) acid, ricinoleic acid, 12-hydroxy octadecanoic acid, distearin, *cis*-7-nonadecenoic acid, *cis*-7-nonadecenoic methyl ester, *cis*-9-*cis*-12- *cis*-15-octadecatrienoic (linolenic) methyl ester, linolenic acid and conjugated octadecadienoic (CLA) methyl ester (*cis*-9, *trans*-11 and *trans*-10, *cis*-12 mixture) all with > 99% purity were purchased from Nu-Chek Prep, Inc. (Elysian, MN). 9, 10-Dihydroxy octadecanoic acid (> 90% purity) was supplied by Pfaltz and Bauer (Waterbury, CT). 2-Amino-2-methyl-1-propanol (95% purity), butyronitrile, 4-methylmorpholine-N-oxide (97% purity), and osmium tetroxide 2.5 wt% solution in 2-methyl-2-propanol were from Sigma Aldrich (St. Louis, MO). HPLC grade solvents (chloroform, methanol, hexane, isopropyl alcohol, acetonitrile, acetic acid) as well as sulfuric acid, formic acid and microbiological media were obtained from Fisher Scientific (Ottawa, Canada).

3.2.2. Strains and growth conditions

Lactobacillus hammesii DSM16381, *Lactobacillus sanfranciscensis* ATCC27651, and *Lactobacillus plantarum* TMW1460 were cultivated in

modified DeMan-Rogosa-Sharpe (mMRS) (de Man *et al*., 1960) containing 10 g L^{-1} maltose, 5 g L^{-1} fructose and 5 g L^{-1} glucose with a pH of 6.2. Lactobacilli were incubated under microaerophilic conditions (1% O_2, balance N_2) at 30 °C for 24 h. The cells were washed twice with 0.85% NaCl, and resuspended in 10 mL of 0.85% NaCl, and 20 mL mMRS broth containing 4 g L^{-1} linoleic acid were inoculated with 5% 10^9 cfu mL^{-1} inoculum. Alternatively, cultures were inoculated in mMRS medium containing 4 g L^{-1} mono-hydroxy octadecenoic acid, linolenic acid, CLA, or ricinoleic acid. Media that were incubated without inoculum and cultures grown without addition of lipid substrates served as controls. Inoculated media were incubated with shaking at 120 rpm for 48 h at 30 °C.

3.2.3. Extraction of lipids

After incubation, cells were removed by centrifugation and filtration with a 0.45 μm filter. Supernatants were extracted three times with two volumes of chloroform/methanol 85:15 (v/v) and the organic phase was collected after each extraction. The organic solvent was removed under vacuum at 30 °C and the residue was stored at -20 °C under nitrogen until analysis.

3.2.4. High-speed counter-current chromatography

HSCCC was performed using a model TBE-300B high-speed counter-current chromatograph with 300 mL column capacity and 0.03 mm i.d. tubing (Tauto Biotech, Shanghai, China), equipped with a 501 PrimeLine solvent delivery module (Analytical Scientific Instruments, El Sobrante, CA), a VUV-24 Visacon

UV-Vis detector (Reflect Scientific Inc., Orem, UT), and a model CHF 122SC fraction collector (Avantec Toyo Kaisha Ltd., Tokyo, Japan). The solvent system consisting of hexane/ethyl acetate/methanol/water 3.5:1.5:3:2 (v/v/v/v) was selected for separation of lipids extracted from cultures of lactobacilli. The solvent mixture was usually prepared in 2 L batches and allowed to separate at room temperature overnight. The upper phase of the biphasic solvent system was used as stationary phase and the lower phase was used as mobile phase. A retention of 70 ± 1.6% (v/v) stationary phase was achieved on introduction of the mobile phase which was pumped at 3 mL min^{-1} in the head-to-tail mode (Ito, 2005). The sample was dissolved to give a concentration of 25 mg mL^{-1} in 10 mL of solvent comprised of 5 mL of the upper phase and 5 mL of the lower phase; the entire 10 mL of sample solution was injected onto the HSCCC column. After a run time of 180 min at a rotor speed of 1000 rpm, all of the hydroxy fatty acids eluted and the mobile phase was switched to be the upper phase to facilitate elution of linoleic acid and CLA. HSCCC separations were monitored at 242 nm with a UV detector. Fractions were collected at 3 min intervals and were analyzed by normal phase LC/APPI-MS as described below. After analysis, fractions containing only the C18:1 mono-hydroxy fatty acid were combined. This combined fraction was used for derivatization, method development analysis and lactobacilli pathway determination.

3.2.5. Preparation of hydroxylated derivatives

The procedure for the vicinal hydroxylation of double bonds was based on the method described by Moe and Jensen (2004). Approximately 1 mg fatty acid was dissolved in 100 µL chloroform and 200 µL of a 1% (w/v) solution of 4-methylmorpholine-N-oxide in methanol and 10 µL of osmium tetroxide were added. The sample was flushed with nitrogen gas and reacted at 60 °C for 2 h. After cooling, the sample was dissolved in 1 mL of chloroform and passed through a conditioned Sep-Pak 500 mg silica solid-phase cartridge (Waters Ltd.; Mississauga, Canada). Hydroxylated fatty acids were then eluted using 20 mL of chloroform/methanol/formic acid (10:1:0.1 v/v/v). The sample was dried under nitrogen gas and resuspended in chloroform/methanol (85:15 v/v) at a concentration of 0.1 mg mL^{-1} prior to LC/APPI-MS/MS analysis.

3.2.6. Preparation of fatty acid methyl ester derivatives

Esterification of free fatty acids was performed using an acid catalyst as outlined by Christie (1989). Fatty acid (10 mg) was dissolved in 1 mL methanol and 2 mL of 1% (v/v) sulfuric acid in methanol was added. The sample was reacted at 50 °C for 2 h with intermittent shaking. After the reaction was complete, 5 mL of a 5% (w/w) sodium chloride solution was added and 5 mL of hexane was used to extract fatty acid methyl esters (FAME) twice. The hexane layer was washed with a 4 mL solution of 2% potassium bicarbonate (w/w). The hexane layer was collected, and dried over anhydrous sodium sulfate, and evaporated under nitrogen gas. FAME were re-suspended to a concentration of

0.1 mg mL^{-1} in isopropyl alcohol for analysis by ozonolysis/APPI-MS or in hexane for silver ion LC/APPI-MS/MS.

3.2.7. Ozonolysis/atmospheric pressure photo ionization - mass spectrometry*

In-line ozonolysis/APPI-MS analysis was performed on the pure mono-hydroxy octadecenoic FAME fraction from HSCCC for the determination of double bond positions as described by Sun *et al*. (2013). Briefly, the in-line system consisted of a 0.5 L Omnifit solvent bottle, a three-valve bottle cap and 15 cm of gas permeable Teflon AF-2400 tubing (0.5 mm o.d., 0.25 mm i.d.; Biogeneral Inc., San Diego, CA). The Teflon tubing was inserted into the ozone filled solvent bottle, with one end connected to the LC autosampler and the other end directly coupled to the APPI ion source of the mass spectrometer. Samples in 3 μL aliquots were injected into a 0.2 mL min^{-1} flow of hexane/isopropanol (98:2, v/v) in the Teflon tubing. After being subjected to ozonolysis, FAME were directly analyzed by APPI/MS operating in positive ion mode.

3.2.8. Combined liquid chromatography/atmospheric pressure photo ionization - tandem mass spectrometry

Un-derivatized fatty acid extracts, hydroxylated fatty acids and FAME were analyzed by LC/APPI-MS/MS. All liquid chromatography was conducted on an Agilent 1200 series LC system (Agilent Technologies, Palo Alto, CA). Separations for un-derivatized and vicinal hydroxylated fatty acid derivatives were conducted on an YMC PVA-Sil column (150 mm × 2.0 mm i.d., 5 μm; Waters Ltd., Mississauga, Canada) at 25 °C. Lipid samples were injected

(injection volume, 2 µL) onto the column and eluted with a gradient of (A) 0.2% acetic acid in hexane and (B) 0.2% acetic acid in isopropanol at a flow rate of 0.2 mL min^{-1}. The gradient was as follows: 0 min 99% A; 20 min 70% A; 20.1 min 99% A; for a total run time of 27 min including equilibration. Silver ion chromatography was performed on lipid fractions as FAME using a Varian ChromSpher 5 Lipids column (250 mm × 2.0 mm i.d., 5 µm; Varian Inc., Palo Alto, CA) at 25 °C. The separation used an isocratic flow of 0.3 mL min^{-1} hexane containing 0.14% butyronitrile, a run time of 20 min and an injection volume of 2 µL.

Negative and positive ion APPI-MS/MS was performed on a QStar® Elite hybrid orthogonal Q-TOF mass spectrometer coupled to a PhotoSpray® source with Analyst® QS 2.0 software (Applied Biosystems/MDS Sciex, Concord, Canada). For negative ion-mode, the source and mass spectrometer conditions were: nebulizer gas 70 (arbitrary units), auxillary gas 20, curtain gas 25, ionspray voltage -1300 V, source temperature 400 °C, declustering potential (DP) -35 V, focusing potential -130 V and DP2 -13 V with a scan range of *m/z* 50 – 700. Collision induced dissociation (CID) was used nitrogen at a collision energy of 35 eV for un-derivatized fatty acids and 40 eV for vicinal hydroxylated fatty acids. The conditions used for positive-ion mode were: nebulizer gas 50 (arbitrary units), auxillary gas 10, curtain gas 25, ionspray voltage 1300 V, source temperature 380 °C, DP 30 V, focusing potential 130 V and DP2 5 V with a scan

range of *m/z* 100 – 1000. CID spectra of FAME were obtained using a collision energy of 20 eV.

Positive ion APPI-MS analysis of the FAME ozonolysis products were run under the same conditions except for a source temperature of 375 °C, DP 35 V, focusing potential 150 V and DP2 10 V and a scan range of *m/z* 100 – 1300. For analysis of FAME CLA, the flow was split 1:1 post-column between a UV detector at 242 nm and the mass spectrometer in order to distinguish CLA from linoleic acid.

3.2.9. Preparation of 4,4-dimethyloxazoline derivatives and gas chromatography/mass spectrometry analysis

4,4-Dimethyloxazoline **(**DMOX) derivatives of fatty acids were prepared as described by Fay and Richli (1991). Briefly, 0.25 g of 2-amino-2-methyl-1-propanol was added to 1 mg of pure fatty acid sample. The samples were flushed with nitrogen gas and reacted at 180 ºC for 2 h. After cooling to ambient temperature, the reaction mixture was dissolved in 5 mL dichloromethane and washed with 3 mL of water. The water layer was then extracted with 2 mL dichloromethane. The combined dichloromethane layer was dried with anhydrous sodium sulphate and evaporated under a stream of nitrogen gas. The derivatized DMOX fatty acid sample was dissolved in hexane to a concentration of 0.1 mg mL^{-1} prior to analysis by GC/MS using an Agilent 7890A gas chromatograph and Agilent 5975C mass-selective detector (Agilent Technologies Inc., Palo Alto, CA). A 2 µL sample volume was injected with a 50:1 split onto a HP-5 capillary column (30 m × 0.32 mm i.d., 0.25 µm film thickness; J & W Scientific Inc.,

Folsom, CA) at 300 ºC. The helium flow was kept constant at 1 ml min^{-1}. The oven program was as follows: the initial column temperature of 100 ºC was increased to 285 ºC at 10 ºC min^{-1}, after which, immediately to a final temperature of 300 ºC at 15 ºC min^{-1} for 3 min. Electron impact ionization was used at 70 eV electron energy and a mass scan range of *m/z* 40 – 500. An Agilent Chem Station E.02.02.1431 was used for data analysis.

3.2.10. Quantification of metabolites

For the quantification of hydroxy fatty acids, 200 µL of the supernatant from the centrifugation and filtration of bacterial cultures was extracted twice with 15% methanol in chloroform (v/v). The samples were spiked with 150 µg of a ricinoleic acid standard to measure extraction recovery. Extracts were adjusted to a volume of 5 mL with chloroform after addition of 25 µg of distearin as an internal standard. Calibration curves were established with 10-hydroxy-12-octadecenoic acid that was purified from *L. hammesii* cultures by HSCCC. The identity and purity of the standard was verified to be >99% by normal phase and reversed phase LC/MS, LC/ELSD and GC/MS. All samples were analyzed by negative ion LC/APPI-MS from triplicate independent experiments with triplicate technical repeats.

For the quantification of CLA in bacterial cultures, 2 mL of the supernatant was extracted twice with 15% methanol in chloroform (v/v) in the presence of 100 µg of *cis*-7-nonadecenoic acid recovery standard. The resulting extract was dried under nitrogen gas and methylated as described above. After the extraction

of FAME, 50 µg of linolenic methyl ester internal standard was added to the 10 mL total volume of hexane. External calibration curves were established with *cis*-7-nonadecenoic methyl ester and CLA methyl ester standards. All samples were analyzed by positive ion LC/APPI-MS from triplicate independent experiments with triplicate technical repeats.

3.3. Results

3.3.1. Development of a high-speed counter-current chromatography method for isolation of antifungal fatty acid

In our previous communication (Black *et al*., 2013) we identified antifungal activity from a hydroxy fatty acid fraction extracted from *L. hammesii*. In order to isolate a sufficient quantity of this antifungal fraction for structural analyses and further testing an HSCCC method was developed. A two phase solvent system was selected on the basis of recommendations by Ito (2005). Various combinations of biphasic solvent systems were equilibrated following this, linoleic, 12-hydroxy octadecanoic or 9, 10-dihydroxy octadecanoic acid standards were added, mixed, and left to settle at room temperature. Aliquots of the upper and lower phases were removed and analyzed by LC/MS and relative peak areas from these analyses were used to determine the partition coefficient ($K_{U/L}$). The $K_{U/L}$ of each analyte was calculated for each solvent system containing different ratios of hexane/ethyl acetate/methanol/water. The solvent system selected for the separation of lipids extracted from cultures of lactobacilli was hexane/ethyl acetate/methanol/water 3.5:1.5:3:2 (v/v/v/v) with a $K_{U/L}$ for linoleic, 12-hydroxy

octadecanoic and 9, 10-dihydroxy octadecanoic acid of 400, 1.4 and 1.0, respectively. Using this solvent system, the separation was monitored at 242 nm (**Appendix 2**, **Figure 2A-1**) and fractions were collected every three minutes and analyzed by flow injection APPI-MS (**Figure 3-1**).

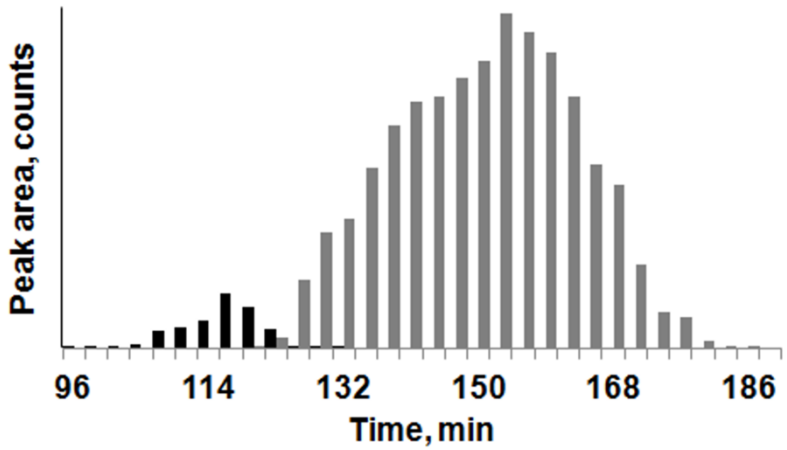

Figure 3-1. Flow injection APPI-MS analysis of fractions collected from HSCCC separations of *L. hammesii* lipid extracts. Black bars represent *m/z* 295.2273 ($C_{18}H_{31}O_3$); grey bars represent *m/z* 297.2403 ($C_{18}H_{33}O_3$), antifungal mono-hydroxy C18:1 fatty acid.

Over the elution times of interest, two major compounds were observed. The first, with a nominal *m/z* of 295, was shown previously to be a lipid oxidation product (Black *et al*., 2013). This eluted close to the antifungal mono-hydroxy fatty acid of *m/z* 297 (**Figure 3-1**). The fractions that did not contain the compound with *m/z* 295 were all combined to give the purified extract containing

the mono-hydroxy fatty acid. A 250 mg injection of crude lipid extract from *L. hammesii* fermentation yielded >55 mg of purified mono-hydroxy fatty acid in each HSCCC separation.

3.3.2. Analysis of hydroxy fatty acid fractions by liquid chromatography/atmospheric pressure photo ionization – mass spectrometry

Since the crude lipid extracts from fermentations of lactobacilli contain complex mixtures of fatty acids with various degrees of hydroxylation and unsaturation, a sophisticated analytical approach was needed to separate and identify individual compounds. Initially, LC separations in both normal and reversed phase modes were compared (**Figure 3-2A & Appendix 2**, **Figure 2A-2)**. It was found that a normal phase separation using hexane and isopropanol with acetic acid on a polymeric (polyvinyl alcohol) silica phase resulted in complete separation of mono-, di- and tri- hydroxy fatty acids and un-hydroxylated compounds (**Figure 3-2A**).

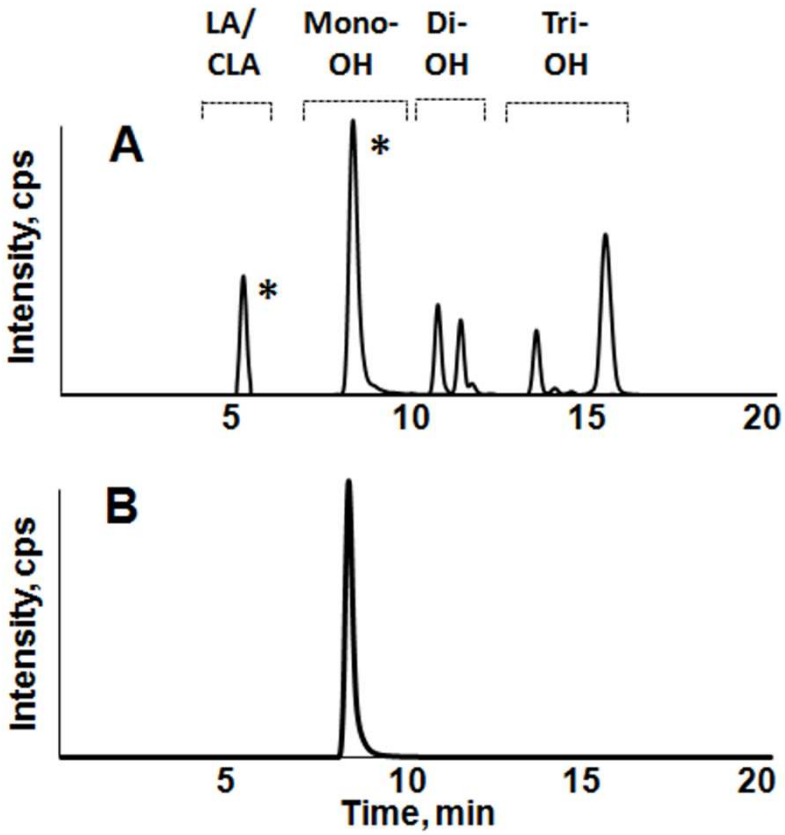

Figure 3-2. Silica LC/APPI-MS analysis of deprotonated un-derivatized fatty acids from *L. hammesii*. (A) XIC of *m/z* 279 – 331.5, encompassing the crude lipid extract profile before fractionation by HSCCC. Compounds that were produced by microbial transformation were labeled with an asterisk. (B) XIC of *m/z* 279 – 331.5, after fractionation by HSCCC. A single peak was collected, with an elution time of 8.4 min and a *m/z* 297.2403 ($C_{18}H_{33}O_3$).

Separation of many isomeric hydroxy fatty acids was also achieved with normal phase, where as reverse phase separation was less adequate for hydroylated fatty acids (**Appendix 2**, **Figure 2A-2**). However, such normal phase separations can be less amenable to analysis using electrospray ionization methods without post-column make-up flows of polar solvents, ideally aqueous. To overcome this, in

the present work we have exclusively used atmospheric pressure photoionization which does not require post-column solvent addition to assist in ionization. In addition, the hexane in the mobile phase acts as an APPI dopant due to its low ionization potential, so no addition of toluene or other dopant is required. Thus, it was found that normal phase LC/APPI-MS was highly suitable for the separation and mass spectrometric identification of hydroxy fatty acids. However, it should be noted that the APPI-MS response to linoleic acid and other non-hydroxylated fatty acids were considerably lower than those for hydroxylated compounds.

The LC/APPI-MS chromatogram of the lipid extract from *L. hammesii* before and after HSCCC fractionation was compared in **Figure 3-2A and 3-2B**. Only a single peak with retention time of 8.4 min was observed in the HSCCC purified material demonstrating the complete separation from the linoleic acid substrate as well as from more highly hydroxylated metabolites. The negative ion APPI mass spectrum averaged across the single peak seen in **Figure 3-2B** showed a single ion of nominal *m/z* 297 in addition to a background ion at *m/z* 59 assigned to acetic acid from the mobile phase. The measured mass for the observed $[M-H]^-$ ion at *m/z* 297.2410, corresponded to the exact mass of a mono-hydroxy octadecenoic acid (calculated *m/z* 297.2403).

3.3.3. Identification of the hydroxyl group location

3.3.3.1. Liquid chromatography/atmospheric pressure photo ionization – tandem mass spectrometry analysis

After collection by HSCCC, the antifungal hydroxy fatty acid produced by *L. hammesii* was analyzed by negative ion LC/APPI-MS/MS to identify the position

of the hydroxyl group. The MS/MS spectrum of the $[M-H]^-$ ion at *m/z* 297 showed two main fragment ions at *m/z* 279 and 185 **(Figure 3-3)**.

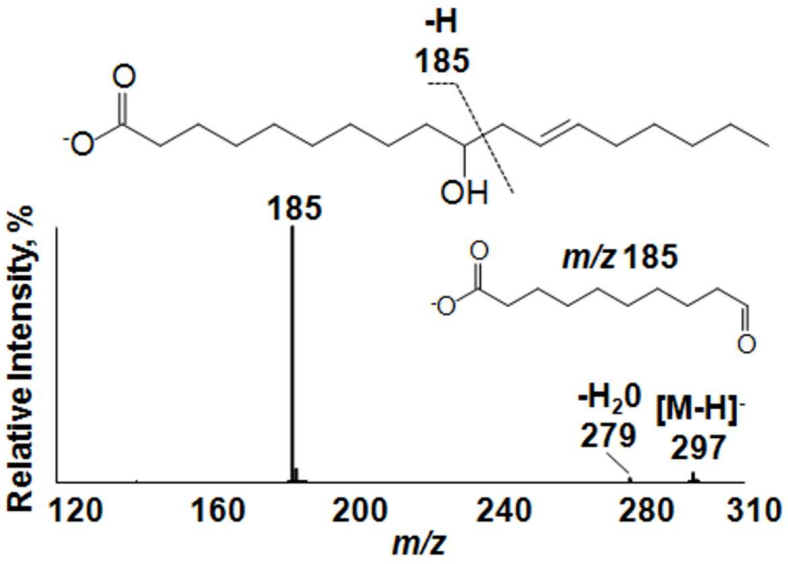

Figure 3-3. Silica LC/APPI-MS/MS spectrum of deprotonated un-derivatized fatty acid compound from *L. hammesii* with a *m/z* 297.2403 ($C_{18}H_{33}O_3$) isolated by HSCCC.

The ion at *m/z* 279 was due to loss of water from $[M-H]^-$, which was consistent with the presence of one hydroxyl group (Zhang *et al*., 2011). The fragment ion of *m/z* 185.1183, identified from the accurate mass as having an elemental composition of $C_{10}H_{17}0_3$, indicated the likely formation of an aldehyde at the Δ^{10} position of the hydroxy fatty acid (Moe *et al*., 2004). This implies that the hydroxyl group position was at Δ^{10}. However, the mass spectrum, which showed limited fragmentation, gave no indication of the location of the double bond.

3.3.3.2. Gas chromatography/mass spectrometry analysis

The hydroxy group position was further confirmed by derivatization of the fatty acid to a DMOX derivative for subsequent analysis by GC/MS in the electron impact mode of ionization. Derivatives such as DMOX, can be used in mass spectrometry to localize the charge, resulting in charge-remote fragmentation along the hydrocarbon chain (Dobson & Christie, 2002). This allows for simple interpretation of the mass spectra to determine the positions of functional groups and unsaturations. The mass spectrum of the DMOX derivative of the hydroxy fatty acid isolated from *L. hammesii* was shown in **Figure 3-4**. The intense ion at *m/z* 113 was characteristic of mass spectra of DMOX derivatives of fatty acids, due to a McLafferty rearrangement ion (Dobson & Christie, 2002). The series of fragment ions separated by 14 mass units going from *m/z* 126 to *m/z* 210 indicate successive CH_2 groups, i.e., a saturated hydrocarbon chain. A 30 mass unit difference occurred between fragmentations *m/z* 210 and 240 (with intermediate ion at *m/z* 224), indicating the presence of a hydroxyl group as shown in **Figure 3-4**.

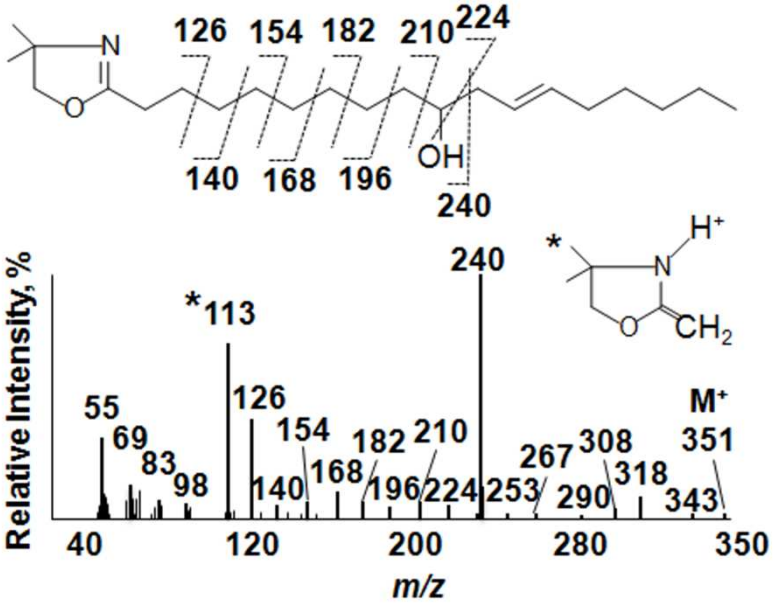

Figure 3-4. GC/MS mass spectra of 4,4-dimethyloxazoline derivative of mono-hydroxy C18:1 fatty acid from *L. hammesii*. The proposed McLaffertty ion at *m/z* 113 is designated with an asterisk.

The general features of this spectrum , including the high abundance of the ion at *m/z* 240 resulting from fragmentation adjacent to the hydroxyl group, are consistent with that seen for the isomeric DMOX derivative of 9-hydroxy-12-octadecenoic acid (Christie, 2013). Thus, analysis of the DMOX derivative of the fatty acid metabolite from *L. hammesii* confirms the hydroxyl group to be located at Δ^{10}. However, although the double bond location was shown to be closer to end of the chain than the hydroxyl group, consistent with the MS/MS spectrum, its location cannot be determined from the spectrum of the DMOX derivative.

3.3.4. Identification of double bond location

3.3.4.1. Liquid chromatography/atmospheric pressure photo ionization – tandem mass spectrometry analysis

The position of the double bond in the hydroxy fatty acid from *L. hammesii* was identified after vicinal hydroxylation. Following this derivatization procedure, analysis by LC/APPI-MS resulted in two peaks from the mono-hydroxy fatty acid with retention times of 13.2 and 13.5 min (**Figure 3-5A**). The MS/MS spectra of the $[M-H]^-$ ions from both peaks showed identical fragmentation patterns (only one of which is shown in **Figure 3-5**), suggesting stereoisomers. The exact mass of the deprotonated molecule of *m/z* 331.2484 and the consecutive triple water loss ions at *m/z* 313, 295 and 277 **(Figure 3-5B)** indicated the presence of three hydroxyl groups, consistent with the addition of hydroxyl groups across the double bond (**Figure 3-5 inset**). The fragment ion at *m/z* 185 in the MS/MS spectrum was the result of fragmentation adjacent to the original hydroxy group, consistent with the un-derivatized fatty acid spectrum in **Figure 3-3**. Considering the overall structure of a hydroxy fatty acid, the fragment ion at *m/z* 99.0815 ($C_6H_{11}O$) can only arise via cleavage that incorporates a single hydroxyl group.

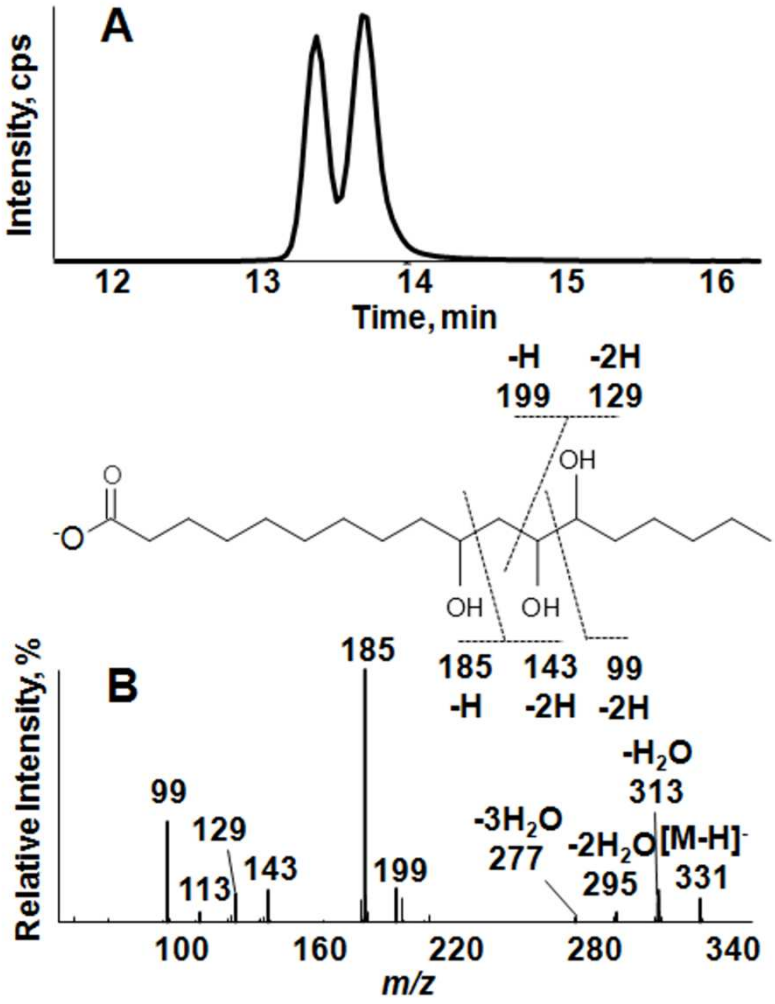

Figure 3-5. LC/APPI-MS analysis of deprotonated vicinal hydroxylation derivatized fatty acid from *L. hammesii*, exact mass *m/z* 331.2484 ($C_{18}H_{35}O_5$). (A) Silica separation of the hydroxylated mono-hydroxy C18:1 fatty acid collected from HSCCC, two peaks eluting at 13.2 and 13.5 min. (B) LC/APPI-MS/MS spectra of 10-hydroxy-12-octadecenoic acid, representative of both peaks.

This cannot be the original hydroxy group since this would have required multiple cleavages along the chain and was thus highly unlikely. Furthermore, the fragment ion at *m/z* 129, and complementary ion at *m/z* 199 located the second of

the vicinal hydroxyl groups in the derivative, as shown in **Figure 3-5**. Hence, the MS/MS spectra indicated that the double bond was located at the Δ^{12} position in the hydroxy fatty acid.

3.3.4.2. Ozonolysis/atmospheric pressure photo ionization – mass spectrometry analysis*

The position of the double bond at Δ^{12} was further confirmed by analysis of the methyl ester derivative of the hydroxy fatty acid using in-line ozonolysis/APPI-MS (Sun *et al*., 2013). In this method, ozonolysis which cleaves double bonds, resulting in characteristic aldehydes that were indicative of double bond positions, was performed in-line with APPI-MS. The degree of ozonolysis was adjusted such that both the molecular ion $[M+H]^+$ and ozonolysis fragments were seen. In the ozonolysis/APPI-MS spectrum of the FAME derivative of the mono-hydroxy fatty acid from *L. hammesii*, the $[M+H]^+$ ion and subsequent water loss indicated a methylated mono-hydroxy fatty acid (**Figure 3-6A)**. The abundant ion at *m/z* 227 corresponded to the formation of an aldehyde following ozonolysis and subsequent neutral loss of water. This was indicative of a double bond at Δ^{12} (**Figure 3-6B)**. The ion at *m/z* 195 was due to a further methanol loss from the protonated aldehyde ion at *m/z* 227, which was often observed in previous ozonolysis experiments (Sun *et al*., 2013) (**Figure 3-6C)**. Since oxidative cleavage of ozonolysis only occurs across carbon-carbon double bonds, in-line ozonolysis/ APPI-MS unambiguously assigned the double bond position at Δ^{12}, in agreement with the vicinal hydroxylation result.

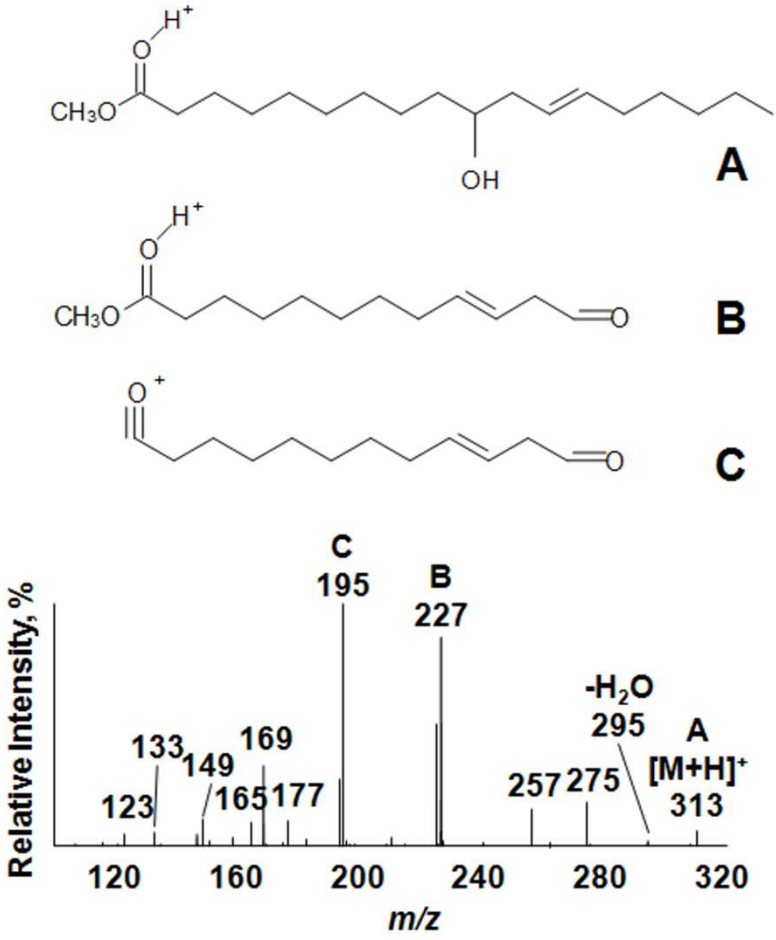

Figure 3-6. Ozonolysis/APPI-MS spectra of protonated methyl esters from *L. hammesii*; (A) $[M+H]^+$ *m/z* 313.2737; (B) fragment ion *m/z* 227.1642, proposed structure; (C) fragment ion *m/z* 195.1380, proposed structure.

In summary, LC/APPI-MS/MS analysis identified the antifungal hydroxy fatty acid produced by *L. hammesii* to be 10-hydroxy-12-octadecenoic acid; the hydroxyl group and double bond locations were confirmed using GC/MS and in-line ozonlysis/MS.

3.3.5. Identification of geometric isomers

As described above, normal phase separation of the purified hydroxylated derivative of 10-hydroxy-12-octadecenoic acid gave a pair of peaks with identical mass spectra (**Figure 3-5**). Therefore, silver ion chromatography was employed to investigate the possibility that these stereoisomers arose through the *syn* addition of hydroxyl groups by OsO_4 to both the *cis* and *trans* geometric isomers of the unsaturated hydroxy fatty acid. The purified 10-hydroxy-12-octadecenoic acid from *L. hammesii* was collected by HSCCC, derivatized to FAME and analyzed by silver ion LC/APPI-MS/MS. The extracted ion chromatogram (XIC) of the $[M+H]^+$ ion at *m/z* 295 gave two well resolved peaks, as shown in **Figure 3-7**. The MS/MS spectra of the $[M+H]^+$ ions were identical for each of the two peaks (**Figure 3-7)**, implying isomeric compounds. It is well known that the interaction between silver ions and double bonds in the *trans* configuration are weaker than those with *cis* double bonds (Dobson *et al*., 1995). Thus, the two peaks eluting at 15.0 and 17.7 min can be identified as methylated *trans* and *cis* 10-hydroxy-12-octadecenoic acid, respectively. The *cis* isomer accounted for 53 ± 5% of the total peak area of the *m/z* 295 extracted ion chromatogram whilst the *trans* isomer accounted for 47.4 ± 5%. This indicates that the 10-hydroxy-12-octadecenoic acid produced by *L. hammesii* was a racemic mixture of *cis* and *trans* isomers.

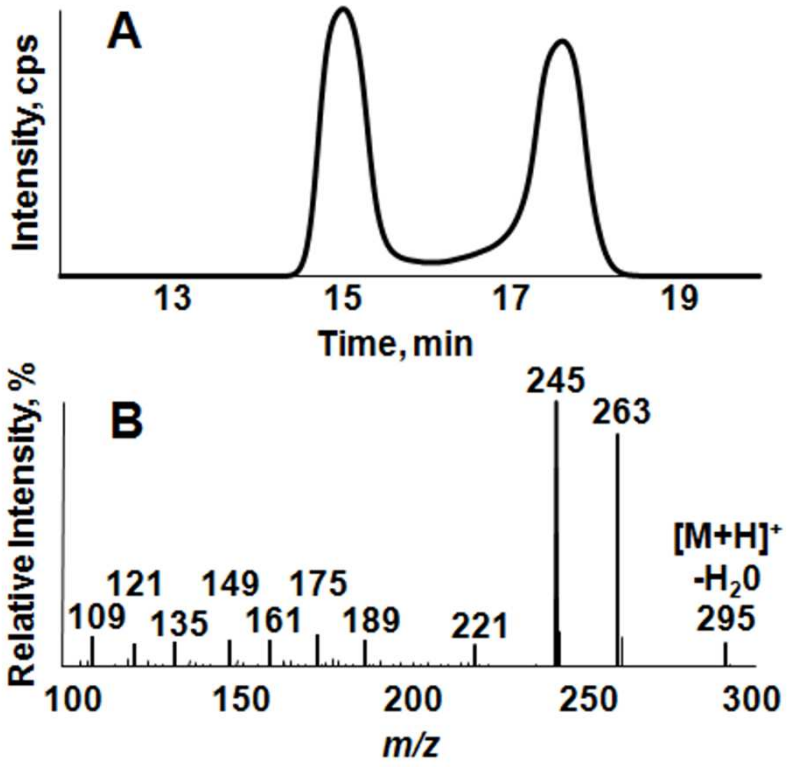

Figure 3-7. (A) XIC of a silver ion LC/APPI-MS chromatogram of methyl ester geometric isomers from *L. hammesii*, product ion of *m/z* 295.2; (B) MS/MS spectra representative of both methylated *trans* isomer at 15.0 min and methylated *cis* isomer at 17.7 min.

3.3.6. Analysis of the lipid extract from *L. plantarum* by liquid chromatography/atmospheric pressure photo ionization – tandem mass spectrometry

The normal phase LC/APPI-MS/MS technique was further applied to analyze the crude lipid extract of *L. plantarum*, fermented in broth supplemented with linoleic acid. Comparison to cell free or substrate free controls revealed that *L. plantarum* produced three hydroxy fatty acids and several CLA isomers. Two of these hydroxy fatty acids (one mono- and one di-hydroxy compound) were not

produced by *L. hammesii* under similar conditions (compare **Figure 3-8A** and **Figure 3-2A)**.

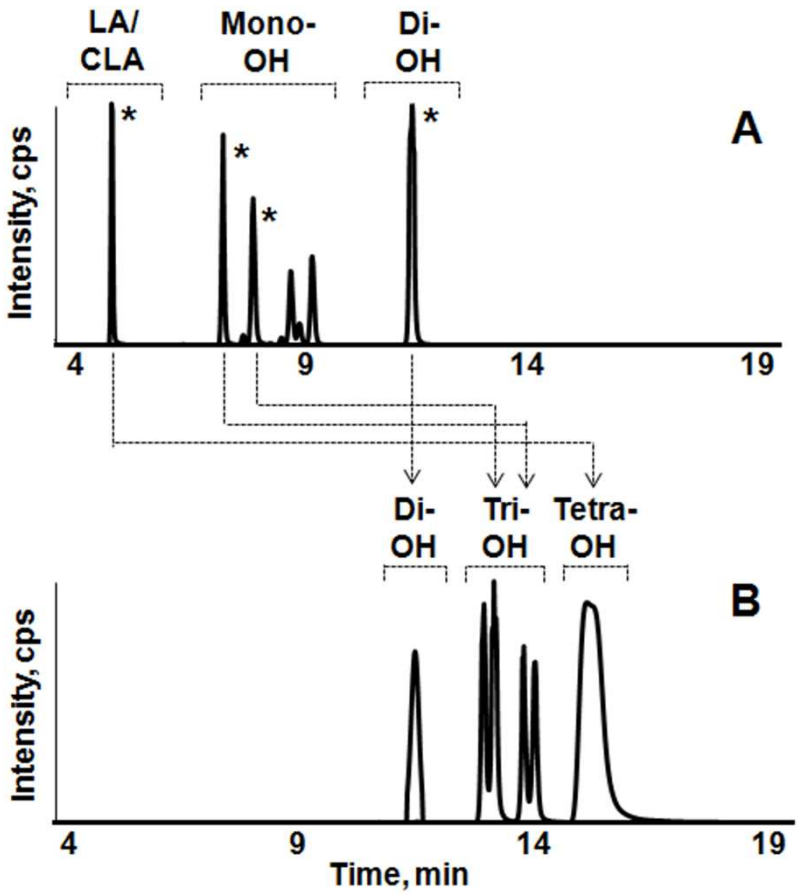

Figure 3-8. Silica LC/APPI-MS chromatograms of crude lipid extract from *L. plantarum*. (A) XIC of *m/z* 279 – 331.5, un-derivatized fatty acids. CLA and linoleic acid (LA) co-eluted at 5.1 min. While compounds produced from microbial transformation were a secondary mono-hydroxy C18:1 fatty acid eluting at 7.8 min and 10-hydroxy-12-octadecenoic acid eluting at 8.4 min, both with an exact mass of *m/z* 297.2403 ($C_{18}H_{33}O_3$), and a saturated di-hydroxy fatty acid eluting at 11.8 min with a mass of *m/z* 315.2535 ($C_{18}H_{35}O_4$). Compounds that were produced by microbial transformation were labeled with an asterisk. (B) XIC of *m/z* 279 – 348.5, hydroxylated derivatives of crude lipid extract. Resulting

peaks consisted of a saturated di-hydroxy fatty acid with a retention time of 11.8 min, *m/z* 315.2535 ($C_{18}H_{35}O_4$); saturated tri-hydroxy fatty acids, two pairs of peaks at 13.2 & 13.5 min and 14.0 & 14.3 min with a *m/z* of 331.2484 ($C_{18}H_{35}O_5$); and an unresolved group of saturated tetra-hydroxy fatty acids with a mass of *m/z* 348.2517 ($C_{18}H_{36}O_6$).

Without fractionation or derivatization, LC/APPI-MS of the *L. plantarum* lipid extract shows a peak at 11.8 min ($[M-H]^-$ at *m/z* 315.2535, $C_{18}H_{35}O_4$); the MS/MS spectrum of this peak (**Figure 3-9**) identifies it as 10, 13-dihydroxy octadecanoic acid.

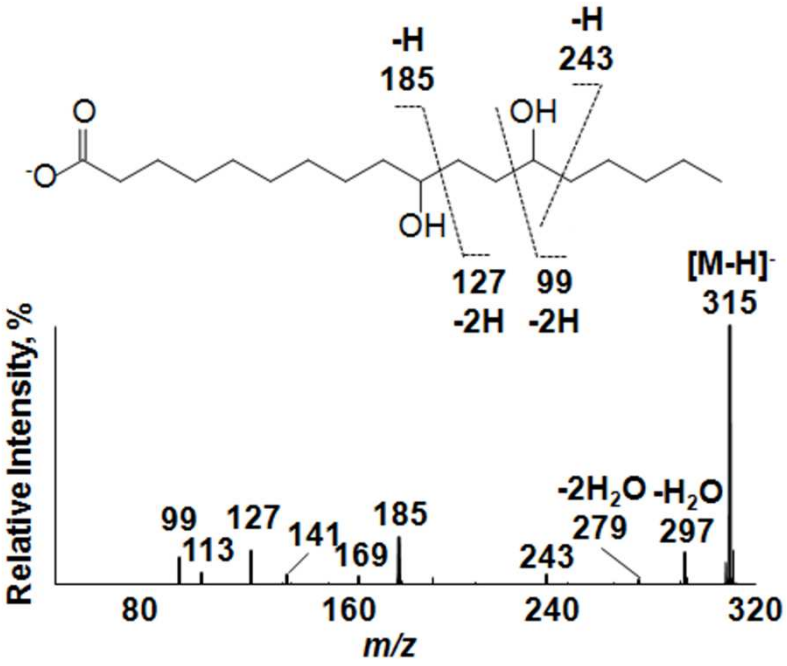

Figure 3-9. APPI-MS/MS spectra of 10, 13-dihydroxy octadecenoic acid from *L. plantarum*, eluting at 11.8 min, *m/z* 315.2535 ($C_{18}H_{35}O_4$).

The second compound eluting at 7.8 min in **Figure 3-8A** was identified as a mono-hydroxy octadecenoic acid isomer (*m/z* 297.2403, $C_{18}H_{33}O_3$); its MS/MS spectrum (**Figure 3-10**) was distinct to that of 10-hydroxy-12-octadecenoic acid (**Figure 3-3**).

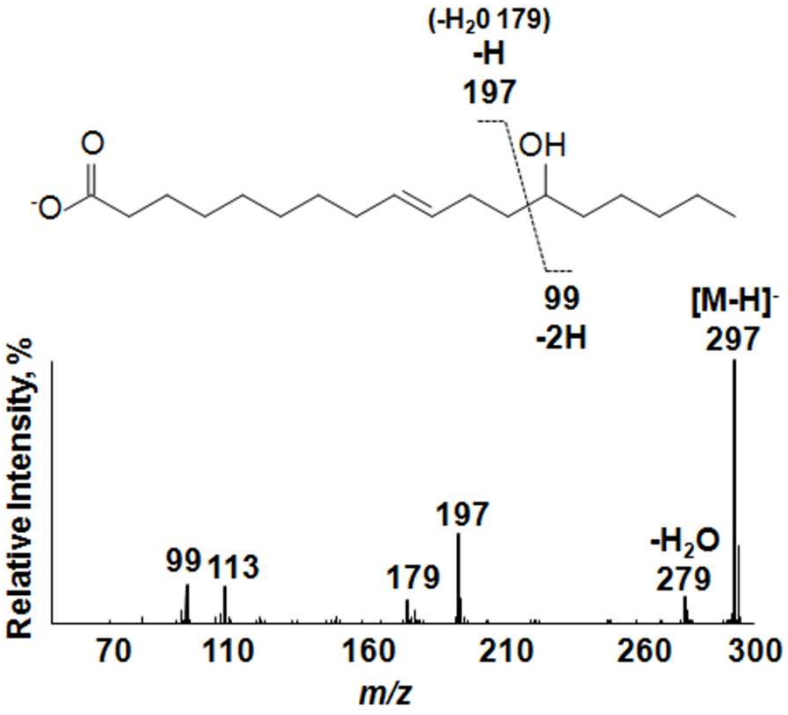

Figure 3-10. APPI-MS/MS spectra of deprotonated un-derivatized mono-hydroxy C18:1 fatty acid from *L. plantarum* eluting at 7.8 min, *m/z* 297.2403 ($C_{18}H_{33}O_3$).

The fragment ion at *m/z* 99.0815 ($C_6H_{11}O$) and complimentary ion at *m/z* 197.1547 ($C_{12}H_{21}O_2$) identified the hydroxyl group position as Δ^{13} but the MS/MS spectrum can only locate the double bond to between Δ^2 and Δ^{12}. Hence, vicinal hydroxylation of the whole crude lipid extract was performed. The LC/APPI-MS chromatogram (**Figure 3-8B)** of the resulting hydroxylated derivatives showed

that while 10, 13-dihydroxy octadecanoic acid was unaffected (retention time 11.8 min), the unsaturated compounds, such as linoleic acid/CLA and the mono-hydroxy fatty acids were shifted to longer elution times due to the addition of hydroxyl groups across double bonds. Thus, mono-hydoxy C18:1 compounds were converted to diastereomers of saturated tri-hydroxy fatty acids (*m/z* 331.2484, $C_{18}H_{35}O_5$). At the same time CLA isomers were converted to tetra-hydroxy derivatives. Additionally, from the *L. plantarum* fermented crude lipid extract, a separate peak pair resulted from the derivatization at retention times of 14.0 and 14.3, each with an identical MS/MS spectrum (**Figure 3-11**) which indicates the double bond position at Δ^9 from diagnostic fragment ions at *m/z* 201, 171 and 157.

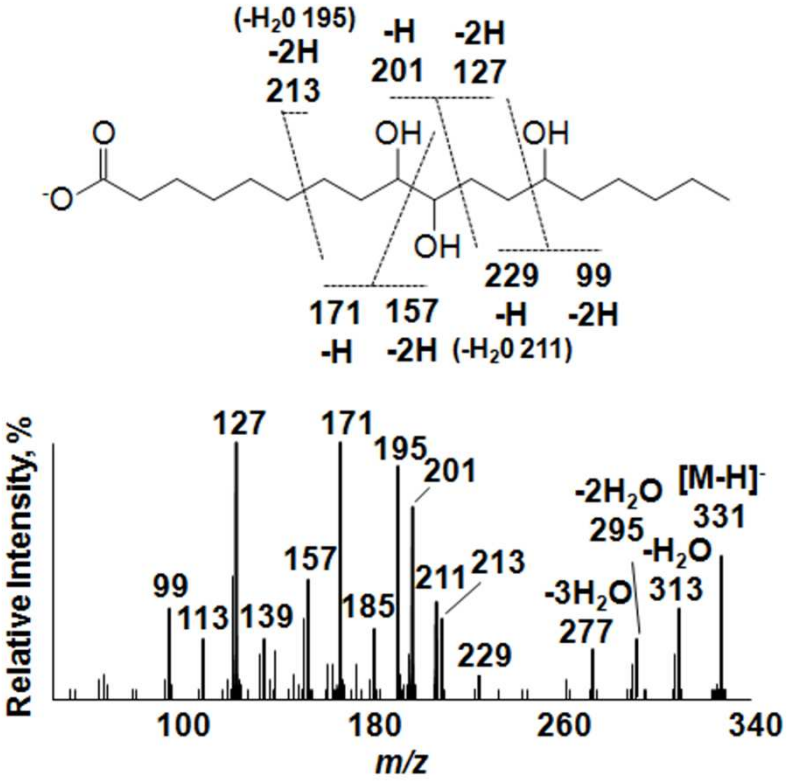

Figure 3-11. APPI-MS/MS spectra of deprotonated vicinal hydroxylation derivatized 13-hydroxy-9-octadecenoic acid from *L. plantarum*, representative of both peaks at 14.0 & 14.3 min, *m/z* 331.2484 ($C_{18}H_{35}O_5$).

Hence, in the case of mono-hydroxy C18:1 fatty acid produced by *L. plantarum*, the combination of normal phase LC/APPI-MS/MS with vicinal hydroxylation was used to identify 13-hydroxy-*cis*/*trans*-9-octadecenoic acid, without fractionation of the crude lipid extract.

3.3.7. Pathway of conversion

Conversion of linoleic acid by lactobacilli produced *cis/trans* 10-hydroxy-12-octadecenoic acid and CLA (**Figure 3-12**).

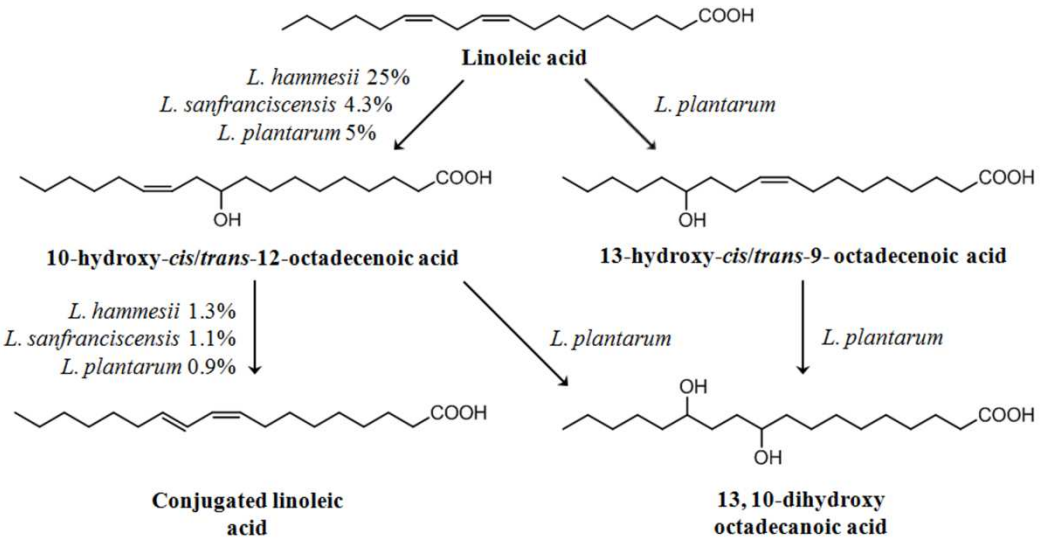

Figure 3-12. A proposed scheme of two alternate pathways for the bio-conversion of linoleic acid by lactobacilli after 2 d. Each percentage represents the amount of compound converted of the original 4 g L^{-1} linoleic acid used for supplementation. Reactions are not reversible.

L. hammesii converted 25% (1.0 ± 0.1 g L^{-1}) of the substrate to 10-hydroxy-12-octadecenoic acid; other lactobacilli tested also produced this metabolite including *L. sanfranciscensis* and *L. plantarum* which converted 4.3% (0.17 ± 0.0 g L^{-1}) and 5% (0.2 ± 0.0 g L^{-1}), respectively. All strains produced racemic mixtures of the *cis* and *trans* isomers. After 48 h of fermentation with *L. hammesii*, *L. sanfranciscensis* and *L. plantarum*, CLA isomers were observed (**Figure 3-13**; **Appendix 2** for structural identification with ozonolysis/MS) and accounted for 1.3% (0.1 ± 0.0 g L^{-1}), 1.1% (0.04 ± 0.01 g L^{-1}), and 0.9% (0.04 ± 0.0 g L^{-1}) of the initial substrate (**Figure 3-12)**.

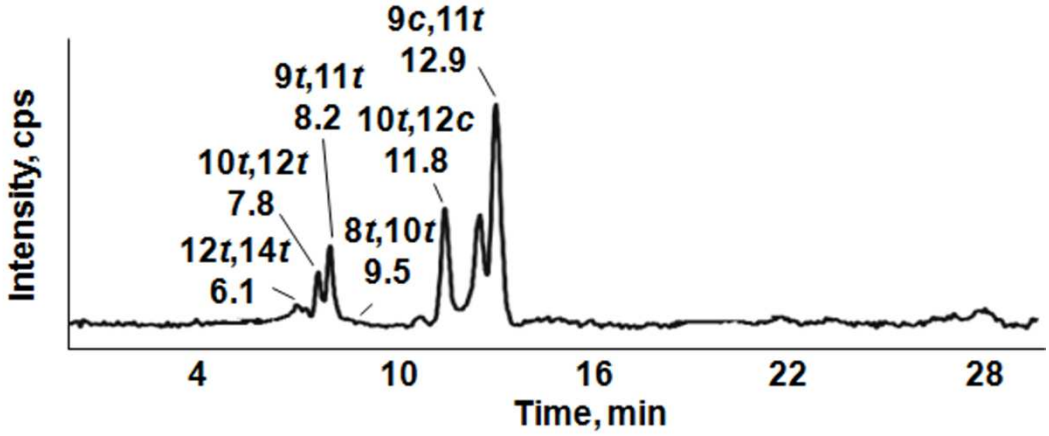

Figure 3-13. Ag^{+} – LC/APPI-MS separation of *L. plantarum* FAME. XIC of *m/z* 295 showing CLA isomers.

L. plantarum converted linoleic acid to *cis/trans* 13-hydroxy-9-octadecenoic acid and 10, 13-dihydroxy octadecanoic acid in addition to 10-hydroxy-12-octadecenoic acid and CLA. These metabolites were not found in lipid extracts from cultures of *L. hammesii* and *L. sanfranciscensis*.

To determine whether the conversion of linoleic acid to hydroxy fatty acids and CLA was reversible, cultures were grown in presence of 4 g L^{-1} CLA or purified *cis/trans* 10-hydroxy-12-octadecenoic acid. 10-Hydroxy-12-octadecenoic acid was converted to CLA by all three strains but formation of linoleic acid from 10-hydroxy-12-octadecenoic acid was not observed. CLA was not further transformed by any of the three strains. Hence, the conversion of linoleic acid to hydroxy-fatty acids and further to CLA was found to be irreversible.

The enzymatic transformation of ricinoleic acid by *L. hammesii*, *L. sanfranciscensis,* or *L. plantarum* resulted in a saturated di-hydroxy fatty as sole

product. Conversion of linolenic acid yielded a 10-hydroxy di-unsaturated fatty acid as the sole product, indicating a preference for hydration of double bond located at Δ^9. These results confirm that hydratases of lactobacilli accept alternative substrates, however, only linoleic acid supported the formation of CLA.

3.4. Discussion

In this study, LC/APPI-MS/MS was used to effectively elucidate the structure of antifungal 10-hydroxy-*cis*-12-octadecenoic and 10-hydroxy-*trans*-12-octadecenoic acids, and to quantify metabolites of linoleic acid. APPI-MS was found to be sufficiently sensitive to allow observation of un-derivatized fatty acids and their hydroxyl-derivatives from bacterial extracts. With the inclusion of hexane in the LC mobile phase, the ionization energy of the mobile phase itself was low enough that a post-column addition of a dopant was not necessary for the photoionization of fatty acids. No derivatization was required for both the identification of hydroxyl group positioning and the quantification of deprotonated fatty acids via silica LC/APPI-MS and tandem mass spectrometry. However, the addition of hydroxyl groups across the unsaturations was necessary to determine the position of the double bonds.

HSCCC was utilized as an efficient semi-preparative separation for antifungal metabolites from lactobacilli extracts. The identification of hydroxy fatty acids with silica LC/APPI-MS/MS did not require a pre-step fractionation

with HSCCC, as the chromatography produced baseline resolution from crude lipid extracts of food and media fermented with lactobacilli (Black *et al*., 2013). Thus, when compared to previous fractionation and spectroscopic identification methods used for lactobacilli extracts (Ogawa *et al*., 2001), much less effort was required for the fractionation and identification of hydroxy fatty acids by the methods described above. In addition, the data were more informative than that obtained from GC/MS methods (Volkov *et al*., 2010; Kishino *et al*., 2011; Yang *et al*., 2013).

10-Hydroxy-12-octadecenoic acid inhibits growth of *Aspergillus niger* and *Penicillium roqueforti* at concentrations of 0.7 g L^{-1} and 0.1 g L^{-1}, respectively, and conversion of linoleic acid to 10-hydroxy-12-octadecenoic acid during growth of *L. hammesii* in sourdough delayed fungal spoilage of bread (Black *et al*., 2013). The *cis/trans* configuration of hydroxy fatty acids did not influence their antifungal activity (Kobayashi *et al*., 1987). However, the positioning of hydroxyl groups and double bonds strongly affected the antifungal activity of hydroxy-fatty acids (Kobayashi *et al*., 1987; Black *et al*., 2013). The LC/APPI-MS/MS methods that were developed in this study were essential for the identification of these antifungal metabolites of linoleic acid.

The first step of the conversion of linoleic acid by lactic acid bacteria is catalyzed by hydratases. Hydratases of lactic acid bacteria recognize oleic, linoleic, linolenic, and ricinoleic acid as substrates. They preferentially hydrate double bonds at Δ^9 and are specific for free fatty acids (Kishino *et al*., 2009;

Volkov *et al*., 2010; Yang *et al*., 2013). Our data thus conforms to known properties of hydratases and additionally demonstrates that the conversion is irreversible. Remarkably, hydratases of lactic acid bacteria do not hydrate *trans* double bonds (Volkov *et al*., 2010; Yang *et al*., 2013) even though unsaturated hydroxy fatty acids were produced as a racemic mixture of *cis* and *trans* isomers (this study). Linoleic acid was converted to 10, 13-dihydroxystearic acid by *L. plantarum* via 10-hydroxy-12-octadecenoic acid and 13-hydroxy-9-octadecenoic acid, but not by the other strains used in this study. It remains unknown whether the observed differences in the profile of fatty acid metabolites from different lactobacilli relate to functional differences of the hydratase enzymes or other species- or strain-specific differences.

Hydroxy fatty acids produced by lactic acid bacteria were previously considered as intermediates of CLA formation. However, a majority of reports on CLA formation by lactobacilli used buffer fermentations with high cell densities (Ogawa *et al*., 2001; Kishimoto *et al*., 2003; Kishino *et al*., 2009). This study provides data on linoleic acid metabolites produced by growing cells, representing physiological conditions. Our observations demonstrate that hydroxy fatty acids are the major product of conversion while CLA levels remain low. Lactobacilli increase the proportion of hydroxy fatty acids in membrane lipids in response to environmental stress (Fernández Murga *et al*., 1999), indicating a physiological role in the protection against heat stress and membrane-active inhibitors (O'Flaherty & Klaenhammer, 2010). A physiological role of CLA, or the

presence of CLA in bacterial membranes, remains to be demonstrated. Moreover, formation of CLA by purified enzymes from lactic acid bacteria has not yet been demonstrated. Recently, CLA formation from hydroxy fatty acids by *L. plantarum* was attributed to the combined activity of an alcohol dehydrogenase and acetolactate decarboxylase (Kishino *et al*., 2011). Taken together, CLA production from hydroxy-fatty acids likely occurs as an enzymatic side reaction of metabolic enzymes that moonlight as linoleic acid isomerases. Comparable to the formation of hydroxy fatty acids by lactobacilli, the formation CLA by lactobacilli yields a mixture of *cis*-9, *trans*-11 and *trans*-9, *trans*-11 geometric isomers (Kishino *et al*., 2003; Kishino *et al*., 2011). However, the previous analytical methods were not sufficiently sensitive and/or specific and many isomers were potentially not accounted for. To date, the biological function of most CLA isomers remains unknown (Park, 2009). In this work, six CLA isomers were shown to be produced by *L. hammesii* and *L. plantarum*. The analytical methods developed in this study can in the future be used in optimizing the production of antifungal fatty acids in food fermentations while monitoring the production of CLA isomers of unknown biological activity.

The antifungal activity of 10-hydroxy-12-octadecenoic acid has initially been demonstrated in sourdough bread (Black *et al*., 2013). However, lactobacilli are commonly utilized in food fermentations (Hammes & Hertel, 2009; Black *et al*., 2013) and production of 10-hydroxy-12-octadecenoic acid was observed for several other food-fermenting lactobacilli (Kishino *et al*., 2009; Black *et al*.,

2013). Antimicrobial metabolites produced by lactobacilli during fermentation are considered as “natural” and have potential to be used in many food applications. Within this study we demonstrate a rapid and sensitive identification method as well as the means to collect increased quantities of 10-hydroxy-12-octadecenoic acid.

3.5. References

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CHAPTER 4

4. Structural identification of novel oligosaccharides produced by *Lactobacillus bulgaricus* and *Lactobacillus plantarum*[3]

4.1. Introduction

Galacto-oligosaccharides (GOS) are recognized as prebiotics that support digestive and immune health. β-GOS consist of one to four β-linked galactose moieties with galactose or glucose located at the reducing end. GOS other than lactose are not digested in the small intestine, and have a low caloric content and prebiotic activity (Macfarlane *et al*., 2008; Oku & Nakamura, 2002). In lactose-intolerant individuals, lactose also remains undigested in the small intestine and is fermented in the colon (Venema, 2012). Prebiotic activity promotes the growth of beneficial bacteria in the gut, which displaces pathogenic bacteria (Macfarlane *et al*., 2008). Dietary indigestible oligosaccharides can also prevent the adhesion of pathogens and toxins to epithelial cell walls by acting as receptor analogues (Kulkarni *et al*, 2010). The interaction between pathogen and glycans on the epithelial surface is highly specific for the carbohydrate structure. For example, both Shiga toxin 1 and Shiga toxin 2e (Stx2e) produced by *Escherichia coli* respond to globotriaosylceramide as a glycan receptor; however, only Stx2e will also recognize globotetraosylceramide (Johannes & Römer, 2010). Owing to the recognition of globotetraosylceramide by Stx2e, this toxin causes disease in

[3] A version of this chapter has been published. Black, B. A., Lee, V. S. Y., Zhao, Y. Y., Hu, Y., Curtis, J. M., & Gänzle, M. G. (2012). Journal of Agricultural and Food Chemistry. 60, 4886-4894.

Asterisks (*) indicate contributions by V.S.Y. Lee & Y.Y. Hu

piglets which are resistant to other Shiga-like toxins as the corresponding glycan receptor on the cell surface is lacking (Johannes & Römer, 2010).

The inhibition of pathogen adhesion by GOS has been demonstrated both *in vitro* and *in vivo* (Shoaf *et al*., 2006). *In vivo*, the oral delivery of GOS produced by *Bifidobacterium bifidum* significantly reduced colonization of *Salmonella enterica* serovar Typhimurium in mice (Searle *et al*., 2009). These benefits could be relevant for human health if the amounts of GOS that are required for prevention of pathogen adhesion do not cause gastrointestinal distress.

GOS of varying chain lengths are synthesized through enzymatic transgalactosylation of lactose via β-galactosidases (β-Gal) (Torres *et al*., 2010; Gänzle, 2012). During lactose conversion by β-Gal, galactose is covalently linked to the active site of the enzyme and subsequently transferred to water. If lactose is present in excess, β-Gal will use lactose, galactose or glucose as an alternative galactosyl-acceptor to form GOS. Enzymatic galactosylation leads to the formation of β-(1→2)-, β-(1→3)-, β-(1→4)-, or β-(1→6)-linked GOS, and bacterial and fungal enzymes generally form more than one linkage type (Torres *et al*., 2010; Gänzle, 2012). The source of β-Gal affects the type of GOS produced, as different enzymes favor the formation of specific linkages, thus creating structurally distinct GOS (Gänzle, 2012). Transglycosylation of acceptor carbohydrates other than glucose or galactose yields galactose-containing oligosaccharides with the acceptor carbohydrates at the reducing end (Torres *et al*., 2010; Li *et al*., 2010; Gänzle, 2012). Composite oligosaccharides formed by

β-Gal are referred to as hetero-oligosaccharides (HeOS). HeOS increase the variation of oligosaccharide structures and may allow for novel applications to prevent pathogen adhesion. HeOS are also non-digestible oligosaccharides but are fermented by bacteria in the colon. Because HeOS remain undigested, they may also have other health benefits similar to GOS or oligosaccharides found in human milk (Kunz *et al*., 2000; Schwab & Gänzle, 2011).

Although no single analytical method can completely structurally identify oligosaccharides present in small amounts, mass spectrometry (MS) is an effective tool offering analytical diversity and high sensitivity. Mass spectrometry, or tandem mass spectrometry (MS/MS) which provides structural information through investigation of ion fragmentation, is often coupled to liquid chromatography, thereby adding an extra dimension of compound separation. Electrospray ionization (ESI) is a technique used in mass spectrometry to ionize biomolecules with limited fragmentation occurring in the ion-source. In particular, ESI can directly ionize underivatized neutral oligosaccharides, in either negative or positive ion modes, to achieve high-sensitivity detection and the generation of informative data used for structural identification (Chai *et al*., 2001; Pfenninger *et al*., 2002). Moreover, ESI can ionize saccharides over a range of molecular weights or degrees of polymerization (DP). For example, picogram levels of oligosaccharides with DP one to eleven were ionized by ESI and detected by an ion trap mass spectrometer (Liu *et al*., 2005). Combined ESI-MS/MS has been used to elucidate branching sequences or partial sequences of,

for example, neutral oligosaccharides from human milk of DP 10 and 11 (Chai *et al*., 2005).

Commercial GOS production relies on microbial *β*-Gal used at lactose concentrations close to saturation. Food grade organisms, including bifidobacteria and lactobacilli, have also been utilized to produce GOS (Nguyen *et al*., 2007; Macfarlane *et al*., 2008; Searle *et al*., 2009; Gänzle, 2012). The use of food-grade enzyme sources facilitates the process development for production of food ingredients (Splechtna *et al*., 2007). However, commercial and experimental GOS preparations are a mixture of mono- and oligosaccharides and are often poorly characterized with regard to their composition and the structure of individual compounds in the preparation (Nguyen *et al*., 2007; Macfarlane *et al*., 2008). LC/MS/MS methods for oligosaccharide analysis have not been employed and validated for characterization of GOS. Particularly, the linkage type and degree of polymerization of HeOS formed by *β*-Gal of lactobacilli remain unknown (Schwab *et al*., 2011). It was therefore the objective of this study to identify the structures of galactosylated N-acetyl-D-glucosamine and galactosylated fucose produced by *β*-Gal of lactobacilli. A LC/ESI-MS/MS method was established to identify oligosaccharides. A crude cellular extract of *Lactobacillus bulgaricus* ATCC 11842 was used as biocatalyst since this previously has been shown to produce GOS and HeOS from lactose (Schwab *et al*., 2011). *Lactococcus lactis* M1363 expressing LacLM of *L. plantarum* FUA3112 was additionally employed to assess the activity of a defined enzyme.

4.2. Materials and methods

4.2.1. Chemicals and standards

Oligosaccharide standards lacto-N-biose (Gal*β*-(1→3)-GlcNAc), N-acetyl-D-lactosamine (Gal*β*-(1→4)GlcNAc), *β*1-6 galactosyl-N-acetyl glucosamine (Gal*β*-(1→6)-GlcNAc) were purchased from Dextra Laboratories (Reading, UK). Fisher Scientific (Ottawa, Canada) supplied HPLC grade acetonitrile, methanol, ammonium acetate and crystalline sodium chloride. All other solvents were of analytical grade unless specified otherwise.

4.2.2. Sample production*

L. bulgaricus ATCC11842 and *L. plantarum* FUA3112 were cultivated under microaerophilic conditions (1% O_2, balance N_2) at 37 °C in modified DeMan-Rogosa-Sharpe (mMRS) (de Man *et al*., 1960) containing 5% w/v lactose with a pH of 6.2 (Schwab *et al*., 2011). *Lc. lactis* MG1363 harboring pAMJ586 with LacLM from *L. plantarum*, as sole source of *β*-Gal activity (Schwab *et al*., 2010) was grown in M17 with the addition of 0.5% glucose and 5 mg L^{-1} erythromycin at 30 °C. Cells were cultured for 12 h, harvested, and washed twice before suspension in 1 mL of 50 mM sodium phosphate buffer (pH 6.8) with 20% glycerol and 1 mM dithiothreitol. The cell suspension was transferred to screw-cap tubes with 0.5 mL of zirconia/silica beads (0.1 mm), and disrupted in a Mini Beadbeater-8 (model 693; BioSpec, Bartlesville, OK) for two passes of 1.5 min and chilled in ice between passes. The disrupted cells were centrifuged at 15 300 x g for 20 min at 4 °C. The supernatant, designated as crude cellular extract

(CCE), was used for GOS synthesis. The protein content of CCE was determined using the Bio-Rad Protein Assay (Bio-Rad); the specific activity (enzyme activity level relative to cell mass) was determined with *o*-nitrophenylgalactoside (Sigma, Oakville, Canada) as described (Schwab *et al*., 2010). In keeping with previous investigations (Schwab *et al*., 2010; Schwab *et al*., 2011), the *β*-Gal activities of CCE of *L. bulgaricus*, *L. plantarum,* and *L. lactis* expressing LacLM from *L. plantarum* ranged from 30 – 60 units (min × mg protein)$^{-1}$.

To analyze transglycosylation with CCE of *L. bulgaricus*, carbohydrate solutions were prepared with 23% w/v lactose in a 50 mM sodium phosphate buffer (pH 6.8) with 100 mM KCl and 2 mM $MgCl_2$ (PB). An acceptor carbohydrate of either N-acetyl-D-glucosamine (GlcNAc) or L-fucose was added in a concentration of 12% w/v to each of the solutions of lactose. Solutions were filter sterilized prior to the addition of 20% v/v CCE. Enzymatic reactions were conducted at 37 °C for 24 h, and terminated by heating to 95 °C for 15 min.

To analyze transglycosylation with CCE of *L. plantarum* or *Lc. lactis* expressing LacLM of *L. plantarum*, 20 µl CCE was mixed with 80 µl PB, 100 mM KCl and 1 mM $MgCl_2$ containing 0.5 M each of lactose and GlcNAc. Reactions were incubated at 45 °C for 16 h and terminated by heating at 95 °C.

4.2.3. High performance anion exchange chromatography with pulsed amperometric detection

High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) analyses were performed using an ICS-3000 system consisting of an AS50 autosampler, and a pulsed electrochemical

detector with a gold electrode and an Ag/AgCl reference electrode (Dionex Corp., Sunnyvale, CA). Sample oligosaccharide solutions were injected in 10 μL aliquots on to a CarboPac PA-20 Dionex carbohydrates column (3 x 150 mm) paired with a CarboPac PA-20 guard column (3 x 30mm) and were separated at a flow rate of 0.25 mL min^{-1} for 42 min at 25 °C. Eluents A (water), B (0.2 M sodium hydroxide), C (1 M sodium acetate) were used in the following gradient: 0 min 30.4% B, 1.3% C; 25 min 30.4% B, 15% C; 28 min 0%B, 50% C; 31 min 73% B,17% C; 31.1 min 30.4% B, 36.3% C; 34 min 73% B,17% C; 37 min 73% B,17% C; 40 min 30.4% B, 1.3% C.

4.2.4. Combined liquid chromatography/electrospray ionization – tandem mass spectrometry

Un-derivatized oligosaccharide fractions were analysed by LC/ESI-MS/MS. Separations were conducted on an Agilent 1200 series LC system (Agilent Technologies, Palo Alto, CA) at 25 °C using a Supelcosil LC-NH_2 column (250 mm x 4.6 mm i.d., 5 μm; Sigma Aldrich, Oakville, Canada). Each solution analyzed contained a final concentration of 0.1 g L^{-1} oligosaccharide in water. When the positive ion mode was used, 10 mM NaCl was supplemented to aid in ionization. Twenty-five microliters of each solution was injected onto the column using an isocratic flow of acetonitrile/water 80:20 (v/v) at a rate of 1 mL min^{-1}. The effluent from the column was split at a ratio of 1:4 (v/v), so that the flow rate to the mass spectrometer was 0.2 mL min^{-1} with the remainder going to waste. A post-column addition of ammonium acetate (40 mM in methanol) was delivered

at a rate of 0.02 mL min^{-1} by an Agilent 1200 series isocratic pump to the flow entering the ESI source.

Positive and negative ion ESI-MS and collision induced dissociation tandem mass spectrometry (CID-MS/MS) were performed on a QStar Elite hybrid orthogonal Q-TOF mass spectrometer coupled to a TurboIon Spray source with Analyst QS 2.0 software (Applied Biosystems/MDS Sciex, Concord, Canada). In negative ion mode, the source conditions were as follows: nebulizer gas, 50 (arbitrary units); ion spray voltage, -4500 V; curtain gas, 25; declustering potential, -45 V; focusing potential, -170 V; and declustering potential 2, -20 V, scanning over a mass range of *m/z* 50-600. Fragmentation was achieved using nitrogen as a collision gas at a collision energy that varied between -10 to -25 eV, optimized for each saccharide. Similarly, conditions for the optimal formation and analysis of positive ions were as follows: nebulizer gas, 45; ion spray voltage, 5000 V; curtain gas, 25; declustering potential, 45 V; focusing potential, 170 V; declustering potential 2, 20 V, with a scan range of *m/z* 50-1100. Collision energy for positive ions was optimal between 25 to 40 eV depending on the analyte. Auxillary gas flow was optimized at 60 arbitrary units and the source temperature at 400 °C for both negative and positive ion modes. Quasi MS^3 spectra were obtained from the MS/MS spectra of in-source fragment ions fromed by increasing the declustering potential by an additional 30 V. Estimation of the amounts of galactosylated GlcNAc compounds present in samples was achieved using calibration curves for the external standards, Galβ-(1→4)-GlcNAc and

Galβ-(1→6)-GlcNAc, with correction for the response of fucose added as an internal standard.

4.3. Results

4.3.1. Separation of galacto-oligosaccharides and hetero-oligosaccharides

HPAEC-PAD was first used to confirm the presence of HeOS formed by transgalactosylation of fucose and GlcNAc. A comparison between the chromatograms of the CCE enzymatic reactions carried out in presence of GlcNAc or fucose with those of a control reaction carried out in presence of lactose only confirmed the successful transfer of galactose to either fucose or GlcNAc as previously observed (Li *et al*., 2010). To obtain structural information of HeOS, LC/MS data was obtained to detect molecular species arising from HeOS produced by transgalactosylation of GlcNAc or fucose. A total of four HeOS formed by transgalactosylation of GlcNAc were observed in addition to five HeOS formed by transgalactosylation of fucose. In reactions containing GlcNAc as an acceptor, two disaccharides and two trisaccharides were identified (**Figure 4-1A**). Additionally, two disaccharides and three trisaccharides isomers were detected in reactions containing fucose as an acceptor (**Figure 4-1B**).

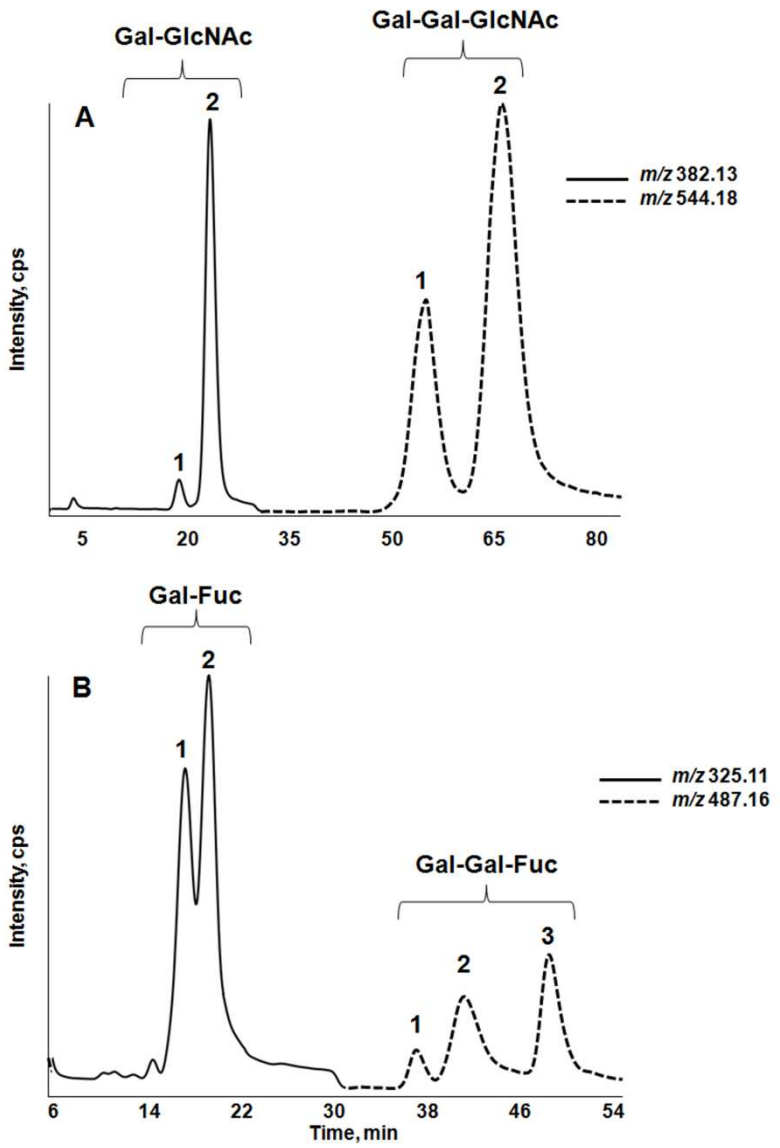

Figure 4-1. LC/ESI-MS extracted ion chromatogram (XIC) overlays of (A) the $[M-H]^-$ ions of Gal-GlcNAc *m/z* 382.13 and Gal-Gal-GlcNAc *m/z* 544.18 and (B) the $[M-H]^-$ ions of Gal-Fuc *m/z* 325.11 and Gal-Gal-Fuc *m/z* 487.16.

The composition of the HeOS species were confirmed by exact mass measurements in both positive and negative ion modes (**Table 4-1)**. The low mass accuracy for the fucose–HeOS molecular ions in negative ionization mode was

likely due to the low signal-to-noise ratio. However, the exact masses for the molecular ions of all compounds of interest were all determined with a better than 5 ppm (**Table 4-1**).

Table 4-1. Mass accuracy (of sodiated adducts and deprotonated molecules) and retention times of all HeOS formed between samples with either GlcNAc or Fucose added as acceptor carbohydrates.

			$[M+Na]^+$			$[M-H]^-$		
Acceptor CHO	**Compound**	**Retention Time (min)**	**Measured mass (Da)**	**Exact mass (Da)**	**Error (mDa)**	**Measured mass (Da)**	**Exact mass (Da)**	**Error (mDa)**
+ GlcNAc	**Gal-GlcNAc**	18.5; 23.2	406.1323	406.1319	0.3	382.1364	382.1354	0.9
	Gal-Gal-GlcNAc	55.2; 67.3	568.1850	568.1848	0.3	544.1883	544.1883	0.0
+ Fuc	**Gal-Fuc**	17.0; 19.1	349.1101	349.1105	-0.4	325.1150	325.1140	1.0
	Gal-Gal-Fuc	37.1; 41.0; 48.2	511.1635	511.1633	0.2	487.1694	487.1668	2.5

4.3.2. Structural identification of oligosaccharides with electrospray - mass spectrometry

Tandem mass spectrometry was then employed to elucidate monomer sequence and glycosidic linkage information. Three external standards (Galβ-(1→3)-GlcNAc, Galβ-(1→4)-GlcNAc, and Galβ-(1→6)-GlcNAc) were available as reference for GlcNAc-derived HeOS, but none were obtained for fucose-derived HeOS. The fragments that were observed from MS/MS experiments were labeled according to the nomenclature of Domon and Costello (1988). In this nomenclature, A-, B- and C-type fragments are those retaining the charge at the nonreducing end of the oligosaccharide, whereas X-, Y- and Z-type

fragments retain the charge at the terminal unit. A and X indicate cross-ring fragmentation; B, C, Y, and Z signify fragmentation of glycosidic linkages.

Both $[M+Na]^+$ and $[M-H]^-$ ions were detected in relatively high intensity for HeOS under either positive or negative ionization polarity, respectively. In general, the mass spectra of $[M-H]^-$ species gave more informative MS/MS spectra than did $[M+Na]^+$ ions. Hence, although, the MS/MS spectra of the $[M+Na]^+$ ions from HeOS samples did in some cases give some complementary fragmentation to that seen in negative ion mode, the more informative negative ion MS/MS analysis is the focus of the data interpretation presented below. Positive ion MS/MS spectra is presented in **Appendix 3**.

4.3.3. Disaccharides formed in the presence of an N-acetylglucosamine galactosyl-acceptor

A total of two isomers could be resolved by amino separation: Gal-GlcNAc peaks 1 and 2 (**Figure 4-1A**). Tandem mass spectrometry data for the two peaks present different fragmentation patterns indicating that Gal-GlcNAc isomers in peaks 1 and 2 have different glycosidic linkages (**Figure 4-2A** and **4-2B**; **Appendix 3**, **Figure 3A-1)**.

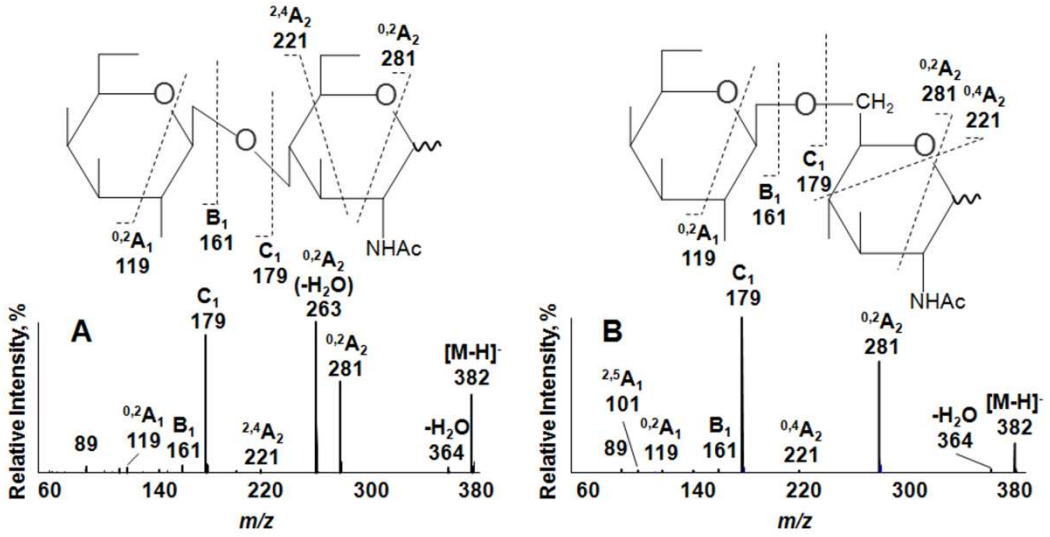

Figure 4-2. ESI-MS/MS spectra of $[M-H]^-$ ions of Gal-GlcNAc isomers. (A) Sample compound, Gal-GlcNAc peak 1; (B) Sample compound, Gal-GlcNAc peak 2.

Interglycosidic B_1 and C_1 fragments at *m/z* of 179 and *m/z* 161 respectively, present in both spectra, consistent with the monosaccharide sequence of Gal-GlcNAc. The retention time of the Gal-GlcNAc peak 1 was congruent with the retention time of a Gal*β*-(1→4)-GlcNAc standard, and the retention time of Gal-GlcNAc peak 2 was also congruent with the standard Gal*β*-(1→6)-GlcNAc. Compounds eluting at retention times corresponding to the Gal*β*-(1→4)-GlcNAc and Gal*β*-(1→6)-GlcNAc standards were also identified in the HPAEC-PAD analysis of total oligosaccharides (data not shown). To gain further information, the three available isomeric standards of Gal-GlcNAc were utilized under identical chromatographic and MS/MS conditions as those of compounds Gal-GlcNAc peaks 1 and 2. Panels A, B and C of **Figure 4-3** show the MS/MS

spectra of $[M-H]^-$ ions from the Galβ-(1→4)-GlcNAc, Galβ-(1→6)-GlcNAc, and Galβ-(1→3)-GlcNAc standards; sodiated spectra are shown in **Appendix 3**, **Figure 3A-2.**

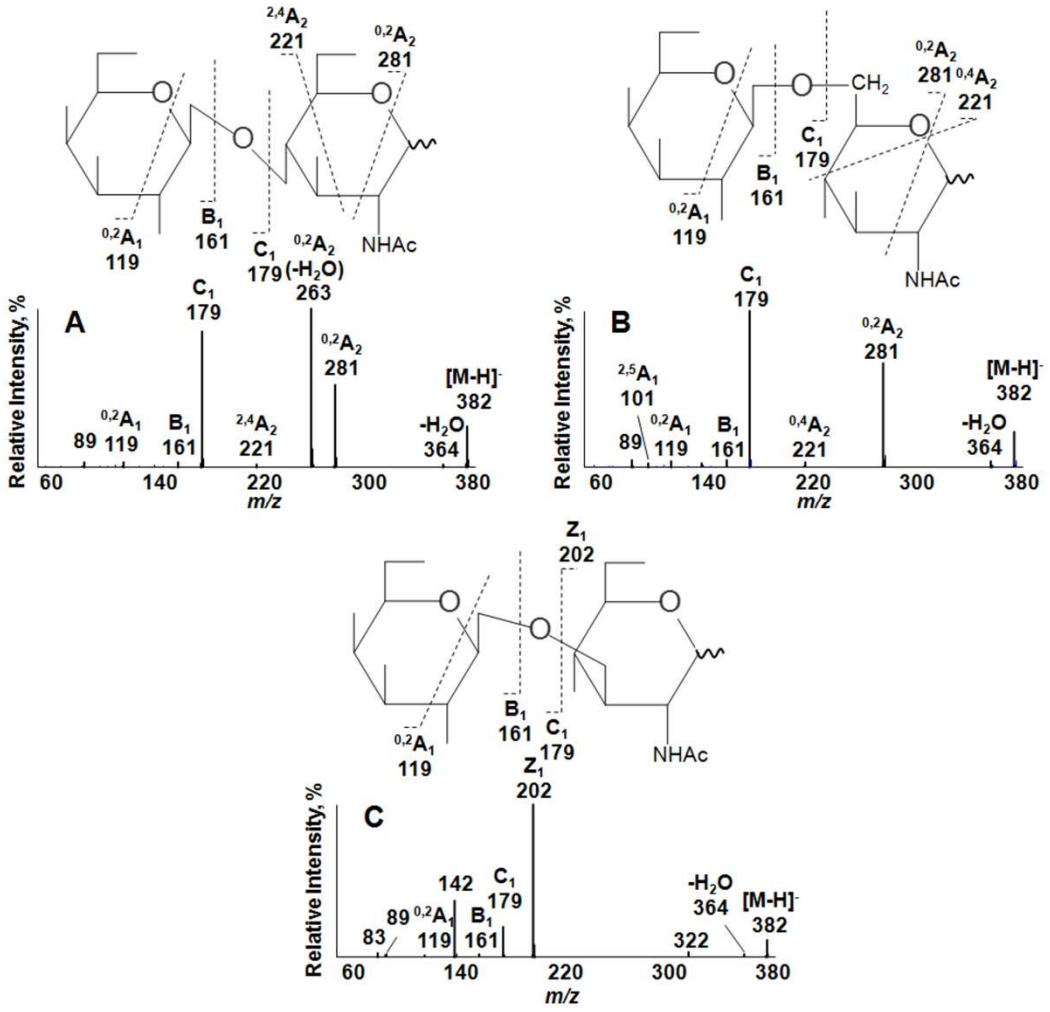

Figure 4-3. ESI-MS/MS spectra of $[M-H]^-$ ions representative of Gal-GlcNAc isomers: (A) Galβ-(1→4)-GlcNAc standard; (B) Galβ-(1→6)-GlcNAc standard; (C) Galβ-(1→3)-GlcNAc standard.

The MS/MS spectra of these isomers are distinctly different (**Figure 4-3**). Fragment ions seen in the MS/MS spectrum of the Gal*β*-(1→4)-GlcNAc standard match, in terms of both the ions present and their relative intensities, those seen in the Gal-GlcNAc peak 1 (**Figure 4-3A** and **Figure 4-2A**). Thus, the Gal-GlcNAc peak 1 was identified as being Gal*β*-(1→4)-GlcNAc. Similarly, fragment ions in the MS/MS spectra of the Gal*β*-(1→6)-GlcNAc standard closely matched those from Gal-GlcNAc peak 2 (**Figure 4-3B** and **Figure 4-2B**), thereby confirming the identity of the latter. In addition, both the retention time and the MS/MS spectra of standard Gal*β*-(1→3)GlcNAc differed from those of the Gal-GlcNAc peaks in samples containing HeOS.

It was noted that the spectra for the Gal*β*-(1→6)-GlcNAc standard was not entirely consistent with literature reports for other 1,6 linked oligosaccharides. In earlier reports, the MS/MS spectra of oligosaccharide anions, it has been indicated that 1,3 linkages display no ^{0,2}A cross-ring fragmentation; the presence of *m/z* 263 and *m/z* 281 and the absence of *m/z* 251 ions indicate a 1,4 linkage; and the presence of *m/z* 251 and *m/z* 281 but the absence of *m/z* 263 indicate a 1,6 linkage (Dallinga & Heerma, 1991; Carroll *et al*., 1993; Guan & Cole, 2008). In this work, the MS^2 spectrum of Gal*β*-(1→4)-GlcNAc exhibited a $^{0,2}A_2$ cross-ring fragment of *m/z* 281 and a water loss of *m/z* 263, indicating a 1,4 linkage and consistent with the above rules. However, the MS/MS spectrum of Gal*β*-(1→6)-GlcNAc presents the $^{0,2}A_2$ cross-ring fragment of *m/z* 281 with the absence of *m/z* 263 as expected, but in addition *m/z* 251 ions were not detected. This

inconsistency in the spectrum of Gal*β*-(1→6)-GlcNAc compared to literature data for 1,6-linked oligosaccharides is likely due to the effect of the N-acetyl group on the fragmentation pattern compared to hexoses moieties without this functional group. Furthermore, this same fragmentation pattern was observed using an authentic standard as reported in **Figure 4-3B** and **Figure 4-2B**.

4.3.4. Trisaccharides formed in the presence of an N-acetylglucosamine galactosyl-acceptor

The two distinct peaks seen in the extracted ion chromatogram of the $[M-H]^-$ ion of Gal-Gal-GlcNAc indicate the presence of two isomeric species (peaks 1 and 2 in **Figure 4-1A**). The MS/MS spectra of each of these isomers were found to have quite different fragmentation patterns (**Figures 4-4A** and **4-5A**; **Appendix 3**, **Figure 3A-3)**.

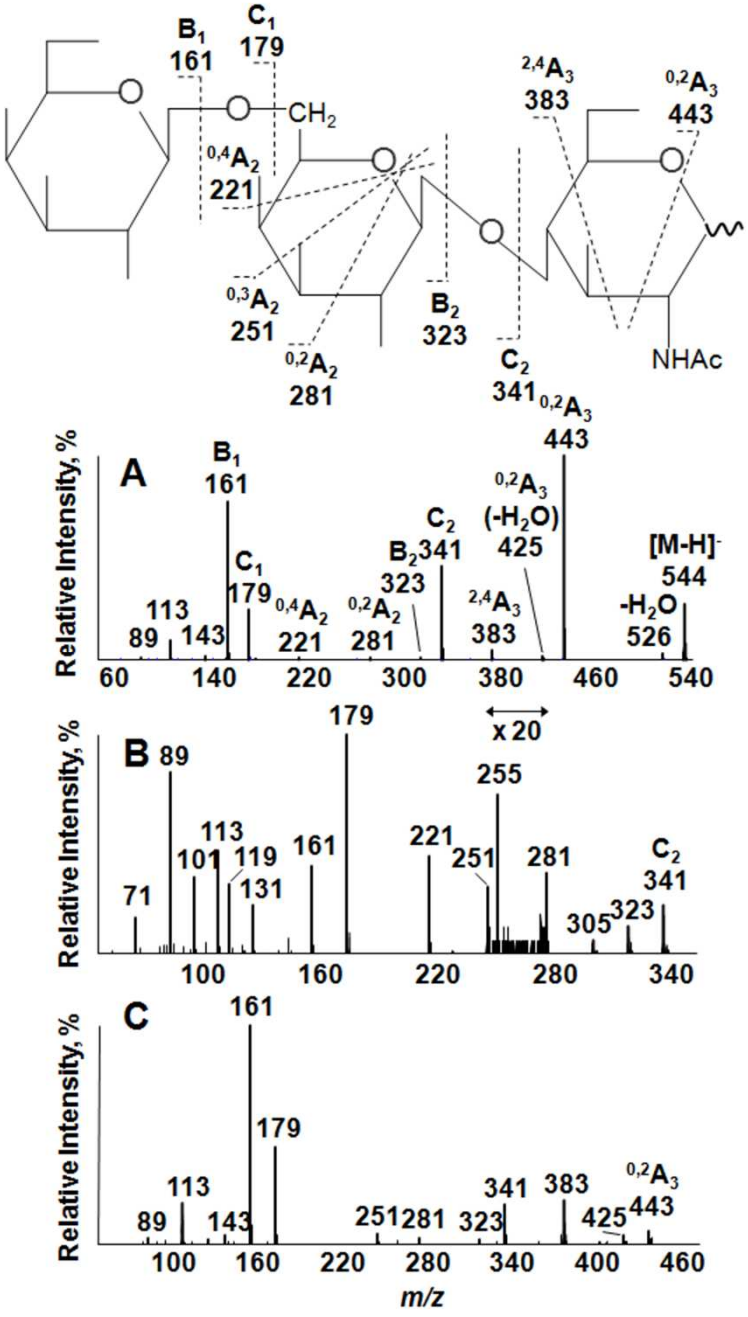

Figure 4-4. ESI-MS/MS spectra of sample compound Gal-Gal-GlcNAc peak 1: (A) $[M-H]^-$ ion at *m/z* 544; (B) in-source fragment ion C_2 at *m/z* 341; (C) in-source fragment ion $^{0,2}A_3$ at *m/z* 443.

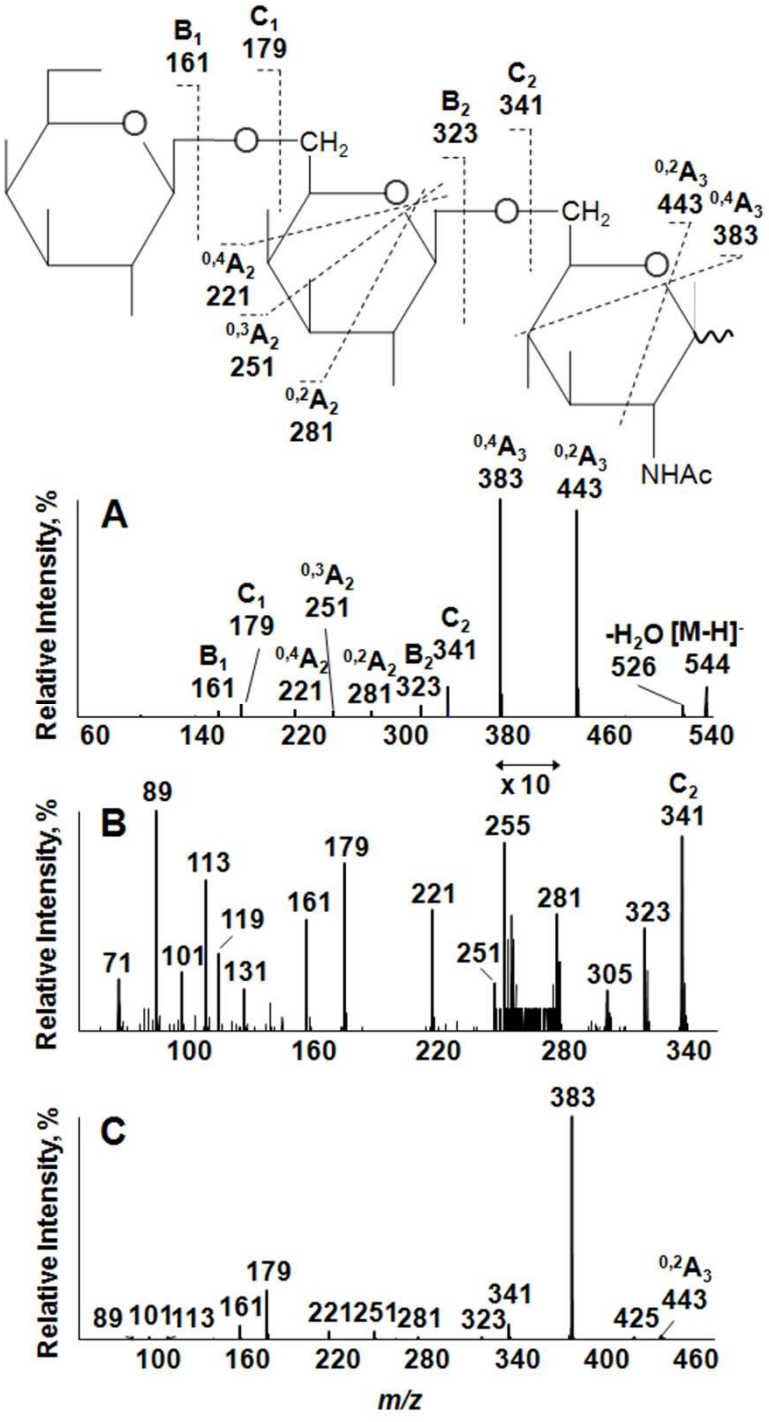

Figure 4-5. ESI-MS/MS spectra of sample compound Gal-Gal-GlcNAc peak 2: (A) $[M-H]^-$ at *m/z* 544; (B) in-source fragment ion C_2 at *m/z* 341; (C) in-source fragment ion $^{0,2}A_3$ at *m/z* 443.

However, both isomers display the fragment ions C_1 at *m/z* 179, C_2 at *m/z* 341 and the corresponding B_1 and B_2 fragment ions at 18 *m/z* units lower. These confirm that the monosaccharide sequence of both peaks 1 and 2 is Gal-Gal-GlcNAc. Due to the lack of authentic standards for the trisaccharide, a quasi MS^3 experiment was implemented to obtain additional linkage information. This was accomplished on a qTOF type instrument by increasing the ESI cone voltage to induce in-source fragmentation; the resulting fragment ions were then selected and subjected to collision-induced dissociation. In this way, the MS/MS spectra of the C_2 fragment ion at *m/z* 341 from Gal-Gal-GlcNAc were obtained for both peaks 1 and 2. The C_2 fragment ion was specifically selected so that the galactose backbone and the first glycosidic linkage of the Gal-Gal-GlcNAc molecule would remain intact, eliminating the N-acetyl glucosamine group. It was found that the MS/MS spectra of these C_2 ions from both Gal-Gal-GlcNAc peaks 1 and 2 are identical (**Figures 4-4B** and **4-5B**), indicating that the first glycosidic linkage in each compound is the same. In these MS/MS spectra the presence of small but significant ion peaks of *m/z* 281 and *m/z* 251 is indicative of a 1,6 linkage, so that the structure of the two isomers can be deduced to be Gal*β*-(1→6)-Gal*β*-(1→X)-GlcNAc.

Next, MS/MS experiments of the $^{0,2}A_3$ fragment ion at *m/z* 443 were obtained (**Figures 4-4C** and **4-5C**). Because the $^{0,2}A_3$ fragment ion arises through fragmentation excluding the N-acetyl glucosamine group, both the first and second glycosidic linkages remain intact and hence the MS/MS spectrum of this

ion is indicative of both linkages. The MS/MS spectra of the $^{0,2}A_3$ fragment ions arising from Gal-Gal-GlcNAc peaks 1 and 2 are different, but the A-type fragments observed do not provide unambiguous information on the second glycosidic linkage. However, it is believed that the presence of the cross-ring fragment $^{0,2}A_3$ through the terminal sugar excludes the possibility of an oligosaccharide with the structure Gal*β*-(1→6)-Gal*β*-(1→3)-GlcNAc (Seymour *et al*., 2006) because the blocked hydroxyl group of a 3-linked saccharide prevents the anomeric ring from opening and undergoing retro-enol rearrangement (Carroll *et al*., 1995). This explanation is well demonstrated in **Figure 4-3C** by the complete absence of an $^{0,2}A$ cleavage from the Gal*β*-(1→3)-GlcNAc standard. Hence a 1,3-linkage between Gal and GlcNAc would not be consistent with the data. On the other hand, during the enzymatic synthesis of HeOS by transgalactosylation of GlcNAc, digalactosylated GlcNAc must originate from transgalactosylation of disaccharides. Thus, under the experimental conditions employed in this study, trisaccharides are formed from Gal*β*-(1→4)-GlcNAc or Gal*β*-(1→6)-GlcNAc as described above. This gives rise to corresponding structures Gal*β*-(1→6)-Gal*β*-(1→4)-GlcNAc and Gal*β*-(1→6)-Gal*β*-(1→6)-GlcNAc as the only possibility that is consistent with the disaccharides produced in the enzymatic reactions. In addition, under the chromatographic conditions used, linkage positioning alone influenced elution times such that Gal*β*-(1→6)-GlcNAc eluted after Gal*β*-(1→3)-GlcNAc or Gal*β*-(1→4)-GlcNAc (**Figure 4-1**). By analogy, it is concluded that the trisaccharide Gal*β*-(1→6)-Gal*β*-

(1→6)-GlcNAc will elute after the trisaccharide Gal*β*-(1→6)-Gal*β*-(1→4)-GlcNAc, which differs only by the linkage at the reducing sugar. Hence, in **Figure 4-1A**, the trisaccharide Gal-Gal-GlcNAc peak 1 is believed to be Gal*β*-(1→6)-Gal*β*-(1→4)-GlcNAc, whereas peak 2 is Gal*β*-(1→6)-Gal*β*-(1→6)-GlcNAc,

4.3.5. Fucosylated hetero-oligosaccharides

Five new compounds were separated by LC/MS after transgalactosylation of fucose with CCE of *L. bulgaricus* **(Figure 4-1B)**. Isomers of the disaccharide and trisaccharide HeOS were analyzed via MS/MS spectra of their $[M-H]^-$ ions, resulting in complementary B-, C-, and Z-type glycosidic fragments to determine the monosaccharide sequence. Sodiated spectra are shown in **Appendix 3**, **Figure 3A-4**. Gal-Fuc peaks 1 and 2 (**Figure 4-6**) both displayed *m/z* 161 (B_1), *m/z* 179 (C_1) and *m/z* 145 (Z_1), confirming that the sequence is indeed Gal-Fuc.

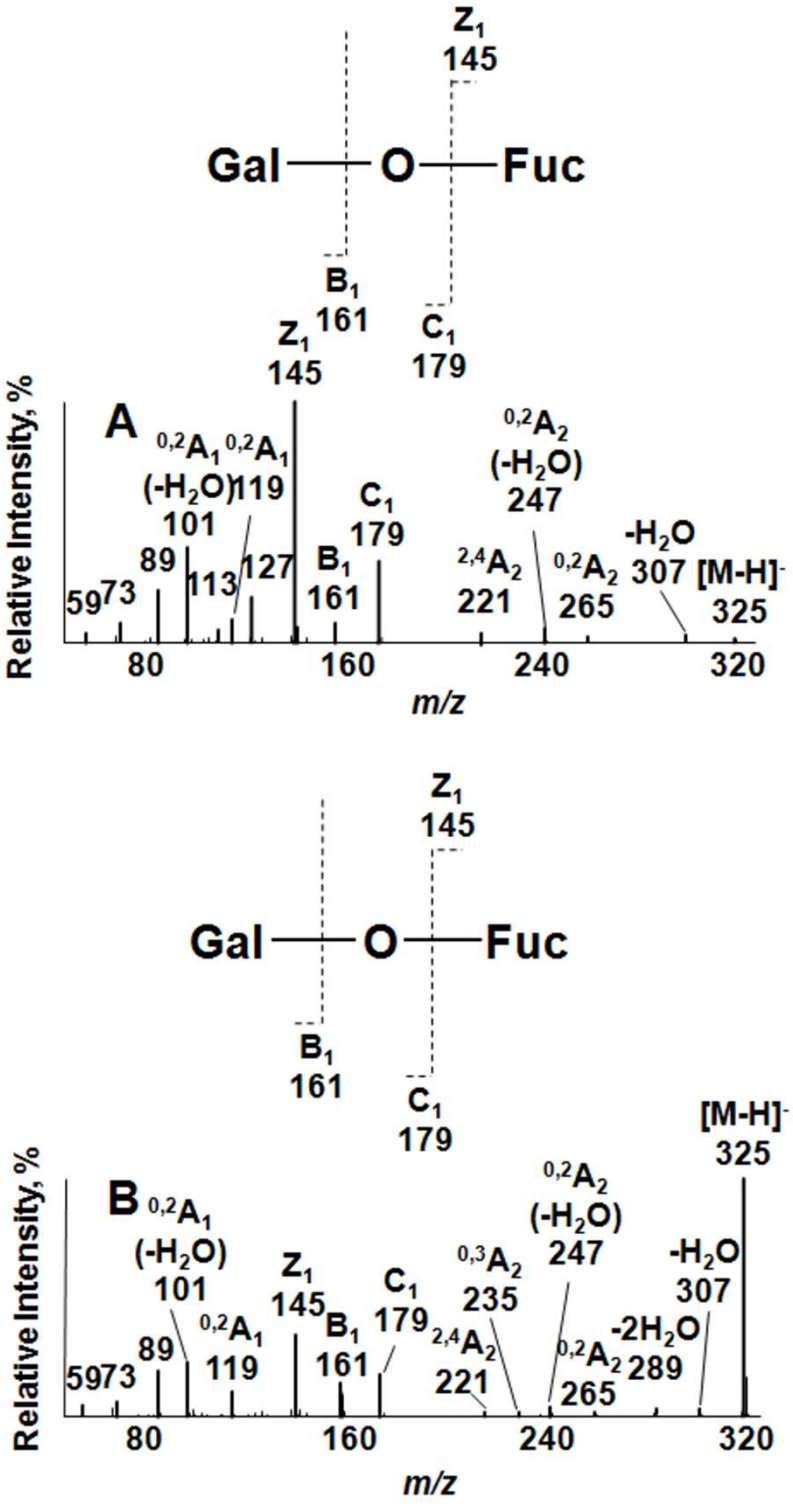

Figure 4-6. ESI-MS/MS spectra of the $[M-H]^-$ ions of sample compound (A) Gal-Fuc peak 1 and (B) Gal-Fuc peak 2.

However, both isomers of Gal-Fuc displayed different fragment ion patterns and relative intensities in the MS/MS spectra, which is indicative of linkage variation because all other structural aspects correspond. MS/MS spectra analysis was also performed on the $[M-H]^-$ ions of the Gal-Gal-Fuc peaks 1 – 3 (**Figure 4-7**), indicating the monosaccharide sequence to be Gal-Gal-Fuc.

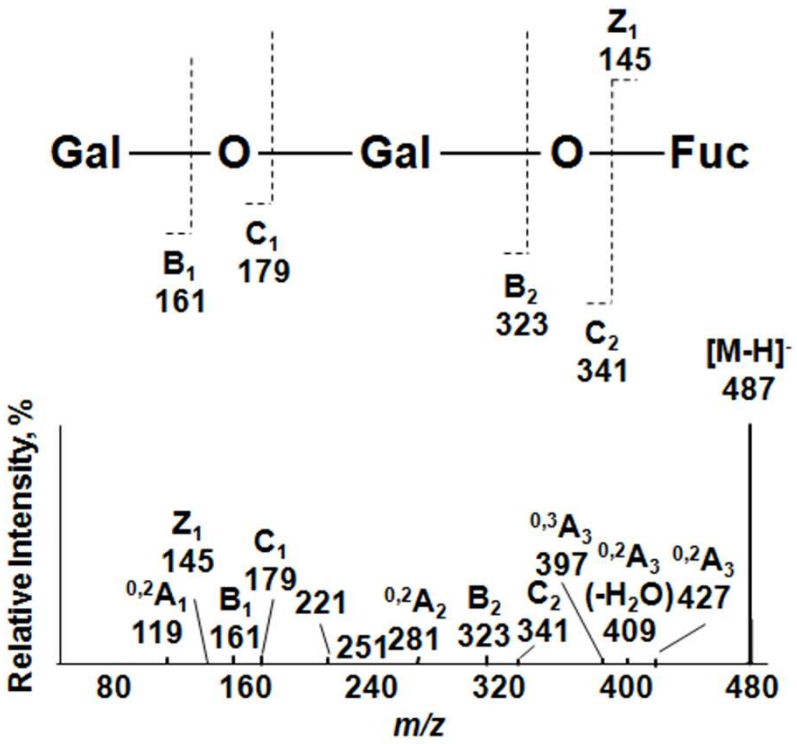

Figure 4-7. ESI-MS/MS spectra of $[M-H]^-$ ions representative of sample compound Gal-Gal-Fuc peak 1–3.

However, there was no discernable difference between the three spectra; therefore, **Figure 4-7** is presented as representative of all three isomers. The intensities of Gal-Gal-Fuc fragments under optimized collision energies in

MS/MS in both positive (**Appendix 3**, **Figure 3A-4C**) and negative ion mode were poor. This could be due to a low concentration of fucosylated HeOS formed in solution, inadequate ionization, or the orientation of the glycosidic linkage. Standards of galactosylated fucose oligosaccharides were not available for comparison.

4.3.6. Disaccharides formed by LacLM of *L. plantarum* FUA3112

The formation of *β*-(1→4) linked acceptor products by *β*-Gal of lactic acid bacteria is in apparent contrast with reports on the linkage type of GOS produced by lactobacilli and *Streptococcus thermophilus* (Gänzle, 2012). To analyze the spectrum of disaccharides formed from a single, defined *β*-Gal, transglycosylation of GclNAc was performed with CCE of *L. plantarum,* and *Lc. lactis* expressing LacLM of *L. plantarum* FUA3112 as sole source of *β*-Gal activity. LC/ESI-MS/MS aimed to identify the linkage type in all disaccharides, including galacto-oligosaccharides. Identification of galactosylated GlcNAc confirmed the exclusive presence of Gal*β*-(1→4)-GlcNAc and Gal*β*-(1→6)-GlcNAc (**Appendix 3**, **Figure 3A-5**); mass spectra of mono- and di-galactosylated GlcNAc obtained with LacLM from *L. plantarum* were identical to those observed in reactions with *L. bulgaricus*. The concentrations of Gal*β*-(1→4)-GlcNAc and Gal*β*-(1→6)-GlcNAc measured in enzymatic reactions with *Lc. lactis* expressing LacLM of *L. plantarum* FUA3112 were 0.71 and 19.5 g L^{-1}, respectively, indicating a preference for formation of the *β*-(1→6) linkages. The analysis of disaccharides carrying glucose or galactose at the reducing end (GOS) revealed the presence of

β-(1→6), *β*-(1→3) and *β*-(1→4) linked GOS (**Appendix 3**, **Figure 3A-6**). The presence of two *β*-(1→4)-linked disaccharides, representing Gal*β*-(1→4)-Gal and lactose, demonstrates that LacLM also forms *β*-(1→4) linkages. However, the relative quantity of *β*-(1→4)-linked products could not be determined because lactose is both substrate and product of the reaction.

4.4. Discussion

This study determined the structure of disacchrides and trisaccharides produced by transglycosylation of *β*-Gal from *L. bulgaricus* and LacLM from *L. plantarum* with lactose as galactosyl donor and GlcNAc as galactosyl acceptor. LC/ESI-MS/MS analysis with the use of external standards identified the HeOS Gal*β*-(1→4)-GlcNAc and Gal*β*-(1→6)-GlcNAc. Gal*β*-(1→6)-Gal*β*-(1→4)-GlcNAc and Gal*β*-(1→6)-Gal*β*-(1→6)-GlcNAc were identified by a combination of LC/ESI-MS/MS analysis, their retention behaviour and the identification of the precursor compounds in the enzymatic synthesis. Fucose also served as a galactosyl acceptor to form two disaccharides and three trisaccharides; however, the limited information obtained from the MS/MS data, the lack of available standards, and the low concentrations of the analytes prevented identification of the linkage types within these compounds.

In keeping with previous reports on GOS and HeOS formation by lactobacilli (Toba *et al*., 1981; Splechtna *et al*., 2006; Schwab *et al*., 2011), linkage types in oligosaccharides produced by enzymes from *L. bulgaricus* and *L. plantarum* were

identical. The identification of *β*-(1→4)-linked disaccharides as products of transgalactosylation of GlcNAc and galactose by *β*-Gal of lactobacilli extends information from previous reports (Toba *et al*., 1981; Splechtna *et al*., 2006; Torres *et al*., 2010; Gänzle, 2012), which identified only *β*-(1→3)- or *β*-(1→6)-linked GOS. Past studies identifying the linkage type of GOS, produced with *β*-Gal from lactobacilli, relied solely on chromatographic methods paired with the use of external standards (Toba *et al*., 1981; Splechtna *et al*., 2006), which left Gal*β*-(1→4)-Gal undetected. The *β*-(1→4)-linked disaccharides observed in this study were a minor product compared to *β*-(1→6)-linked oligosaccharides, but the sensitivity of the MS/MS method nevertheless allowed their identification. The specificity of *β*-Gal from *L. bulgaricus* and *L. plantarum* thus differs from the *β*-Gal from *Bacillus circulans* (Li *et al*., 2010). Tri- and tetrasaccharides formed with GlcNAc as acceptor carbohydrate by *B. circulans β*-Gal exhibited exclusively *β*-(1→4) linkages (Li *et al*., 2010). The absence of Gal*β*-(1→4)-GlcNAc may be attributable to steric hindrance by the N-acetyl moiety at the C2 position. Because the linkage type of oligosaccharides is an important determinant for their ability to prevent pathogen adhesion (Kulkarni *et al*., 2010; Johannes & Römer, 2010), this study increased the variation of oligosaccharide structures formed in the acceptor reaction of *β*-Gal.

These results can be applied to the food-grade conversion of whey permeate, a lactose-containing product of the ultrafiltration of whey, to novel oligosaccharides. GOS production and *β*-Gal activity in whey permeate was 1.8

fold higher when compared to a lactose solution (Splechtna *et al*., 2007). This difference is attributable to the presence of monovalent and divalent cations in whey permeate, which enhance *β*-Gal activity (Vasiljevic & Jelen, 2002). Whey permeate is a readily available source of lactose which can be used for GOS and HeOS production. Additionally, GlcNAc is one of the most abundant polysaccharides on earth, derived from the hydrolysis of chitin, an extracellular polymer found in invertebrates, fungi, and algae (Kurita, 2001).

Possible physiological functions of HeOS produced by lactobacilli are also related to structural similarities to human milk oligosaccharides (HMOS). The core molecule of HMOS consists of galactose and GlcNAc monomers *β*(1→3/4)-linked repetitively with lactose at the reducing end. Additional fucosylation and sialylation of these core molecules also creates other composite structures (Kunz *et al*., 2000; Boehm & Stahl, 2007). Different functions are attributed to individual types of HMOS, which relates to their wide variety of structures (Kunz *et al*., 2000; Zivkovic *et al*., 2011). Currently, with their structural and compositional complexity, HMOS cannot be industrially produced, and infant formula is supplemented with simpler GOS structures to mimic the bifidogenic effect of human milk oligosaccharides (Macfarlane *et al*., 2008). N-acetyllactosamine, the Gal*β*-(1→4)-GlcNAc core structure of HMOS, was one of the products of transgalactosylation of GlcNAc by *β*-Gal from lactobacilli. Enzymatic synthesis of Gal*β*-(1→4)-GlcNAc could thus be employed act as a step in chemoenzymatic synthesis of HMOS. Moreover, N-acetyllactosamine is a

preferred substrate for bifidobacteria (Zivkovic *et al*., 2011), and was identified as a competitive inhibitor to enteropathogenic *Escherichia coli* (Hyland *et al*., 2008). Thus, HeOS formed in food grade conversions by the *β*-Gal of lactobacilli could be used as food additives, particularly to supplement infant formulas.

4.5. References

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5. Characterization of *α*-galactooligosaccharides formed via heterologous expression of *α*-galactosidases from *Lactobacillus reuteri* in *Lactococcus lactis*[4]

5.1. Introduction

Bacterial infection is initiated by adherence to skin or mucosal surfaces in mammalian hosts (Finlay & Falkov, 1997). Adherence can be prevented by soluble oligosaccharides that resemble surface glycans of eukaryotic cells and act as receptor analogues (Kulkarni *et al*., 2010). An improved understanding of the interactions between surface glycans of eukaryotic cells and adhesion mechanisms of viruses and between pathogenic bacteria and their toxins has resulted in increased interest in applications of oligosaccharides to treat infections in farm animals and humans (Rozeboom *et al*., 2005; Arslanoglu *et al*., 2007; Liu *et al*., 2008; Bruzzese *et al*., 2009). Preventative treatments utilizing oligosaccharide analogues are also motivated by the need to replace antibiotics as growth promoters in animal production (Fox, 1988; Verstegen & Williams, 2002).

Human milk oligosaccharides (HMOS) inhibit bacterial adhesion to epithelial surfaces in infants (Kunz *et al*., 2000). HMOS are constituted of glucose (Glc), galactose (Gal), N-acetylglucosamine (GlcNAc), fucose (Fuc) and sialic acid and are joined by a variety of linkage types and branching which collectively gives rise to highly diverse and complex structures (Bode, 2006). *β*-Galacto-

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Asterisks (*) indicate contributions by Y. Wang

oligosaccharides (*β*-GOS) consist of *β*-linked galactose moieties with galactose or glucose located at the reducing end. Although they differ structurally from HMOS, *β*-GOS were also shown to prevent pathogen adhesion. For example, *β*-GOS were used to prevent the adhesion of enteropathogenic *Escherichia coli* to tissue culture cells, and prevented *Salmonella enterica* adhesion both *in vitro* and *in vivo* (Shoaf *et al*., 2006; Searle *et al*., 2010).

β-GOS are produced by transgalactosylation of glucose, galactose, or lactose, and the mechanisms of enzymatic transgalactosylation by microbial *β*-galactosidases (*β*-Gal) are well understood (Torres *et al*., 2010; Gänzle, 2012). Transgalactosylation with microbial *β*-Gal produces hetero-oligosaccharides (HeOS) with potential applications in food and therapeutics if suitable acceptor carbohydrates or sugar alcohols are present (Gänzle, 2012). *β*-Gal from lactic acid bacteria and bifidobacteria were employed to produce *β*-HeOS, with structural similarity to HMOS. These *β*-HeOS have the potential to mimic receptor glycan structures of enteropathogenic *E. coli* (Schwab *et al*., 2011; Black *et al*., 2012). Similarly to *β*-Gal, *α*-galactosidases (*α*-Gal) [EC 3.2.1.22] cleave Gal*α*-(1→6)-Glc bonds and subsequently transfer the liberated galactose moiety to suitable acceptors forming *α*-galacto-oligosaccharides (*α*-GOS). Comparable to *β*-GOS, *α*-GOS have the potential to mimic glycan receptors on eukaryotic cells. For example, Gal*α*-(1→4)-Gal and Gal*α*-(1→4)-Gal*β*-(1→4)-Glc, act as receptors for P-fimbriae of *E. coli* and Shiga toxin I/II, respectively (Strömberg *et al*., 1991; Lingwood, 1996). *α*-Gal activity is widespread within prokaryotes and relatively

common among lactobacilli (Gänzle & Follador, 2012); however, applications for α-Gal from lactic acid bacteria are almost exclusively aimed to eliminate raffinose-and stachyose in food and feed fermentations (Alazzeh *et al*., 2009; Teixeira *et al*., 2012). Only a few reports describe α-GOS formation by α-Gal (Mital *et al*., 1973; Tzortzis *et al*., 2003). The resulting α-GOS, however, were not characterized on a structural level, and the formation of HeOS with α-Gal was not explored.

It was therefore the aim of this study to employ α-Gal from lactobacilli for production of α-GOS with different acceptor carbohydrates, and to structurally characterize the α-GOS formed from various acceptor sugars. *Lactobacillus reuteri* was chosen as the source of α-Gal because α-GOS formation by enzymes of this organism was previously described (Tzortzis *et al*., 2003). Genes coding for α-Gal in *L. reuteri* 100-16 and 100-23 were cloned into an α-Gal negative strain of *Lactococcus lactis* and transgalactosylation was achieved using the crude cell extracts (CCE) of the transgenic α-Gal active *Lc. lactis*. α-GOS were characterized by liquid chromatography/electrospray ionization tandem – mass spectrometry (LC/ESI-MS/MS) (Black *et al*., 2012).

5.2. Materials and methods

5.2.1. Chemicals and standards

Oligosaccharide standards including melibiose, raffinose, and stachyose were purchased from Sigma Aldrich (Oakville, Canada); globotriose was purchased

from Carbosynth (Berkshire, United Kingdom). Fisher Scientific (Ottawa, Canada) supplied HPLC grade acetonitrile, methanol, and ammonium acetate. Other solvents were of analytical grade.

5.2.2. Bacterial strains and growth conditions*

E. coli TG1 was purchased from Statagene (Amsterdam, the Netherlands). *L. reuteri* 100-23 (FUA3030, DSM17509), *L. reuteri* 100-16 (FUA3032), and *Lc. lactis* MG1363 (FUA3016) were obtained from the Food Microbiology strain collection of the University of Alberta (FUA) and were grown in modified DeMan-Rogosa-Sharpe (mMRS) medium (de Man *et al*., 1960) containing 10 g L^{-1} maltose, 5 g L^{-1} fructose and 5 g L^{-1} glucose with a pH of 6.5. *Lc. lactis* was grown in M17 medium with the addition of 0.5% glucose (mM17). *E. coli* was grown in Luria-Bertani (LB) medium. Agar plates contained 15 g L^{-1} agar for each medium and strains were incubated at 37 °C.

5.2.3. Cloning of *α*-galactosidase and transformation of *E. coli* and *Lc. lactis**

The *E. coli – Lc. lactis* expression shuttle vector pAMJ586 was used for cloning the *α*-Gal gene (*aga*) (Israelsen *et al*., 1995). *α*-Gal genes from the genome-sequenced strain *L. reuteri* 100-23 and *L. reuteri* 100-16 (Accession numbers NZ_AAPZ02000002) were amplified by PCR using primers listed in **Table 5-1**. Restriction digests of the PCR products were then conducted to generate the insert with FastDigest® restriction enzymes *Sma*I and *Sal*I (Fermentas, Burlington, Canada). The same restriction digests were performed for the pAMJ586 shuttle vector and all *Sma*I and *Sal*I restriction fragments were gel

purified using the PureLink™ Quick Gel Extraction Kit (Life Technologies Inc., Burlington, Canada) after electrophoresing in a 1% agarose gel at 90 V for 45 min. Ligation using the T4 DNA ligase (Fermentas) was done to produce the final constructs, which were named as pAMJ586-*aga*23 and pAMJ586-*aga*16.

Table 5-1. Primers used in *α*-galactosidase amplification

Primer name	5' – 3'	Reference sequence accession number	Restriction enzyme site included
LR-*aga*F-*Sma*+RBS	TCCCCCGGGTCTAGATTAGGGTAACTTTGAAAGGATATTCCTC**ATG**ATTACATTTGATGAACAGC	NZ_AAPZ02000002 (241775- 243964)	*Sma*I
LR-*aga*R-*Sal*	ACGCGTCGAC*CTA*TTCACCTTTAAAGTAATGC		*Sal*I

Underlined: restriction site; bold: start codon; italics: stop codon; highlighted: *α*-galactosidase (*aga*) gene sequence.

Recombinant plasmids were electro-transformed into *E. coli* TG1 and subsequently electro-transformed into the *α*-Gal negative host strain *Lc. lactis* MG1363. The electroporation conditions used were 25 μF, 1.7 kV, and 200 Ω in 0.1 cm Gene Pulser® cuvettes (Biorad, Mississauga, Canada). Electroporated *E. coli* were recovered in SOC medium (Life Technologies); electroporated *Lc. lactis* were recovered in mM17. Both strains were incubated for at least 2 h at 37 °C. After recovery, transformed strains were grown on their respective media with erythromycin as the selective agent at 5 mg L^{-1} in LB for *E. coli* and at 100 mg L^{-1}

in mM17 for *Lc. lactis*. Competent cells of *Lc. lactis* were prepared as described (Schwab *et al*., 2010), except overnight cultures were inoculated 2% in 500 mL mM17 supplemented with 1% glycine. Cloning of the gene was confirmed by PCR and sequencing of the amplicon (Macrogen, Rockville, MD). The sequence of *aga*23 matched the corresponding sequence in the genome of *L. reuteri* 100-23; the sequence of *aga*16 was deposited to Genbank with the accession number KF410950. Gene expression was confirmed with the observation of blue coloured transformants growing on mM17 agar supplemented with erythromycin at 5 mg L^{-1} and 20 μL of 20 g L^{-1} 5-bromo-4-chloro-3-indoxyl-*α*-D-galactopyranoside (X-*α*-D-Galactoside) (Gold Biotechnology Inc., St. Louis, MO) that forms a blue colour upon hydrolysis by *α*-Gal. The transformants carrying *aga*23 and *aga*16 from *L. reuteri* 100-23 and *L. reuteri* 100-16, respectively, were designated as *Lc. lactis* FUA 3376 and FUA 3377.

5.2.4. Preparation of crude cell extracts*

Single colonies of *Lc. lactis* MG1363, FUA 3376 or FUA 3377 were used to inoculate 10 mL cultures in mM17 with 0.0274% manganese sulphate added (Ibrahim *et al*., 2010). The cultures were incubated overnight and were used to inoculate 500 mL of the same medium at 2%. The cultures were then incubated until the pH was reduced to values between 5.0 and 5.2, and cells were harvested by centrifugation at 5525 × g for 20 min. Cells were washed once in McIlvaine buffer (0.1 M citric acid and 0.2 M disodium phosphate, pH 5.66), and resuspended in approximately 10 mL of the same buffer additionally

supplemented with 10% glycerol and 0.0274% manganese sulphate. Cells were disrupted with a bead beater at 4 °C and crude cell extracts were collected by centrifuging at 12, 000 × g for 10 min at 4 °C to remove cellular debris.

5.2.5. Determination of *α*-galactosidase activity of AGA23 and AGA16 in crude cell extracts*

Enzymatic assays were conducted as described (Church *et al*., 1980) by adding 5 μL of CCE to 95 μL of 4 g L^{-1} 4-nitrophenyl-α-D-galactopyranoside (PNPG). The reaction was stopped after 1, 2, 3, 4 or 5 min by the addition of 130 μL of 1 M sodium carbonate. Absorption at 400 nm was determined using a Varioskan Flash Multimode Reader (Fisher Scientific Limited, Ottawa, Canada). Relative *α*-Gal activity of each CCE was defined as the liberation of 1 mmol PNPG per min per mg of CCE at 35 °C and pH 4.7. The total protein concentrations of CCEs were determined with the Bio-Rad Protein Assay reagent (Bio-Rad) and bovine serum albumin (New England Biolabs, Missisauga, Canada) as a standard.

The optimal pH was determined using McIlvaine buffer with PNPG dissolved at 4 g L^{-1}. The pH of the buffer was adjusted to 3.34, 3.64, 4.03, 4.47, 4.64, 4.75, 5.48, 6.10, 6.67, and 7.03. The optimal temperature was determined by conducting the reaction at temperatures between 20 °C to 55 °C. In all enzymatic reactions, CCE from the *α*-Gal negative *Lc. lactis* MG1363 was used as the negative control.

5.2.6. Synthesis of oligosaccharides in acceptor reactions*

Reactions were conducted at 30 °C for 24 h with 600 g L^{-1} melibiose or raffinose, or with 300 g L^{-1} melibiose or raffinose and 300 g L^{-1} lactose, fucose or GlcNAc. Reactions without CCE, or reactions with CCE from *L. lactis* MG1363 were included as negative controls. CCE of *L. lactis* FUA 3376 or FUA 3377 were added to achieve a final α-Gal activity of 151 ± 39 μmol × (min × mg)$^{-1}$. All carbohydrates were dissolved in McIlvaine buffer at pH 4.7.

5.2.7. High performance anion exchange chromatography with pulsed amperometric detection*

Sugar standards of glucose, galactose, melibiose, raffinose, fucose, GlcNAc and lactose were prepared by dissolving 0.6 g L^{-1} of each sugar in distilled water. Standards and samples were analyzed with high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using a CarboPac PA-20 column (3 × 150 mm; Dionex, Oakville, Canada) as described (Black *et al*., 2012). Because GlcNAc co-eluted with glucose and galactose, acceptor reactions with GlcNAc were analyzed with an Aminex HPX-87H column (7.8 × 300 mm, 9 μm; BioRad, Mississauga, Canada) coupled to an RI detector eluted at 70 ºC with 5 mM sulfuric acid at 0.4 mL min^{-1} (Schwab *et al*., 2007).

5.2.8. Combined liquid chromatography/electrospray ionization tandem mass spectrometry

Oligosaccharides were analyzed by LC/ESI-MS/MS. Separations were conducted on an Agilent 1200 series LC system (Agilent Technologies, Palo

Alto, CA) at 25 °C using a Supelcosil LC-NH_2 column (250 mm × 4.6 mm, 5 μm; Sigma Aldrich, Oakville, Canada). Samples were injected onto the column at 3 μL each time, using an isocratic flow of acetonitrile/water 80:20 (v/v) at a rate of 1 mL min^{-1}. The effluent from the column was split at a ratio of 1:3 (v/v). A post-column addition of ammonium acetate (40 mM in methanol) at 0.03 mL min^{-1} was delivered by an Agilent 1200 series isocratic pump that was added to the ESI source.

Negative ion ESI-MS and collision induced dissociation tandem mass spectrometry (CID-MS/MS) were performed on a QStar® Elite hybrid orthogonal Q-TOF mass spectrometer coupled to a TurboIon Spray® source with Analyst® QS 2.0 software (Applied Biosystems/MDS Sciex, Concord, Canada). The conditions were as follows: nebulizer gas, 50 (arbitrary units); auxillary gas, 60 (arbitrary units); ionspray voltage, -4500 V; curtain gas, 25 (arbitrary units); declustering potential (DP), -50 V; focusing potential, -150 V; DP2, -10 V; and a source temperature of 400 °C, scanning over a mass range of *m/z* 100-1000. Fragmentation was achieved using nitrogen as a collision gas at a collision energy that varied between -10 to -35 eV and was optimized for each saccharide.

5.3. Results

5.3.1. Enzyme sequence alignment*

α-Gal from *L. reuteri* 100-16 and 100-23 was initially compared to other *α*-Gal from lactic acid bacteria that are characterized on the protein level. Alignment

of the protein sequences with CLUSTALW multiple sequence Alignment software (version 2.1) revealed that AGA23 and AGA16 were 97% identical (data not shown). AGA23 had 62% sequence identity with *melA* of *L. plantarum* (AF189765) (Silvestroni et al. 2002); 42% with the α-Gal of *L. brevis* (Q03PP7), 37% with *melA* of *L. fermentum* (Q6IYF5), and 51% match with *aga*A of *Carnobacterium maltaromaticum* (AAL27305.1) (**Figure 5-1** and data not shown). Overall, AGA23 and AGA16 were highly similar in sequence, had comparable enzyme activities, and acceptor reaction results. While CCE containing both types of enzymes were used in most cases during our study, results obtained with either one of the enzymes are presented.

Figure 5-1. Multiple sequence alignment analyses of putative α-Gal active sites in lactobacilli and related lactic acid bacteria (adapted from Silvestroni *et al*., 2002). Conserved amino acids are shaded in grey; the number at the right end of each sequence indicates the last amino acid number. MelA of *L. plantarum* and *L. fermentum* and AgaA of *C. maltaromaticum* were characterized on the protein level.

5.3.2. Optimum pH and temperature of *α*-galactosidase reactions from *L. reuteri**

The pH and temperature for optimum activity of *α*-Gal was determined with PNPG as a substrate. *α*-Gal activities of AGA23 and AGA16 were optimal at a pH 4.7 and at temperatures between 30 °C – 35 °C (**Figure 5-2** and data not shown). In order to determine whether the presence of sugars alters the optimal temperature of *α*-Gal, sucrose or melibiose were incorporated into enzyme assays at a concentration of 300 g L^{-1}. Sucrose and melibiose reduced enzyme activities below 35 °C when compared to the control, but enzymes were stabilized in the presence of the sugars at higher temperatures. Addition of melibiose shifted the optimal temperature of *α*-Gal from 35 °C to 40 °C.

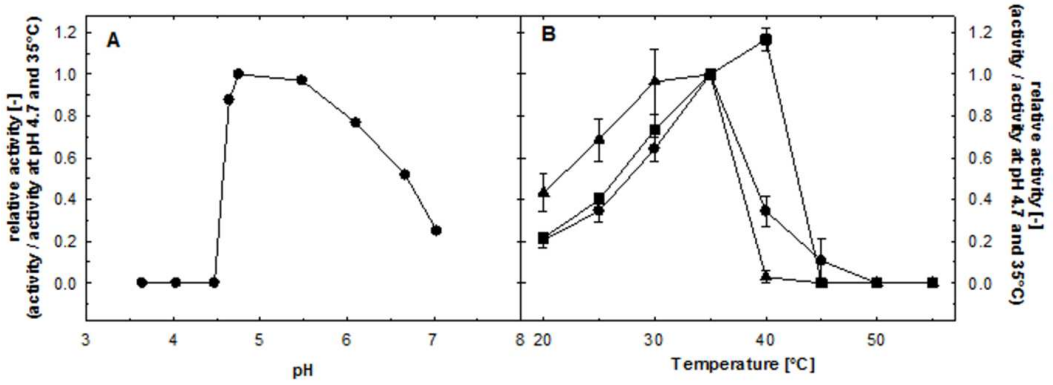

Figure 5-2. (Panel A) Relative activity of AGA16 in *Lc. lactis* at 35 °C and different pH values. Data are means of three independent replicates and the experimental error is smaller than symbol size. (Panel B) Relative activity of AGA16 in *Lc. lactis* at pH 4.7 and temperatures ranging from 20 – 50 °C. Reactions were carried out with PNPG as a substrate in McIlvaine buffer (▲), McIlvaine buffer containing 300 g L^{-1} melibiose (■), or McIlvaine buffer

containing 300 g L^{-1} sucrose (●). Data represent means ± standard deviation of three independent experiments.

5.3.3. Acceptor reactions*

Transgalactosylation of acceptor carbohydrates was initially assessed by analysis of acceptor reactions with HPAEC-PAD. The formation of putative products was observed in the reactions with lactose, fucose, or GlcNAc as galactosyl-acceptors (**Figure 5-3**, **Appendix 4 Figure 4A-1** and data not shown).

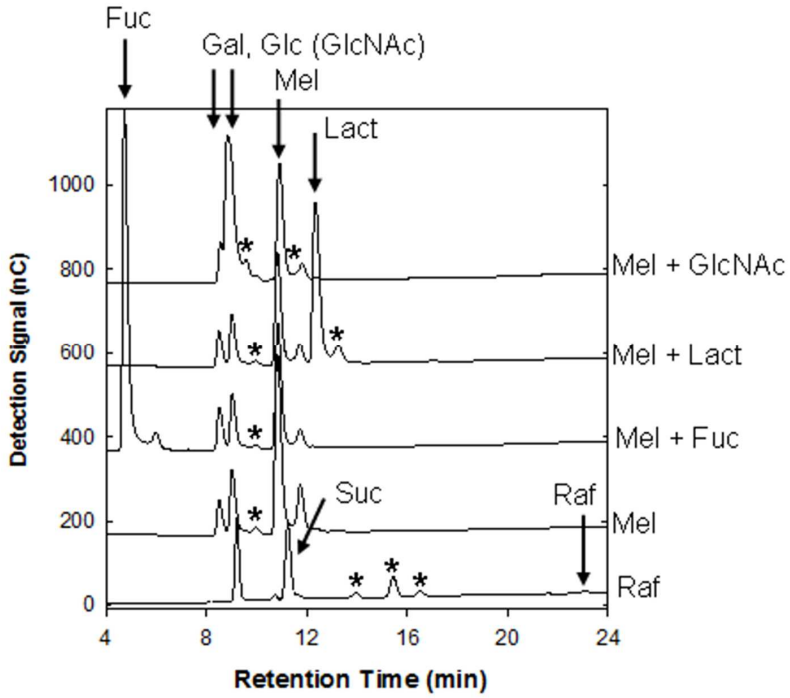

Figure 5-3. HPAEC-PAD chromatograms of acceptor reactions with AGA23. Reactions were carried out at pH 4.7 and 30 °C for 24 h at a total carbohydrate concentration of 600 g L^{-1} with sugars indicated to the right (Raf, raffinose; Mel, melibiose; Fuc, fucose; Lact, lactose; GlcNAc, N-acetylglucosamine). Sugars that were identified by external standards are indicated by arrows (Gal, galactose; Glc,

glucose); unknown peaks representing putative acceptor carbohydrates are indicated by asterisks. Data are representative of triplicate independent experiments. Comparable results were obtained with AGA16 (data not shown).

Transferase and hydrolase activities were calculated from the release of glucose (representing total enzyme activity) and the release of galactose (representing hydrolase activity), and the difference between glucose and galactose release (representing transferase activity) (**Table 5-2**).

Table 5-2. Comparisons between enzyme activities among different acceptor reactions.

Acceptor reactions {n = 3}	Glucose liberated (mol L^{-1})	Percent hydrolase activity	Percent transferase activity
M + AGA16[a)]	0.52 ± 0.17	55.1 % ± 0.5	44.9 % ± 0.5
M + Fuc + AGA16	0.26 ± 0.13	80.2 ± 6.3	19.8 % ± 6.3
M + Lactose + AGA16	0.36 ± 0.12	65.0 % ± 1.1	35.0 % ± 1.1
M + GlcNAc + AGA16	0.22 ± 0.03	77.9 % ± 13.7	22.1 % ± 13.7

[a)]Note: M, melibiose, Fuc, fucose, GlcNAc, N-acetylglucosamine. Reactions were conducted at a total sugar concentration of 600 g L^{-1} (300 g L^{-1} melibiose and 300 g L^{-1} acceptor carbohydrate). For reactions with melibiose only, 600 g L^{-1} melibiose were used.

No significant differences were observed when different acceptor carbohydrates were present. However, when melibiose was present at 600 g L^{-1}, the total activity and contribution of transferase activity was higher, likely in consequence of the higher substrate concentration.

5.3.4. Characterization of oligosaccharides produced from melibiose with liquid chromatography/electrospray ionization tandem mass spectrometry

LC/ESI-MS was employed to confirm the presence of oligosaccharides formed by the transferase reaction of α-Gal. All exact masses of observed compounds are shown in **Table 5-3**.

Table 5-3. Mass accuracy of deprotonated molecular ions and retention times of oligosaccharides formed with either GlcNAc, fucose or lactose added as acceptor carbohydrates.

Acceptor	Compound	Retention Time (min)	Measured Mass (Da)	Error (mDa)
		Melibiose		
None	$C_{12}H_{21}O_{11}$	16.3; 18.2; 19.1; 21.9	341.1099	1.0
	$C_{18}H_{32}O_{16}$	39.9; 53.3	503.1637	1.9
+ Fuc	$C_{12}H_{21}O_{10}$	11.5; 14.0	325.1156	1.6
	$C_{12}H_{21}O_{11}$	16.3; 18.2; 19.1; 21.9	341.1076	-1.3
	$C_{18}H_{31}O_{15}$	26.1; 33.4; 36.4	487.1689	2.0
	$C_{18}H_{32}O_{16}$	39.9; 53.0	503.1609	-0.9
+ Lactose	$C_{12}H_{21}O_{11}$	18.7; 21.8	341.1081	-0.8
	$C_{18}H_{32}O_{16}$	37.5; 53.2	503.1632	1.4
+ GlcNAc	$C_{14}H_{24}O_{11}N$	15.6	382.1375	2.0
	$C_{12}H_{21}O_{11}$	16.3; 18.3; 19.1; 21.8	341.1099	1.0
	$C_{18}H_{32}O_{16}$	39.5; 53.2	503.1638	2.0
		Raffinose		
None	$C_{18}H_{32}O_{16}$	24.2; 31.2; 33.0	503.1629	1.1
	$C_{24}H_{41}O_{21}$	48.5; 75.7	665.2164	1.8
		Standards		
N/A	Lactose	18.8	341.1082	-0.7
	Melibiose	21.9	341.1095	0.6
	Raffinose	31.3	503.1628	1.0
	Globotriose	33.9	503.1626	0.8
	Stachyose	75.8	665.2155	0.9

In order to determine the structural identity of oligosaccharides, tandem mass spectrometry (MS/MS) was performed. This allowed for the comparison of fragmentation patterns from substrates or products in the samples to those of known standards, in cases where these were available. Additionally, matching retention times between analytes and standards confirmed structural identity (**Appendix 4**, **Figure 4A-2**). Based on the extact mass measurements and MS/MS spectra, a total of five oligosaccharides were identified that were formed by *α*-Gal with melibiose as the substrate. The linkage identifications were based partly on the standards used within this study and by adherence to the fragmentation rules described earlier by Black *et al*. (2012). Specifically, the absence of both ^{0,2}A(-$H_2$0) and ^{0,3}A cross-ring fragment ions indicates a *β*/*α*-1→3 linkage; the presence of ^{0,2}A(-$H_2$0) and the absence of ^{0,3}A cross-ring fragment ions indicates a *β*/*α*-1→4 linkage; and the presence of ^{0,3}A and the absence of ^{0,2}A(-$H_2$0) cross-ring fragment ions indicate *β*/*α*-1→6 linkage. Thus, the disaccharide Gal*α*-(1→3)-Gal/Glc at retention time 16.3 min was identified by the absence of both *m/z* 263 $^{0,2}A_2$(-$H_2$0) and *m/z* 251 $^{0,3}A_2$ ions; Gal*α*-(1→4)-Gal/Glc at retention time 18.2 min was identified by the presence of *m/z* 263 $^{0,2}A_2$(-$H_2$0) and the absence of *m/z* 251 $^{0,3}A_2$ ions; Gal*α*-(1→6)-Gal at retention time 19.1 min was identified by the presence of *m/z* 251 $^{0,3}A_2$ and the absence of *m/z* 263 $^{0,2}A_2$(-$H_2$0) ions (**Figure 5-4A-C**). The linkages for the trisaccharide products were determined similarly; Gal*α*-(1→3)-Gal*α*-(1→6)-Gal/Glc eluted at 39.9 min; Gal*α*-(1→6)-Gal*α*-(1→6)-Gal/Glc eluted at retention time 53.3 min (**Figure 5-4D-E**).

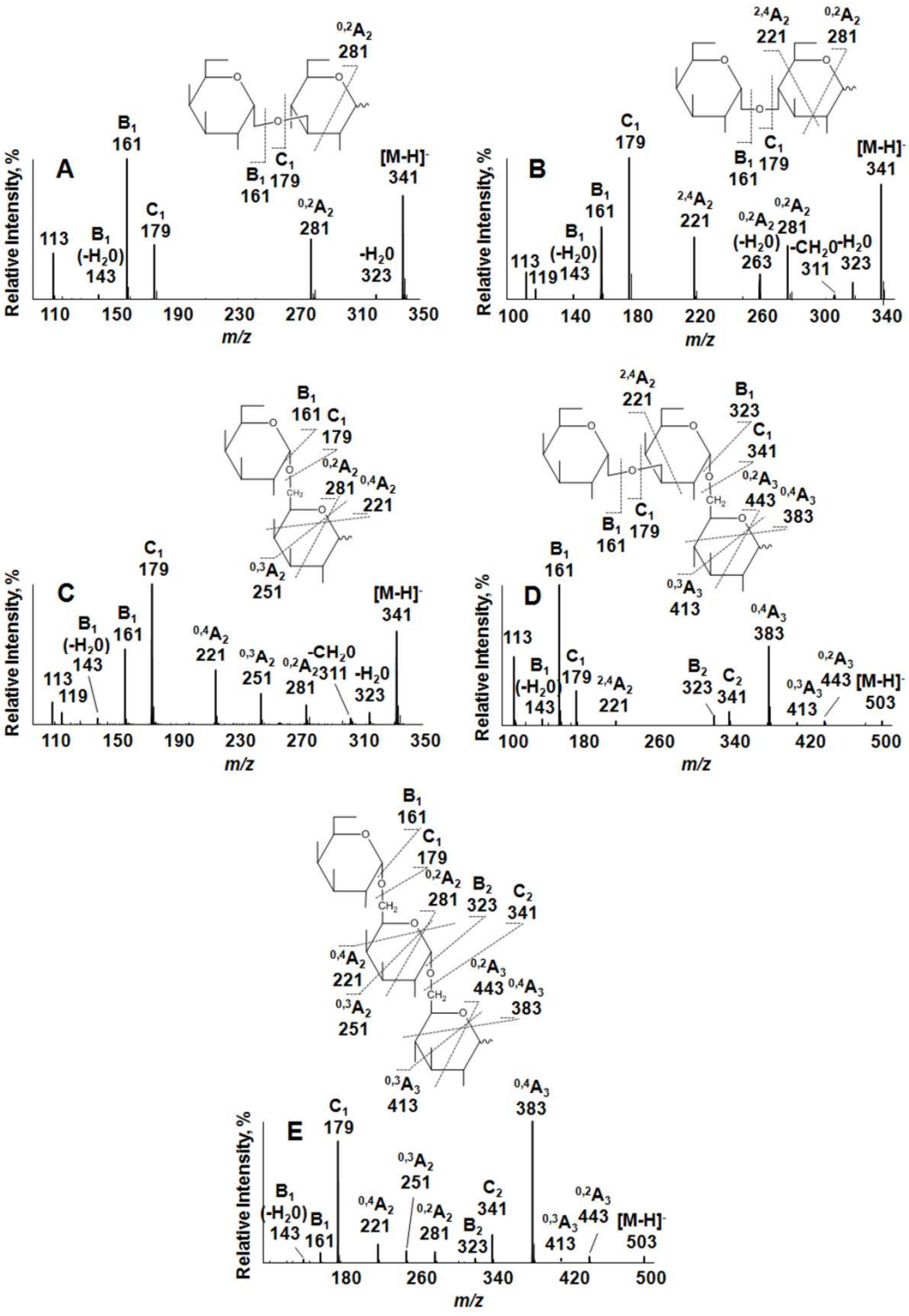

Figure 5-4. ESI-MS/MS spectra of $[M-H]^-$ ions of oligosaccharide products from *α*-Gal with melibiose as galactosyl-donor and -acceptor. (A) Gal*α*-(1→3)-Gal/Glc

m/z 341 at retention time 16.3 min; (B) Gal*α*-(1→4)-Gal/Glc *m/z* 341 at retention time 18.2 min; (C) Gal*α*-(1→6)-Gal *m/z* 341 at retention time 19.1 min; (D) Gal*α*-(1→3)-Gal*α*-(1→6)-Gal/Glc product *m/z* 503 at retention time 39.9 min; (E) Gal*α*-(1→6)-Gal*α*-(1→6)-Gal/Glc product *m/z* 503 at retention time 53.3 min. Melibiose eluted at 21.9 min, its spectrum is shown in the online supplemental material.

5.3.5. Characterization of oligosaccharides produced from melibiose and fucose, lactose, or GlcNAc

When lactose, fucose or GlcNAc were present as acceptor sugars, *α*-Gal formed hetero-oligosaccharides in addition to the products produced from melibiose alone. In the presence of fucose, a total of eleven oligosaccharides were obtained. Disaccharides resulting from transgalactosylation of fucose were identified as Gal*α*-(1→3)-Fuc, which eluted at a retention time of 11.5 min and Gal*α*-(1→4)-Fuc at a retention time 14.0 min (**Figure 5-5** and **Table 5-3**). The low abundance of ions in the LC/MS/MS analysis of trisaccharides formed by galactosylation of fucose was sufficient for the monosaccharide sequence to be established but did not provide enough information to positively determine the linkages (**Appendix 4**, **Figure 4A-3**).

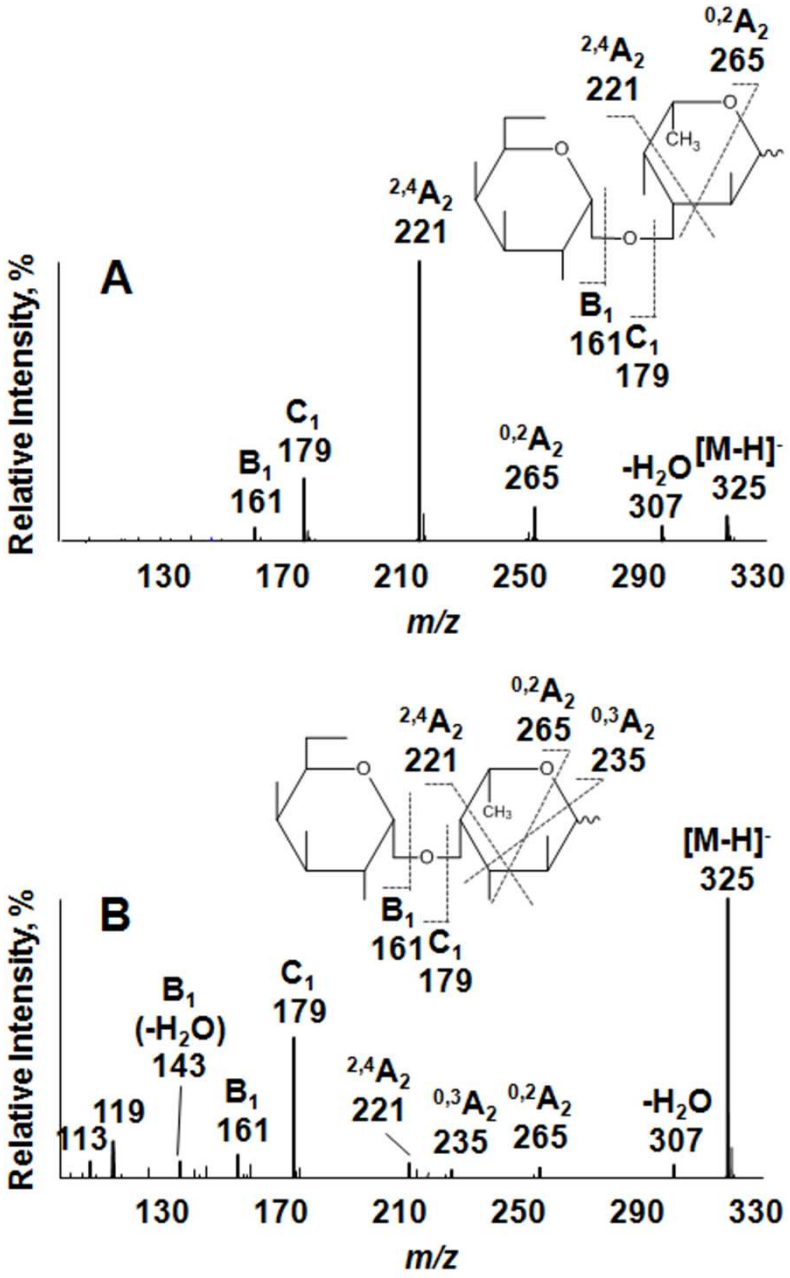

Figure 5-5. ESI-MS/MS spectra of $[M-H]^-$ ions of oligosaccharides from *α*-Gal with melibiose as galactosyl-donor and fucose as galactosyl-acceptor. (A) Gal*α*-(1→3)-Fuc *m/z* 325 at retention time 11.5 min; (B) Gal*α*-(1→4)-Fuc *m/z* 325 at retention time 14.0 min.

In the presence of lactose, one additional trisaccharide eluting at 37.5 min was formed; this product was identified as Gal*α*-(1→6)-Gal*β*-(1→4)-Glc (**Figure 5-6A**). Disaccharides formed as the result of reactions with melibiose and lactose could not be identified, due to co-elution with lactose. In addition, the intense peak due to Gal*α*-(1→6)-Gal*β*-(1→4)-Glc co-eluted at the tail end over the Gal*α*-(1→3)-Gal*α*-(1→6)-Gal/Glc product (retention time 39.9 min) formed from melibiose and could not be distinguished in an extracted ion chromatogram of *m/z* 503 (**Table 5-3**, **Appendix 4 Figure 4A-1**).

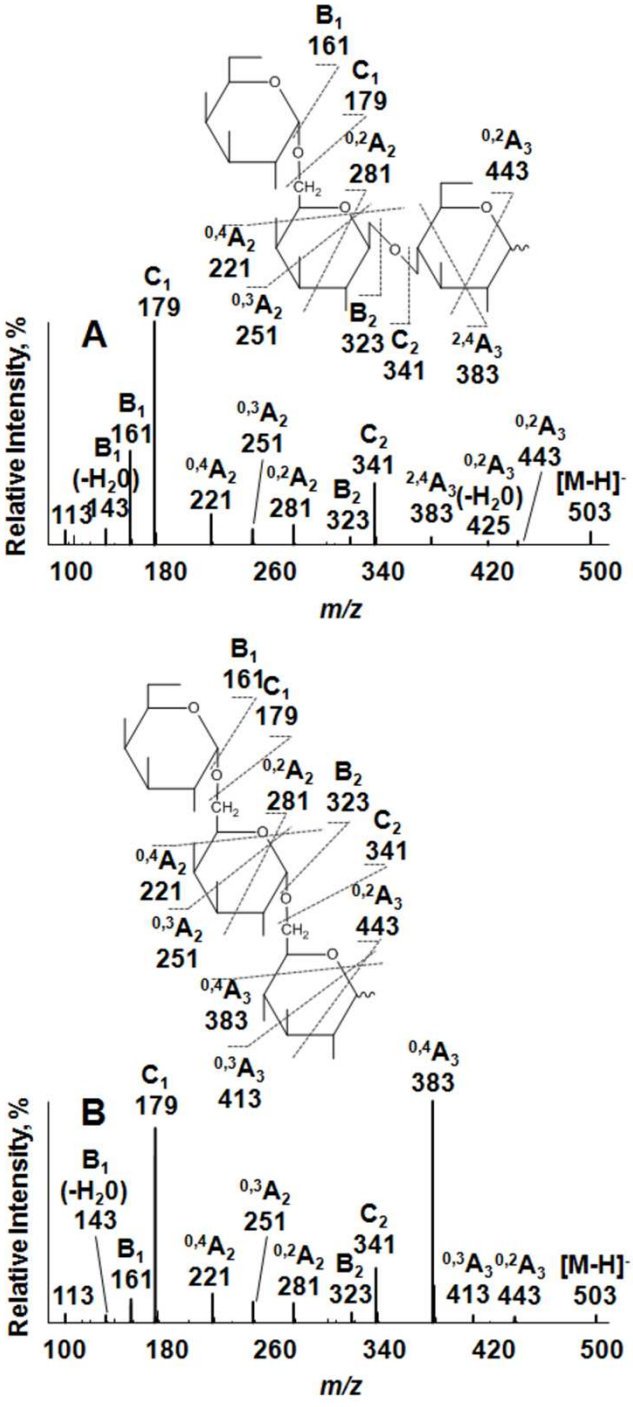

Figure 5-6. ESI-MS/MS spectra of $[M-H]^-$ ions of oligosaccharides from *α*-Gal with melibiose as galactosyl-donor and lactose as galactosyl-acceptor. (A) Gal*α*-(1→6)-Gal*β*-(1→4)-Glc *m/z* 503 at retention time 37.5 min; (B) Gal*α*-(1→6)-Gal*α*-(1→6)-Gal/Glc *m/z* 503 at retention time 53.2 min.

Finally, in reactions with GlcNAc, one new disaccharide eluting at 15.6 min was identified as Gal*α*-(1→6)-GlcNAc (**Figure 5-7**). As with Gal*β*-(1→6)-GlcNAc, Gal*α*-(1→6)-GlcNAc did not display the characteristic *m/z* 251 fragmentation ion (Black *et al*., 2012).

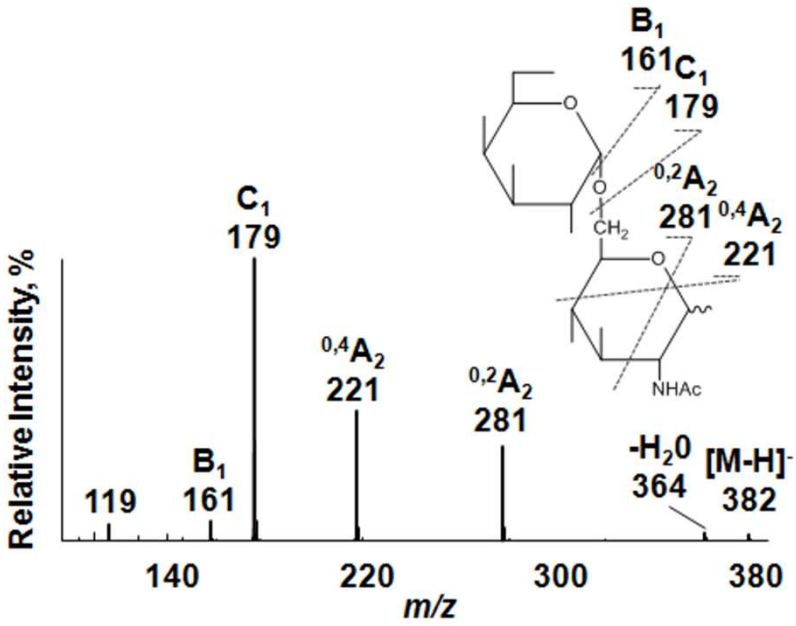

Figure 5-7. ESI-MS/MS spectra of $[M-H]^-$ ions of Gal*α*-(1→6)-GlcNAc *m/z* 382 at retention time 15.6 min from *α*-Gal with melibiose as galactosyl-donor and GlcNAc as galactosyl-acceptor.

5.3.6. Compositional analysis of oligosaccharides produced from raffinose

α-Gal formed a total of four products when raffinose was present as a substrate. Trisaccharides were identified as Gal*α*-(1→3)-Glc*α*-(1→2)-Fru

(retention time 24.2 min) and Galα-(1→4)-Glcα-(1→2)-Fru (retention time 33.0 min) (**Figure 5-8A-B**).

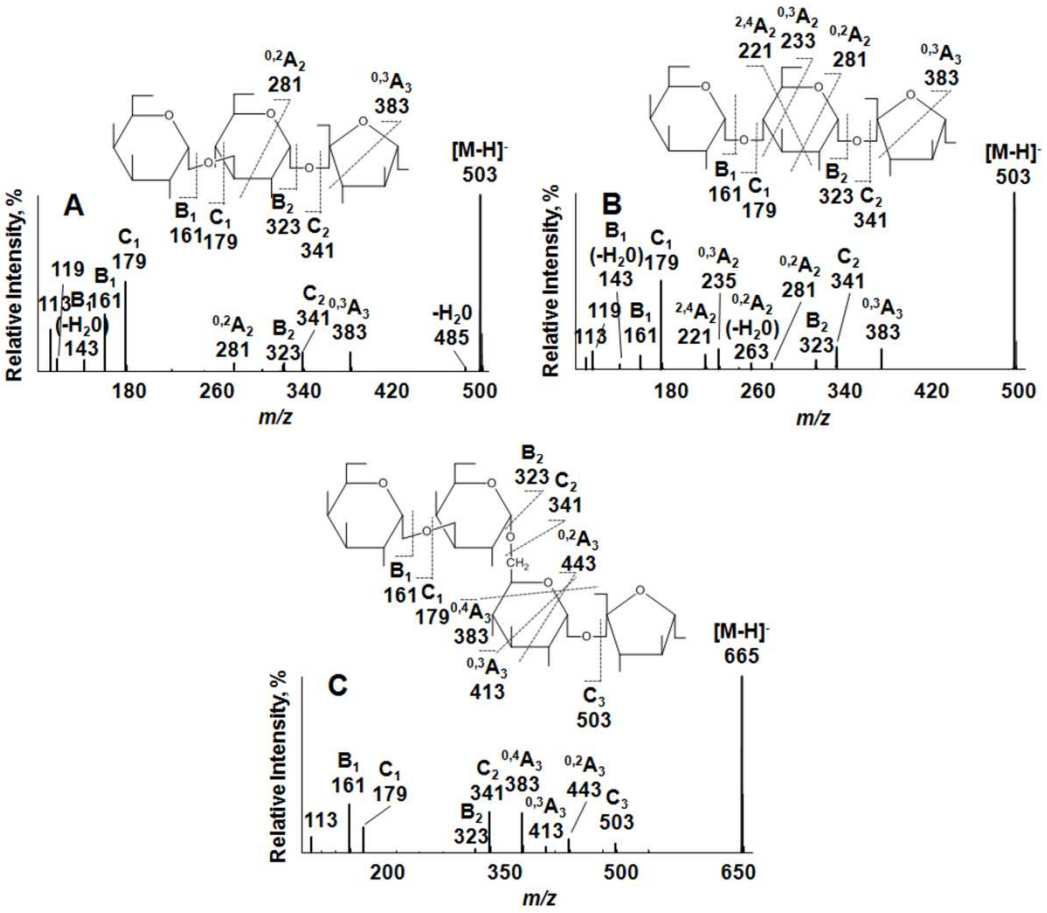

Figure 5-8. ESI-MS/MS spectra of [M-H]$^-$ ions of oligosaccharides from α-Gal with raffinose galactosyl-donor and -acceptor. (A) Galα-(1→3)-Glcα-(1→2)-Fru *m/z* 503 at retention time 24.2 min; (B) Galα-(1→4)-Glcα-(1→2)-Fru *m/z* 503 at retention time 33.0 min; (C) Galα-(1→3)-Galα-(1→6)-Glcα-(1→2)-Fru *m/z* 665 at retention time 48.5 min. Raffinose reactant eluted at 31.2 min, its spectrum is shown in Appendix 5, Figure 5A-1.

Likely, *α*-(1→6)-linkages also formed, producing raffinose (retention time 31.2 min); in this case, reactant and product were indistinguishable. Tetrasaccharides were identified as Gal*α*-(1→3)-Gal*α*-(1→6)-Glc*α*-(1→2)-Fru product (retention time 48.5 min) (**Figure 5-8C**) and stachyose (Gal*α*-(1→6)-Gal*α*-(1→6)-Glc*α*-(1→2)-Fru, retention time 75.7 min) (**Figure 5-9**). Stachyose production by *α*-Gal from raffinose was confirmed by comparison of the retention time and the fragmentation pattern of acceptor carbohydrates with an authentic standard (**Figure 5-9**).

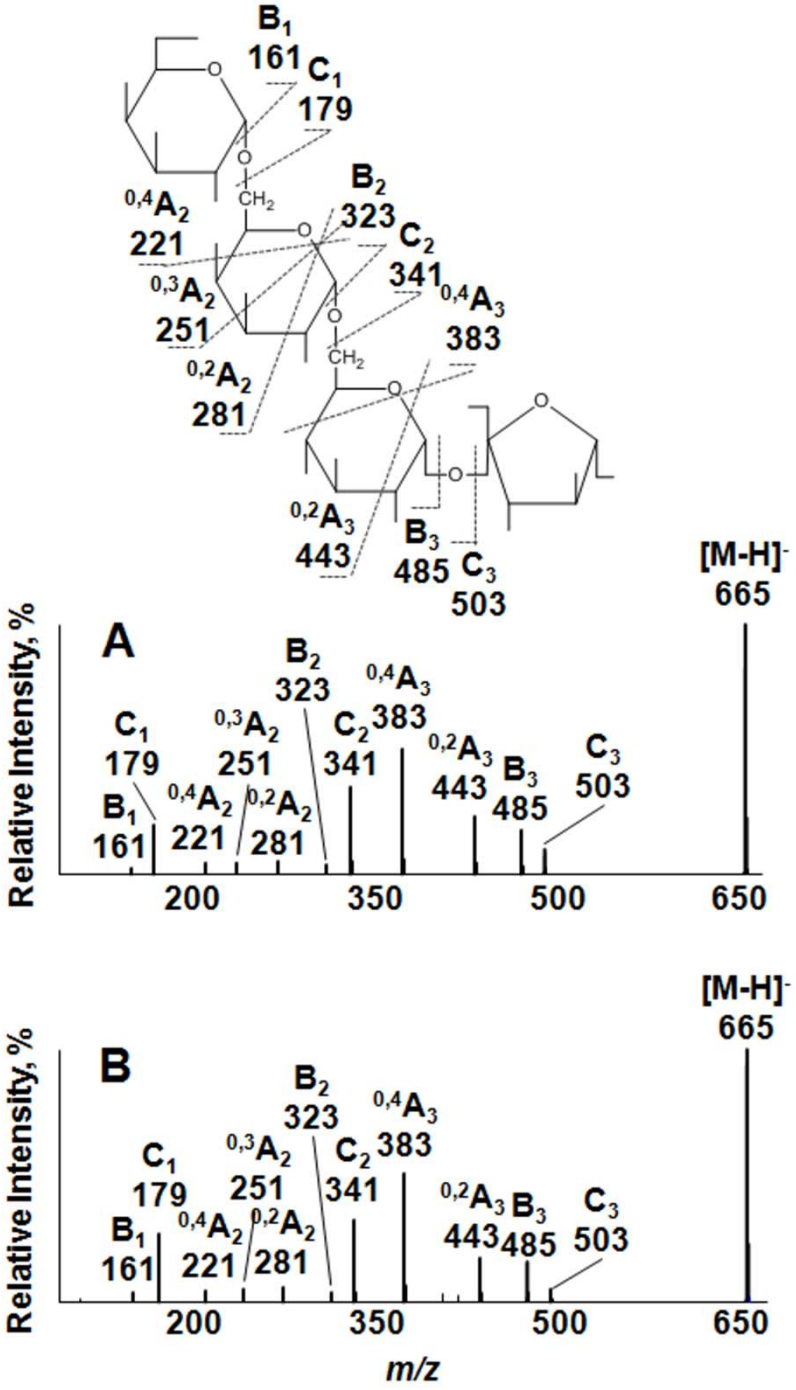

Figure 5-9. ESI-MS/MS spectra of $[M-H]^-$ ions representative of stachyose. (A) stachyose standard; (B) Gal*α*-(1→6)-Gal*α*-(1→6)-Glc*α*-(1→2)-Fru (*m/z* 665 at retention time 75.7 min) produced by *α*-Gal with raffinose galactosyl-donor and -acceptor.

5.4. Discussion

Lactic acid bacteria are excellent sources for food grade α-Gal (Alazzeh *et al*., 2009; Donkor *et al*., 2007). α-Gal gene sequences are present in genomes of most lactobacilli (Gänzle & Follador, 2012) but α-Gal has been characterized only in few species, including *L. reuteri*, *L. plantarum*, *L. fermentum*, *L. brevis*, and *L. buchneri* (Mital *et al*., 1973; Tamura & Matsushita 1992; Garro *et al*., 1993). Past studies reported α-Gal activity and oligosaccharide formation, however, no connection between observed activity and protein sequences were made and the potential of transgalactosylation of acceptor carbohydrates with α-Gal was not explored. Silverstroni *et al*., (2002) previously aligned the putative active site for α-Gal, which was biochemically and physiologically characterized in *L. plantarum*. The comparison of the amino acid sequences of the uncharacterized hydrolases of *L. reuteri* 100-16 and 100-23, in combination with biochemical characterization, confirmed that AGA16 and AGA23 are fully functional α-Gal.

The optimum pH of α-Gal from *L. reuteri* 100-16 and 100-23 match the growth optimum of the organism and prior reports on α-Gal activity of lactobacilli (Mital *et al*., 1973; Garro *et al*., 2003; Carrera-Silva *et al*., 2006). However, the observed temperature optimum of α-Gal from *L. reuteri* was below the temperature optimum of α-Gal from *L. fermentum* and other lactobacilli (Mital *et al*., 1973; Garro *et al*., 2003; Carrera-Silva *et al*., 2006), and below the growth optimum of *L. reuteri*, 37 °C – 42 °C (Gänzle *et al*., 1995; van Hijum *et al*., 2002). Moreover, the optimal temperature of α-Gal shifted when high

concentrations of melibiose were incorporated into the enzyme reactions. Therefore, melibiose and sucrose may stabilize heat-labile enzymes above the optimal temperature and behave as compatible solutes for microorganisms in a physiological context (Brown & Simpson, 1972).

Past studies on oligosaccharide formation of *α*-Gal have employed melibiose or raffinose as galactosyl-donor and galactosyl-acceptor (Mittal *et al*., 1973; Tzortzis *et al*., 2003). Manninotriose (Gal*α*-(1→6)-Gal*α*-(1→6)-Glc) was tentatively identified as acceptor product, indicating that oligosaccharide formation by *α*-Gal introduces *α*-(1→6) linkages (Mittal *et al*., 1973). The present study confirmed the formation of *α*-(1→6) in oligosaccharides by identification of manninotriose and stachyose produced from melibiose and raffinose, respectively. Additionally, *α*-(1→3) and *α*-(1→4) linkages were formed by *α*-Gal. Comparable to *β*-Gal of lactic acid bacteria (Gänzle, 2012; Black *et al*., 2012), *α*-Gal of lactobacilli thus produce different linkages in the transgalactosylation reaction. While the production of *α*-GOS or raffinose family oligosaccharides with *α*-Gal of lactic acid bacteria is possible, it is unlikely to have practical applications. Raffinose family oligosaccharides widely occur in nature, and the conversion of raffinose-family oligosaccharides to *α*-GOS by levansucrase activity is straightforward and attained with a high yield (Teixeira *et al*., 2012).

This study is the first to clearly demonstrate the formation of oligosaccharides using acceptor sugars such as fucose, lactose and GlcNAc by *α*-Gal in lactic acid bacteria. HPLC analyses not only allowed quantification of

hydrolase and transferase activities, but also demonstrated the ability of *α*-Gal from *L. reuteri* to produce oligosaccharides. From LC/ESI-MS/MS analyses, the composition, sequence and in many cases the linkage types of formed *α*-GOS were determined. Linkage-type information of reaction products was achieved since the characteristic fragment ions differentiating (1→2)-, (1→3)-, (1→4)- and (1→6)-linkages are similar for both *β*-GOS and *α*-GOS (Dallinga & Heerma, 1991; Mulroney *et al*., 1995). The linkage-types of HeOS formed by *β*-Gal were previously characterized using a similar LC/ESI-MS/MS method along with authentic *β*-HeOS standards (Black *et al*., 2012). Hence, these fragmentation patterns were applied in the present work in order to characterize the linkage types of HeOS formed with *α*-Gal. In case of stachyose, the MS/MS spectra of acceptor products could also be compared to authentic standards, further confirming that the characteristic ions to determine linkage-type are transferable between *β*-GOS and *α*-GOS. Similar to HeOS produced by *β*-Gal (Black *et al*., 2012), *α*-Gal of *L. reuteri* exhibited a preference for the formation of (1→6)-linkages, although (1→3)- and (1→4)-linkages were also observed in acceptor reactions with fucose or lactose. The formation of (1→3)-, (1→4)-, and (1→6)-linkages in transgalactosylation of melibiose and acceptor carbohydrates may allow the targeted synthesis of oligosaccharides for use as receptor analogues to prevent pathogen adhesion. For example, the formation of *α*-(1→4) linkages by *α*-Gal may allow formation of Gal*α*-(1→4)-Gal*β*-(1→4)-Glc (globotriose) by transgalactosylation of lactose. Globotriose and globo-series oligosaccharides are

abundantly present in on the surface of animal and human mucosal cells, and are widely used by several bacterial pathogens or their toxins for establishing adherence (Jacewicz *et al*., 1986; Stromberg *et al*., 1990; Samuel *et al*., 1990; Leach *et al*., 2005) However, in the present study, formation of globotriose from melibiose and lactose could not be confirmed by HPLC or MS data and comparison with an authentic standard.

In conclusion, heterologously expressed *α*-Gal from *L. reuteri* was used to produce novel oligosaccharides; additionally, LC/ESI-MS/MS was used to characterize composition, monosaccharide sequence, and the linkage type of (most) acceptor products. Our work leads to improving industrial processes to produce oligosaccharides with physiological functionality, particularly with applications to prevent pathogen adhesion to mammalian hosts. Our findings may contribute to the future incorporation of *α*-GOS as therapeutic functional food ingredients to reduce the incident of gastrointestinal infections and to improve the health of farm animals and humans.

5.5. References

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6. Structural and functional characterization of galactosylated chitin-oligosaccharides and chitosan-oligosaccharides[5]

6.1. Introduction

Chitin is one of the most abundant natural polymers, commonly found in the exoskeletons of many invertebrates and in the cell walls of most fungi. Chitin is extracted commercially from shellfish wastes (Mathur & Narang, 1990; Barreteau *et al*., 2005). Chitin is a linear homopolymer of *β*-(1→4)-linked N-acetylglucosamine (GlcNAc) monomers. Larger polymers of chitin are not utilized as additives for food products due to their insoluble nature (Barreteau *et al*., 2005). However, chitosan, the deacetylated form of chitin, is more soluble than native chitin in neutral aqueous solutions and is more soluble if depolymerized to chitosan-oligosaccharides (COS) (Jeon & Kim, 2000).

COS are inexpensive to produce, biodegradable and non-toxic to mammals (Qin *et al*., 2006). They possess a number of functional properties which have led to considerable interest in developing food applications. For example, COS promote the growth of beneficial gastrointestinal microbiota *in vitro* (Lee *et al*., 2002). The antimicrobial activity of COS against pathogenic bacteria and fungi also has been widely studied (Xia *et al*., 2011). Gram-positive and -negative bacterial cultures including *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Shigella dysenteriae*, and *Vibrio cholerae* are susceptible to the antibacterial activity of COS at concentrations of

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Asterisks (*) indicate contributations by Y. Hu

50 to 1000 mg L^{-1} (Shahidi *et al*., 1999; Lin *et al*., 2009). Antifungal activities of COS have been reported at a minimum concentration of 150 mg L^{-1} for *Aspergillus niger*, *Rhizopus apiculatus*, and *Mucor circinelloides* (Wang *et al*., 2007). Although the mechanism of antimicrobial activity is unknown, it has been proposed that the positively charged chitosan molecules, solubilized in a pH below 6, interact with the negatively charged microbial membranes leading to leakage of intercellular constituents or prevention of nutrient uptake. The antimicrobial activity is dependent upon the degree of polymerization and concentration of oligosaccharides (Dutta *et al*., 2012). More recently, COS were shown to inhibit the adhesion of enteropathogenic *E. coli* (EPEC) by interfering with bacterial-host cell interactions as receptor analogues (Quintero-Villegas *et al*., 2013). Receptor analogues prevent viruses, pathogenic bacteria and bacterial toxins from interacting with surface glycans of eukaryotic cells by acting as molecular decoys for adhesion, thus preventing infection. Since *β*-linked GlcNAc moieties often occur at the reducing end of mammalian cell surface glycans, similarly structured *β*-galactosylated-GlcNAc oligosaccharides can act as decoys. For example, N-acetyllactosamine, Gal*β*-(1→4)-GlcNAc, the core structure of human milk oligosaccharides, was identified as a competitive inhibitor to EPEC (Hyland *et al*., 2008). Gal*β*-(1→4)-GlcNAc also acts as a receptor for *P. aeruginosa*, *Salmonella typhimurium* and *Neisseria gonorrhoea* (Ramphal *et al*. 1991; Shoaf-Sweeney & Hutkins 2006). However, *β*-galactosylated N-glucosamine (GlcN) has yet to be produced and tested for activity.

CHAPTER 6

Galactosidases from lactobacilli form β-(1→4)- and α/β-(1→6)-linked oligosaccharides with GlcNAc as a galactosyl-acceptor (see Chapter 5; Black *et al*., 2012). However, it remains undetermined whether oligosaccharides, which are inexpensive to produce, are suitable galactosyl-acceptors. Moreover, the effect of acetylation on transgalactosylation reactions is unknown.

Lactobacillus species are widely used in food fermentations (Hammes & Hertel, 2009) and are a source of galactosidase to produce transgalactosylated oligosaccharides for food applications (Gänzle & Follador, 2012). Extending the range of products formed by transgalactosylation reactions, by using COS as acceptors, will increase the potential for products to act as molecular decoys to prevent adhesion and ultimately infection. Therefore, it was the aim of this study to determine if chitin- and chitosan-oligosaccharides are suitable acceptors for β-galactosidases. To investigate this, pure chitinbiose, chitintriose, chitinpentaose, and COS of various DPs, were tested to determine acceptor suitability. Resulting transgalactosylated oligosaccharides were identified by liquid chromatography-mass spectrometry (LC/MS) following procedures described earlier (Black *et al*., 2012). In addition, the galactoyslated COS were tested *in vitro* for pathogen adhesion activity (Wang *et al*., 2010).

6.2. Materials and methods

6.2.1. Chemicals and standards

Oligosaccharide standards including lacto-N-biose (Gal*β*-(1→3)-GlcNAc), N-acetyl-D-lactosamine (Gal*β*-(1→4)GlcNAc), *β*1-6 galactosyl-N-acetyl glucosamine (Gal*β*-(1→6)-GlcNAc), chitinbiose, chitintriose, and chitinpentose were purchased from Dextra Laboratories (Reading, United Kingdom). GlcNAc and *o*-nitrophenyl-*β*-galactoside were purchased from Sigma Aldrich (Oakville, Canada). COS (90% deacetylated) were enzymatically prepared from shrimp shells and were provided by Yumin Du, Department of Environmental Science at the University of Wuhan (Wuhan, China). Fisher Scientific (Ottawa, Canada) was used as a supplier of HPLC grade acetonitrile, methanol, and ammonium acetate. Other solvents were of analytical grade.

6.2.2. Bacterial strains and preparation of crude cell extract*

Lactobacillus plantarum FUA3112 was cultivated under microaerophilic conditions (1% O_2, balance N_2) at 30 °C in modified DeMan-Rogosa-Sharpe (mMRS) (de Man *et al*., 1960) containing 2% (w/v) lactose with a pH of 6.2. *Lactococcus lactis* MG1363, a *β*-Gal negative strain, was grown in a modified M17 with the addition of 0.5% (w/v) glucose at 30 °C. *Lc. lactis* MG1363 harboring pAMJ586 with LacLM from *L. plantarum*, as the sole source of *β*-Gal activity (Schwab *et al*., 2010), was grown similarly to *Lc. lactis* MG1363 with the addition of 5 mg L^{-1} erythromycin. Overnight cultures from single colonies were used to inoculate 500 mL mMRS or respective mM17 broth at a 1% (v/v).

Cultures were incubated until the medium was acidified to a range between pH 5.0 and 5.2. Cultured cells were harvested and washed twice before suspension in 1 mL of 50 mM sodium phosphate buffer (pH 6.5) with 10% glycerol and 1 mM magnesium chloride. The cell suspension was transferred to screw-cap tubes with 0.5 g of Zirconia/Silica beads (0.1 mm), disrupted in a Mini Beadbeater-8 (model 693, BioSpec, Bartlesville, OK) for 2 min and chilled in ice for a minimum of 5 min. The supernatant was collected by centrifugation of disrupted cells (15 300 × g for 10 min at 4 °C) and designated crude cell extract (CCE). The CCE was then used for oligosaccharide synthesis. The CCEs of *L. plantarum*, *Lc. lactis* and *Lc. lactis* expressing LacLM from *L. plantarum*, were collected from three independent culture fermentations. The protein content of CCEs was determined with the Bio-Rad protein assay reagent (Bio-Rad, Mississauga, Canada). *o*-Nitrophenyl-*β*-galactoside was used to determine specific enzymatic activity of CCEs calculated as enzyme activity relative to cell protein mass as described by Schwab *et al*. (2010). Similar to previous studies synthesizing oligosaccharides using *β*-Gal, activities of CCE ranged from 25 – 30 units (min × mg protein)$^{-1}$ (Schwab *et al*., 2010; Schwab *et al*. 2011; Black *et al*., 2012). CCE of *Lc. lactis* MG1363 displayed no *β*-Gal activity and was used as a negative control.

6.2.3. Synthesis of galactosylated chito-oligosaccharides in acceptor reactions*

To prepare saccharide solutions for reactions, lactose, GlcNAc, chitinbiose, chitintriose, chitinpentaose and COS were dissolved into 50 mM sodium phosphate buffer (pH 6.8) with 100 mM potassium chloride and 2 mM

magnesium chloride (PB) at 90 °C for all except COS, which was dissolved at 50 °C. The addition of 10% ethanol was used to improve the solubility of chitinbiose, chitintriose and chitinpentaose without impacting enzyme activity (data not shown). Chitinpentaose remained insoluble and was not used in reactions. Lactose was used at a concentration of 1 M to produce galacto-oligosaccharides (GOS) with 20% CCE of *L. plantarum*, *Lc. lactis* and *Lc. lactis* expressing LacLM from *L. plantarum* in three separate reactions. Similarly, acceptor reactions were performed using a 1 M total saccharide concentration of 1:1 (w/w) acceptor/lactose ratio with 20% CCE of *L. plantarum*, *Lc. lactis*, and *Lc. lactis* expressing LacLM from *L. plantarum*. All reactions were conducted at 45 °C for 16 h and terminated by heating to 95 °C for 10 min. Triplicate independant experiments were conducted.

6.2.4. Combined liquid chromatography/electrospray ionization tandem mass spectrometry

Un-derivatized oligosaccharide samples were analysed by liquid chromatography / electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) for structural characterization and for confirmation of the composition of saccharide solutions prior to transgalactosylation reactions. Separations were conducted on an Agilent 1200 series LC system (Agilent Technologies, Palo Alto, CA) at 60 °C using a Supelcosil LC-NH_2 column (250 mm x 4.6 mm i.d., 5 μm; Sigma Aldrich, Oakville, Canada). Each solution analysed contained a final concentration of 0.1 g L^{-1} oligosaccharide in water, with the exception of quasi

MS^3 experiments, where the concentration was increased to 1 g L^{-1}. Each solution was injected in 25 μL volumes onto the column using a gradient of acetonitrile and water at a flow rate of 1.5 mL min^{-1}, starting with an acetonitrile concentration of 80%; decreasing to 75% at 20 min; 60% at 35 min; then returning to 80% at 37 min. The effluent from the column was split at a ratio of 1:4 (v/v), so that the flow rate to the mass spectrometer was 0.4 mL min^{-1} with the remainder going to waste. A 10% (v/v) post-column addition of 40 mM ammonium acetate in methanol was delivered by an Agilent 1200 series isocratic pump to the flow entering the ESI source.

Negative ion ESI-MS and collision induced dissociation tandem mass spectrometry (CID-MS/MS) was performed on a QStar® Elite hybrid orthogonal Q-TOF mass spectrometer coupled to a TurboIon Spray® source with Analyst® QS 2.0 software (Applied Biosystems/MDS Sciex, Concord, Canada). The source conditions were as follows: nebulizer gas, 50 (arbitrary units); auxillary gas, 60 (arbitrary units); ionspray voltage, -4500 V; curtain gas, 25 (arbitrary units); source temperature, 400 °C; declustering potential, -50 V; focusing potential, -190 V; declustering potential-2, -10 V and a mass range of *m/z* 100-900. Fragmentation was achieved using nitrogen as a collision gas at a collision energy that varied between -10 to -25 eV, decreasing in energy as the degree of polymerization increased. Quasi MS^3 spectra were obtained from the MS/MS spectra of in-source fragment ions formed by increasing the declustering potential by an additional 30 V.

6.3. Results

6.3.1. Analysis of oligosaccharides by liquid chromatography tandem mass spectrometry

LC/ESI-MS experiments were performed to measure elution times and exact masses of galactosylated products formed from *β*-transgalactosylation of lactose, GlcNAc, chitinbiose, chitintriose and COS (**Table 6-1**).

Table 6-1. Mass accuracy of deprotonated molecular ions and retention times of galactosylated products.

Sample	Oligosaccharide	Retention Time (min)	Measured Mass (Da)	Error (mDa)
Lactose + Chitinbiose	Gal – GlcNAc – GlcNAc	10.4	585.2148	-0.06
	Gal – Gal – GlcNAc – GlcNAc	16.1; 17.1	747.2692	1.51
Lactose + Chitintriose	Gal – GlcNAc – $(GlcNAc)_2$	15.7	788.2942	-0.03
Lactose + COS	Gal – GlcN	8.0	340.1235	-1.41
	Gal – GlcN – GlcN	14.8	501.1931	-0.63
	Gal – GlcN – GlcNAc	11.0	543.2030	-1.29
	Gal – GlcN – $(GlcN)_2$	29.3	662.2623	-0.24
	Gal – Gal – GlcN – GlcN	26.2	663.2466	0.05
	Gal – $(Gal)_2$ – GlcN	25.1	664.2301	-0.47
	Gal – GlcN – GlcN – GlcNAc	20.4	704.2745	1.40
	Gal – GlcN – $(GlcN)_3$	31.6	823.3326	1.26
	Gal – Gal – GlcN – $(GlcN)_2$	30.8	824.3154	0.04
	Gal – $(Gal)_2$ – GlcN – GlcN	27.6	825.2933	-2.05
	Gal – Gal – GlcN – GlcN – GlcNAc	26.2	866.3223	-3.62

Nomenclature: Galactose, Gal; N-acetylglucosamine, GlcNAc; Deacetylated N-glucosamine, GlcN; Chitosan-oligosaccharides, COS

β-Gal reactions with lactose only and GlcNAc with lactose formed GOS and galactosylated-GlcNAc oligosaccharides, respectively, as described by Black *et al*. (2012). Additionally, these LC/ESI-MS experiments confirmed that chitinbiose, chitintriose and COS act as acceptors for *β*-Gal reactions from *L. plantarum* and *Lc. lactis* expressing LacLM from *L. plantarum* (**Table 6-1**). In

contrast, reactions involving *Lc. lactis* formed no identifiable hydrolyzed- or galactosylated-products, confirming no galactosidase activity. Three products were formed with chitinbiose as a galactosyl-acceptor in *β*-Gal reactions and one product with chitintriose as a galactosyl-acceptor. These products were first identified by their elution order and by the accurate determination of *m/z* for deprotonated molecular ions using high resolution mass spectrometry.

Tandem MS (MS/MS) and quasi MS^3 experiments were performed on galactosylated-chitinbiose and –chitintriose to determine monosaccharide sequence and glycosidic bond positions. The Gal–GlcNAc–GlcNAc compound, formed from chitinbiose as a galactosyl-acceptor, presented *m/z* 179 and 382 C-type glycosidic cleavages in MS/MS confirming the monosaccharide sequence (**Figure 6-1A**). Additionally, $^{0,2}A$ and $^{0,2}A(-H_2O)$ fragments, along with the absence of a $^{0,3}A$ cross-ring fragment, indicate the presence of 1→4 linkages (Black *et al*., 2012). To further confirm the linkage formed by *β*-Gal in the transfer of galactose, the C_2 ion at *m/z* 382 was further fragmented in a quasi MS^3 experiment (**Figure 6-1B**). Similar to the MS/MS spectrum of the trisaccharide molecular ion (**Figure 6-1A)**, $^{0,2}A$ and $^{0,2}A(-H_2O)$ fragment ions were formed with the absence of a $^{0,3}A$ fragment ion. Furthermore, the *m/z* 382 fragmentation from the quasi MS^3 experiment matched the fragmentation of an authentic Gal*β*-(1→4)-GlcNAc standard (Black *et al*., 2012). In summary, the compound that eluted at 10.4 min with a *m/z* 585.2149 was clearly identified as the product of *β*-transgalactosylation with chitinbiose as an acceptor as Gal*β*-(1→4)-GlcNAc*β*-

(1→4)-GlcNAc. However, the abundance of *m/z* 747.2692 ions, indicative of Gal-Gal-GlcNAc-GlcNAc products eluting at 16.1 and 17.1 min, was too low to obtain reliable MS^2 spectra for linkage determination (**Appendix 5**, **Figure 5A-1A**).

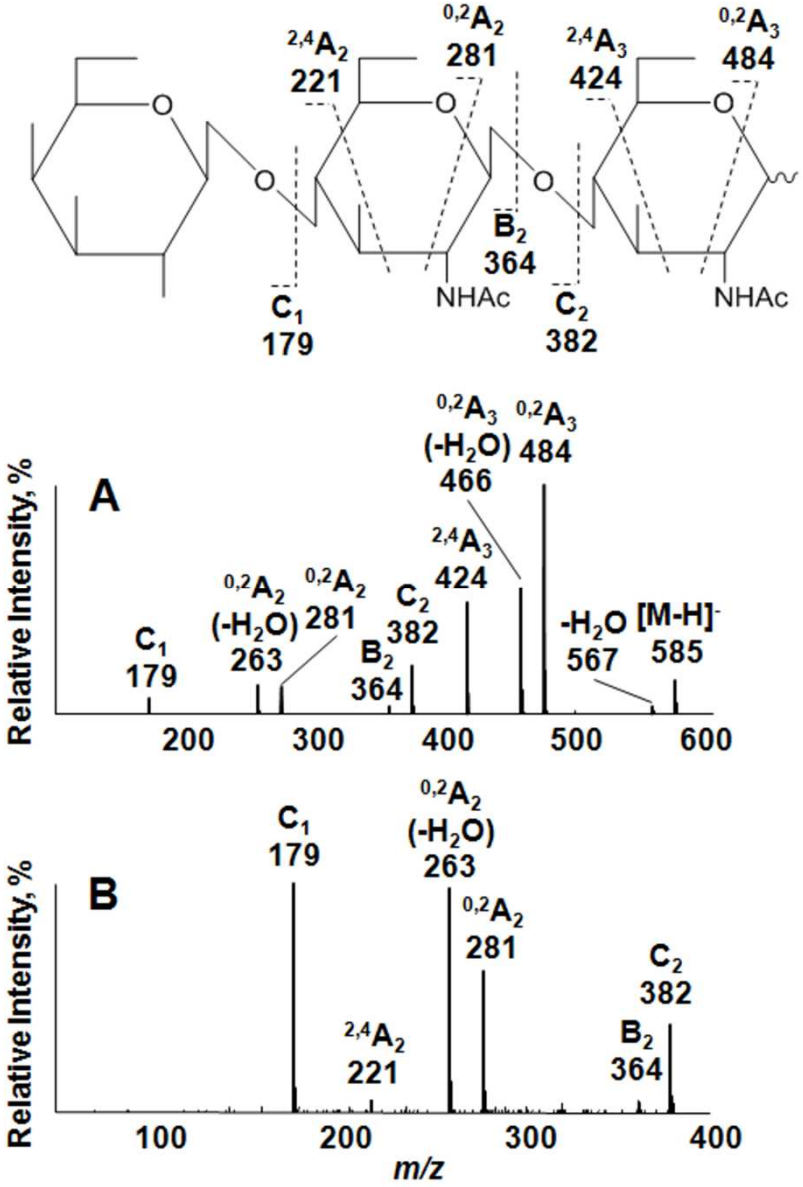

Figure 6-1. LC/ESI-MS/MS spectra of sample compound Galβ-(1→4)-GlcNAcβ-(1→4)-GlcNAc with elution time of 10.4 min. (A) $[M-H]^-$ ion at *m/z* 585.2149 ($C_{22}H_{37}O_{16}N_2$); (B) In-source fragment ion C_2 at *m/z* 382.1355 ($C_{14}H_{24}O_{11}N$).

Compounds formed with chitintriose as a galactosyl-acceptor in β-Gal reactions were also analyzed via MS/MS and quasi MS^3 experiments. The C-type

fragments arising from the tandem mass spectra of Gal–GlcNAc–$(GlcNAc)_2$ confirm part of the monosaccharide sequence (**Figure 6-2A**). Moreover, the accompanying A-type cross-ring fragments of $^{0,2}A$ and $^{0,2}A(-H_2O)$ along with the absence of a $^{0,3}A$ fragment, confirmed all linkages as 1→4, except where galactose was transgalactosylated. In order to determine the remaining structural information, a quasi MS^3 experiment was completed again targeting fragment C_2 at *m/z* 382 (**Figure 6-2B**). The sequence order for the remaining monosaccharides could be ascertained from the resulting C_1 fragmentation at *m/z* 179 indicating a galactose moiety at the non-reducing end. The reoccurring quasi MS^3 fragmentation of *m/z* 382 concluded the presence of a 1→4 linkage. Overall, the compound produced by *β*-transgalactosylation of lactose and chitintriose, eluting at 15.7 min with a *m/z* 788.2942 was identified as Gal*β*-(1→4)-GlcNAc*β*-(1→4)-GlcNAc*β*-(1→4)-GlcNAc (**Appendix 5**, **Figure 5A-1B**). Equivalent results were obtained with *L. plantarum* and *L. lactis* expressing LacLM of *L. plantarum*.

COS were supplied as 90% deacetylated and were predominately composed of DP 2 and 3 oligosaccharides, although mono- and tetra-saccharides were also detected by LC/MS. Owing to the diversity of acceptor carbohydrates, many galactosylated-COS product ions were observed in LC/MS experiments, but at low abundances that were inadequate for sequence and linkage analysis in the context of the tandem MS experiments used.

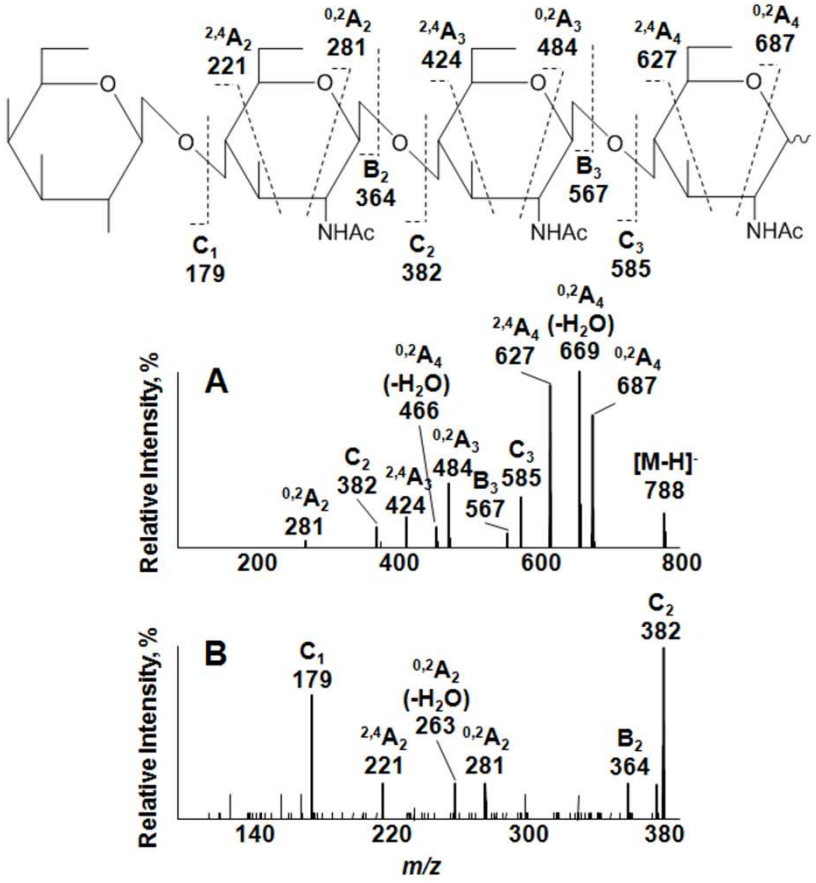

Figure 6-2. LC/ESI-MS/MS spectra of sample compound Gal*β*-(1→4)-GlcNAc*β*-(1→4)-GlcNAc*β*-(1→4)-GlcNAc with elution time of 15.7 min. (A) $[M-H]^-$ ion at *m/z* 788.2942 ($C_{30}H_{50}O_{21}N_3$); (B) In-source fragment ion C_2 at *m/z* 382.1355 ($C_{14}H_{24}O_{11}N$).

6.4. Discussion

Galactosylated- GlcNAc oligosaccharides are often targets for different biological functions, including recognition by pathogenic toxins, microorganisms and antibodies (Varki, 1993; Kunz *et al*., 2000; Ofek *et al*., 2013). LC/MS was used to determine that chitinbiose, chitintriose and COS were both suitable acceptors in *β*-Gal transgalactosylation reactions. Galactosylated-chitinbiose and -

chitintriose were identified indicating that oligosaccharides as well as GlcNAc monomers act as galactosyl-acceptors in *β*-Gal reactions (Black *et al*., 2012).

From LC/MS/MS analyses, lactobacilli *β*-Gal formed specifically *β*-(1→4)-linkages during the galactosylation of chitinbiose and chitintriose. The *β*-(1→4)-linkage of Gal*β*-chitinbiose and -chitintriose contrasts with the predominant *β*-(1→6)-linkage formed with the monomer GlcNAc as a galactosyl-acceptor of *β*-Gal (Black *et al*., 2012). This result is promising, as galactosylated *β*-(1→4)-linked GlcNAc often demonstrates anti-adhesion activity (Ramphal *et al*. 1991; Shoaf-Sweeney & Hutkins 2006; Hyland *et al*., 2008).

Galactosylated-COS, ranging from DP1 to DP4 with various degrees of acetylation, were also identified by LC/MS, demonstrating that the deacetylated amine content of reactant COS did not prevent galactosylation. During *β*-Gal reactions, the protonation of glucosamine was reduced by maintaining the pH at 6.8, which may have increased the suitability of COS as galactosyl-acceptors. However, protonated COS were not tested as acceptors in this study. Overall, the variation of COS has been extended by transgalactosylation reactions of *β*-Gal from lactobacilli to produce new compounds.

Gal*β*-COS inhibited agglutination of porcine erythrocytes by ETEC strains at a concentration of 2.5 g L^{-1} (Yan *et al*., 2013), thereby effectively mimicking mammalian receptors and competitively binding with pathogenic bacterial cells. Because lactose was used as a source of galactose for transgalactosylation reactions, GOS were preferentially formed over Gal*β*-COS in these samples tested

for haemagglutination (data not shown). Gal*β*-COS exhibited increased anti-adhesion activity compared to that of GOS, but less compared to COS (Yan *et al*., 2013). The anti-agglutination activity of COS was effective at lower concentrations than expected, as Quintero-Villegas *et al*., (2013) reported a concentration of 0.5 g L^{-1} COS had only 30% inhibition of EPEC adherence. Our results deviate from previous studies due to the possible activity of impurities remaining from purification steps taken to remove proteins in samples prior to haemagglutination testing. Additionally, *E. coli* strains differed, as this work tested COS adherence on ETEC, while the previous study reported a lower activity on EPEC (Quintero-Villegas *et al*., 2013).

The conversion of lactose with *β*-Gal from lactobacilli and chito-oligosaccharides as acceptors allows for the formation of novel oligosaccharides with potential anti-diarrheal effects. This is the first report on the formation of galactosylated-chito-oligosaccharides and their ability to mimic glycoconjugate receptors to pathogenic *E. coli*, thus preventing the first step in infection. The concept of COS as a versatile food ingredient is not new (Shahidi *et al*., 1999); additionally, COS is an inexpensive, abundant waste product of shellfish. Galactosylation of chito-oligosaccharides increases the structure variation for functional components that can be used for novel food product development.

6.5. References

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7. General Discussion and Conclusions

Liquid chromatography/mass spectrometry (LC/MS) was used to characterize isomeric bioactive fatty acid and oligosaccharide compounds produced by lactobacilli with no or little derivatization. Preparative steps were unnecessary and hydroxylation was only required to induce MS/MS fragmentation in order to determine double bond location within the fatty acid structure. Base-line resolution of isomeric compounds was achieved for all hydroxylated fatty acids and most oligosaccharides with simple and reproducible LC separations compatible with MS ionization sources. This allowed for the sensitive detection and identification of additional isomers from the preliminary work of others.

A normal phase LC/atmospheric pressure photo ionization (APPI)-MS method was developed in this study to analyze un-derivatized fatty acids and was advantageous over both GC/MS and LC/electrospray ionization (ESI)-MS analysis methods. Hydroxy fatty acid analysis by GC/MS has previously required samples to be derivatized to fatty acid methyl esters (FAME) and additionally to trimethylsilyl ethers (TMS) (Christie & Han, 2010). TMS-FAME derivatives are analyzed by GC/MS with electron impact and the resulting spectra are routinely compared to existing libraries to identify their structure. However, comparing ions to library spectra is not possible for novel compound identification. Alternatively, 4, 4-dimethyloxazoline (DMOX) derivatives, or other charge localizing derivatives, have been prepared for GC/MS analysis for the structural identification of unsaturated fatty acids; however, as outlined in this work for

hydroxy fatty acid analysis, if the hydroxyl group is located proximal to the derivatized carboxyl group, the double bond location(s) will remain elusive. In this work, the separation of lipid extracts of *Lactobacillus* sp. by normal phase LC was greatly resolved and the total run time was reduced by 10 min compared to previous GC analysis of the same lipid extracts (Shahzadi, 2011). Normal phase LC/APPI-MS allowed for isomeric separation of un-derivatized hydroxy fatty acids compared to reverse phase LC/ESI-MS analysis. Normal phase LC/APPI-MS was two times more sensitive than reverse phase LC/ESI-MS, with a linear range of three orders of magnitude for antifungal hydroxy fatty acid analysis. LC/APPI-MS/MS was suitable for the positional determination of hydroxyl groups; however, vicinal hydroxylation derivatization was required for the location of double bonds, as minimal fragmentation resulted from fatty acids with less than three unsaturations (this work; Kerwin & Wiens, 1996). Regardless, the normal phase LC/APPI-MS/MS presented in this work is the most rapid method for the complete structural identification of novel hydroxy fatty acids to date (Hou, 1995; Ogawa *et al*., 2001; Kishimoto *et al*., 2003; Christie & Han, 2010; Volkov *et al*., 2010; Shahzadi, 2011; Kishino *et al*., 2011; Yang *et al*., 2013a; Yang *et al*., 2013b).

LC/ESI-MS/MS was used to elucidate new information about galactosidases of lactobacilli and the oligosaccharides that are formed. Although LC/ESI-MS/MS for structural analysis of un-derivatized oligosaccharides is not a new concept, oligosaccharide analysis by GC/MS and/or nuclear magnetic resonance

(NMR) is still common practice (Dass, 2007; Ruas-Madiedo *et al*., 2012). By using LC/ESI-MS/MS in this work, the specificity of linkage formation by *β*-galactosidases (Gal) of lactobacilli was extended. Previously, *β*-Gal of lactobacilli were known to form only *β*(1→3)- or *β*(1→6)-linked galacto-oligosaccharides (GOS) (Toba *et al*., 1981; Splechtna *et al*., 2006; Torres *et al*., 2010; Gänzle, 2012). This work was the first to identify *β*(1→4)-linked GOS produced by *β*-Gal activity from lactobacilli. Additionally, the structural characterization of hetero-oligosaccharides (HeOS) from *β*-Gal of lactobacilli was the first of its kind. The LC/ESI-MS/MS method for structural analysis was extended to *α*-Gal activity of lactobacilli, where *α*-GOS and *α*-HeOS were characterized fully, including linkage type, which had not been previously reported (Mital *et al*., 1973; Donkor *et al*., 2007; Alazzeh *et al*., 2009). Similarly, LC/ESI-MS/MS was utilized to structurally identify galactosylated *β*-chito-oligosaccharides (COS) produced by lactobacilli, which is also the first report of glycosylation of COS. Hence, this work demonstrates the significance of LC/ESI-MS/MS as a method for the characterization of isomeric compounds produced from enzymatic conversion of saccharides by lactobacilli. No derivatizations or purification steps were required.

LC/MS methods developed in this work were shown to be efficient and sensitive. With further improvements to LC/MS systems including increased resolution, sensitivity, accuracy and compatible chromatography (Xie *et al*., 2012), and the results presented within this work, demonstrates that LC/MS

should be utilized as the primary analysis for structural characterization of bioactive carbohydrates and lipids produced from enzymatic conversion by lactobacilli.

High resolution separation of isomeric compounds for complete structural identification within this work allowed for advancements in structure-function relationships and metabolic pathway determination of lactobacilli. Substrates of enzymatic conversion often have similar structures to the active products, and/or the active products may be similarly structured to non-active products. As analytical techniques advance, additional information is obtained compared to previous studies that allow for greater understanding of the function of active fatty acid and oligosaccharide compounds, which previously may have evaded proper detection. Thus, in this work structural analysis methods by LC/MS, combined with high-speed counter-current chromatography as a preparative step, were able to advance the literature for active fatty acid compounds and novel insight for lactobacilli pathway elucidation for conversion of linoleic acid to CLA. LC/MS/MS analysis of oligosaccharides in this work extended, or presented for the first time, the linkage-types formed by β- and α-Gal enzymes in lactobacilli. Once the structures of active fatty acid and oligosaccharide compounds were determined and enzymatic pathways elucidated, studies were performed to determine whether active compound conversion directly in foods was practical, or whether the application should be limited to biotechnology alone. A functional

amount of antifungal hydroxy fatty acids was formed in sourdough bread, from the conversion of linoleic acid by both *L. hammesii* and lipoxygenase enzymes found within the wheat flour, to delay the growth of some mould species. Lactobacilli conversion of saccharides into functional oligosaccharides up to therapeutic levels has not been determined in a food system to date; however, whey permeate containing lactose, a waste product from cheese manufacture, may be used as a source for conversion (Lee, 2009).

Structure elucidation and bioactivity assays ultimately assist in attributing function to genome sequences by way of enzyme activity. If homology exists between specific lactobacilli sequences, predictions may be inferred about the formation of active compounds by other lactobacilli with similar genomes (Gänzle & Follador, 2012; Yang *et al*., 2013a). Lactobacilli contain many enzymes for their own growth and metabolism, and ultimately these food-related bacteria offer a unique toolset which may be exploited to produce biologically active compounds in order to promote the health of the consumer beyond basic nutrition.

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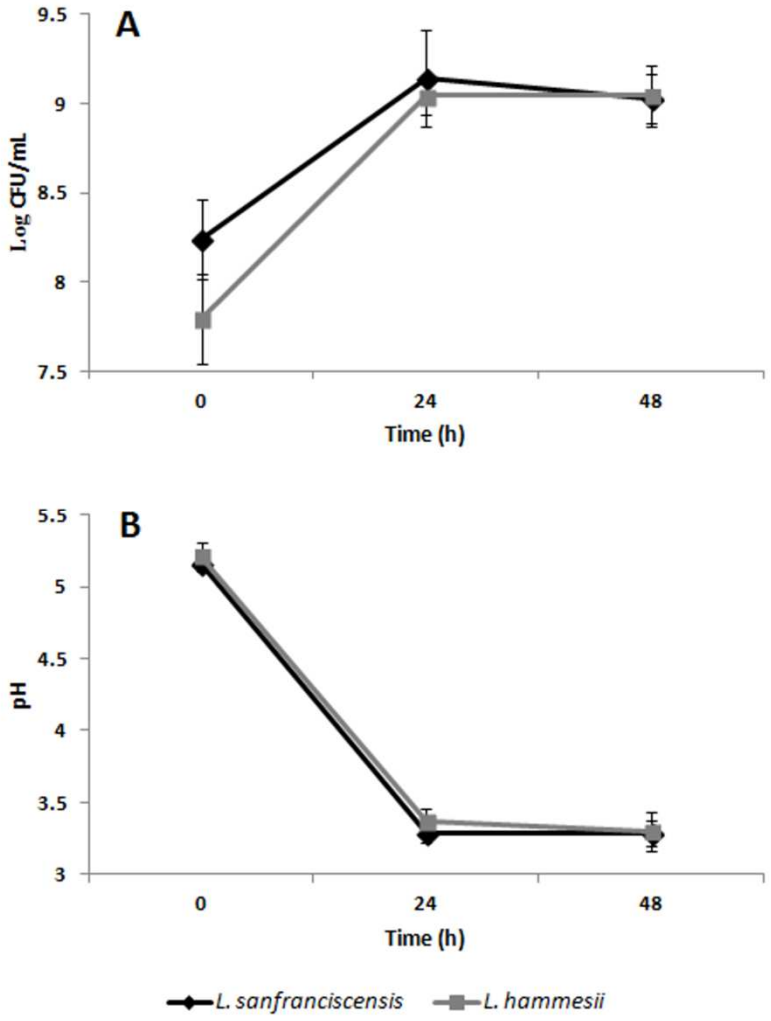

Figure 1A-1. Plate counts and pH of sourdough fermentations over a 48 h incubation period. Cell counts were established from 10-fold serial dilutions plated on mMRS agar with an automatic spiral plater (Don Whitley Scientific, Shipley, England). Plates were incubated overnight, whereupon individual colonies were counted and colony morphology was observed visually to verify strains as lactobacilli. At day 2, the end of the fermentation period, three colonies were picked from the plates containing 100 to 300 colonies, purified and subjected to random amplified polymorphic DNA (RAPD) analysis. For the determination of pH, sourdough samples were diluted 10-fold (w/w) with deionized water and measured with a glass electrode. Organic acid and ethanol

analysis was performed using an Agilent 1200 series LC system (Agilent Technologies; Palo Alto, CA) including a refractive index detector. Separations were achieved using an Aminex HPX-87H column (7.8 × 300 mm i.d., 9 μm, BioRad, Mississauga, Canada) at 70 ºC with an isocratic flow of 5 mM H_2SO_4 at 0.4 mL min^{-1}.

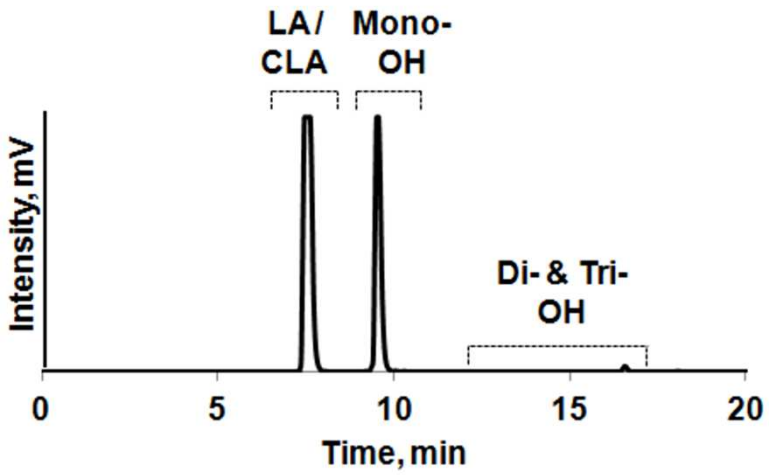

Figure 1A-2. LC/ELSD chromatogram of organic extract of mMRS fermented with *L. hammesii* in the presence of 4 g L^{-1} linoleic acid. Separation performed using Zorbax Rx-SIL semi-preparative column without pre-fractionation. The ELSD temperature was set to 60 ^{o}C, with N_2 gas flow of 3L/min at pressure of 3.5 bar, and all data was collected by Agilent Chemstation software (version G2180BA).

APPENDIX 1

Table 1A-1. Relative quantitation of C18 hydroxy fatty acids in mMRS and sourdough by LC/MS. Hydroxy fatty acid concentrations are expressed relative to the concentration of the same compound in the *L. hammesii* mMRS + LA sample. Data are shown as means ± standard deviation of triplicate independent experiments. LA, addition of 4 g L^{-1} linoleic acid.

No. of hydroxyl groups[a)]	**Mono-OH C18 fatty acids**		**Di-OH C18 fatty acids**	
No. double bonds[b)] **Strain / matrix**	**2**	**3**	**2**	**3**
***L. hammesii* / mMRS + LA**[c)]	**100±12**	**100±3**	**100±11**	**100±4**
L. hammesii / mMRS	0±0	2±1	2±0	2±0
L. sanfranciscensis / mMRS + LA	49±3	17±2	13±2	14±5
L. sanfranciscensis / mMRS	0±0	2±1	2±0	2±1
L. hammesii / dough + LA	7±1	5±1	10±1	7±2
L. hammesii / dough	8±1	3±0	3±0	5±1
L. sanfranciscensis / dough + LA	1±0	3±1	12±0	7±1
L. sanfranciscensis / dough	0±0	2±0	3±1	2±0
Chemically acidified / mMRS + LA	92±5	233±28	81±5	97±13
Chemically acidified / dough + LA	3±1	18±2	13±1	9±2

[a)] Number of hydroxy groups on C18 fatty acids. [b)] Number of double bonds on C18 fatty acids. [c)] The concentration of fatty acids extracted from *L. hammesii* grown in mMRS in presence of linoleic acid was used as a reference (100%).

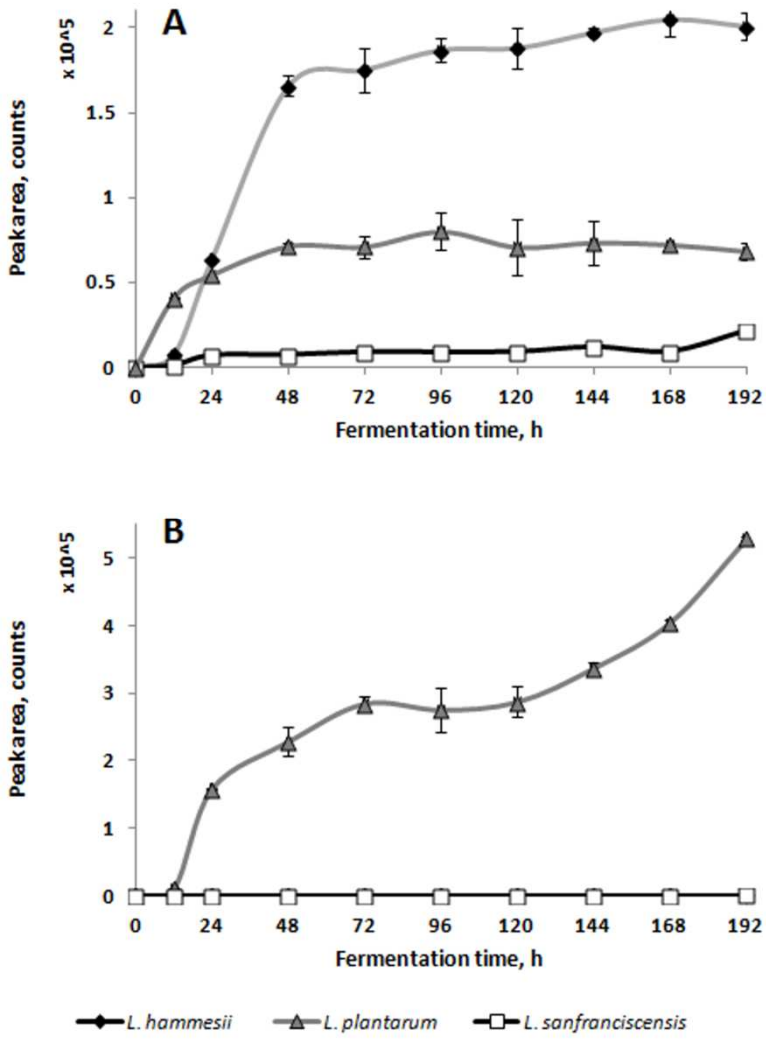

Figure 1A-3. LC/APPI-MS analysis of peak areas from separations performed on a Waters YMC silica column. Samples were fermented with linoleic acid and analysed at 24 h intervals. (A) XIC of *m/z* 297.2403 ($C_{18}H_{33}O_3$) mono-hydroxy C18:1 fatty acid. (B) XIC of *m/z* 315.2535 ($C_{18}H_{35}O_4$) di-hydroxy C18:0 fatty acid, where the plot for *L. hammesii* was consistent at 0 counts. n=3

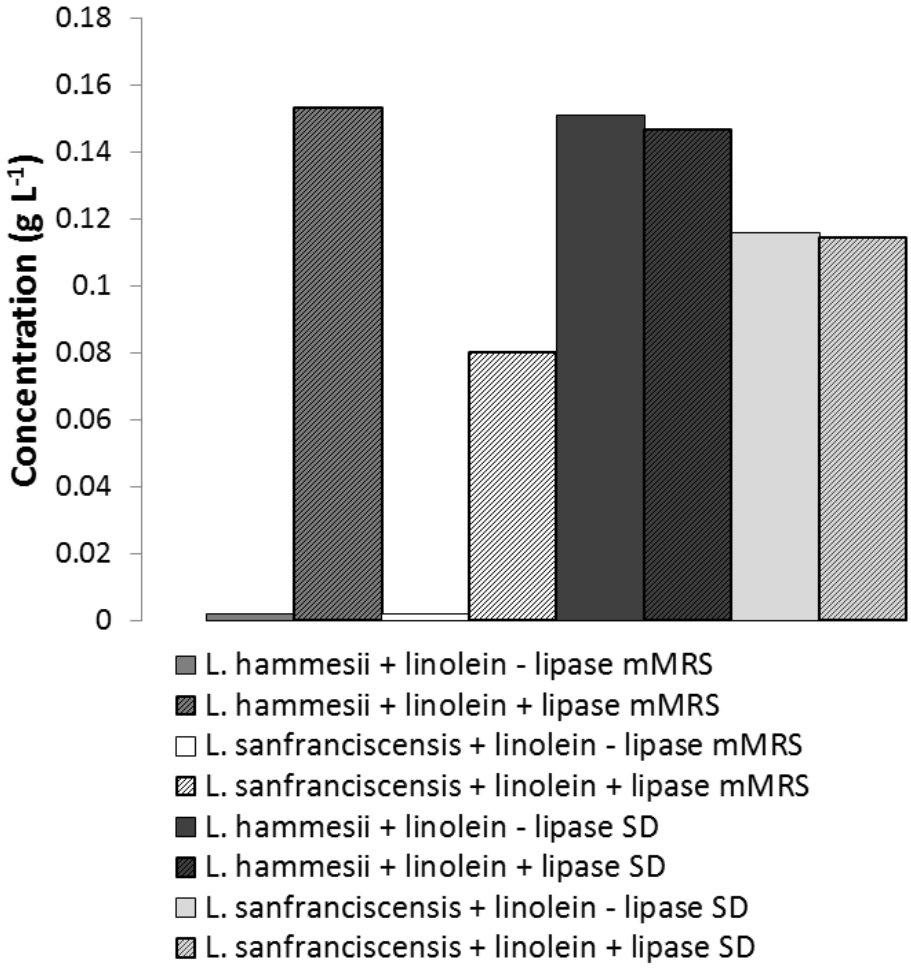

Figure 1A-4. LC/APPI-MS quantification of 10-hydroxy-12-octadecenoic acid from mMRS broth or sourdough (SD) supplemented with 4 g kg^{-1} linolein triacylglyceride (> 99% purity; Nu-Chek Prep, Inc., Elysian, MN) and fermented with *L. hammesii* or *L. sanfranciscensis* for 2 d at 30 °C. Lipase (*Candida rugosa* ≥900 unit mg^{-1}; Sigma Aldrich, Oakville, Canada) was added at a 0 or a 10 g kg^{-1} level and reacted at 37 °C for 2 h, prior to the addition of lactobacilli. All samples were fermented and extracted similarly to sourdough and mMRS samples in Chapter 2. n=1

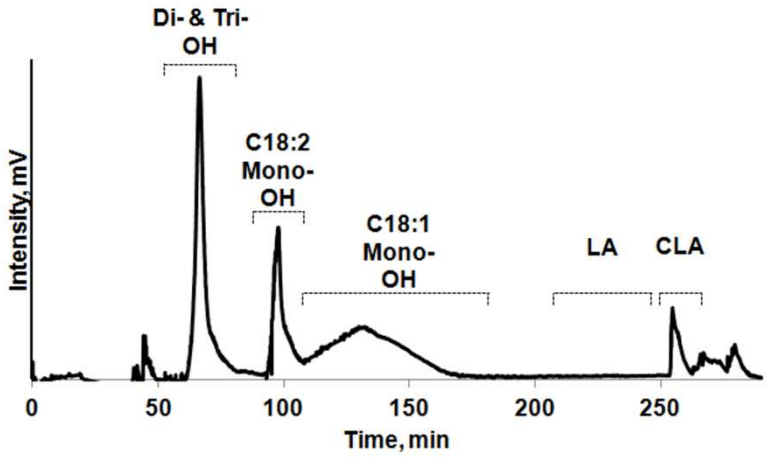

Figure 2A-1. HSCCC separation of *L. hammesii* lipid extract detected with UV at 242 nm. Conjugated-dienes primarily absorb this wavelength. Linoleic acid and conjugated linoleic acid are abbreviated as LA and CLA, respectively.

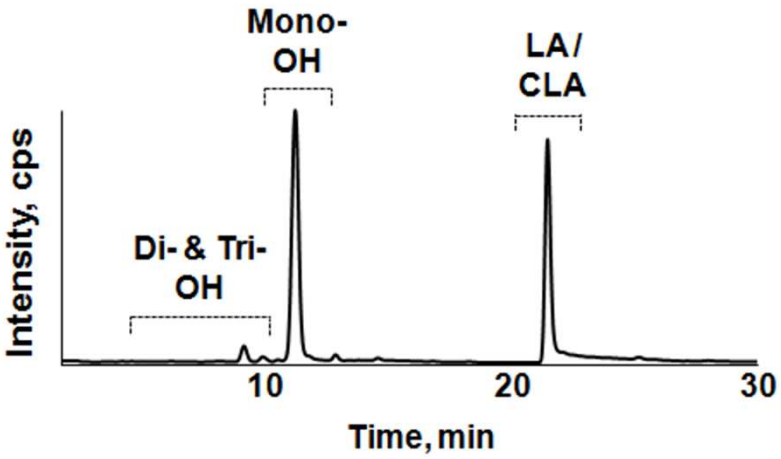

Figure 2A-2. C18 LC/ESI-MS analysis of deprotonated un-derivatized fatty acids from *L. hammesii*. XIC of *m/z* 279 – 331.5, encompassing the crude lipid extract profile. Separations were conducted on an Luna C18 column (250 mm × 4.6 mm i.d., 5 µm, Phenomenex Inc.; Torrance, CA) at 25°C. Lipid samples were injected (injection volume, 5 µL) onto the column and eluted with a gradient of (A) acetonitrile and (B) water at a flow rate of 1.0 mL min^{-1}. The gradient started at 50% A and transitioned to 100% A after 30 min, for a total run time of 30 min.

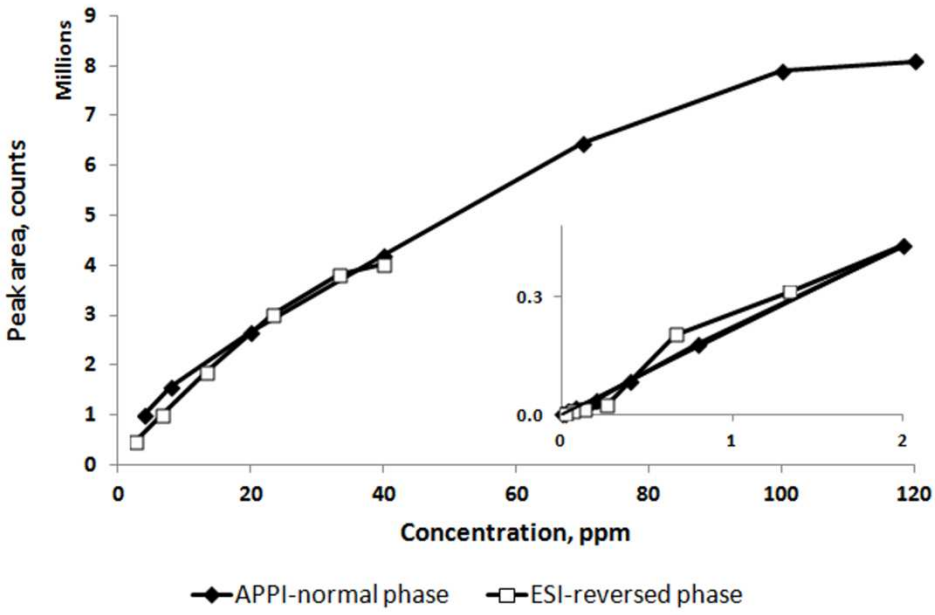

Figure 2A-3. Linearity and detection limits of 10-hydroxy 12-octadecenoic acid compound using normal phase LC/APPI-MS and reversed phase LC/ESI-MS. The normal phase – APPI method (as described in section 3.2.8.) had a quantification range 0.35 µg (70 ppm) and 0.2 ng (0.04 ppm) with a detection limit of 40 pg (0.008 ppm) on column. The reversed phase – ESI method (as described in Figure 3A-2 and below) has a quantification range of 0.167 µg (33.3 ppm) and 3.3 ng (0.67 ppm) with a detection limit of 0.33 ng (0.067 ppm). The limit of quantification and detection were determined by a signal-to-noise ratio of *m/z* 297 of greater than 10 and 3, respectively; additionally, the quantification range was linear. Injection volumes for each experiment were 5 µL and n=1.

Mass spectrometer conditions for negative ion ESI were as follows: nebulizer gas 40 (arbitrary units), auxillary gas 20, curtain gas 25, ionspray voltage -4500 V, source temperature 425 °C, declustering potential (DP) -50 V, focusing potential -250 V and DP2 -15 V with a scan range of *m/z* 50 – 700. The incoming LC effluent was split 1:3 (v/v; mass spectrometer to waste).

APPENDIX 2

2A. Identification of conjugated linoleic acid isomers by silver ion - liquid chromatography/in-line ozonolysis/mass spectrometry[2]

2A.1. Introduction

Conjugated linoleic acid (CLA) is the group of octadecadienoic acid isomers (18:2) with conjugated double bonds, as distinct from the more abundant non-conjugated $\Delta^{9,12}$ linoleic acid. Each CLA positional isomer may also exist in *cis,cis*-, *cis,trans*-, *trans,cis*- and *trans,trans*- configurations. In the fat of ruminants, such as in milk or beef, rumenic acid (*cis*-9, *trans*-11-18:2) is the most abundant CLA, formed by bacterial biohydrogenation of linoleic and linolenic acid (Fritsche *et al*., 2000; Mendis *et al*., 2008). It has been reported that some of CLA isomers may result in anti-carcinogenic and anti-atherogenic effects, and may bring about changes in body composition (Ip *et al*., 1994; Park *et al*., 1999; Lee *et al*., 2005). Although the exact biological mechanisms for CLA activity are still under investigation, current research has shown that individual CLA isomers may have different impacts on lipid metabolism, cancer and diabetes (Pariza *et al*., 2001; Evans *et al*., 2002; Belury *et al*., 2003; Corl *et al*., 2003; Kelley *et al*., 2007).

Due to the diversity of individual CLA positional and geometric isomers and their isomer-specific biological effects, an analytical method is required that readily allows for accurate identification of CLA isomers. Gas chromatography coupled with flame ionization detection (GC/FID) is widely used for analysis of fatty acid methyl esters (FAME) including CLA methyl esters (Kramer *et al*., 2001; Prandini *et al*., 2011). However, even with the best separation achieved

using long (100 m) polar columns with cyanopropyl type stationary phases CLA isomers, especially the *trans,trans*- positional isomers, are still not well resolved from each other. Furthermore, interferences, such as the coelution of 20:1 FAME with CLA methyl esters, may occur (Roach *et al*., 2000). In addition, GC/FID requires a standard mixture of CLA for identification of isomers in food and biological samples, but only a limited number of CLA isomers are available as pure standards.

For the unambiguous identification of each CLA positional isomer, GC combined with electron impact ionization mass spectrometry (GC/EI-MS) has been used following the specific derivatization that is necessary for double bond localization. The latter is required since the EI mass spectra of positional isomers of CLA methyl ester are indistinguishable. Commonly employed derivatives for CLA analyses include picolinyl esters, Diels-Alder adducts (Christie, 1998) and especially dimethyloxazolines (DMOX) (Spitzer, 1997), which are easily formed and can be well separated by GC. The fragment ions in the EI mass spectra of DMOX derivatives of unsaturated fatty acids directly indicate the location of the double bonds and the diagnostic fragment ions from the DMOX derivative of CLA positional isomers from $\Delta^{6,8}$ to $\Delta^{13,15}$ have all been reported (Christie *et al*., 2007). Reconstructed ion chromatograms of these diagnostic ions have been used for CLA isomer identification (Sehat *et al*., 1998). However, the abundance of these ions may be too low for their use in the identification of minor CLA isomers.

APPENDIX 2

Separation by silver ion liquid chromatography (Ag^+- LC) has been used as a complementary tool along with GC for better separation of CLA isomers, especially the *trans,trans*- positional isomers (Christie *et al*., 2007). Since conjugated dienes show characteristic absorptions at 233 nm, a UV detector is normally coupled with the Ag^+- LC (Liu *et al*., 2012). The reported Ag^+- LC separation of CLA isomers has often used an isocratic separation with 0.1- 1.0 % acetonitrile in hexane on a ChromSpher 5 Lipids column (Sehat *et al*., 1998; Yurawecz *et al*., 1998; Christie *et al*., 2007). In this way, CLA isomers can be separated into *trans,trans*-, *cis/trans*- (*cis,trans*- or *trans,cis*-) and *cis,cis*- geometric groups in this order of increasing retention time. Furthermore, studies have shown that CLA positional isomers with conjugated double bonds located closer to carboxyl group elute later within each geometric group (Sehat *et al*., 1998; Yurawecz *et al*., 1998; Christie *et al*., 2007). Multiple Ag^+- LC columns, even up to six, have also been connected in series in order to improve peak resolution (Yurawecz *et al*., 1998). Recently, the relative retention order of all *cis,trans*- and *trans,cis*- CLA isomers from $\Delta^{6,8}$ to $\Delta^{13,15}$ were established using three Ag^+- LC columns in series (Delmonte *et al*., 2005). Although it may be possible to resolve *trans,trans*-, *cis,cis*- and most of *cis/trans*- positional isomers using multiple Ag^+- LC columns under optimal conditions, there is still a significant challenge to identify each peak, especially closely eluting peaks. This is made worse by the instability of retention times on Ag^+- LC columns and the very different concentrations of CLA isomers in natural samples (Eulitz *et al*.,

1999). Since *cis*-9, *trans*-11-CLA is the most abundant CLA isomer existing in nature, the peak of highest intensity in the Ag^+-LC chromatogram is normally assumed to be *cis*-9, *trans*-11-CLA, and from this the identification of the other CLA isomers can be made by their relative retention times. Furthermore, in research on the biological function of specific CLA isomers, CLA isomers other than *cis*-9, *trans*-11-CLA have been used. This gives rise to the situation where *cis*-9, *trans*-11-CLA is no longer the most abundant CLA isomer in these sample (Kramer *et al*., 1998).

The ambiguity in CLA positional isomer identification is also present when mass spectrometry and tandem mass spectrometry (MS/MS) is used. For example, when three Ag^+- LC columns in series were coupled to atmospheric pressure photo ionization mass spectrometry (APPI-MS), the ion $M^{+\cdot}$ at *m/z* 294 was used to identify CLA methyl esters, but the assignment of each specific CLA positional isomer could only be made based on the elution order in Ag^+- LC (Muller *et al*., 2006). To date, there is no single definitive method for the identification of CLA positional isomers.

Ozone can specifically react with carbon-carbon double bonds producing cleavage products of predictable mass and hence the ozonolysis of unsaturated lipids has been used to determine double bond locations (Harrison *et al*., 1996; Thomas *et al*., 2008). Blanksby *et al*. have developed ozone induced dissociation (OzID-MS) in which gas phase ozonolysis takes place within a mass spectrometer to allow for the elucidation of double bond locations (Thomas *et al*., 2008; Poad

et al., 2010). In recent OzID research on the identification of CLA positional isomers, sodium adducts of aldehydes from ozonolysis at each double bond were observed under ESI (+), and used for the assignment of double bond positions (Pham *et al*., 2013). However, this technique requires the specialized introduction of ozone into a mass spectrometer; furthermore it may not be suitable for the identification of low abundance CLA isomers in complex samples.

It is highly desirable to have a single technique that could determine double bond positions, and also achieve *de novo* identification of CLA positional isomers even in complex lipid mixtures. Recently, we reported a simple approach for the direct determination of double bond positions in FAME by coupling ozonolysis in-line with mass spectrometry (in-line O_3-MS) (Sun *et al*., 2013). In that method, the unsaturated FAME within the LC mobile phase passed through gas permeable, liquid impermeable tubing housed within a vessel containing ozone. With this arrangement, ozone passed through the tubing wall resulting in the ozonolysis of unsaturated FAME directly within the mobile phase. The aldehydes, that were the products of ozonolysis and were characteristic of FAME double bond positions, were then analyzed in real-time by APPI-MS. A great advantage of this technique is that it readily allows the coupling of liquid chromatography with O_3-MS (LC/O_3-MS). Hence, the complete identification of each FAME in the chromatogram of complex lipid mixtures can be achieved.

In this study, we explore the feasibility of using in-line O_3-MS for the *de novo* identification of CLA positional isomers. Then, we describe the

development of the LC/O_3-MS method for the identification of CLA isomers in complex lipid samples. Using this approach, we demonstrate the identification of CLA isomers in natural matrices through the examples of a commercial CLA supplement, bovine milk fat and a lipid extract from a bacterial culture.

2A.2. Materials and methods

2A.2.1. Material

HPLC grade hexanes, acetonitrile, isopropanol were purchased from Fisher Scientific Company (Ottawa, Canada). All the CLA methyl ester standards (*cis*-9, *trans*-11-; *cis*-9, *cis*-11-; *trans*-9, *trans*-11-; and *trans*-10, *cis*-12-18:2) were purchased from Matreya Inc. (Pleasant Gap, PA). FAME standards (*cis*-9-18:1 and *trans*-11-18:1) were purchased from Nu-Chek Prep Inc. (Elysian, MN). Each standard solution was prepared in hexane at a concentration of 200 μg mL^{-1}. A solution of porcine renin substrate tetetradecapeptide at 10 pmol μL^{-1} in acetonitrile/water (1:1, v/v) from a chemical standards kit (Applied Biosystems, Foster City, CA) was used for the tuning and calibration of mass spectrometer. The Teflon AF-2400 tubing (0.5 mm o.d., 0.25 mm i.d.) was purchased from Biogeneral Inc. (San Diego, CA).

2A.2.2. Lipid extraction and methylation

2A.2.2.1. Supplement*

The commercial CLA supplement was obtained from a local supermarket. It was manufactured from safflower oil and sold in capsule form for weight control

purposes. Approximately 0.05 g of CLA supplement was dissolved in toluene and methylated using 1% sulfuric acid in methanol at 50 ºC overnight (Christie, 1989). The methylated lipid was dissolved in hexane to a concentration of approximately 50 μg mL^{-1} for analysis.

2A.2.2.2. Milk*

Bovine milk with 3.25% fat content was purchased from local market. Lipid extraction was performed on 1 g of milk using the Bligh and Dyer method (Bligh & Dyer, 1959). The chloroform layer that contained lipid was dried under a flow of nitrogen, and the residual lipid was dissolved in toluene for methylation as above. The entire methylated lipid extract was dissolved in 10 mL hexane and diluted a further 20 times in hexane before analysis.

2A.2.2.3. Bacterial fermentation

Lactobacillus plantarum TMW1460 was incubated in modified DeMan-Rogosa-Sharpe broth, supplemented with 4 g L^{-1} linoleic acid, at 30 °C for 48 h and extracted according to Black *et al*., (2013). After extraction, the fatty acids were methylated as above. The resulting FAME was resuspended in a total volume of 2 mL hexane. Each of two 1 mL FAME aliquots was loaded onto a separate conditioned silver ion-solid phase extraction (Ag^{+} - SPE) cartridge (750 mg 6 mL^{-1}, Supelco Inc., Bellefonte, PA) and 6 mL hexane/acetone (99:1, v/v) was used to elute the unretained FAME. Following this, fractions were eluted

using 6 mL hexane/acetone (96:4, v/v) and 6 mL hexane/acetone (90:10, v/v) (Kramer *et al*., 2008). The fractions collected from each of the two 1mL FAME aliquots were combined, dried under nitrogen and dissolved into 1 mL of hexane.

2A.2.3. In-line ozonolysis/mass spectrometry analysis of conjugated linoleic methyl ester standard*

The in-line O_3-MS method was described in detail in our previous study (Sun *et al*., 2013). In this study, a 10 cm length of gas permeable tubing passed through a chamber filled with oxygen and ozone gas at a concentration of 35.6 g $(m^3)^{-1}$ at room temperature. A 3 μL volume of the 200 μg mL^{-1} CLA standard solution was delivered through the gas permeable tubing by an Agilent 1200 series HPLC system (Agilent Technologies Inc, Palo Alto, CA) with hexane as the mobile phase and at the flow rate of 0.2 mL min^{-1}. The ozonolysis products were analyzed using an APPI ion source in the positive ion mode attached to a hybrid quadrupole time - of - flight mass spectrometer (QSTAR Elite, Applied Biosystems/MDS Sciex, Concord, Canada). The APPI ion source temperature was held at 375 °C while the source region gas flows in arbitrary units assigned by the data system were as follows: curtain gas 25; auxiliary gas 10; nebulizing gas 50. In all cases, high purity nitrogen was the gas used. The ionspray voltage, declustering potential (DP), focus potential (FP), and DP2 were 1300 V, 35 V, 130 V and 10 V, respectively. The mass spectrometer was tuned using the ion at *m/z* 879.9723 and fragment ion at *m/z* 110.0713 obtained by infusing porcine renin substrate tetetradecapeptide into the ESI ion source in the positive ion mode

and at a resolution of above 10,000 (full width at half maximum). This solution was also used for calibration of the mass range *m/z* 100-1,300.

2A.2.4. Silver ion – liquid chromatography/ozonolysis - mass spectrometry analysis of fatty acid methyl ester mixtures from lipid extracts*

Isocratic separation on a ChromSpher 5 Lipids column (2 mm i.d. × 250 mm, 5 μm; Agilent Technologies Inc, Lake Forest, CA) was used with all of the samples. The mobile phase consisted of 20% hexane: isopropanol: acetonitrile (100:1:0.1, v/v/v) and 80% of hexane. The sample injection amount was 2 μL and the flow rate was 0.2 mL min^{-1}. The in-line O_3-MS conditions were the same as above except that only 5 cm tubing was used for sample analysis.

2A.3. Results and discussion

2A.3.1. In-line ozonolysis - mass spectrometry analysis of conjugated linoleic acid standard*

In our previous research, we showed that the ozonolysis product aldehydes resulting from the oxidative cleavage at each double bond can be used as indicators of double bond positions for monounsaturated and non-conjugated polyunsaturated FAME (Sun *et al*., 2013). Here, in-line O_3-MS analysis is performed on a *cis*-9, *trans*-11- and *trans*-10, *cis*-12- CLA methyl ester standard, in order to see whether conjugation will have any affect on double bond assignment by ozonolysis. For both CLA positional isomers, protonated

molecular ions $[M+H]^+$ at *m/z* 295 are observed in the O_3-MS mass spectra under positive ion APPI (**Figure 2A-4**).

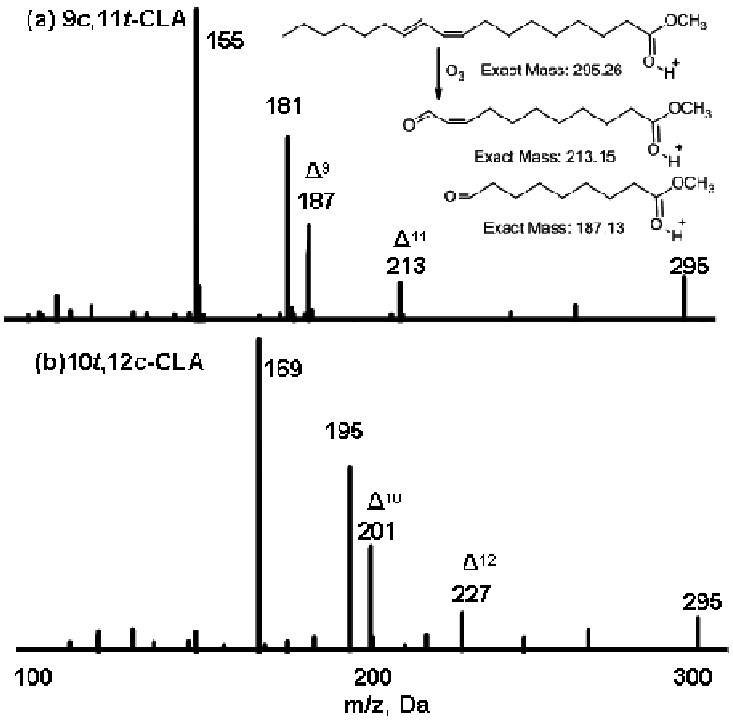

Figure 2A-4. In-line O_3/APPI(+)-MS spectrum of (A) 9*c*,11*t*-CLA methyl ester and (B) 10*t*,12*c*-CLA methyl ester.

From the in-line O_3-MS analysis of *cis*-9, *trans*-11- CLA methyl ester, we observe product ions at *m/z* 213, 181, 187 and 155 (**Figure 2A-4A**). The ions at *m/z* 213 and 187 correspond to the protonated aldehyde ions from the ozonolysis cleavage at Δ^{11} and Δ^{9} position, and the ions at *m/z* 181 and 155 are due to methanol loss from *m/z* 213 and 187. In contrast, for the *trans*-10, *cis*-12- CLA methyl ester the ions at *m/z* 227, 195, 201 and 169 are observed by in-line O_3-MS (**Figure 2A-4B**). The ions at *m/z* 227 and 201 are indicative of double bonds located at Δ^{12} and Δ^{10}

positions and the ions at *m/z* 195 and 169 are due to the methanol loss from these ions.

In-line O_3-MS analysis of *cis*-9, *cis*-11- and *trans*-9, *trans*-11- CLA methyl ester (not shown) gives the same ozonolysis product ions as those seen for *cis*-9, *trans*-11- CLA methyl ester. This result demonstrates that double bond geometry does not affect the ozonolysis product ions observed. However, ozonolysis of CLA isomers is generally seen to proceed at an accelerated rate compared to the non-conjugated *cis*-9, *cis*-12-18:2 methyl ester. Thus, under the same reaction condition (tubing length of 20 cm, ozone concentration 35.6 g $(m^3)^{-1}$, 3 µl of 200 µg ml^{-1} standard solution), only ozonolysis product ions of CLA methyl esters can be observed, whereas the $[M+H]^+$ ion at *m/z* 295 of intact non-conjugated *cis*-9, *cis*-12-18:2 methyl ester still exists. For this reason, only a 10 cm length of the semi-permeable tubing is used here for the in-line O_3-MS analysis of CLA methyl esters so that some CLA molecular ions can still be seen in the mass spectrum. An enhanced reaction rate for gas phase ozonolysis of CLA was also observed in the OzID/MS experiment (Pham *et al.*, 2013).

In summary, the in-line O_3-MS spectra of two CLA positional isomers indicate that the observed ozonolysis product aldehydes are indicative of double bond locations even when the double bonds are conjugated. Even though pure standards of every CLA positional isomer are not available, the pair of diagnostic aldehyde ions arising from ozonolysis cleavage at each double bond can still be used to reliably differentiate CLA positional isomers. All of the predicted

diagnostic aldehyde ions and their corresponding methanol loss fragment ions for CLA positional isomers from $\Delta^{6,8}$ to $\Delta^{13,15}$ are listed in **Table 2A-1**.

Table 2A-1. In-line O_3/APPI(+)-MS diagnostic ions for CLA positional isomer identification

CLA Isomer	**A**. *m/z* of aldehyde ions from O_3 cleavage at Δ^a position	*m/z* of ions due to methanol loss from **A**	**B**. *m/z* of aldehyde ions from O_3 cleavage at Δ+2 position	*m/z* of ions due to methanol loss from **B**
6, 8	145	113	171	139
7, 9	159	127	185	153
8, 10	173	141	199	167
9, 11	187	155	213	181
10, 12	201	169	227	195
11, 13	215	183	241	209
12, 14	229	197	255	223
13, 15	243	211	269	237

[a] Position of double bond counted form the carboxyl group end

2A.3.2. Detection limit of silver ion – liquid chromatography/ ozonolysis - mass spectrometry method*

Since CLA naturally occurs as a low abundance component of lipid, it is important that the method is sensitive enough for CLA isomer detection in the presence of other fatty acids in high amount. Hence, to demonstrate the applicability of the present approach, an estimation of the limit of detection for an individual CLA isomer (*cis*-9, *trans*-11-CLA) has been made. A 1 μL of a 0.20 μg ml^{-1} solution of *cis*-9, *trans*-11- CLA standard was analyzed by Ag^+-LC/O_3-MS using identical ozonolysis conditions to those used for sample analysis (see materials and methods section). The least intense of the diagnostic aldehyde ions

at *m/z* 213 was selected and its extracted ion chromatogram (XIC) was plotted. The signal-to-noise ratio of the peak in this XIC slightly exceeded 3:1, taken as the limit of detection (LOD). Thus, the LOD in this experiment is 0.2 ng for a single CLA isomer. A more realistic situation than a pure standard is the analysis of a lipid mixture that contains CLA as a minor component. As an example, consider the Ag^+-LC/O_3-MS analysis of a 1 μL injection of a 1 mg mL^{-1} solution of this lipid mixture. In this example, 1 μg of total lipid would be analyzed on-column. Given the observed LOD of 0.2 ng for a single component, this corresponds to a minimum abundance 0.02% of the total lipid in order to observe the diagnostic O_3-MS ions. This rough calculation indicates that the Ag^+-LC/O_3-MS method should be sufficiently sensitive for the identification of most CLA isomers in natural lipid samples even without the pre-concentration steps that are often used for GC analysis. Furthermore, higher column loadings, and hence lower LODs are certainly possible.

2A.3.3. Silver ion – liquid chromatography/ozonolysis - mass spectrometry analysis of fatty acid methyl ester mixtures from lipid extracts*

In order to demonstrate the relative retention order of *cis,cis*-, *cis/trans*- *and trans,trans*- CLA isomers in silver ion chromatography, Ag^+-LC/APPI(+)-MS analysis was performed on a standard mixture of CLA isomers (*cis*-9, *trans*-11-; *cis*-9, *cis*-11-; *trans*-9, *trans*-11- and *trans*-10, *cis*-12- CLA methyl esters), methyl oleate (*cis*-9-18:1) and methyl vaccenate (*trans*-11-18:1) (**Figure 2A-5**).

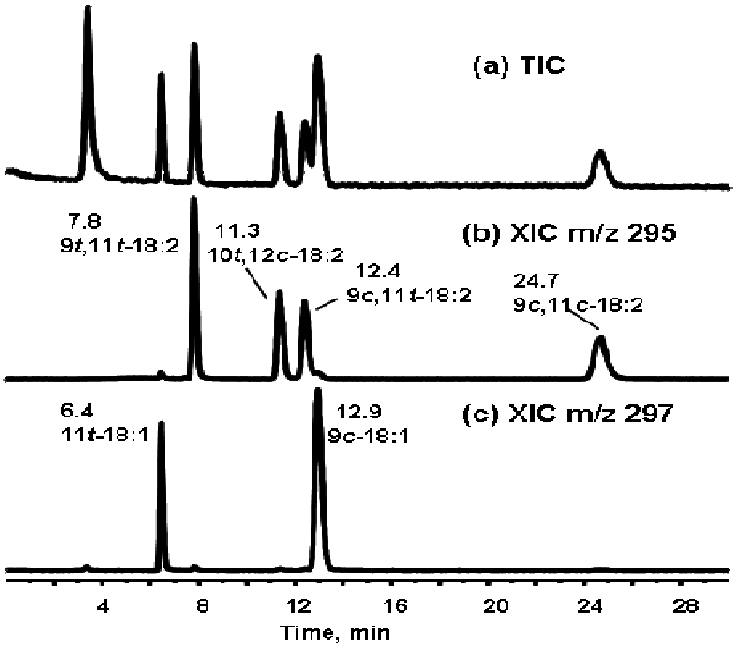

Figure 2A-5. (A) Ag^+-LC/APPI(+)-MS TIC of a mixture of FAME standards including 11*t*-18:1, 9*c*-18:1, 9*t*,11*t*-18:2, 9*c*,11*t*-18:2, 9*c*,11*c*-18:2 and 10*t*,12*c*-18:2 methyl esters; (B) XIC of *m/z* 295; (C) XIC of *m/z* 297.

In the extracted ion chromatogram (XIC) of *m/z* 297 (**Figure 2A-5C**), the peaks at 6.4 and 12.9 min represent *trans*-11-18:1 and *cis*-9-18:1. In the XIC of *m/z* 295, the first eluting peak is *trans*-9, *trans*-11- CLA, the last eluting peak is *cis*-9, *cis*-11-CLA, and the two peaks in the middle are *trans*-10, *cis*-12- and *cis*-9, *trans*-11- CLA, respectively (**Figure 2A-5B**). Within the 30 min isocratic separation, CLA geometric isomers (with the same double bond positions) are well separated from each other and also separated from *cis*- and *trans*- 18:1 methyl esters. Hence, coupling Ag^+-LC to in-line O_3-MS provides the extra dimension of

information needed to determine the double bond geometries in addition to identifying the double bond positions from the mass spectra.

In the following sections, we demonstrate the application of the Ag^+-LC/O_3-MS method for the identification of CLA isomers in a commercial CLA supplement, bovine milk fat and the lipid extract of *L. plantarum* culture. These examples include distinctly different CLA sources having different levels and distributions of CLA isomers and possible matrix interferences. In order to observe the diagnostic aldehyde ions from the ozonolysis of CLA that is present in low amounts in the samples, a shorter length of semi-permeable tubing (5 cm) was used, reducing the extent of ozonolysis.

2A.3.4. Commercial conjugated linoleic acid supplement*

Because of the proven and potential health benefits of CLA consumption, foods enriched in CLA and CLA supplements have become available to consumers. CLA supplements can be manufactured from soybean and safflower oil that are rich in linoleic acid through either photo- or alkali-induced isomerization (Ma *et al*., 1999; Gammill *et al*., 2010). Since these isomerization processes are not isomer specific, multiple CLA isomers are believed to exist in these synthetic CLA mixtures.

The FAME mixture from the CLA supplement was first analyzed by Ag^+-LC/APPI(+)-MS without ozonolysis (**Figure 2A-6A**). In the XIC for $[M+H]^+$ ions at *m/z* 295 of linoleic acid isomers (**Figure 2A-6B**), there are two major peaks at 10.8 and 11.8 min with four other minor peaks at 6.4, 6.7, 23.1 and 24.9

min. The mass spectra of these peaks are identical and thus the double bond positions in these isomers cannot be distinguished directly.

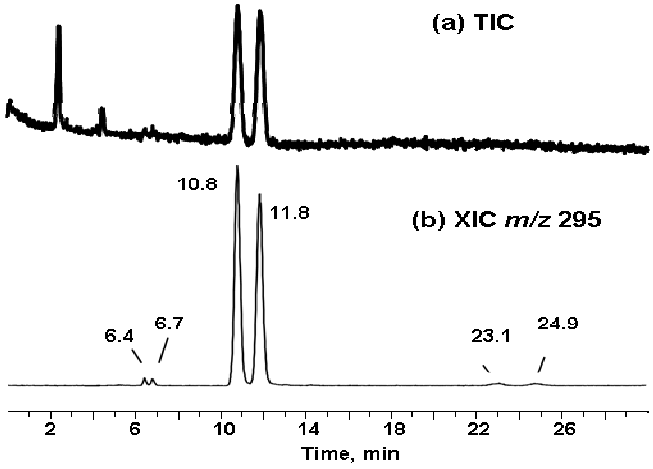

Figure 2A-6. Ag^+-LC/APPI(+)-MS analysis of a CLA supplement (A) TIC; (B) XIC *m/z* 295.

Figure 2A-7A is the total ion current chromatogram (TIC) of the same sample after in-line O_3-MS; the mass spectra of each visible peak at 6.8, 7.2, 11.2 and 12.3 min are also shown.

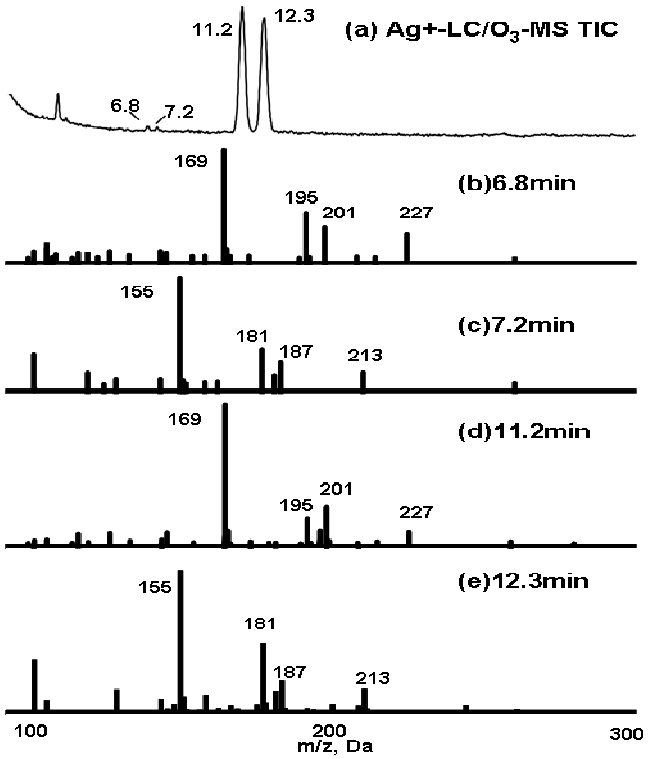

Figure 2A-7. Ag^{+}-LC/O_3-APPI(+)-MS analysis of a CLA supplement (A) TIC; (B) mass spectrum averaged at 6.8 min; (C) mass spectrum averaged at 7.2 min; (D) mass spectrum averaged at 11.2 min; (E) mass spectrum averaged at 12.3 min.

The mass spectra of the peaks at 6.8 (**Figure 2A-7B**) and 11.2 min (**Figure 2A-7D**) are almost the same; the pair of diagnostic ions at *m/z* 227 (methanol loss ion at *m/z* 195) and 201(methanol loss ion at *m/z* 169) can be used to unambiguously assign the double bond position at Δ^{12} and Δ^{10}. Similarly, in **Figure 2A-7C** and **2A-7E** the same pair of diagnostic ions at m/z 213 (methanol loss ion at *m/z* 181) and 187 (methanol loss ion at *m/z* 155) can be seen, which identify these peaks as $\Delta^{9,11}$ CLA isomers. In all of these mass spectra, the $[M+H]^{+}$ ion at *m/z* 295 is not

seen, partly because of the accelerated ozonolysis reaction rate of CLA compared to non-conjugated isomers, as described above. In addition, the CLA content of a natural sample such as milk, cheese and butter is only up to 2% of the total fatty acids (Dhiman *et al*., 2000; Dhiman *et al*., 2005). This much lower abundance also contributes to the failure to observe CLA molecular ions in the mass spectra after ozonolysis.

Even though there are two small peaks at 23.1 and 24.9 min in the XIC of *m/z* 295 (**Figure 2A-6B**), these peaks are difficult to see in Ag^+-LC/O_3-MS TIC trace in **Figure 2A-7A** and hence some CLA isomers might be overlooked. However, a simple screening for all possible CLA positional isomers can be performed by generating XICs for each pair of diagnostic ions listed in **Table 2A-1**. Peaks appearing at the same retention time (t_R) in these XICs correspond to the specific CLA positional isomer. For example, in **Figure 2A-8A**, the three peaks at 7.2, 12.3 and 25.1 min in the XIC of *m/z* 213 are also present in the XIC of *m/z* 187 meaning that these peaks are all due to CLA isomers with double bonds located at Δ^{11} and Δ^{9}.

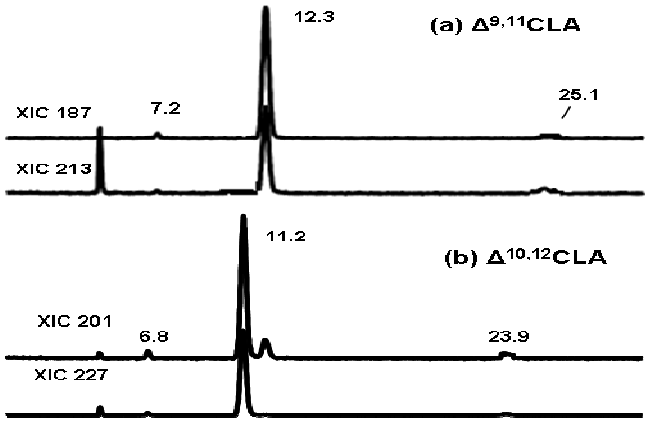

Figure 2A-8. The Ag^{+}-LC/O_3-APPI(+)-MS analysis of a CLA supplement (A) XIC of *m/z* 187 and 213; (B) XIC of *m/z* 201 and 227; (*c.f.* TIC trace in **Figure 2A-7A**).

From the known elution order of CLA geometric isomers in Ag^{+}-LC, the peaks at 7.2 min and 25.1 min must be *trans*-9, *trans*-11- and *cis*-9, *cis*-11- CLA. The peak at 12.33 min could be cis-9, *trans*-11- or *tran*-9, *cis*-11- CLA which are not readily distinguished by their retention time. However, in this particular case, the peak at 12.3 min is identified as *cis*-9, *trans*-11-CLA which is confirmed by addition of *cis*-9, *trans*-11- CLA standard to the sample (data not shown). Similarly, the XICs of another pair of diagnostic aldehyde ions at *m/z* 227 and 201 reveals three geometric isomers of $\Delta^{10,12}$ CLA at 6.8, 23.9 and 11.2 min (**Figure 2A-8B**), which are identified as *trans*-10, *trans*-12-, *cis*-10, *cis*-12- and *trans*-10, *cis*-12- CLA (the latter confirmed by addition of *trans*-10, *cis*-12- CLA standard to the sample). In summary, the results show that the commercial CLA supplement sample contains primarily *cis*-9, *trans*-11- and *trans*-10, *cis*-12-CLA,

along with minor amounts of their geometric isomers *trans*-9, *trans*-11-, *cis*-9, *cis*-11-, *trans*-10, *trans*-12- and *cis*-10, *cis*-12-CLA. This finding is consistent with a previous publication that used Ag^+-LC and GC/(EI) MS to analyze a commercial CLA mixture (Yurawecz *et al*., 1999).

It is important to clarify that as in this example, in-line O_3-MS can directly identify CLA positional isomers without requiring standards, but it is not able to differentiate the geometric isomers. On the other hand, the elution order in Ag^+-LC provides complementary information on double bond geometry, especially when both double bonds are in the same configuration (*trans,trans*- and *cis,cis*-). However, the elution order of a positional isomer having double bonds in *cis,trans*- and *trans,cis*- configurations is less definite, and may depends upon column conditions, temperature and mobile phase composition (Eulitz *et al*., 1998; Delmonte *et al*., 2005). The addition of a CLA standard can help the identification of *cis/trans*- geometric isomer as shown above, but only if the CLA isomer is available as a pure standard. Overall, this example demonstrates that the Ag^+-LC/O_3-MS method is capable of *de novo* identification of CLA positional isomers in lipid mixtures, despite the fact that it is not in itself able to differentiate *cis,trans*- and *trans,cis*- CLA geometric isomers.

2A.3.5. Bovine milk fat*

Milk fat is complicated mixture that contains up to 20 CLA isomers, among them rumenic acid (*cis*-9, *trans*-11-18:2) is normally the most abundant CLA isomer (Deng *et al*., 2003). CLA content and isomer distribution in cow milk is

greatly influenced by the season, feeding practice and by the diet of the cow. Identification of CLA isomers in milk is important for the dairy industry and also for nutritional research (Jiang *et al*., 1996; Kelsy *et al*., 2003).

The XICs of $[M+H]^+$ ions at *m/z* 295 and 297 for 18:2 and 18:1 FAME from Ag^+-LC/APPI(+)-MS analysis of a milk fat FAME mixture is shown in **Figure 2A-9**.

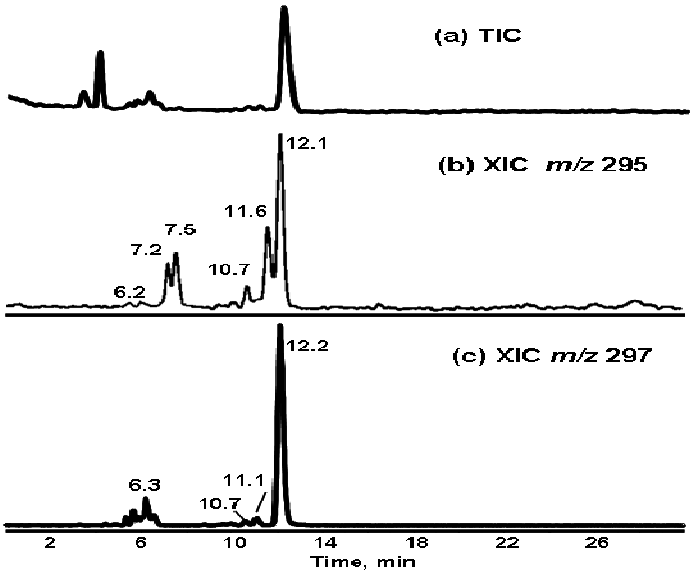

Figure 2A-9. The Ag^+-LC/APPI(+)-MS analysis of milk fat (A) TIC; (B) XIC of *m/z* 295; (C) XIC of *m/z* 297.

The peak at 12.2 min in the XIC trace of *m/z* 297 that is present at high intensity is due to methyl oleate (*cis*-9-18:1), the most abundant 18:1 fatty acid in milk. Due to this high abundance of methyl oleate, the peak at 12.2 min in the TIC appears to be a single component. However, the separation of all minor CLA

species can still be observed in the XIC of *m/z* 295 because of the high selectivity achieved in this trace.

It is apparent from **Figure 2A-9B** that more than one CLA isomer must exist in the sample, so Ag^+-LC/O_3-MS analysis was performed for the elucidation of the double bond positions in the CLA positional isomers. In **Figure 2A-10**, both the traces showing the characteristic aldehyde ions and methanol loss fragment ions are overlaid in order to avoid false positives - especially important for complex samples like the milk fat.

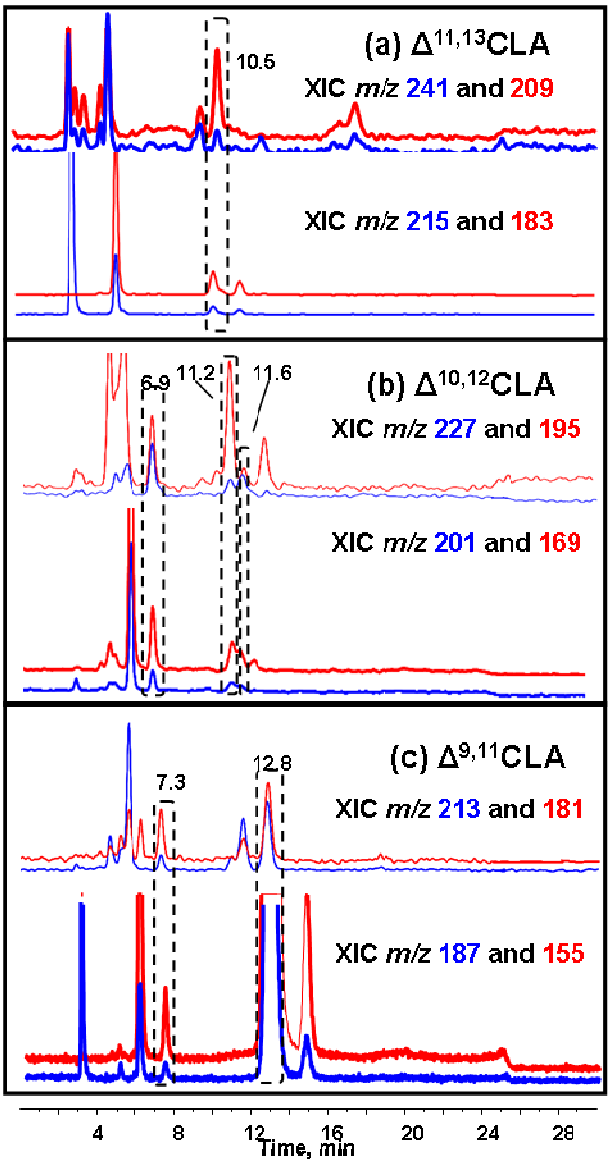

Figure 2A-10. The Ag^{+}-LC/O_3-APPI(+)-MS analysis milk fat (A) XIC of *m/z* 183 and 209, *m/z* 215 and 241; (B) XIC of *m/z* 169 and 195, *m/z* 201 and 227; (C) XIC of *m/z* 155 and 181, *m/z* 187 and 213.

For example, in **Figure 2A-10A** there is a peak at around 3.0 min in the XIC of *m/z* 215 (diagnostic aldehyde ions of Δ^{11} double bond), 241 (diagnostic aldehyde ions of Δ^{13} double bond) and 209 (methanol loss from ion at *m/z* 241), but this

peak is missing from the XIC of *m/z* 183 (methanol loss from ion at *m/z* 215). As seen in the O_3-MS of two CLA standards (**Figure 2A-4**) and also the CLA supplement sample (**Figure 2A-7**), methanol loss from the ozonolysis product aldyhyde ions occurs during APPI ionization and the fragment ions have even higher intensity than their aldehyde ion precursors. Therefore, the peak at 3.0 min in XIC of *m/z* 215 is not due to cleavage of double bond at Δ^{11} by ozonolysis. The peaks in the XIC of ions at *m/z* 241, 209, 215 and 183 overlay each other at 10.5 min (**Figure 2A-10A**), which is thus identified as $\Delta^{11,13}$ CLA in *cis,trans*- or *trans,cis*- configuration. The peaks that superimpose at 6.9, 11.2 and 11.6 min in the XICs of ions at *m/z* 227, 195, 201 and 169 (**Figure 2A-10B**) are identified as *tran*s-10, *trans*-12-, *tran*s-(or *cis*-)10, *cis*-(or *trans*-)12-, *cis*-(or *trans*-)10, *trans*-(or *cis*-)12- CLA.

We can also confirm the existence of $\Delta^{9,11}$ CLA positional isomers at 7.3 min as *tran*s-9, *trans*-11- CLA and at 12.8 min as *cis*-9, *trans*-11- CLA (proved by the addition of a standard of *cis*-9, *trans*-11- CLA in the sample). We notice that the peak at 10.8 min in XIC of *m/z* 187 and 155 are much wider and of much higher intensity than the corresponding peak in XIC of *m/z* 213 and 181 (**Figure 2A-10C**). This is because the abundant methyl oleate (*cis*-9-18:1) elutes just behind *cis*-9, *trans*-11- CLA and the characteristic ions indicating Δ^9 double bond are also *m/z* 187 and 155. Despite this coelution, extracting the two pairs of characteristic ions still allows for the correct identification of *cis*-9, *trans*-11-CLA.

Thus, by using Ag^+-LC/O_3-MS we are able to positively identify six CLA isomers including *trans*-(or *cis*-)11, *cis*-(or *trans*-)13- CLA at 10.5 min, *trans*-10, *trans*-12- CLA at 6.9 min, *trans*-(or *cis*-)10, *cis*-(or *trans*-)12- CLA at 11.2 min, *cis*-(or *trans*-)10, *trans*-(or *cis*-)12- CLA at 11.6 min, *trans*-9, *trans*-11- CLA at 7.3 min and *cis*-9, *trans*-11- CLA at 12.8 min (**Figure 2A-10**) in the milk fat sample.

Unlike other reports that used multiple Ag^+-LC columns in series, resulting in long separations for CLA isomers (Christie *et al*., 2007), only a 30 min isocratic separation on a single column is needed in this study to provide adequate separation of the CLA isomers. Then, the in-line O_3-MS results can be used for the *de novo* identification of CLA positional isomers irrespective of any coelution or interference. In addition, the poor retention time stability often seen with Ag^+-LC and also observed in our study (as seen in **Figure 2A-5B**, **Figure 2A-6B** and **Figure 2A-9B**), has no impact on CLA positional isomer identification since the *de novo* assignment only depends on the extraction of the two pair of characteristic ions listed in **Table 2A-1** instead of a comparison against the retention times of CLA standards. This is especially an advantage for complex lipid samples containing a low abundance of CLA isomers.

2A.3.6. Lipid extract from *L. plantarum* culture

Although animal studies testing the activity of CLA show promise, conflicting results have arisen in studies attempting to show human health benefit when natural sources of CLA were used, for example CLA from food grade

bacteria (McCrorie *et al*, 2011). This inconsistency could relate to the fact that different CLA isomers may elicit a different biological response, and at the same time suitable methods for identification of CLA isomers at low concentration are still lacking. *Lactobacillus* species have a safe history of use in food production and can be utilized to transform linoleic acid to CLA for human consumption (McCrorie *et al*., 2011; Andrade *et al*., 2012). However, a majority of studies do not report on the geometric isomers produced by *Lactobacillus* species and other food-grade bacteria (McCrorie *et al*., 2011; Andrade *et al*., 2012). Here, we have employed *L. plantarum* for bioconversion of linoleic acid as it is known to produce *trans*-9, *trans*-11-, *cis*-9, *trans*-11-, and *trans*-10, *cis*-12-CLA (Ogawa *et al*., 2001; Kishino *et al*., 2011).

A lipid extraction from *L. plantarum* culture was purified and converted to FAME for analysis by the Ag^{+}-LC/O_3-MS method. The XICs of the two pairs of characteristic ions clearly show the presence of $\Delta^{8,10}$, $\Delta^{9,11}$, $\Delta^{10,12}$ and $\Delta^{12,14}$ CLA positional isomers (**Figure 2A-11**), and the retention times allow for the partial assignment of CLA geometric isomers.

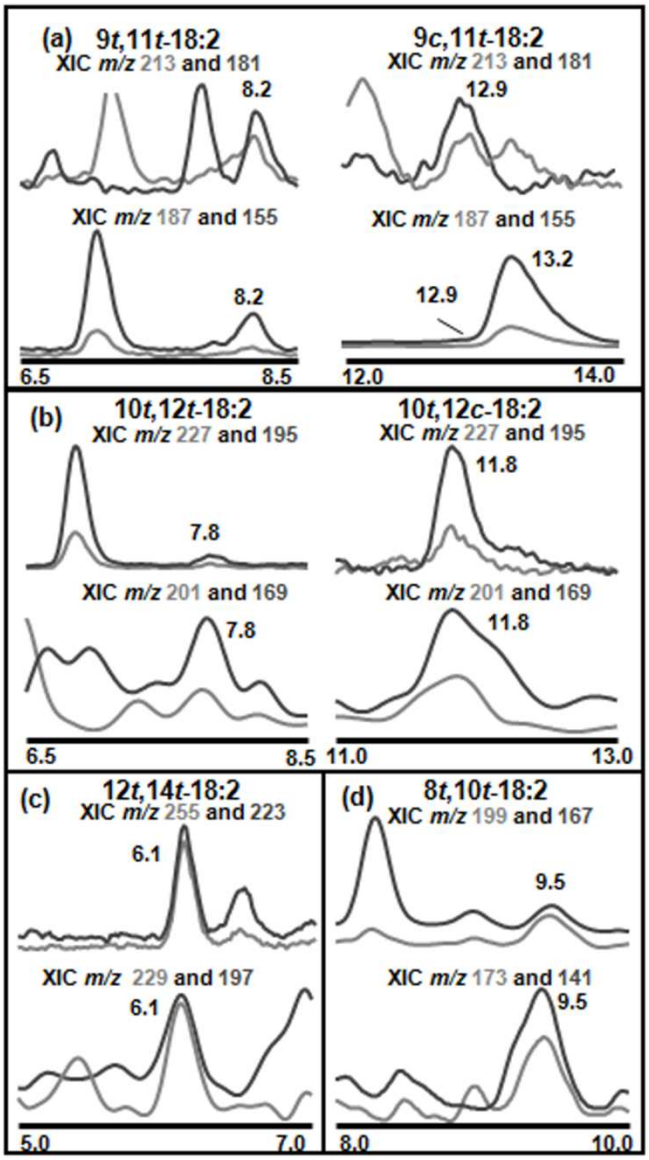

Figure 2A-11. The Ag^{+}-LC/O_3-APPI(+)-MS analysis of a lipid extract from *L. plantarum* culture. Each identified CLA isomer is indicated by its retention time. (A) XIC of *m/z* 155 and 181, *m/z* 187 and 213; (B) XIC of *m/z* 169 and 195, *m/z* 201 and 227; (C) XIC of *m/z* 197 and 223, *m/z* 229 and 255; (D) XIC of *m/z* 141 and 167, *m/z* 173 and 199.

Finally, it can be concluded that *trans*-9, *trans*-11- (8.2 min), *cis*-9, *trans*-11- (12.9 min), *trans*-10, *trans*-12- (7.8 min), *trans*-10, *cis*-12- (11.8 min), *trans*-12, *trans*-14- (6.1 min) and *trans*-8, *trans*-10- CLA (9.5 min) are found in *L. plantarum* culture sample. Hence, in addition to CLA isomers previously elucidated from the conversion of linoleic acid by *L. plantarum*, the use of the Ag^+-LC/O_3-MS method in this study has revealed that three additional CLA isomers are also produced. These additional, previously unidentified isomers may partially be responsible for the lack of definitive and reproducible results in human studies involving CLA from lactobacilli cultures.

2A.4. Conclusions

In this study, we have demonstrated that ozonolysis product aldehyde ions from in-line O_3-MS can be used for the identification of CLA positional isomers. Coupling Ag^+-LC to in-line O_3-MS successfully identifies CLA positional isomers and most of geometric isomers in complex samples, which has been shown in the analysis of CLA isomers in a CLA supplement and even complex lipid mixture such as milk fat and lipid extract of *L. plantarum* culture. Since the diagnostic ozonolysis aldehyde ions and methanol loss fragment ions are predictable, it is easy to extract these target ions (**Table 2A-1**) for the *de novo* identification of CLA positional isomers. Furthermore, the Ag^+-LC/ O_3-MS method is unaffected by the retention time instability of Ag^+-LC columns because the identification solely relies on extracting the diagnostic ions without reference

to the retention times of CLA standards. The estimated LOD of 0.20 ng on-column for the identification of the diagnostic aldehyde ions from a single component, also makes the identification of relatively low abundance CLA isomers possible. Future work will apply Ag^+-LC/ O_3-MS for quantitation of CLA, dependent on the availability of suitable isotopically labeled internal standards. In summary, the Ag^+-LC/ O_3-MS method described here could be expected to facilitate the fast and direct identification of the CLA isomers present in lipid extracts from any source.

2A.5. References

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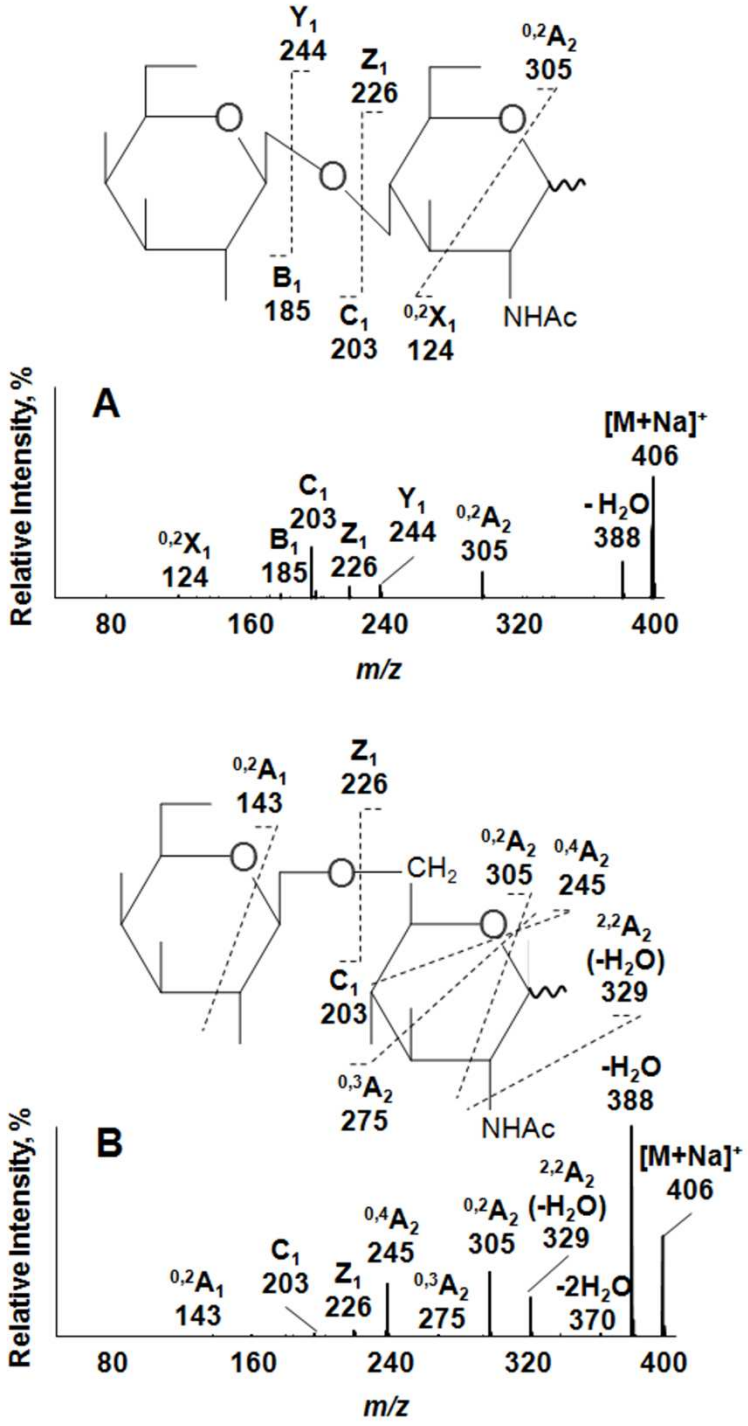

Figure 3A-1. ESI-MS/MS sodiated spectra of Gal-GlcNAc sample compounds, $[M+Na]^+$ at *m/z* 406. (A) Gal-GlcNAc peak 1; (B) Gal-GlcNAc peak 2; Structures are shown to indicate proposed fragmentation.

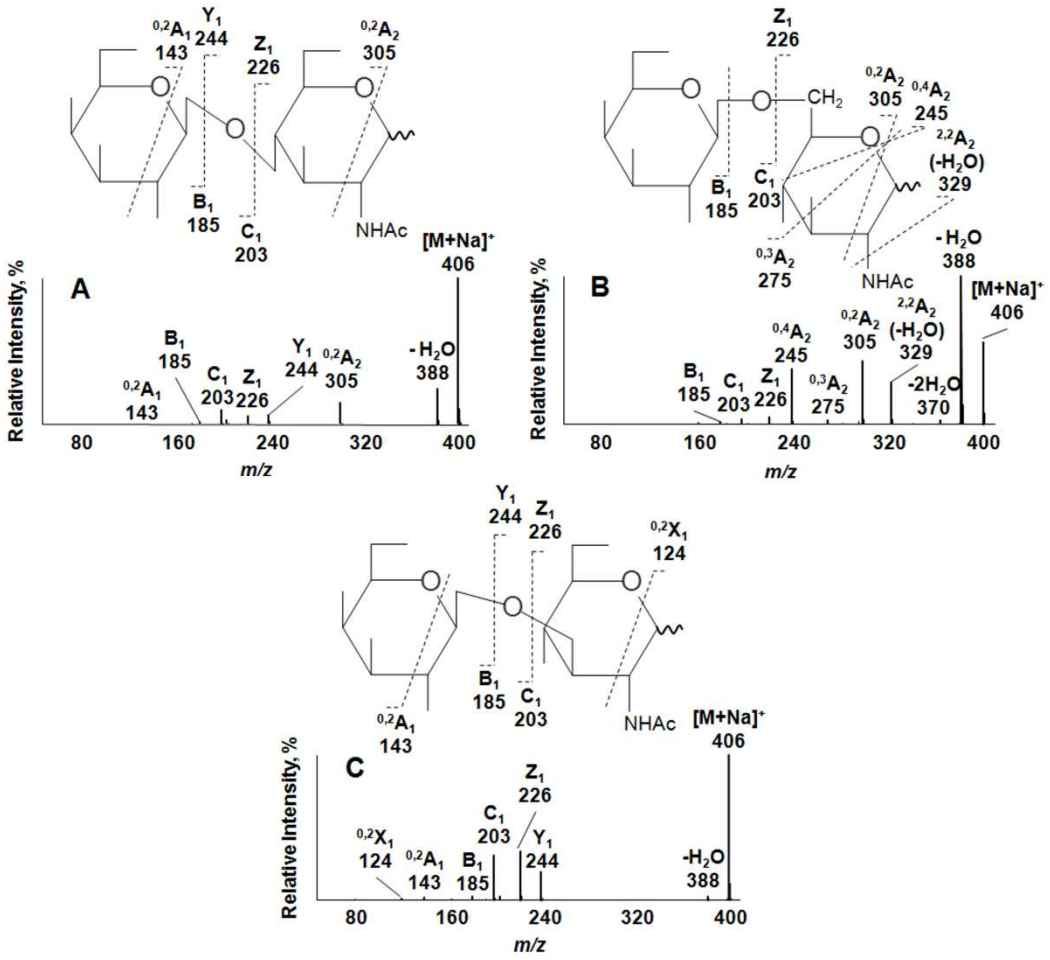

Figure 3A-2. ESI-MS/MS sodiated spectra of Gal-GlcNAc standards, $[M+Na]^+$ at *m/z* 406. (A) Gal*β*-(1→4)-GlcNAc standard; (B) Gal*β*-(1→6)-GlcNAc standard; (C) Gal*β*-(1→3)-GlcNAc standard. Structures are shown to indicate proposed fragmentation.

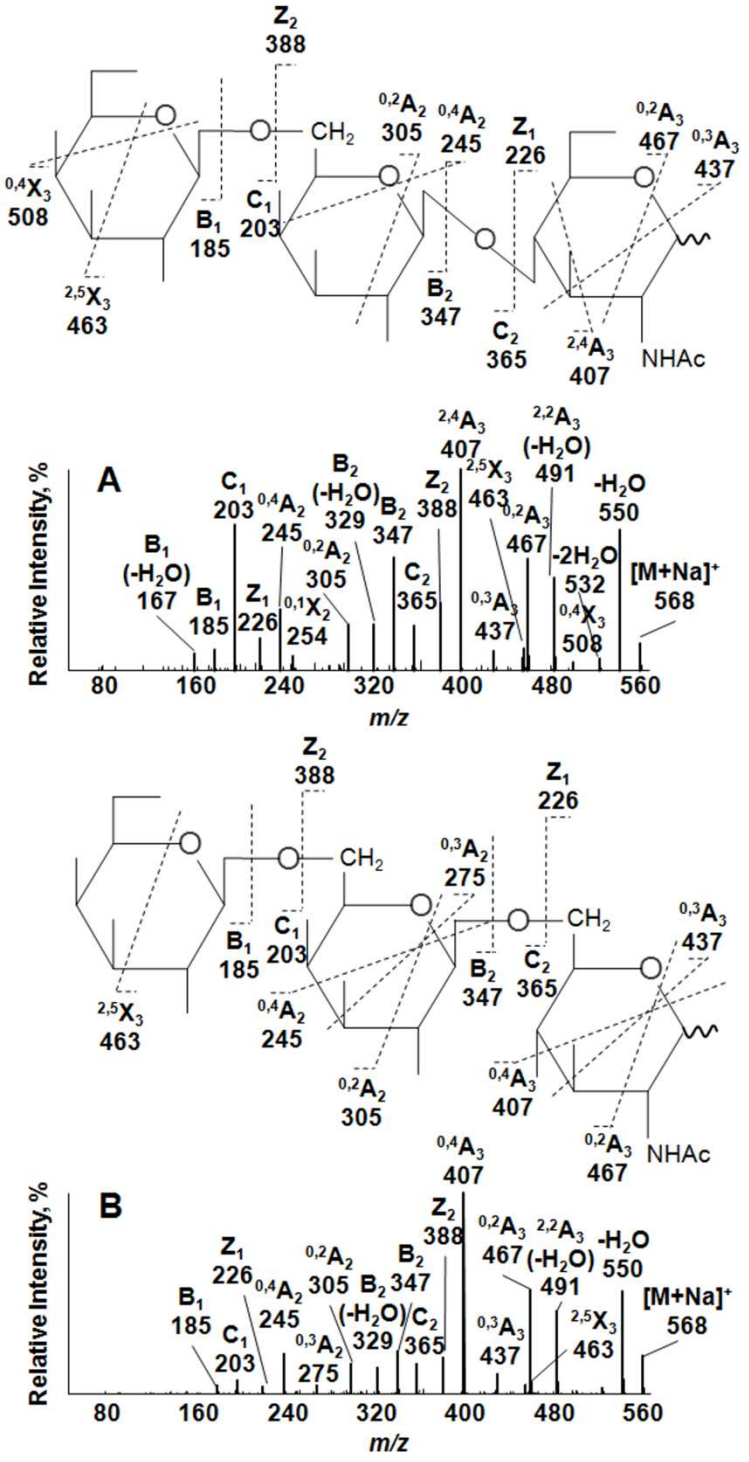

Figure 3A-3. ESI-MS/MS sodiated spectra of Gal-Gal-GlcNAc sample compounds, $[M+Na]^+$ at *m/z* 568. (A) Gal-Gal-GlcNAc peak 1; (B) Gal-Gal-GlcNAc peak 2; Structures are shown to indicate proposed fragmentation.

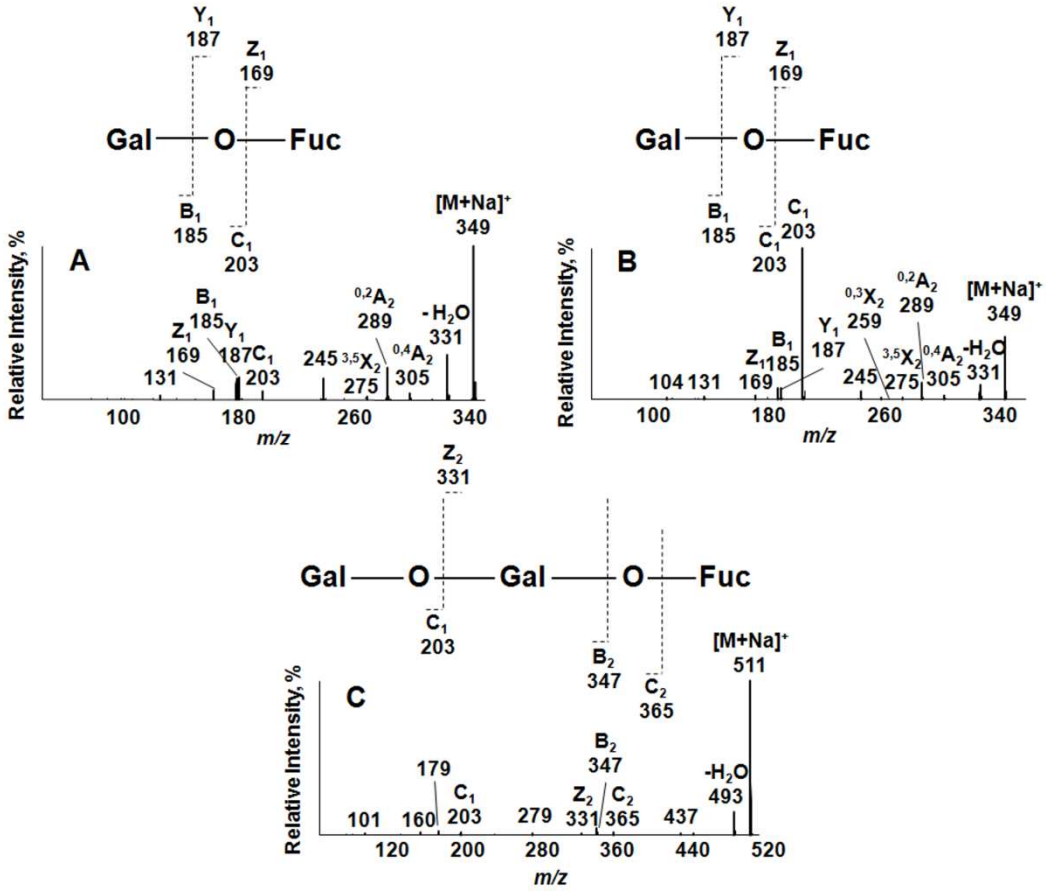

Figure 3A-4. ESI-MS/MS sodiated spectra of sample compounds. (A) Gal-Fuc peak 1, $[M+Na]^+$ at *m/z* 349; (B) Gal-Fuc peak 2, $[M+Na]^+$ at *m/z* 349; (C) Representative of sample compound Gal-Gal-Fuc peak 1, 2 and 3, $[M+Na]^+$ at *m/z* 511; Structures are shown to indicate proposed fragmentation.

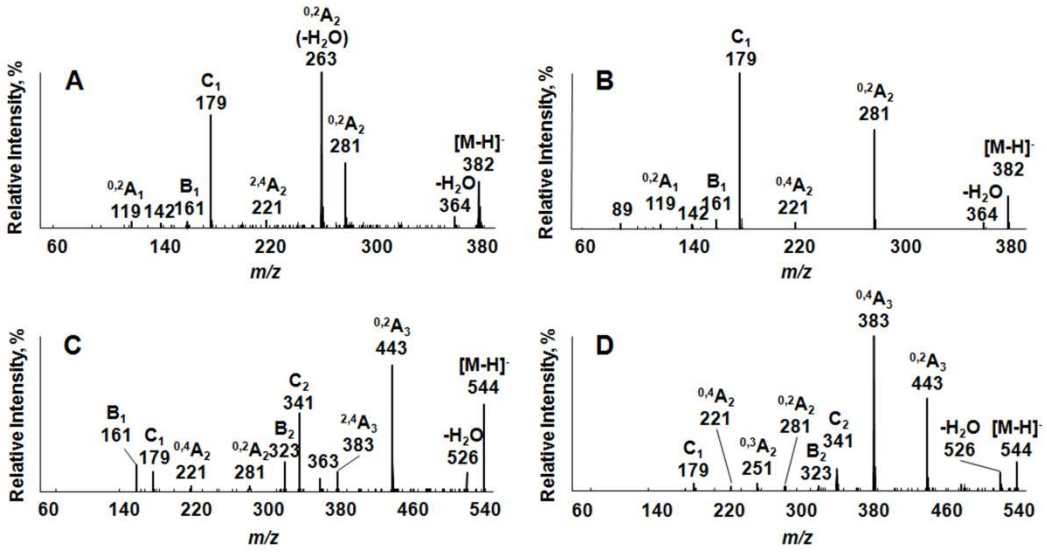

Figure 3A-5. ESI-MS/MS spectra of $[M-H]^-$ ions of galactosylated GlcNAc formed by CCE of *Lactococcus lactis* MG1363 expressing LacLM of *L. plantarum* FUA3112. (A) Gal-GlcNAc *m/z* 382 with a retention time of 18.1 minutes; (B) Gal-GlcNAc *m/z* 382 with a retention time of 22.9 minutes; (C) Gal-Gal-GlcNAc *m/z* 544 with a retention time of 54.8 minutes; (D) Gal-Gal-GlcNAc *m/z* 544 with a retention time of 66.8 minutes.

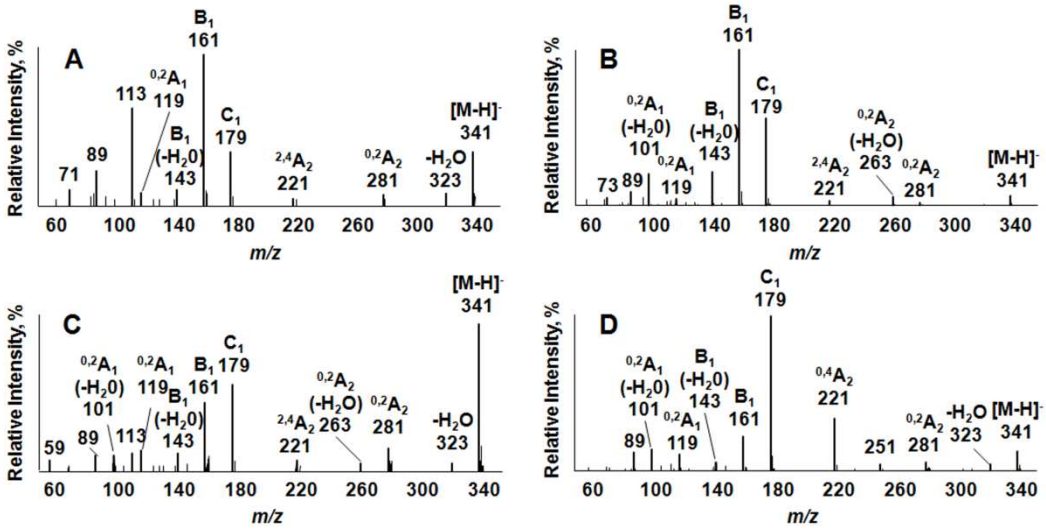

Figure 3A-6. ESI-MS/MS spectra of $[M-H]^-$ ions of galacto-oligosaccharides formed by CCE of *Lactococcus lactis* MG1363 expressing LacLM of *L. plantarum* FUA3112. (A) Gal*β*-(1→3)-Glc/Gal *m/z* 341 with a retention time of 15.5 minutes; (B) Lactose *m/z* 341 with a retention time of 17.5 minutes; (C) Gal*β*-(1→4)-Gal *m/z* 341 with a retention time of 20.1 minutes; (D) Gal*β*-(1→6)-Glc/Gal *m/z* 341 with a retention time of 22.0 minutes.

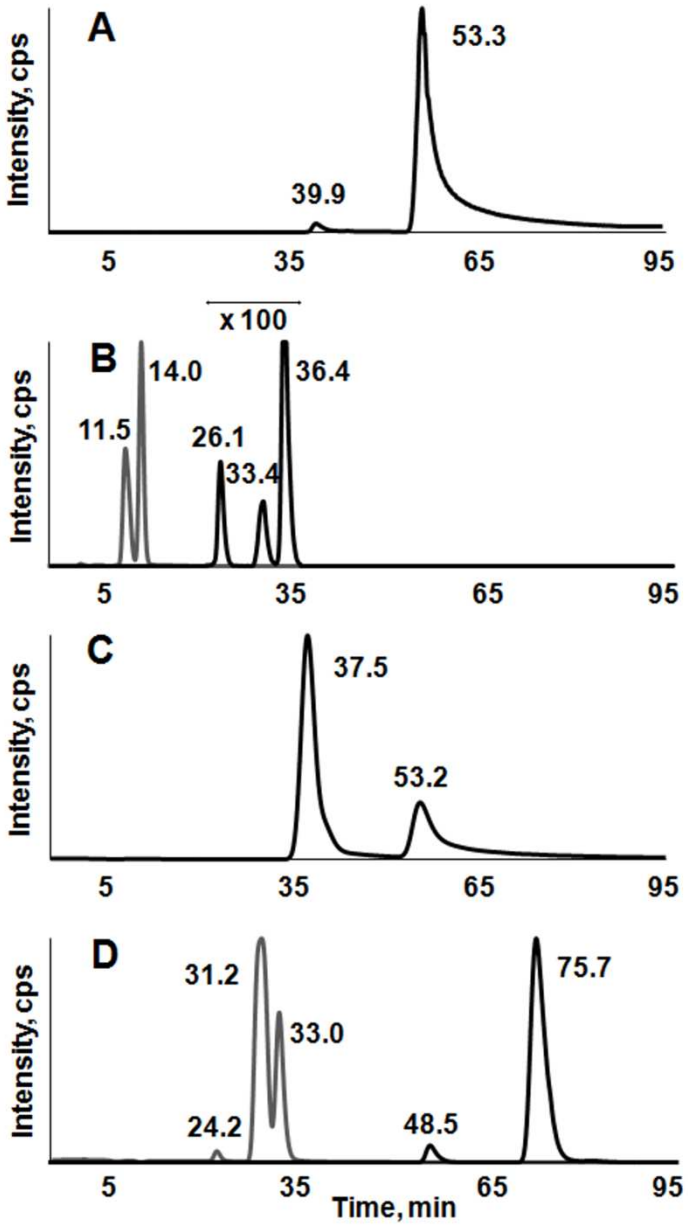

Figure 4A-1. Extracted ion chromatograms from NH_4-LC/ESI-MS of galactosylated product $[M-H]^-$ ions. (A) *m/z* 503 of trisaccharides formed with melibiose as galactosyl-donor and -acceptor; (B) *m/z* 325 of galactosylated-Fuc disaccharides (grey) and *m/z* 487 galactosylated-Fuc trisaccharides (black); (C) *m/z* 503 of trisaccharides formed with melibiose as galactosyl-donor and lactose as galactosyl-acceptor; (D) *m/z* 503 of trisaccharides (grey) and *m/z* 665 of tetrasaccharides (black) formed with raffinose as galactosyl-donor and -acceptor.

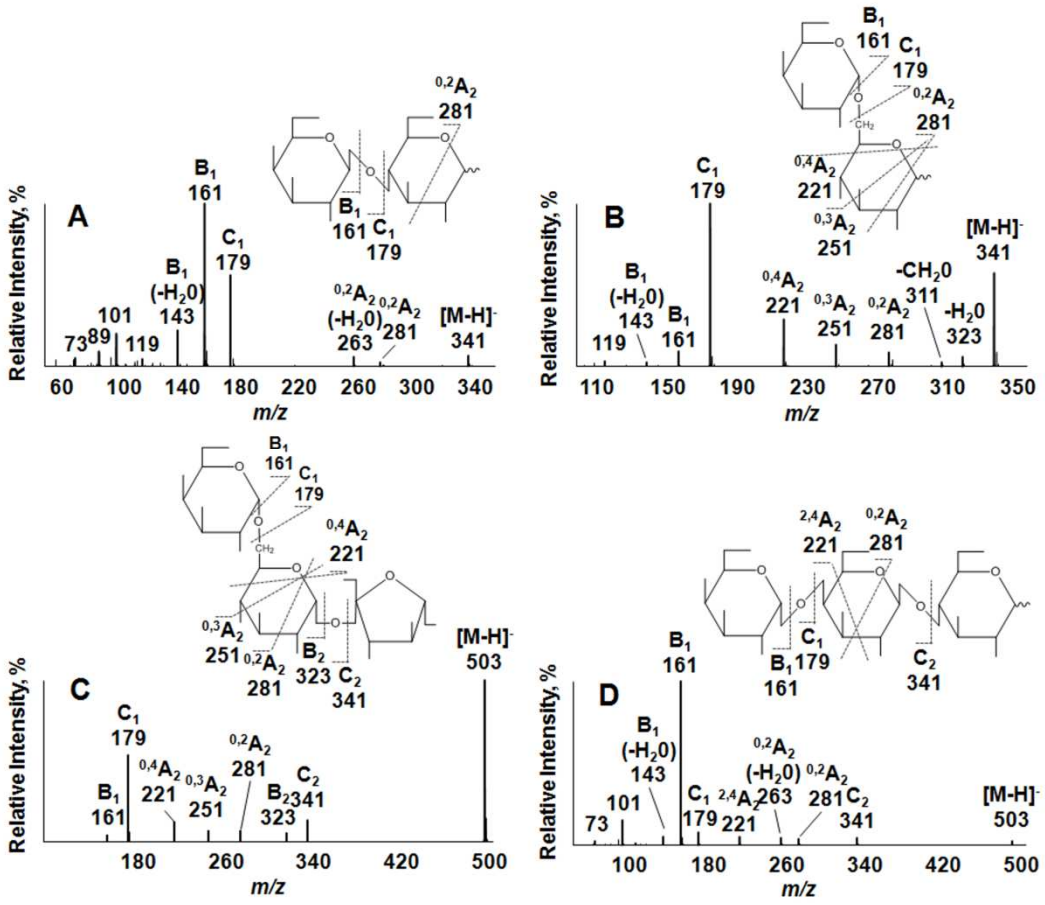

Figure 4A-2. ESI-MS/MS spectra of $[M-H]^-$ ions representative of authentic standards. (A) lactose, Gal*β*-(1→4)-Glc; (B) melibiose, Gal*α*-(1→6)-Glc; (C) raffinose Gal*α*-(1→6)-Glc*α*-(1→2)-Fru; (D) globotriose, Gal*α*-(1→4)-Gal*β*-(1→4)-Glc.

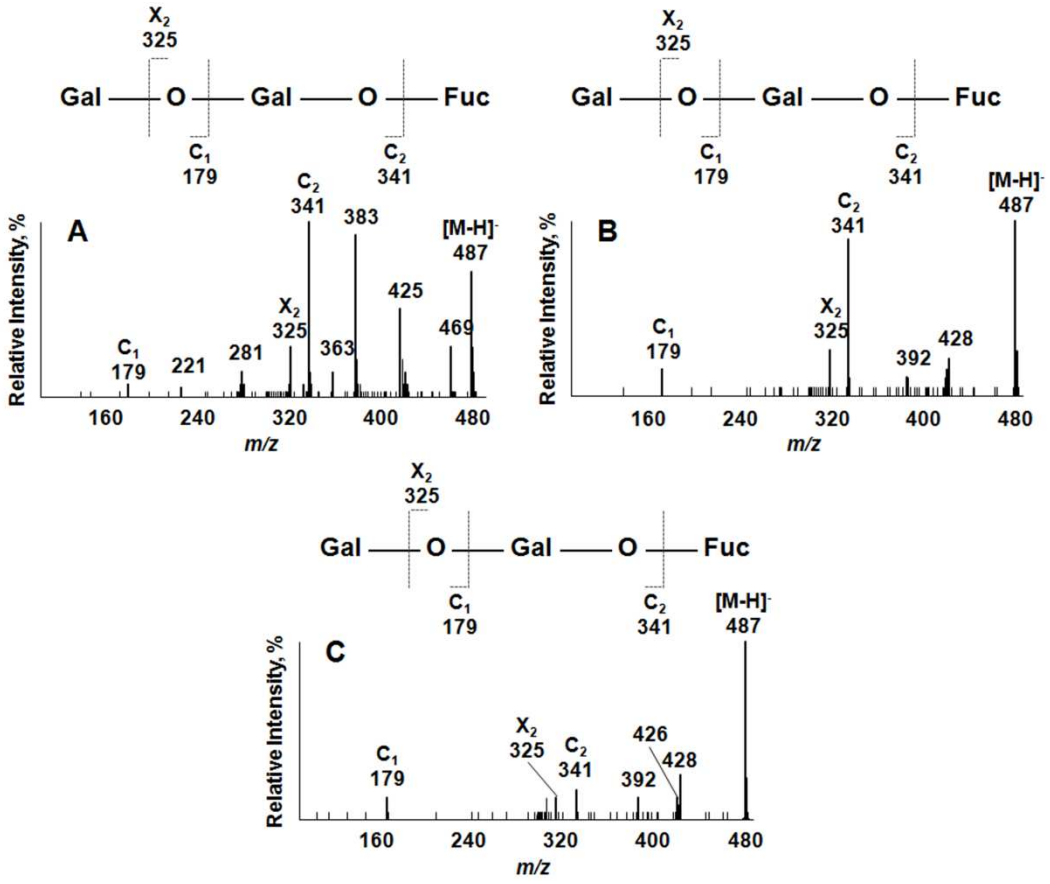

Figure 4A-3. ESI-MS/MS spectra of $[M-H]^-$ ions of sample with α-Gal acting on melibiose as galactosyl-donor and fucose as galactosyl-acceptor. Representative of the monosaccharide sequence of (A) product *m/z* 487 at retention time 26.1 min; (B) product *m/z* 487 at retention time 33.4 min; (C) product *m/z* 487 at retention time 36.4 min.

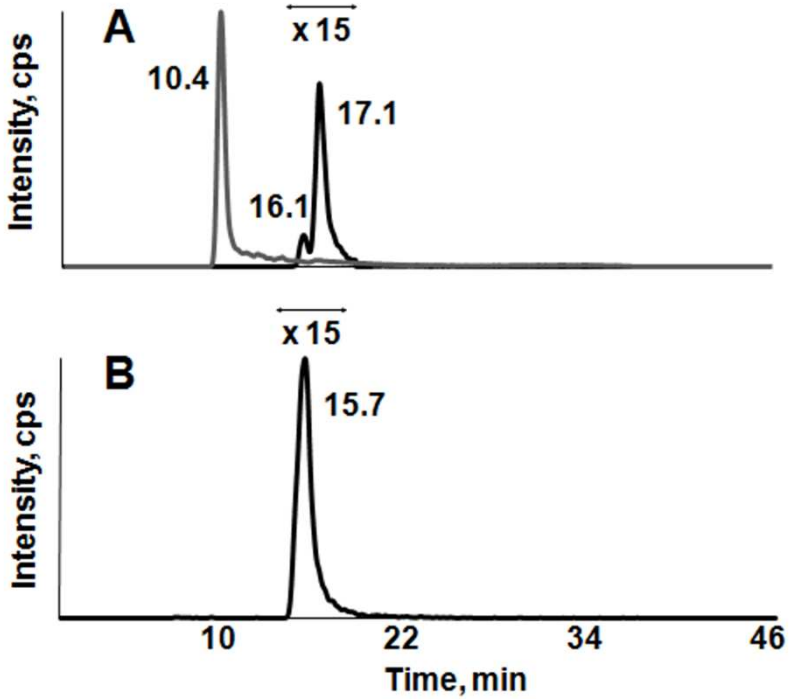

Figure 5A-1. LC/MS extracted ion chromatograms of galactosylated chitin-oligosaccharide $[M-H]^-$ ions. (A) chitinbiose as galactosyl-acceptor *m/z* 585 Gal-GlcNAc-GlcNAc (grey) and *m/z* 747 Gal-Gal-GlcNAc-GlcNAc (black); and (B) chitintriose as galactosyl-acceptor *m/z* 788 Gal- GlcNAc-$(GlcNAc)_2$.

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Authors: Brenna A. Black, Vivian S. Y. Lee, Yuan Yuan Zhao, Ying Hu, Jonathan M. Curtis, and Michael G. Gänzle

Publication: Journal of Agricultural and Food Chemistry

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Date: May 1, 2012

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Title: Identification of Conjugated Linoleic Acid (CLA) Isomers by Silver Ion-Liquid Chromatography/In-line Ozonolysis/Mass Spectrometry (Ag+-LC/O3-MS)

Authors: Chenxing Sun, Brenna A. Black, Yuan-Yuan Zhao, Michael G. Gänzle, and Jonathan M. Curtis

Publication: Analytical Chemistry

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Title: Characterization of α-galacto-oligosaccharides formed via heterologous expression of α-galactosidases from Lactobacillus reuteri in Lactococcus lactis

Authors: Yvonne Wang, Brenna A. Black, Jonathan M. Curtis and Michael G. Gänzle

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