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ENZYMATIC BIOTRANSFORMATION OF NATURAL INTENSE SWEETENERS: STRUCTURAL CHARACTERIZATION AND EVALUATION OF SENSORIAL PROPERTIES AND PREBIOTIC POTENTIAL

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That **Ana Muñoz-Labrador**, has performed, under their supervision, the research work entitled: **"Enzymatic biotransformation of natural intense sweeteners: structural characterization and evaluation of sensorial properties and prebiotic potential"**.

This thesis is submitted in fulfilment of the requirements for the degree of doctor at the Autonomous University of Madrid (UAM).

Madrid, May 2021

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ACRONYMS & ABBREVIATIONS

Ace-K	Acesulfame-K
ADI	Acceptable Daily Intake
CAC	Codex Alimentarius Comission
CFU	Colony-Forming Unit
BMI	Body-Mass Index
DNA	Deoxyribonucleic acid
DP	Degree of Polymerization
EFSA	European Food Safety Authority
ES	Equivalent Sweetness
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FOS	Fructo-oligosaccharides
Fru	Fructose
Fuc	Fucose
Gal	Galactose
GC	Gas Chromatography
GC-FID	Gas Chromatography with Flame Ionization Detector
GI	Glycaemic Index
Glc	Glucose
GlcNAc	N-acetylglucosamine
GRAS	Generally Regarded As Safe
HIS	High-Intensity Sweeteners
HMDS	Hexamethyldisilazane
HMOs	Human Milk Oligosaccharides
IL	Interleukin
IS	Internal Standard
JECFA	Joint FAO/WHO Expert Committee on Food Additives
Lac	Lactose
LC	Liquid Chromatography
LC-ESI-MS	Liquid Chromatography-Mass Spectrometry
LC-RID	Liquid Chromatography with Refractive Index Detector
LHG	Luo Han Guo
MALDI-TOF-MS	Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry
MTBSTFA	N-(ter-butyldimethysilyl)-N-methyltrifluoroacetamine
NCDs	Non-Communicable Diseases
NDOs	Non-Digestible Oligosaccharides
o-NPG	*o*-nitrophenyl-*β*-D-galactopyranoside
p-NPG	*p*-nitrophenyl-α-glucopyranoside
qPCR	quantitative Polymerase Chain Reaction
RE	Relative percentage Error
Rha	Rhamnose
RP-LC-DAD	Reversed-Phase Liquid Chromatography couple to Diode-Array Detector
Suc	Sucrose

TMSOs	Trimethylsilylated Oximes
TNF-α	Tumour Necrosis Factor
TPA	12-O-Tetradecanoyl- Phorbol-13-Acetate
UDP	Uridine-5'-Diphospho
UGTs	Uridine-5'-Diphospho dependent Glucosyltransferases
VLDL	Very Low-Density Lipoprotein
WHO	World Health Organization
Xyl	Xylose

SUMMARY

The rapid increase in the prevalence of non-communicable diseases (NCDs) worldwide has been mainly attributed to an excess of sugar consumption. From a nutritional standpoint, high-intensity sweeteners have fewer calories than sugars while providing a major sweet potency which are in the spotlight as valuable alternatives to sugar. This thesis reviews the state-of-the-art of sweeteners as well as some background information on health and sugar replacements. Continuing consumer interest and growing markets have led to a significant increase in the focus on the sweet diterpenoid glycosides present on the leaves of *Stevia rebaudiana* and, more recently, on the cucurbitane triterpene glycosides present on the fruits of *Siraitia grosvenorii*. However, the taste experience delivered by these natural sweet compounds is not identical to those delivered by sucrose. Although the potential benefits of blending sweeteners have been known for many years, recent development based on synthesis strategies to produce sucrose-like taste profiles is emerging. Biocatalyst alternatives may be preferred as promising green approaches to produce and supply specific sweetener compounds.

The main focus of this PhD Thesis is the enzymatic modification of the above-mentioned natural sweeteners using different carbohydrate-active enzymes in order to improve their sensorial profile and being more feasible for consumption and formulation. Moreover, together with these modifications, the syntheses carried out with specific disaccharides as donor substrates led to the production of prebiotic oligosaccharides fibres which are not only more suitable regarding their organoleptic and nutritional characteristics but also provide different bioactivities. The enzymatic reactions were properly optimized in order to give rise to high synthesis yields. A comprehensive structural analysis was paramount and liquid chromatography coupled to a diode-array detector (LC-DAD), gas chromatography with a flame ionization detector (GC-FID), liquid chromatography-mass spectrometry (LC-ESI-MS) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) were used to determine and identify the chemical modifications that occurred along with optimization. These optimizations provided high-efficiency and easy to scale-up procedures to produce target compounds that could be applicable in industrial-scale

processes. The optimal samples were synthesized and purified on a larger scale to evaluate sensorial profiling, and subsequently, to make an *in vitro* fermentation study of the synthesized dietary oligosaccharide-based sweeteners in order to provide crucial preclinical data of their prebiotic potential.

In summary, this thesis has addressed three main key points: the enzymatic synthesis and analysis of the enzymatic biotransformations, their sensorial evaluation and the study of their bioactivity, resulting in a highly multidisciplinary scientific work. The enzymatic reactions led to the formation of other sweet compounds that were not naturally present on the terpenoid glycosides from *Stevia rebaudiana* and *Siraitia grosvenorii* as well as an enhancement of the flavour profiles consequently to those modifications. The prebiotic-based sweeteners showed a beneficial effect by selectively modulating the microbiota which could be considered as a novel generation of sweeteners with potential biological functionality.

RESUMEN

La incidencia de enfermedades no transmisibles está sufriendo un incremento rápido a nivel mundial, lo cual se ha atribuido especialmente al consumo excesivo de azúcar, entre otros factores. Los edulcorantes de alta intensidad son aditivos alimentarios que, desde un punto de vista nutricional, aportan menos calorías que los azúcares a la vez que proporcionan un mayor poder edulcorante, encontrándose en el punto de mira como alternativas al conocido azúcar de mesa, la sacarosa. En esta tesis se lleva a cabo una revisión de los últimos avances en el estudio de los edulcorantes, haciendo especial hincapié en la evidencia científica existente sobre su impacto en la salud, así como de las posibles alternativas para reemplazar el azúcar. El creciente interés por parte de los consumidores y de la industria ha dado lugar a un gran desarrollo científico que está poniendo el foco en el uso de edulcorantes naturales como son los glicósidos diterpénicos presentes en las hojas de la especie *Stevia rebaudiana* y más recientemente, los glicósidos triterpénicos del fruto de la especie *Siraitia grosvenorii*. Sin embargo, el sabor de estos compuestos dulces difiere de las propiedades sensoriales del azúcar y presenta ciertas limitaciones. A pesar de que durante muchos años la industria alimentaria ha recurrido a la combinación de diferentes edulcorantes con el fin de obtener sinergias en la mejora de parámetros de aceptabilidad sensorial, cada vez salen a relucir más estrategias para producir compuestos con propiedades organolépticas similares a los tradicionales edulcorantes nutritivos como es la sacarosa. En este contexto, el empleo de biocatalizadores de origen microbiano y grado alimentario para desarrollar compuestos específicos de una manera más ecológica es una de las alternativas más prometedoras en la actualidad.

Esta tesis doctoral se centra principalmente en la modificación enzimática de los edulcorantes naturales mencionados anteriormente utilizando diferentes enzimas con actividad carbohidrasa, para así mejorar el perfil sensorial y hacerlos más adecuados para su formulación o para ser directamente consumidos. Además, junto con esas modificaciones, se llevaron a cabo otras síntesis enzimáticas utilizando disacáridos como sustratos donantes, lo cual dio lugar a la producción de oligosacáridos prebióticos, cuyas fibras no son solo más apropiadas por sus características sensoriales y nutricionales, sino que además proporcionan

propiedades bioactivas. Las reacciones se optimizaron adecuadamente con la finalidad de obtener altos rendimientos. Además, se realizó un análisis estructural que permitió determinar e identificar las modificaciones enzimáticas que tuvieron lugar durante la optimización. Esta caracterización estructural se llevó a cabo mediante técnicas analíticas como la cromatografía líquida de alta resolución con detector de diodo array (LC-DAD), la cromatografía de gases con detección de ionización de llama (GC-FID), la cromatografía de líquidos acoplada a espectrometría de masas (LC-MS) y la espectrometría de masas desorción/ionización láser asistida por una matriz con detección de masas por tiempo de vuelo (MALDI-TOF). Como resultado, se obtuvieron procedimientos eficaces y adecuados permitiendo que las reacciones pudieran ser escalables a nivel industrial. De este modo, las muestras óptimas se sintetizaron y purificaron a una mayor escala con el fin de evaluar el perfil sensorial y las propiedades de fermentación *in vitro* de las muestras basadas en oligosacáridos.

En resumen, esta tesis aborda tres aspectos esenciales: la síntesis enzimática de edulcorantes, la evaluación sensorial y el estudio de la bioactividad centrada en sus propiedades prebióticas, dando como resultado un trabajo científico multidisciplinar. Además, las reacciones enzimáticas dieron lugar a la formación de otros compuestos dulces que no están naturalmente presentes en la *Stevia rebaudiana* ni en la *Siraitia grosvenorii*, así como una mejora en los perfiles sensoriales como consecuencia de las modificaciones. Los edulcorantes sintetizados en conjunto con los oligosacáridos mostraron un efecto beneficioso al modular el crecimiento de la microbiota, pudiendo ser considerados como una nueva generación de edulcorantes con propiedades beneficiosas para la salud.

CONTENT

1

General Introduction

OUTLINE

1 INTRODUCTION

1.1 Obesity and related diseases: an overview of an epidemic

Over the last four decades, worldwide obesity has nearly tripled with an alarming obesity prevalence of eight-fold in children and adolescents, and three-fold in adults over this period, reaching epidemic proportions (Bentham *et al.* 2017; Di Cesare *et al.* 2016). Currently, all malnutrition forms are generally measured by the body-mass index (BMI), and their caused factors mainly rely on the imbalance between the energy intake and energy expenditure, physical inactivity, and even undernutrition (Blüher, 2019; Lee *et al.* 2012; Zhang *et al.* 2018). In addition, societal factors are also associated with a prevalence of obesity, with differing tendencies between rural and urban areas (Bixby *et al.* 2019; Popkin *et al.* 2012; Swinburn *et al.* 2019). A plateauing increment is occurring simultaneously in all low-, middle-, and high-income countries mainly led by global changes in the food system, socio-economic climate, and lifestyle (Bentham *et al.* 2017; Hruby *et al.* 2015; Swinburn *et al.* 2011).

Obesity is one of the leading risk factors contributing to the development of NCDs such as cardiovascular diseases, accounting for most of NCD deaths (48%), followed by cancers (21%), chronic respiratory diseases (12%) and diabetes (3.5%), which cause annually more than 63% of global deaths (WHO, 2020). NCDs constitutes one of the greatest global health challenges of the 21st century. Recent proposal are calling to monitor progress and trends of NCDs and their risk factors, outcomes and treatment for guiding policy and priorities in order to achieve a substantial reduction of the chronic disease burden. Efforts are undertaken to implement effective international plans that emphasize the prevention of NCD risk factors (Di Cesare *et al.* 2013; Lozano *et al.* 2010). The World Health Organization (WHO) Global Action Plan for The Prevention and Control of NCDs 2013-2020 proposed a 25% relative reduction in premature mortality from NCDs by 2025 (WHO, 2014; WHO, 2018; WHO, 2020). Likewise, numerous reports have evidenced an interplay between chronic diseases and high nutrient intake-based diets. Other ongoing initiatives aim to reduce the salt, fat, and free sugar content, such as the White Paper on a Strategy for Europe on Nutrition, Overweight and Obesity-Related Health Issues from the European Commission, and EU countries' national programs, whose

initiatives have been developed ensuing the WHO recommendations to promote the implementation of improved food reformulations that contribute to ceasing the burden of risk factors (Belc *et al.* 2019; Commission of the European Communities, 2007).

1.2 Dietary Carbohydrates - Sugar status and its role in food processing and human nutrition

The Joint Food and Agriculture Organization of the United Nations (FAO) and the WHO Expert Meeting in 1979, first reported the role of carbohydrates on human health. The updated FAO/WHO Expert Consultation on Carbohydrates in Human Nutrition in 2006 concluded that among the macronutrients, carbohydrates are the major source of food energy provided in the human diet, accounting for between 55 and 75% of the total energy requirements (FAO, 1980; FAO, 2007).

Dietary carbohydrates make up the largest component of plant-based foods formed by a complex diversity of substances that possess intrinsic biological activities of differing importance to health (Amicucci *et al.* 2019). They are highly structurally diverse and can be classified on the basis of their molecular size or degree of polymerization (DP; number of monosaccharides units combined) as seen in **Table 1**, which are subdivided by subgroups that typically present broadly similar physiological effects. Thus, sugars may be divided into mono-, disaccharides, and sugar alcohols that are rapidly absorbed providing a ready source of energy (Cummings *et al.* 1997; Sako *et al.* 1999).

Table 1. Classification of the principal dietary carbohydrates. Table adapted from Sako *et al.* (2011).

Carbohydrate classes	Subclasses	Examples	Physiology
Monosaccharides (1 monomer)	Sugar	Glucose, fructose, galactose	Absorbed in the small intestine Glucose gives a rapid glycemic response
	Sugar alcohol	Sorbitol, xylitol, mannitol	Absorbed in the small intestine
Disaccharides (2 monomers)	Digestible sugar	Sucrose, maltose, trehalose, lactose	Absorbed in the small intestine Digestible by endogenous hydrolyzing enzymes Rapid glycemic response
	Non-digestible disaccharides	Lactulose	Not absorbed Non-digestible, but fermented in the large intestine
	Sugar alcohol	Isomaltitol, maltitol, lactitol	Poorly digested and absorbed in the small intestine Partly or fully fermented in the large intestine
Oligosaccharides (3-10 monomers)	α-glucans	Maltooligosaccharides	Digestible but partly undigested in the small intestine and give a rapid glycemic response
	Non-digestible oligosaccharides (NDO) or Partially digested oligosaccharides	Fructo-oligosaccharides, galacto-oligosaccharides	Fermented in the large intestine No glycemic response
	Human milk oligosaccharides	Fucosyllactose, sialyllactose, lacto-*N*-tetraose, etc.	Nondigestible Partly fermented in the large intestine
Polysaccharides (>10 monomers)	Starch (α-glucans)	Amylose, amylopectin, pullulan	Digested and absorbed in the small intestine Rapid glycemic response
	Resistant starch (α-glucans)		Digested but indigested in the small intestine Fermented in the large intestine
	Nonstarch polysaccharides (β-glucans, β-fructans, etc.)	Cellulose, hemicellulose, inulin, pectin etc.	Nondigestible Partly fermented in the large intestine

Consumption of free-sugars such as fructose and sucrose, especially in the form of sugar-sweetened beverages, has been steadily increasing, which is proven to be associated with weight gain and obesity-related comorbidities. Moreover, the global production for 2020-21 is forecast for up to 22-188 million tons due to the growth in market production in Brazil, India, and Thailand, expecting consumption to have risen to a new record (FAO, 1998; FAO, 2007; Faruque *et al.* 2019; Pia *et al.* 2017; USDA, 2020). Therefore, current guidelines have proposed to limit consumption of simple sugars to less than 10% of total energy intake, with a conditional recommendation for further reduction to below 5% of total energy intake (WHO, 2015).

It is acknowledged that sweet taste typically provokes hedonic responses in individuals. The flavour profile of sugars plays an important role in the acceptance of formulated products, mainly due to their sweet taste (Bellisle *et al.* 2007; Kim *et al.* 2017). However, sugars also contribute to other functional roles such as texture profiles and other quality properties (Di Monaco *et al.* 2018; Gibson *et al.* 2017a). Biologically, sweeteners induce several hormonal and metabolic effects that influence health and disease parameters (Stowell *et al.* 2007). The glycaemic response is a physiological effect that contributes to satiety and body weight regulation. The glycaemic index (GI) describes the blood-glucose-raising potential and provides useful means of helping to select the most appropriate carbohydrate-containing food, and is influenced by different chemical factors such as the type and quantity of carbohydrates; and physical properties such as the preservation or cooking methods, texture, etc. Numerous meta-analyses have established the inverse association between GI and satiety. High-GI sugars, sweeteners and carbohydrates-based foods trigger different potential effects such as hyperglycaemia and hyperinsulinaemia, and henceforth the appearance of metabolic diseases including heart-related diseases, metabolic syndrome, certain cancers, type II diabetes, and other associated complications. Otherwise, low-GI foods are characterized by a slower rate of digestion and absorption and are considered to confer benefits as a result of the relatively low glycemic response (Aston 2006; Mann *et al.* 2007; O'Donnell *et al.* 2012; Roberts *et al.* 2000; Venn *et al.* 2007). Hence, the acute glycaemic response varies from different sweeteners or carbohydrate-containing foods, which is important to take into account the potential effect on health. Similarly, relationships between dietary GI and blood lipogenesis and cholesterol, among other metabolic effects observed, have been equally related to the development of obesity, NCDs, and their consequences.

1.3 Sweeteners and other sugar alternatives

1.3.1 Bulk (nutritive) sweeteners

Common sugars used as both nutritive sweeteners or added in food formulations are of plant origin and include monosaccharides, such as glucose or fructose, and disaccharides, such as sucrose. Their orosensory and versatile technological functions such as their physical (*e.g.*, solubility, bulking agent, crystallization, and viscosity), microbial, (*e.g.*, preservation and fermentation), and chemical (*e.g.*, caramelization and antioxidation) characteristics make them promising for applications in the food industry (Das *et al.* 2015).

Glucose is the major starch-derived sweetener by acid-enzyme hydrolyzation and is commercially known as dextrose. Fructose is present in fruits and is also produced after the isomerization of glucose. It is added to food and beverage as high-fructose corn syrup or in its crystalline form. The term *sugar* is generally assigned to sucrose which is generated in the leaves of plants by photosynthetic processes and is commercially processed from sugarcane and sugar beets. Sucrose, as a sweetening agent, is the most consumed table sugar in the world and is the usual standard assigned for sensorial analyses. Sugar alcohols or polyols are low-calorie sweeteners found in the vegetable kingdom, but they are manufactured industrially by catalytic hydrogenation of the corresponding reducing sugars. Polyols are considered food additives and are mainly used to texturize, supply dry matter and, act on colouring properties (Hicks 1991). Due to its sweetening power and lower calorific content compared to other monosaccharides and disaccharides, are also categorized as sugar substitutes. The most used polyols are sorbitol (E420), mannitol (E421), lactitol (E966) and xylitol (E967), among others (**Figure 1**) (Carocho *et al.* 2017).

1.3.2 Non-nutritive sweeteners

These non-caloric sweeteners present a higher sweetening power than sucrose with the peculiarity that their energy contribution is very low or even virtually zero. Additionally, no glycaemic responses were designated and consequently, the sole function is to provide an intense sweetening effect, which means that low doses are needed. They are typically used either individually or blended with other nutritive sweeteners in order to bring up synergistic effects to overcome possible limitations (Hanger *et al.* 1996; Massoud *et al.* 2005; Oleson *et al.* 2017). Their blending would suppose a reduction of sugars and a positive manner to take advantage of their benefits when it comes to treat or decrease the development of obesity, body weight, diabetes, or prevention of dental caries. There has been an increasing demand for new highly sweet, noncaloric, and noncariogenic sucrose substitutes in the market, possessing at least equal sensory properties to sucrose. According to their origin, these sweet-tasting sucrose substitutes may be categorized by either synthetic or natural origin.

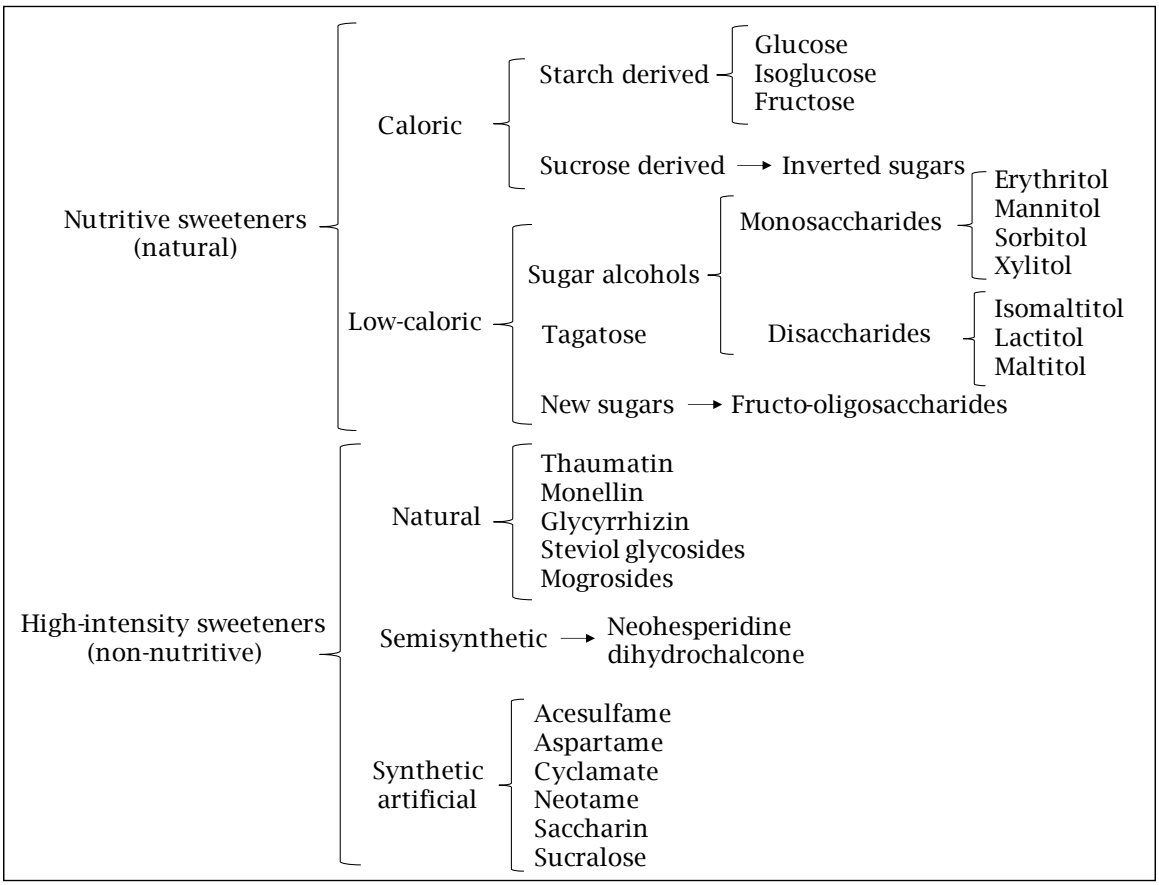


Figure 1. Classification of sweeteners (Carocho *et al.* 2017; Yebra-Biurrun 2004).

1.3.2.1 Synthetic intense sweeteners

Most of these sweeteners were accidentally discovered while some others were artificially created in order to obtain commercially viable high-intensity sweeteners (HIS). Examples of the synthetic sweeteners most used worldwide are aspartame, acesulfame potassium (Ace-K), saccharin, sucralose, neotame, and advantame, which are considered "generally regarded as safe" (GRAS) food additives. Their potential benefits rely on the assumption that they elicit no (or incomplete) energy compensation. However, the use of synthetic sweeteners is being questioned, owing to concerns about potential mechanisms with adverse metabolic outcomes since recent studies suggested the association between their use and weight gain and type 2 diabetes risk, by interfering in the gut microbiota, inducing glucose intolerance, and by stimulating sweet-taste receptors which could theoretically increase appetite and trigger insulin secretion (Azad *et al.* 2017; Borges *et al.* 2017; Carocho *et al.* 2017; Edwards *et al.* 2016; Pepino 2015).

Aspartame (E951) was the first artificial sweetener approved by the Food and Drug Administration (FDA). It is a di-peptide type sweetener that became a widespread commercial success when discovered due to its similar tasting quality in comparison with sucrose, being used in many food, beverages, and pharmacological formulations. However, aspartame and its metabolites may produce adverse effects, such as neurotoxic and neurobehavioral, allergic-type reactions, cancer and others (O'Mullane *et al.* 2014; Rycerz *et al.* 2013; Lindseth *et al.* 2014). Similarly, Ace-K (E950) is one of the most used synthetic sweeteners due to its low calorie, high sweetness, and excellent stability. However, studies *in vitro* found that its consumption could affect cognitive functions altering neuro-metabolic functions (Cong *et al.* 2013) and the perturbation of the gut microbiota (Bian *et al.* 2017; Bandyopadhyay *et al.* 2008). Saccharin (E954) is a sweetener widely manufactured around the world in four commercial forms: acid saccharin, sodium saccharin, potassium saccharin, and calcium saccharin. Around 80% of the saccharin is used in food and pharmacological preparations. It is approximately 400 times sweeter than sucrose; however, apart from that sweetness rate, it also presents a bitter metallic aftertaste (O'Mullane *et al.* 2014). Sucralose (E955) is industrially prepared utilizing sucrose as the starting material. Although is widely used in foods and beverages all

over the world, its safety is being put in doubt by several studies revealing the gut damage and inflammation observed in animal models (Bian *et al.* 2017; Hicks 1991; Li *et al.* 2020). Neotame (E961) and advantame (E969) have been the most recently FDA-approved sweeteners and flavour enhancers. They present similar peptide-based structures and are rapidly metabolized, but incompletely absorbed (Otabe *et al.* 2011; Romo-Romo *et al.* 2017). Finally, another sweetener used is sodium cyclamate (E952), which is a low-calorie sweetener mostly used in low-sodium diets and combined under the synergism offered by saccharin. However, it has been banned in the USA after controversial toxicity studies (Yebra-Biurrun 2004).

1.3.2.2 Natural intense sweeteners

For many decades, natural high-intensity sweeteners have been the subjects of extensive phytochemical studies; however, the consumer interest in natural high-potency sweeteners has grown dramatically, fuelled on one hand by concerns about the use of artificial additives in foods, that have been proved to present a potential risk, and on the other hand, to meet the growing consumer demand for organic products. How far the use of natural potent sweeteners will go is yet to be seen, but there is a growing and continuing effort to find new natural sweeteners to be developed and investigated as they could suppose an alternative to sugar.

Many high-potency sweeteners of diverse chemical structures are known to occur naturally. The presence of potent sweeteners in plants, in some instances, are presumably formed as secondary metabolites and are mostly terpenoids, flavonoids, and proteins. Amongst the sweetener substances, such as glycyrrhizin, thaumatin or phyllodulcin, two types of terpenoid glycosides stand out and have been submitted to GRAS notice status by FDA; several steviol glycosides from *Stevia rebaudiana* (Bertoni) and extracts obtained from the *Siraitia grosvenorii* fruit, containing 12.5 – 90% mogroside V. To date, these sweeteners have been launched in countries such as United States, Canada, Japan and China, while in many other countries, ongoing studies are assessing whether to implement them as a food ingredient.

1.3.2.2.1 *Stevia rebaudiana* (Bertoni)

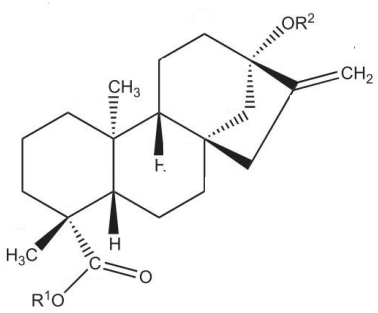
Natural source and background

The perennial herb plant *Stevia rebaudiana* (Bertoni) is a shrub of the family *Asteraceae* native to Paraguay and Brazil. It is one of the approximately 230 species that belong to the genus *Stevia*, specially featured by the production of natural sweet-tasting compounds as secondary metabolites in its leaves, which were originally used by indigenous ancestors for centuries for sweetening and medicinal purposes. *S. rebaudiana* was first noted in the scientific literature by M. Bertoni in 1899 and more fully described in 1905 (Hanson *et al.* 1993). Its final structure elucidation occurred in 1963 and only in the mid-1970s, it began to be commercially utilized for sweetening and flavouring foods and beverages in Japan, in order to substitute several synthetic sweeteners which were banned from the market at that time. Later on, many other sweet-tasting glycoside constituents have been isolated and identified from *S. rebaudiana*. *Stevia* leaves and relatively crude extracts have been used extensively as dietary supplements in the United States and elsewhere since the mid-1990s (Carakostas *et al.* 2012; Kinghorn *et al.* 2010; Perrier *et al.* 2018).

Structural characteristics

S. rebaudiana is formed by *ent*-kaurenoid diterpenoid glycosides that contain a common aglycone, steviol (*ent*-13-hydroxykaur-16-en-18-oic acid), and that differ from each other by the number of glycosidic constituents attached to C-13 and/or C-19. In most cases, steviol is substituted with single β-D-glucopyranose units or relatively small oligosaccharides composed of D-glucopyranose units, whether or not containing additional single sugars such as D-fructofuranose, L-rhamnopyranose, D-quinovopyranose, or D-xylopyranose units at C-13 and C-19 positions via 1,2-; 1,3-; 1,4- or 1,6- α or β-glycosidic linkages (**Figure 2**). The sweet components are known as steviol glycosides which account for 4-20% of the dry weight of the leaf tissues; however, the relative concentration of the different glycosides may vary depending on the *Stevia* leaf origin (genotype, phenological stage), regional location, climatic conditions, and extraction processes (Ceunen *et al.* 2013; Kinghorn *et al.* 2002; Lindley 2012; Purkayastha *et al.* 2016). To date, more than 40 different steviol glycosides have been

identified in *S. rebaudiana*, of which stevioside (4-13%), containing a β-D-glucosyl moiety at the C-19 position of the aglycone steviol as an ester and a 2-substituted β-D-diglucosyl unit (sophorose) at the C-13 position; and rebaudioside A (2-4%), containing a β-D-glucosyl moiety at the C-19 position of the aglycone, in addition to 2,3-substituted β-D-triglucosyl unit at the C-13 position, as the major sweetening agents of *S. rebaudiana* (Kurek *et al.* 2019; Prakash *et al.* 2012a; Sehar *et al.* 2008; Seki *et al.* 2018). The sweetness of sweet diterpenoid glycosides is around up to 100-400 times sweeter than sucrose (0.4% solution). Other minor glycosides (0.1-1%) with lower sweetness intensity include rebaudiosides C, D, E, and F, rubusoside, and dulcoside A (Gerwig *et al.* 2016; Pawar *et al.* 2013).



Steviol glycoside compound	**R_1**	**R_2**
Steviol	H-	H-
Steviolmonoside	H-	Glc(1-
Stebiolbioside	H-	Glc(1-2)Glc(1-
Rubusoside	Glc(1-	Glc(1-
Stevioside	Glc(1-	Glc(1-2)Glc(1-
Rebaudioside A	Glc(1-	Glc(1-2)[Glc(1-3)]Glc(1-
Rebaudioside B	H-	Glc(1-2)[Glc(1-3)]Glc(1-
Rebaudioside C	Glc(1-	Rha(1-2)[Glc(1-3)]Glc(1-
Rebaudioside D	Glc(1-2)Glc(1-	Glc(1-2)[Glc(1-3)]Glc(1-
Rebaudioside E	Glc(1-2)Glc(1-	Glc(1-2)Glc(1-
Rebaudioside F	Glc(1-	Xyl(1-2)[Glc(1-3)]Glc(1-
Dulcoside A	Glc(1-	Rha(1-2)Glc(1-

Figure 2. Chemical structures of *Stevia rebaudiana* compounds (Carakostas *et al.* 2012; Fry 2012; Gerwig *et al.* 2016).

Sensory properties

Structural features are related to their flavour, thus, the individual steviol glycosides exhibit different sweet potencies. The structure-sweetness intensity relationship was early described by Phillips *et al.* (1987). Rebaudioside A is considered the sweetest steviol glycoside reported to be 200-400 times sweeter than sucrose, while stevioside is 150-250 times sweeter than sucrose (Brandle *et al.* 1998; Seki *et al.* 2018; Singla *et al.* 2016).

However, the sweetness of most high-potency sweeteners as for steviol glycosides is generally accompanied by other negative taste attributes such as bitterness or an off-taste, which may constitute an impediment to their use for human consumption and application in food and pharmaceutical products. Moreover, individual consumers' differences in sweetness and bitterness sensitivity and acceptance have been reported along with evidence suggesting that most of the variation in sensitivity to chemical stimuli may have a genetic basis. Gustatory responses to sweet and bitter compounds are mediated by G protein-coupled receptors expressed by taste receptor cells (Mennella *et al.* 2005; Prakash *et al.* 2008). The specific sweet taste is commenced by the activation of the heterogenic receptor, made up of a combination of hTAS1R2 and hTAS1R3 protein. In contrast to the single receptor-based detection of sweet taste, bitter taste receptors belong to frizzled receptor family of G-protein coupled receptor that exhibits unique but partially overlapping molecular receptive ranges because the transduction of bitter taste in humans is mediated by 25 receptors of hTAS2R gene although the mechanism behind is still unknown (Simons *et al.* 2008; Singla *et al.* 2016; Risso *et al.* 2014). Nonetheless, Hellfritsch *et al.* (2012) identified hTAS2R4 and hTASR14 as the receptors that specifically mediate the bitter off-taste of most steviol glycosides (except for steviolbioside and rebaudioside D) *in vitro* and, more recently, Risso *et al.* (2014) found a genotype-phenotype association between stevioside and bitterness perception on TAS2R4, TAS2R12 and TAS2R38 (receptor commonly used to assess variations in bitter sensitivity) in a panel of candidate subjects. Much of the variation across individuals can be explained by those polymorphisms in bitter taste receptors (Allen *et al.* 2013; Oleson *et al.* 2017).

Moreover, the glycosyl residues at both C-19 (R_1) and C-13 (R_2) of the steviol core are essential to determine the taste profile (Ohta *et al.* 2010). Hence, the number of glucose units

at the C-13 tertiary hydroxy group (R_2) seems to influence the sweetness as well as the quality of taste, while the extension of the C-19-ester-linked glycosyl unit led to an increase of sweetness. The sweetness increases with the number of β-glycosyl residues, and the bitterness perception are also correlated with the total number of attached glucose units (Gerwig *et al.* 2016). Steviol glycosides bearing only a few β-glucosyl residues have higher bitter intensities than steviol glycosides with more β-glucosyl residues, and the substitution of one of the three glucosyl units with a rhamnosyl unit results in a reduction in sweetness (Gerwig *et al.* 2016; Pawar *et al.* 2013). Consequently, rebaudioside A, which present three glucose units at the C-13 position, is sweeter and less bitter compared to stevioside (Adari *et al.* 2016; Carakostas *et al.* 2012; Tanaka *et al.* 1982). In agreement with this observation, Singla *et al.* (2016) revealed through a molecular simulation study that the extra sugar residue at the C-13 position in rebaudioside A was incapable to enter into the bitter receptor, thereby suppressing the signalling cascade responsible for the bitter taste.

Physiological and nutritional properties

Nutritional benefits have been associated with *Stevia* leaves and roots to be considered as a source of inulin-type fructo-oligosaccharides, which are known to promote wellness and reduce the risk of certain diseases (De Oliveira *et al.* 2011; Lopes *et al.* 2017). Moreover, it is proven to be a rich source of essential fatty acids such as linolenic acid. Also, *Stevia* leaves contained almost all the indispensable amino acids and significantly high quantities of water-soluble vitamins and minerals. The energy value on a dry weight basis is 2.7 Kcal/g, which grants it the status of a low-calorie sweetener (Kroyer 2010; Lemus-Mondaca *et al.* 2012; Singh *et al.* 2019).

In addition to its sweetening and nutritional properties, numerous reports concerning the potential therapeutic and health-promoting benefits of *Stevia* extract and its pure compounds, have described biochemical and functional aspects after *in vitro* and *in vivo* models and human trials. The numerous reported beneficial health properties include antimicrobial, antioxidant, antitumoral, antihyperglycemic, antidiabetes, immunomodulatory, noncariogenic, cardioprotective, gastroprotective, and other correlated effects (Kurek *et al.*

2019; Soejarto 2002; Zhou *et al.* 2014). Some of the most relevant studies have been listed in **Table 2** with regard to the potential beneficial effects tested.

Table 2. Functional properties of *S. rebaudiana* and related steviol glycosides.

Reference	Tested substance	Health-promoting effect	Model	Findings
Casas-Grajales *et al.* 2019	Stevioside	Anti-inflammatory / immunomodulatory	*In vitro* *In vivo* – rats (liver sections) *In silico*	• Stevioside prevented the diminution of glycogen caused by thioacetamide, a strong hepatotoxin that induces fibrosis and cirrhosis on the liver. • Stevioside diminished the elevation of proinflammatory cytokines (IL-17a, IL-1β, TNF-α, IL-6 and IL-10). • The liver functionality was preserved.
Puri *et al.* 2011	Stevioside	Antimicrobial	*In vitro*	• Stevioside exhibited antimicrobial activity to *Serratia marcescens*, *Klebsiella pneumoniae*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Alcaligenes denitrificans* and *Salmonella typhimurium*.
Ghanta *et al.* 2007	*Stevia* leaf extract	Antioxidant	*In vitro*	• *Stevia* leaves exhibited preventive activity against DNA strand scission.
Ritu *et al.* 2016	*Stevia* leaf extract	Antidiabetic	Human trials	• After oral administration of *stevia* leaf powder, the fasting and postprandial blood glucose levels significantly decreased showing potential to regulate the biochemical parameters associated with type 2 diabetes mellitus. • *Stevia* leaf powder lowered the levels of VLDL cholesterol and triglycerides.
Hsieh *et al.* 2003	Stevioside	Cardiovascular properties	Human trials	• A treatment consisting of capsules of stevioside administered orally by subjects with hypertension significantly decreased both the systolic and diastolic blood pressure compared to placebo.

Metabolism, regulatory status, and future prospects

Despite the previously noted therapeutical benefits, the use of the different steviol glycosides has been initially questioned due to the deficiency of safety-related studies. Metabolism and analysis in humans and animals are important to support the interpretation of the safety of high purity steviol glycosides (Pawar *et al.* 2013; Perrier *et al.* 2018).

Steviol glycosides pass through the gastrointestinal tract completely or partially intact without virtual absorption. They are subsequently hydrolysed by the gut microbes into the common aglycone steviol by the colonic and/or cecal bacteria (Carakostas *et al.* 2012). *In vitro* and *in vivo* studies have shown that the digestive enzymes, such as α-amylase, pepsin, and pancreatin, and acids found in the mouth, stomach and small intestine are unable to hydrolyse the β-glycosidic bonds, whereas the glucose moieties resulting from the microbial metabolism that takes place in the large intestine are used as energy by colonic bacteria (mainly *Bacteroides* sp.) (Gardana *et al.* 2003; Purkayastha *et al.* 2016; Wingard *et al.* 1980). The conversion rate differs on the steviol glycosides, stevioside being more rapid and preferentially hydrolyzed over rebaudioside A, which is explained by the extra glucose unit present in the structure. However, no major evidence was reported regarding the difference in the intestinal microbiota metabolism of the *stevia* mixture and/or the *stevia*-related compounds between human and rats (Koyama *et al.* 2003a; Koyama *et al.* 2003b). Consequently, steviol is absorbed via the portal vein before undergoing the conjugated glucuronidation process in the liver to yield the final metabolite steviol glucuronide, to then being excreted by the faeces or urine (Ramos-Tovar *et al.* 2017; Roberts *et al.* 2008; Zou *et al.* 2020).

The oral lethal dose has been investigated through *in vitro* and *in vivo* studies to examine the acute toxicity of several steviol glycosides and the aglycone steviol (Carakostas *et al.* 2012). As a result, an extensive toxicological database on steviol glycosides has been evaluated by several government authorities finding no evidence of genotoxicity, carcinogenicity, or reproductive toxicity (Mullane *et al.* 2014; Zou *et al.* 2020). From 2008, steviol glycoside preparations with major individual steviol glycosides (stevioside and rebaudiosides A, C, D and M) were categorized as GRAS by the United States FDA (Perrier *et al.* 2018). Since achieving the GRAS status, steviol glycosides have entered the mainstream of

sweeteners for tabletop products, juices, dairy products and bakery items (Pawar *et al.* 2013). In 1998, 2004, 2007, 2008 and 2010, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) reviewed the safety of steviol glycosides and established specifications and an acceptable daily intake (ADI; 4 mg/kg body weight), currently including rebaudiosides A, B, C, D, F, stevioside, dulcoside A, rubusoside, steviolbioside and rebaudioside A and stevioside as the primary sweetener molecules (JECFA, 2010, Purkayastha *et al.* 2016). In 2011, the Codex Alimentarius Commission (CAC) adopted proposed draft maximum levels for steviol glycosides in foodstuffs for some Codex member countries and such as Australia, Malaysia, Paraguay, Costa Rica, United States, Colombia, Mexico and Japan, and other associations (FAO, 2010). The European Commission permitted the use of steviol glycosides as sweetening agent under Commission Regulation (EU) No 1131/2011 and established a steviol glycoside specification containing 95% or more of nine steviol glycosides (rebaudiosides A, B, C, D, E, F, stevioside, dulcoside A, rubusoside and steviolbioside), with a total rebaudioside A and/or stevioside content of not less than 75% and with a maximum level of 30-3300 mg/L or mg/kg as appropriate in the different food categories (European Commission, 2011). A recent 2020 report from The European Food Safety Authority (EFSA) highlighted that stevioside is neither carcinogenic nor genotoxic with no risks associated with reproductive or developmental toxicity (EFSA, 2020).

It has been over a century since the discovery of S. *rebaudiana*. China and the North American region have been the largest producers of *Stevia*; however, its cultivation has been introduced to some regions of Asia, Europe, and Canada. Worldwide, 80,000 acres of land is under *Stevia* cultivation, 75% of which lies in China alone. Global production of *Stevia* is estimated to be around 40,000 million tons (Yadav *et al.* 2012). Yet, researchers are still trying to study every chemical constituent isolated from the plant (Lemus-Mondaca *et al.* 2012; Zou *et al.* 2020). S. *rebaudiana* extracts and the purified steviol glycoside sweeteners have been approved as food additives in Argentina, Brazil, Japan, South Korea, China, Peru, and Russia for many years (Chaturvedula *et al.* 2011a; JECFA, 2005; Lindley 2012). Presently in Europe, the safety of steviol glycosides as a food additive (E960) has been approved, including a minimum of 95% of purity for the total content of steviol glycosides (EFSA, 2020).

In 2017 the number of new commercial products sweetened with steviol glycosides outpaced those using aspartame. In 2020, the global *Stevia* market size was valued at USD 476.46 million, and it is expected to reach USD 1.1 billion by 2026, rising at a market growth of 10.3% compound annual growth rate during the forecast period. However, with the sudden break of Covid-19 in the late months of the year 2019, the markets worldwide are experiencing a declining trend in market sizes and are expected to sustain and even amplify themselves in the following years (Ciriminna *et al.* 2019; Research And Markets, 2020).

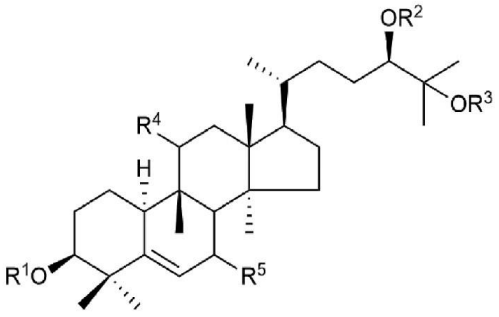
1.3.2.2.2 Siraitia grosvenorii

Natural source and background

The perennial vine *Siraitia grosvenorii* belongs to the Cucurbitaceae family that is indigenous to Guangxi, a province of China. For centuries, the *Siraitia* fruits have been cultivated in southern China and used as a traditional folk medicine for the treatment of pulmonary congestion, colds, and sore throats (Jin *et al.* 2012; Luo *et al.* 2016). In addition to medicinal uses, these species have been used as supplement sweeteners due to the sweet constituents of the plant known as triterpene glycosides (Lindley 2012). In 1941 Walter Tennyson Swingle published the original botanical description and the plant was officially named after him in 1979 as *Siraitia grosvenorii* (Swingle) after several renamings (Lindley 2012; Swingle 1941). In 1975, Lee *et al.* first reported chemical studies establishing the cucurbitane-type triterpene glycosides structures which were initially isolated and characterized by Takemoto *et al.* in 1983. In 1987, *Siraitia* fruit was listed as a medicinal and edible species by the China Ministry of Health (Li *et al.* 2014). Consequently, the sweet glycosides from this fruit have become the focus of increasing scientific and commercial attention for being currently proposed as a potential source of pharmaceutical and sweetener compounds for a wide range of food products (Kinghorn *et al.* 2010; Lu *et al.* 2012).

Structural characteristics

Common names for *S. grosvenorii* include: Luo Han Guo, Luo Han Kuo, Monk Fruit, among others (Soejarto *et al.* 2019). The triterpenoid glycosides are known as mogrosides and their chemical structures consist of mogrol (10α-cucurbit-5-ene-3β,11α,24(R),25-tetraol), the common aglycone, with glycosylated sugar moieties linked to C-3 and C-24 by β-linkage (Wang *et al.* 2018). The extract of the Luo Han Guo (LHG) fruit contains several sweet triterpene glycosides as shown in **Figure 3**: mogroside V, which has a disaccharide composed of glucose-glucose linked β-1,6 and three additional glucose units β-1,2 and β-1,6 attached to the mogrol (aglycone); and mogroside IV, composed by a glucose less in the trisaccharide position, both being the main sweet compounds of the dried fruits (Lindley 2012; Yoshikawa *et al.* 2005). Several investigators studied the specific mogrosides in the LHG extract revealing their different content regarding the cultivation area, the extractions procedure and the ripening process of the extract (Chun *et al.* 2014; Jin *et al.* 2012; Wang *et al.* 2018).



Triterpenoid compound	R_1	R_2	R_3	R_4	R_5
Mogrol	H-	H-	H-	-OH	H_2-
Mogroside IA	H-	Glc(1-	H-	H_2-	H_2-
Mogroside IE1	Glc(1-	H-	H-	-OH	H_2-
Mogroside IIA1	H-	Glc(1-6)Glc(1-	H-	-OH	H_2-
Mogroside IIA2	Glc(1-6)Glc(1-	H-	H-	-OH	H_2-
Mogroside IIE	Glc(1-	Glc(1-	H-	-OH	H_2-
Mogroside IIIA1	H-	Glc(1-6)[Glc(1-2)]Glc(1-	H-	-OH	H_2-
Mogroside IIIA2	Glc(1-6)Glc(1-	Glc(1-	H-	-OH	H_2-
Mogroside IIIE	Glc(1-	Glc(1-2)Glc(1-	H-	-OH	H_2-
Mogroside IVA	Glc(1-6)Glc(1-	Glc(1-6)Glc(1-	H-	-OH	H_2-
Mogroside IVE	Glc(1-6)Glc(1-	Glc(1-2)Glc(1-	H-	-OH	H_2-
Mogroside V	Glc(1-6)Glc(1-	Glc(1-6)[Glc(1-2)]Glc(1-	H-	-OH	H_2-
Mogroside VI	Glc(1-6)[Glc(1-2)]Glc(1-	Glc(1-6)[Glc(1-2)]Glc(1-	H-	-OH	H_2-
Siamenoside I	Glc(1-	Glc(1-6)[Glc(1-2)]Glc(1-	H-	-OH	H_2-

Figure 3. Chemical structures of *Siraitia grosvenorii* compounds (Gong *et al.* 2019; Soejarto *et al.* 2019).

Sensory properties

Although the content of mogrosides fluctuates during the ripening process, a dried extract of LHG fruits contains approximately 0.5-1% of mogrosides, mainly formed by mogroside V, which is confirmed to be rated as 250-425 times sweeter than sucrose respectively, depending on the concentration found (Wang *et al.* 2018; Zhang *et al.* 2012). Reported studies showed a relationship between the structure and the taste of the triterpene glycosides derivatives. It was suggested that the oxygen function at the 11-position of the aglycone is responsible for the occurrence of taste. Likewise, the number of glucose units included in the aglycone moiety influences the taste perception; the presence of at least three glucose units or greater is essential for the occurrence of sweetness while less than three glucose units result in being tasteless or having a bitter taste. Furthermore, the allocation of the glycosyl units is similarly noteworthy on the sweetness. The hydroxyl group with alpha configuration also contributes to the sweet taste, while the beta configuration turns into a bitter taste. However, the interglycosidic linkage of the glucose units was found to only have a minor effect on the taste (Kasai *et al.* 1989; Matsumoto 1990; Wang *et al.* 2014; Xia *et al.* 2008).

Physiological and nutritional properties

In addition to the well-known traditional pharmacopoeia, in terms of nutrients, *S. grosvenorii* is a zero-calorie sweetener that contains large amounts of cucurbitane-type triterpenoid glycosides. Other several classes of minor compounds such as carbohydrates; polysaccharides composed of rhamnose, arabinose, xylose among others, phenolic compounds possessing the aglycone of kaempferol or quercetin, proteins, amino acids and vitamins (Jin *et al.* 2012; Li *et al.* 2014; Qing *et al.* 2017). These components represent the biochemical basis of various medicinal properties of mogrosides. Indeed, to date, pharmacological and clinical investigations encompassing *in vitro* and *in vivo* studies have shown specific biological effects of both extracts and individual compounds of mogrosides. These potential biological activities include hypoglycemic, blood lipid-lowering, antioxidant, anti-inflammatory, anticarcinogenic and antitumor targeting effects, whose examined

activities are gathered in **Table 3**; and others physiological effects such as anti-tussive, immunostimulatory, antiallergic, hepatoprotective, etc. (Gong *et al.* 2019; Jin *et al.* 2012; Li *et al.* 2014; Liu *et al.* 2018).

Table 3. Functional properties of *S. grosvenorii* and related mogrosides.

Reference	Tested substance	Health-promoting effect	Model	Findings
Qi *et al.* 2008	Mogroside extract Mogroside V Mogroside IV Siamenoside I 11-oxo-mogroside V	Antidiabetic effect	*In vitro* *In vivo* – alloxan-induced diabetic mice	• *S. grosvenorii* mogrosides lowered serum total cholesterol and triglyceride content and improved high-density lipoprotein cholesterol content, thereby normalizing the blood lipid levels. • Mogrosides regulate lipid metabolism being helpful in the prevention of diabetic complications associated with oxidative stress and hyperlipidemia.
Takasaki *et al.* 2003	Mogroside V 11-oxo mogroside V	Anticarcinogenic activity	*In vivo* - mice	• A strong inhibitory effect was found on the primary screening test indicated by the induction of Epstein-Barr virus early antigen (EBV-EA) by tumour promoter 12-O-tetradecanoyl-phorbol-13-acetate (TPA). • The tested mogrosides exhibited significant inhibitory effects in the two-stage carcinogenesis test of mouse skin tumours induced by peroxynitrite as an initiator and TPA as a promoter.
Qi *et al.* 2008	*S. grosvenorii* extract (> 98% total mogroside) Mogroside V	Antioxidative effects	*In vitro* *In vivo* – rats	• The components displayed significant inhibitory effects on the oxygen-free radical, hemolysis of red blood cells, and lipid peroxidation induced by Fe_2^+ or H_2O_2 *in vitro* indicating antioxidant effects.
Di *et al.* 2011	*S. grosvenorii* extract (> 98% total mogroside)	Anti-inflammatory activity/effect	*In vitro* *In vivo* – Ear oedema mice model	• Lipopolysaccharide-induced macrophages (RAW264.7) to induce inflammation was incubated with mogrosides at a different concentration resulting in an inhibition of the release of cyclooxygenase-2 and mRNA expression of inducible nitric oxide synthase and IL-6 in murine macrophages in a dose-dependent manner and indicating an anti-inflammatory activity by inhibiting the release of inflammatory mediators, such as prostaglandins, IL-6 and NO, by macrophages. • Mogrosides inhibited the phorbol ester-induced inflammation. Following administration, there was a reduction in the expression of cyclooxygenase-2 and IL-6 in the murine inflammation model, indicating that mogrosides exerted an anti-inflammatory effect by inhibiting the release of inflammatory factors and mediators of inflammation.
Chun *et al.* 2014	*S. grosvenorii* extract (> 98% total mogroside)	Immunomodulatory	*In vivo* - rats	• There was a significant increase in the percentage of acid α-naphthyl acetate acid enzyme-positive lymphocytes in the peripheral blood, as well as in the ratio of rosette-forming cells. • LHG could significantly improve both cellular and humoral immunity processes, with no effect on the non-specific immunity of the rats.

Metabolism, regulatory status, and future prospects

Recent literature has addressed the study of the biotransformation and the metabolic fate of mogrosides during digestion *in vitro* and *in vivo*. An extract of *S. grosvenorii*, mainly formed by mogroside V (> 72%) and orally administrated to rats is degraded by the digestive enzymes and the intestinal microbiota into its aglycone mogrol and its mono- and diglucosides (Murata *et al.* 2010). From the original mogrosides, different metabolic pathways were identified, which also varied depending on whether they were carried out in animal or human models. Metabolic reactions such as deglycosylation, hydroxylation, dehydrogenation, isomerization, glucosylation and methylation gave rise to the formation of different mogrosides. While studies found that mogroside V is partially converted during digestion, detecting mostly unchanged mogroside V in faeces after excretion without being absorbed (Murata *et al.* 2010), other study results indicated the absorption of mogroside V and systemic bioavailability along with its extensive conversion by digestive enzymes and the intestinal microbiota (Zhou *et al.* 2018). Mogroside V and several of its metabolites were found to be distributed unevenly in urine and various rat organs such as in liver, heart, spleen, intestine, kidney, and lung, suggesting that they may contribute to the organ-specific bioactivities (Xu *et al.* 2015). A recent *in vitro* study using human faecal homogenate assay indicated that mogrosides share a common metabolic pathway to mogrol, the aglycone of mogrosides (Bhusari *et al.* 2021). However, very little is currently known about the impact of mogrosides on the human gut microbiota composition and activity.

Some safety studies on extracts of *S. grosvenorii* and the mogrosides have been conducted in animal models since 1999. Toxicity studies have shown that mogrosides are non-toxic and do not have mutagenicity, genotoxic nor other adverse effects (Qin *et al.* 2006; Marone *et al.* 2008). Commercially, despite its traditional use, it was in the 1990s when the China Food and Drug Administration (CFDA) approved the use of *S. grosvenorii* as a sweetener in foods, and given the clearing toxicology and food safety tests, mogrosides were granted with GRAS status to be used as food additives by the FDA, for what since early 2010 in United States products containing mogrosides have been available commercially (FDA, 2017). A current update of the scientific opinion on the safety of Monk fruit extract of the FAF EFSA

Panel concluded that the toxicity database on Monkfruit extract is insufficient to conclude on the safety of the use of monk fruit extract as a food additive (EFSA, 2019).

Hence, despite its safety and being recognized to have numerous molecular targets, further studies are needed to be explored to understand the complex pharmacological actions of mogrosides and the potential clinical applications. Nevertheless, LHG extract and mogrosides are both available on the market in many countries and it is still expected to expand the market for sweeteners. Several *S. grosvenorii* products based in mogrosides are patented such as Monk Fruit in the Raw®, PureLo®, SweetMonk™, Go-Luo™, among others. In addition, there is now an increasing number of conventional foods on the market using monk fruit extract, including soft drinks, juice concentrate and infant foods that are notified as GRAS (Bechtel *et al.* 2011; Bhusari *et al.* 2021; Cho *et al.* 2015; Heimbach *et al.* 2016; Kinghorn *et al.* 2002).

1.3.2.2.3 Biotransformation methods for natural, non-sugar, high-intensity sweeteners compounds from *Siraitia grosvenorii* and *Stevia rebaudiana*

Only a few sweet-tasting plant-derived natural products, such as steviol glycosides and mogrosides, have been launched commercially as sucrose substitutes to date. These HIS have served as lead compounds for extensive structural modifications in attempts to produce analogues that either possess better edulcorating qualities or present specific functions to health. The complexities of each structure hinder the purification or synthesis, resulting in difficulties for further production of specific compounds. Available methods, especially for food applications, based on the biotransformation to convert starting compounds into the target ones are a key issue. The conversion of mogrosides or steviol glycosides can be achieved by both chemical and enzymatic modifications.

Chemical modifications

Chemical conversions using organic synthesis are very common and have been carried out over the years (Gerwig *et al.* 2016; Wang *et al.* 2018). Fletcher was the first in studying the behaviour of stevioside with an alkali (NaOH), which resulted in producing another compound, steviolbioside (Fletcher 1955). More recently, Chaturvedula *et al.* (2011b) carried out both acid and alkaline hydrolysis experiments of two major steviol glycosides: stevioside and rebaudioside A. Under alkaline conditions (NaOH) two compounds, steviolbioside and rebaudioside B were respectively produced by the cleavage of the β-D-glucopyranosyl unit present at their C-19 positions; whereas acid hydrolysis (H_2SO_4) produced furnished D-glucose for both compounds. Subsequently, the following studies developed the total synthesis of certain analogues of stevioside not present in nature. With the purpose of modifying the sweetness, some studies applied a synthetic stereoselective glycosylation method using different monosaccharides such as xylose, arabinose, mannose, glucose resulting in new components that did not present bitterness (Esaki *et al.* 1984), and disaccharides fragments, *i.e.* 2-*O*-α-L-rhamnopyranosyl-β-D-glucopyranosyl, 2-*O*-α-L-rhamnopyranosyl-β-D-galactopyranosyl, 2-*O*-α-quinovopyranosyl-β-D-glucopyranosyl, respectively, which also did not have a bitter taste but additionally maintained the sweetness (Kamiya *et al.* 1979; Ogawa

et al. 1980). Similarly, Zhou *et al.* (2014) carried out a glycosylation of *ent*-kaurene derivatives in order to produce compounds considered as potential anticancer candidates. Likewise, Prakash *et al.* (2012) carried out a catalytic hydrogenation of stevioside, rebaudiosides A, B, C and D, and rubusoside using $Pd(OH)_2$, and the corresponding dihydro derivatives reduced or lost the sweetness with respect to original compounds. Dubois *et al.* (1984) reported that bitterness is correlated with molecular hydrophilicity, thus they carried out an alkaline saponification of stevioside and rebaudioside A to modulate hydrophobicity and to produce a compound with no bitter-taste character.

Typically, mogrosides hydrolysis is carried out with various acids which have specific hydrolysing properties. In the reactions, different parameters are carefully considered such as acid concentration, acid-to-substrate ratio, reaction temperature and time (Wang *et al.* 2018). Several attempts have been made to perform mogroside transformation. Hydrolysis with sulphuric acid (H_2SO_4) afforded glucose units which support the understanding of the diversity of the triterpenoid glycosides isolated from *S. grosvenorii* (Prakash *et al.* 2014b; Chaturvedula 2015). Also, hydrochloric acid ($HCl_{(aq)}$) is employed in order to isolate different cucurbitane triterpenoids (Chaturvedula *et al.* 2011). Chen *et al.* (2011) employed the same acidic hydrolysis to hydrolyse crude LHG extract, forming various types of mogrosides, including mogrosides IV, III, II and I, to identify the anti-diabetic principles of this medicinal herb. Phosphoric acid (H_2PO_4) and trifluoroacetic acid (TFA) are other acids employed for the modification of mogrosides (Wang *et al.* 2018).

Although a substantial amount of work has gone into the chemistry of this class of sweeteners, it would be too expensive to produce through chemical synthesis for commercial profit (Soejarto 2002). Furthermore, it is important to take into account that these organic conversions use toxic chemical reagents that not only harm the environment but also raise concerns in accepting these derivatives in the food industry due to the uncertain safety. These chemical treatments also include other general limitations such as low extraction efficiencies and variations in the product quality because of a bitter aftertaste caused by remnants of solvents (Puri *et al.* 2012).

Enzymatic modifications

Biocatalyst alternatives may be preferred to be more in line with the objectives of greener chemistry assisting in the extraction, modification, or synthesis of complex biofunctional substances of natural origin. For a long time, enzymatic conversion using several types of carbohydrate-active enzymes has been used to produce various steviol glycosides and mogrosides, including some compounds that are not naturally occurring on the extracts or only present in low concentrations. The first studies consisted of enzymatic modifications using α-glucosidases to convert mogroside V further into mogrosides IV E, III E, II E, I E1 and 11-oxo-mogroside I E1 (Takemoto *et al.* 1983), and to convert stevioside, using a β-amylase, into different deglucosylated products such as steviolbioside, and others, with enhanced sensory profiles (Lobov *et al.* 1991). Other modifications of stevioside and mogroside have been reported by using different donor substrates and other commercial enzymes with glucosidase (*i.e.*, α-glucosidase and β-glucosidase) functions (**Figures 4** and **5**).

The use of enzymes may provide the biotransformation of HIS with a high yield of efficiency and production. However, the different enzymes are distributed in living organisms including microbes and plants, and therefore there are different degrees of specificity towards the substrates, in addition to the cost of the commercial enzymes, as to process large quantities. Advanced development of enzyme immobilization techniques is offering an alternative solution to lower the cost of enzymes. For the first time, Wang *et al.* (2018) applied an immobilized enzymes system using β-glucosidase to LHG in order to produce specific types of mogrosides namely siamenoside I, mogrosides IV, IIIE, and IIA, respectively, with different functions such as the suppression of the rise in blood glucose, reducing pulmonary fibrosis, etc. In the same line, Chiu *et al.* (2020) converted mogroside V into mogroside IV, siamenoside I, mogrosides IIIE and IIA by using a group of fungi (*Ganoderma lucidum*) with higher β-glucosidase activity among others). Other biotransformations of mogroside V with β-glucosidases from 16 mutant yeasts were screened (Chiu *et al.* 2013). Likewise, the use of β-glucosidases leads to the hydrolyzation of glucose moieties of the sophoroside moiety at C-13 in stevioside, yielding to the production of steviolbioside, steviol mono-glucoside, steviol mono-glucosyl ester, rubusoside and even the aglycone steviol (Ko *et al.* 2013; Nguyen *et al.*

2019; Wan *et al.* 2012; Wang *et al.* 2015). However, based on several reports, it was described that α-glucosidase and β-glucosidase enzymes are also implied in the synthesis of oligomers by transglycosylation (Kusama *et al.* 1986; Lobov *et al.* 1991; Saibi *et al.* 2007).

Other approaches include secondary enzymatic activities as transglycosylation by other commercially available glycoside hydrolases such as α-amylase (Tanaka *et al.* 1977; Ye *et al.* 2013), pullulanase (Lobov *et al.* 1991), β-amylase, alternansucrase (Musa *et al.* 2014), β-fructofuranosidase (Kitahata *et al.* 1989), and cyclodextrin glucosyltransferases (CGTases) (Yoshikawa *et al.* 2005) using oligosaccharides or polysaccharides as donors and with the purpose of fully or partially removing the bitter taste and aftertaste of the respective acceptors, either steviol glycosides or mogrosides (De Oliveira *et al.* 2007; Gerwig *et al.* 2016). In a recent work, mogroside IIE was converted into a sweet triterpenoid saponin mixture using an enzymatic glycosyl transfer method based in CGTases, with starch as the glucose donor substrate. The new saponins with the α-glucose group exhibited a sweet taste whilst bitter taste had disappeared, which is in agreement with the exposed sensory aspects in the *Sensory properties* section. Also, the newly synthesized mogrosides kept the same favourable physiological and safety characteristics consisting of the same antioxidant properties as the natural mogrosides (Wang *et al.* 2014). Transglycosylation of stevioside by CGTases of various groups of microorganisms as biocatalysts were carried out under controlled parameters in order to improve the edulcorant quality (Abelyan *et al.* 2004; Fukunaga *et al.* 1989). Likewise, a mutant glucansucrase enzyme elongated the stevioside at the Glc-(β1→C-19) site of the steviol core with Glc-(α1→4)-Glc-(α1→6) element, forming novel α-glucosylated stevioside products and gluco-oligo/polysaccharides synthesis, while significantly improving the edulcorant and organoleptic properties (Devlamynck *et al.* 2019). Only in a patent application (Zhou *et al.* 2014), a β-galactoside from *Aspergillus oryzae*, among other studied enzymes, was used to apply enzymatic digestion of mogrosides, resulting in a redistribution of different mogrol glycoside contents under carefully screened conditions (Wang *et al.* 2018).

In *S. rebaudiana*, β-galactosidase from *Kluyveromyces lactis* was found to specifically catalyse the hydrolysis of the glycosyl ester linkage of stevioside, to yield steviolbioside, which presented a promising anticancer activity due to the inhibition rate obtained for human breast

cancer cells (Chen *et al.* 2016). Similarly, another β-galactosidase from *Aspergillus oryzae* exerted as deglycosylation system by forming rubusoside from stevioside, nevertheless, it presented a weak transglycosylation rate and no transglycosylated products were characterized (Wan *et al.* 2012). Although β-galactosidases are widely used in the industry to improve sweetness (Husain 2010), no related taste studies of steviol glycosides or mogrosides were found with this group of enzymes. In the same way, a few reports studied the modification of steviol glycosides and mogrosides compounds involving the use of cytochrome P450 enzymes using uridine-5'-diphospho (UDP) dependent glucosyltransferases (UGTs) (Liu *et al.* 2018; Prakash *et al.* 2014a).

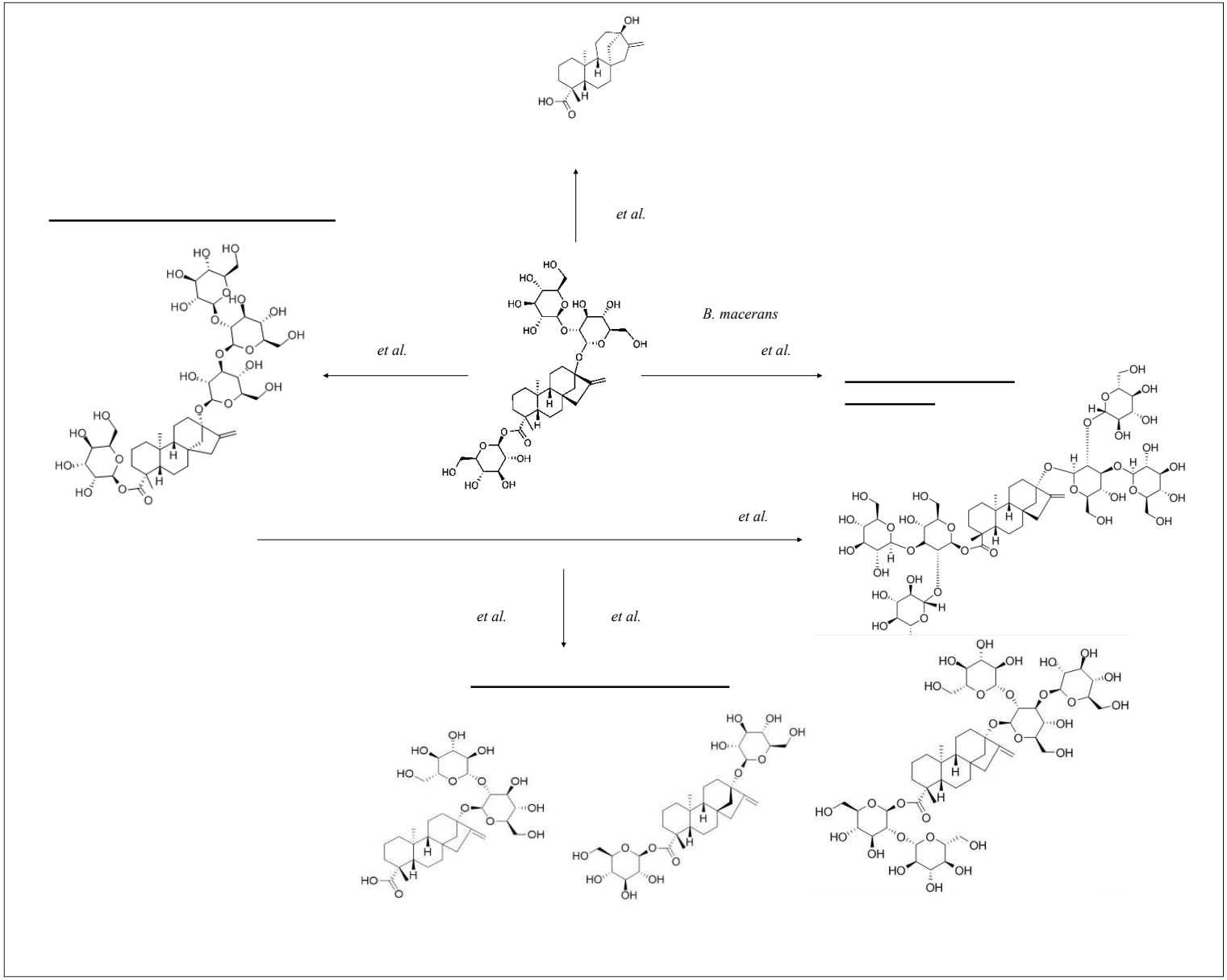


Figure 4. Bioconversion pathways of major compound stevioside into different steviol glycosides through enzymatic modifications.

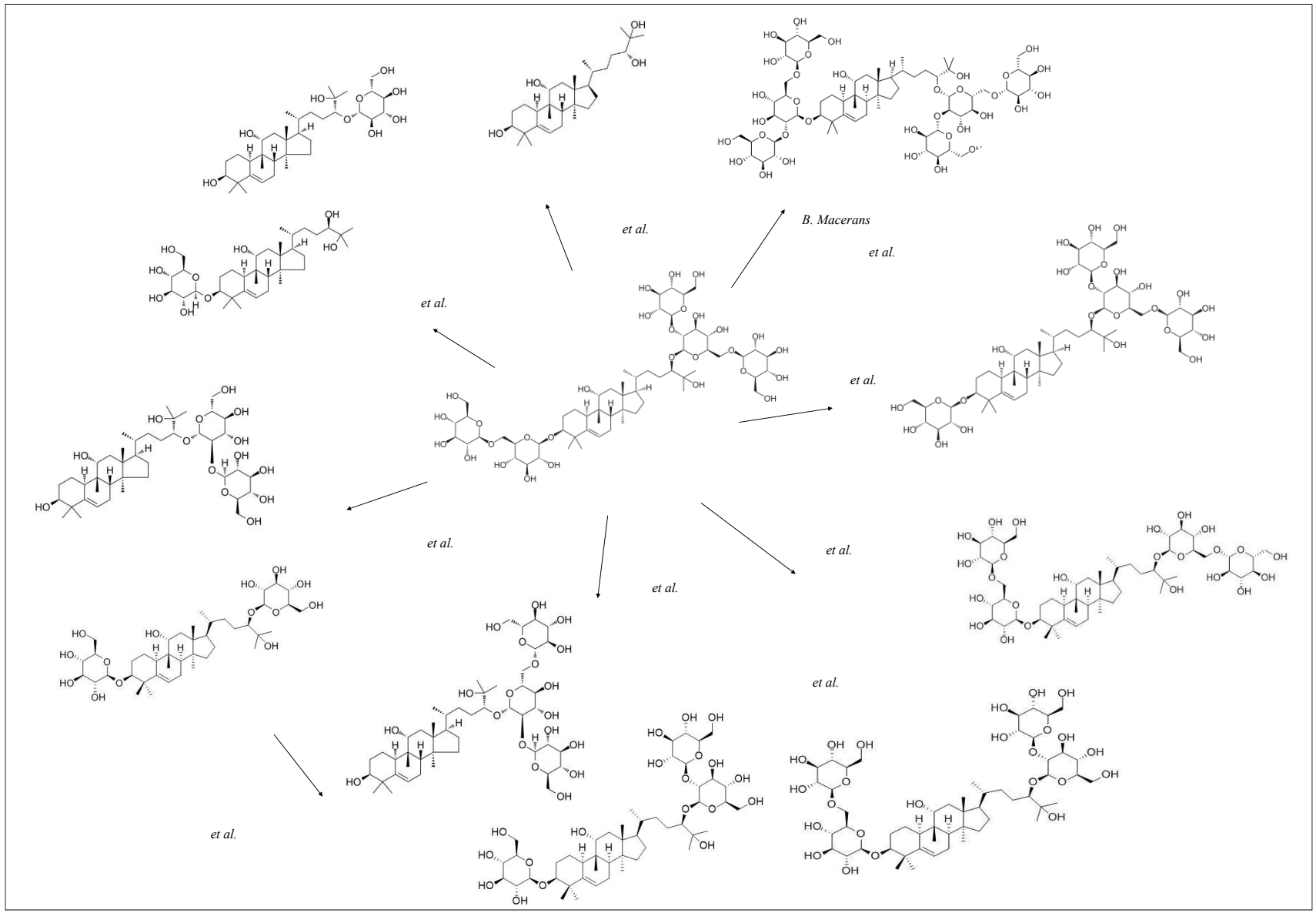


Figure 5. Bioconversion pathways of major compound mogroside V into different mogrosides through enzymatic modifications.

1.3.3 Non-digestible oligosaccharides and properties

Oligosaccharides are low molecular weight carbohydrates with a low DP containing 3 to 10 monomeric units linked together, as previously seen in **Table 1**, although some have a greater chain length and could go up to 70 units. Within this group, on the basis of their physicochemical and physiological properties, they can be classified as digestible or non-digestible. NDOs are characterized as resistant to salivary and gastric acidity and digestion by mammalian enzymes in the small intestine, and they are potential substrates for the bacteria that colonize the large intestine, belonging to the category of dietary fibre, with the nutritional and physiological importance that it provides such as the increment of the bulking effect. The indigestibility of NDOs results either from the configuration of their glycosidic bond between monomeric sugar units or from the substrate selectivity of gastrointestinal digestive enzymes. Most NDOs have a β-configuration and cannot be degraded by human salivary and gastrointestinal digestive enzymes, which are mainly specific for α-glycosidic bonds substrates (Delzenne *et al.* 1994; Van Loo *et al.* 1999; Roberfroid *et al.* 2000a).

It is in this context that the prebiotic concept is found and defined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit", in which are found NDOs, among the carbohydrate-based compounds, together with other substances such as polyphenols and polyunsaturated fatty acids. Such classification requires to meet three criteria: (i) the ability to resist host digestion (gastric acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption); (ii) that they are fermented by intestinal microbiota; and (iii) that they selectively stimulate the growth and/or activity of intestinal bacteria associated with health and wellbeing (Gibson *et al.* 2017b).

Most NDOs are hydrolysed in the caecum-colon into small oligomers and monomers, which are further metabolized by either one, a few, or most of the anaerobic bacteria providing energy for proliferation, short-chain fatty acids (SCFA), mainly acetate, propionate, and butyrate of which acids themselves elicit metabolic benefits, gases (H_2, CO_2, CH_4); and indirectly serving as energy substrates and metabolic regulators. The prebiotic effect that NDOs exert by the stimulation of the growth of bifidobacteria and lactobacilli in complex microbial communities that exist in the colon was demonstrated *in vitro*, and most importantly in human

subjects *in vivo*. This particular effect is regarded as potentially beneficial for health because it leads to an increase in epithelial barrier function and a reduction in the risk of intestinal infections. Moreover, a consequence of the NDOs colonic fermentation is a decrease in pH, a phenomenon partly responsible for hindering of development of certain pathogenic bacterial strains like Clostridia class and *Escherichia coli* (Cummings *et al.* 2002; Gibson *et al.* 1995a; Roberfroid *et al.* 2010; Topping *et al.* 2001).

In addition to their prebiotic effect, NDOs also have interesting systemic effects such as acting as modulators of the metabolism of lipids and carbohydrates, leading to a decrease in cholesterol and triglycerides and/or endocrine secretions, which present a positive relationship with the carcinogenesis process, immune system response, obesity and other associated diseases such as gastrointestinal disorders, diabetes, atherosclerosis, etc. (**Figure 6**) (Gibson *et al.* 1995a; Loo *et al.* 1999; Nie *et al.* 2020).

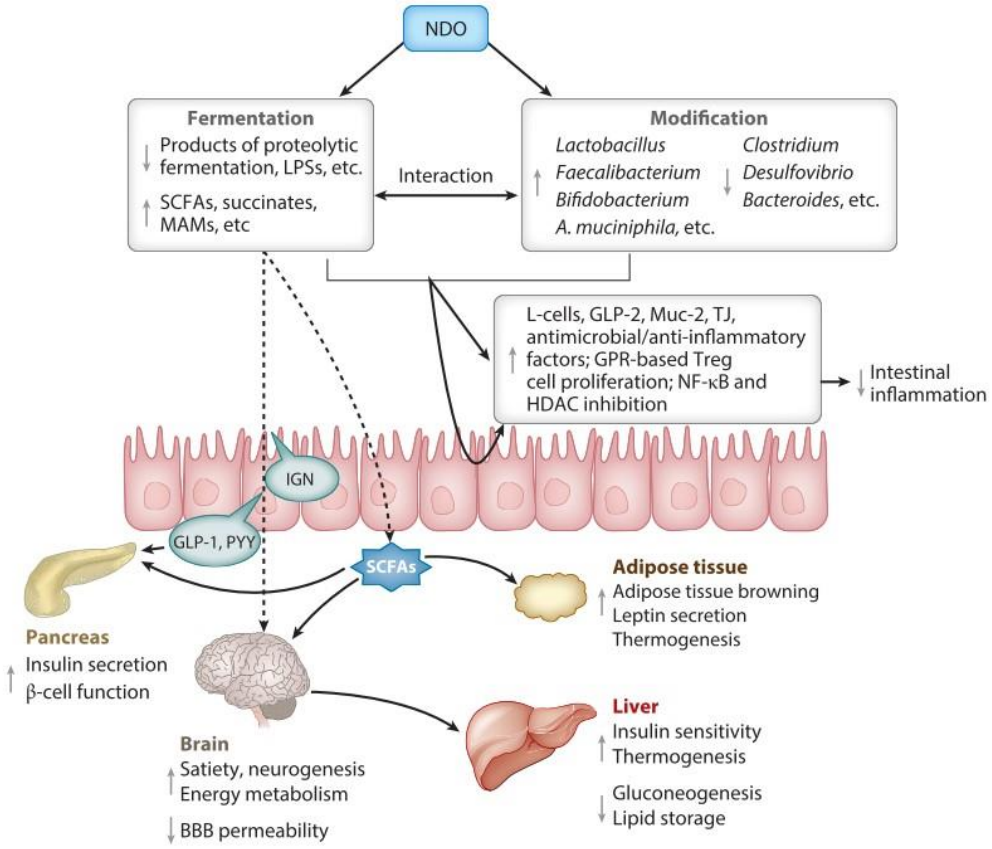


Figure 6. Potential mechanism of non-digestible oligosaccharides (NDOs) on obesity and other related diseases (Nie *et al.* 2020).

Oligosaccharides occur naturally in several foods from which NDOs preparations can be extracted, but can also be commercially produced through partial enzymatic hydrolysis of polysaccharides (*e.g.* dietary fibres, starch…) or catabolic enzymatic reactions from lower molecular weight sugars (Grizard *et al.* 1999; Scott *et al.* 2020). The main categories of NDOs presently available or in development as food ingredients include carbohydrates in which the monosaccharides unit can be fructose, galactose, glucose and/or xylose (**Table 4**) (Crittenden *et al.* 1996; Mussatto *et al.* 2007; Montilla *et al.* 2014).

Table 4. Non-digestible oligosaccharides (NDOs) with bifidogenic functions commercially available. Table adapted from Mussatto *et al.* (2007).

Compound	Molecular structure[a]	Main linkages
Lactulose	Gal-β(1,4)-Fru	
Lactosucrose	Gal-β(1,4)-Glc-α(1,2)β-Fru	
Isomaltulose (or palatinose)	Glc-α(1,6)-Fru	
Glucosylsucrose (or erlose)	Glc-α(1,4)-Glc-α(1,2)β-Fru	
Soybean oligosaccharides	$(Gal\text{-}\alpha/1,6))_n$-Glc-α(1,2)β-Fru	
2'-Fucosyllactose (2'-FL)	Fuc-α(1,2)-Gal-β(1,4)-Glc	
Lacto-*N*-neotetraose (LNnT)	Gal-β(1,4)-GlcNAc-β(1,3)-Gal-β(1,4)-Glc	
Raffinose	Gal-α(1,6)-Glc-α(1,2)β-Fru	
Cyclodextrins	$(Glc)_n$	α(1,4)
Fructo-oligosaccharides	$(Fru)_n$-Glc	β(2,1)
Galacto-oligosaccharides	$(Gal)_n$-Glc	β(1,4); β(1,6); β(1,3); β(1,2); β(1,1)
Gentio-oligosaccharides	$(Glc)_n$	β(1,6)
Isomalto-oligosaccharides	$(Glc)_n$	α(1,6)
Malto-oligosaccharides	$(Glc)_n$	α(1,4)
Xylo-oligosaccharides	$(Xyl)_n$	β(1,4)

[a]Gal, galactose; Glc, glucose; GlcNAc, *N*-acetylglucosamine; Fru, fructose; Fuc, fucose; Xyl, xylose.

NDOs that are confirmed prebiotics, meeting the above criteria, and which have consistent evidence with beneficial proven effects in human studies are fructans and galactans (Cummings *et al.* 2002; Scott *et al.* 2020). Additionally, human milk oligosaccharides (HMOs) denote a structurally diverse group of well-studied galactose-based oligosaccharides structures — as well as in GOS — and are also considered to exert prebiotic effects especially influencing infants health (**Table 4**) (Zeuner *et al.* 2019; Ayechu-Muruzabal *et al.* 2018; Akkerman *et al.* 2019). However, the most abundantly supplied and utilized group of NDOs as food ingredients are FOS and β-GOS, whose specific yields are optimized taking into account the bacteriological source of the enzymes, the respective donors and acceptor substrates, and synthesis conditions such as pH, temperature and time (Sangeetha *et al.* 2005b; Zeuner *et al.* 2014).

1.3.3.1 Fructo-oligosaccharides

Fructo-oligosaccharides (FOS) are linear fructose oligomers linked by β-2,1-glycosidic blonds either with a terminal glucose residue linked by α-2-1 (GFn-type) or without it (FFn-type). Based on the DP they are usually divided into subcategories with a relatively short (3 to 5 units), medium (6 to 10 units) and long-chain length (11 to 60 units), which mainly depends on the plant source and harvest conditions. In most cases, FOS are mixtures of short-chain oligosaccharides namely 1-kestose (DP3), nystose (DP4) and 1F-fructofuranosylnystose (DP5) (Romano *et al.* 2016).

FOS can be extracted from plants produced from partial hydrolysis of inulin or synthesized enzymatically from sucrose (**Figure 7**) (Liu *et al.* 2020; Man *et al.* 2021). Currently, commercial FOS are generally produced either from inulin hydrolysis using β-fructosidases (EC 3.2.1.7) or by synthesis from sucrose using β-fructofuranosidases (EC 3.2.1.26) and fructosyltransferases (EC 2.4.1.9) (Lorenzoni *et al.* 2014; Mao *et al.* 2019; Roberfroid 2007). Production of FOS from sucrose by enzymes derived from microorganisms like *Aspergillus phoenicis, A. japonicus, A. niger, Fusarium oxysporum, Scopulariopsis brevicaulis, Penicillium frequentens, P. rugulosum, Aureobasidium pullulans* and *Arthrobacter* sp. has also been reported (Sangeetha *et al.* 2005a).

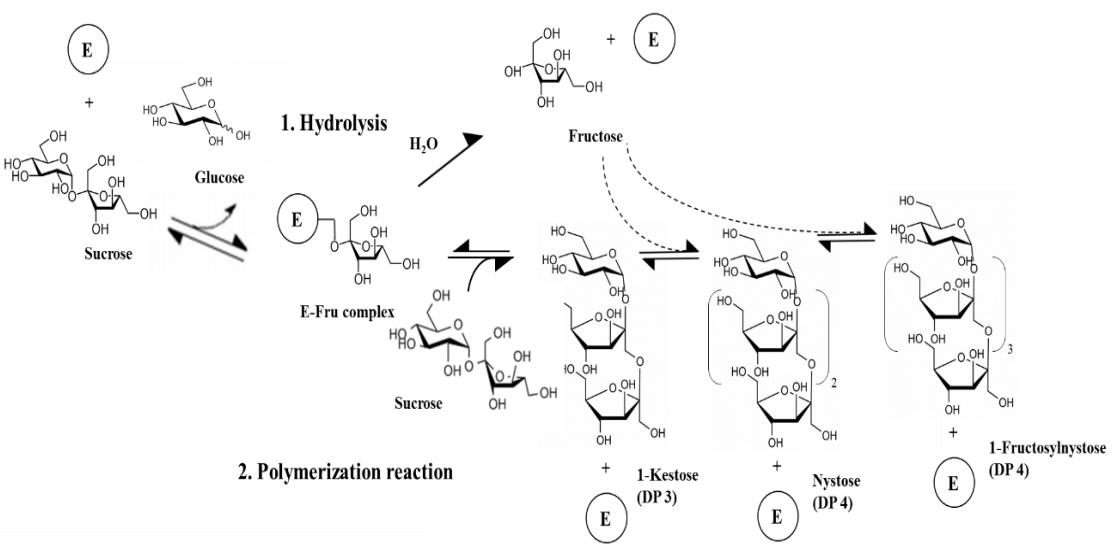


Figure 7. Schematic representation of the mechanism of FOS synthesis through transfructosylation reactions. Figure adapted from Martins *et al.* (2019).

1.3.3.2 Galacto-oligosaccharides

Another type of NDOs with prebiotic activities are galacto-oligosaccharides (GOS) composed of different galactosyl residues (2 to 9 units) and a terminal glucose or fructose linked by β- or α-glycosidic bonds, respectively. Therefore, they can be divided into two subcategories: α-GOS, isolated from natural sources such as from soybeans and other kind of pulses, by transgalactosylation reactions of α-galactosidase, or by conversion of raffinose family oligosaccharides by levansucrase, however either the transgalactosylation reactions of α-galactosidase or the human studies for the prebiotic effects are scarce (Meyer *et al.* 2015; Martins *et al.* 2019; Mitmesser *et al.* 2017); and β-GOS, derived by enzymatic synthesis from lactose using galactosyltransferases as well as β-galactosidases (Julio-González *et al.* 2019; Rastall *et al.* 2010; Sako *et al.* 1999; Gosling *et al.* 2010; Wang *et al.* 2014). Both the structure, regiochemistry and the DP will interfere differently on the biological properties and food applications.

β-GOS synthesis requires a kinetically controlled reaction based on a 2-step mechanism: (i) the formation of an enzyme-galactosyl complex, with simultaneous liberation of glucose, and (ii) the transfer of the enzyme-galactosyl complex to a nucleophilic acceptor containing a hydroxyl group (**Figure 8**). Specifically, during this conversion, the thermodynamically favoured hydrolysis of lactose, which generates D-galactose and D-glucose, competes with the transferase activity that generates a complex mixture of various galactose-based di- and oligosaccharides of different structures (Contesini *et al.* 2018; Villamiel *et al.* 2014). Commercial β-GOS have been typically synthesized by the action of microbial β-galactosidases (EC 3.2.1.23) produced by *Kluyveromyces lactis, Bacillus circulans, Bifidobacterium bifidum, Aspergillus oryzae, A. aculeatus, A. niger* or *Streptococcus thermophiles* (Martins *et al.* 2019; Torre *et al.* 2010).

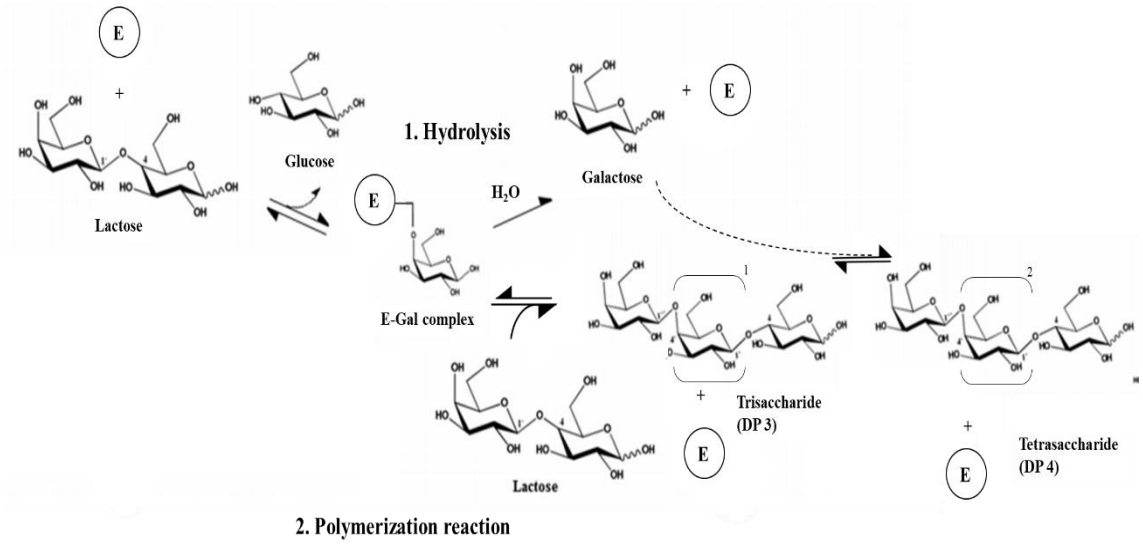


Figure 8. Schematic representation of the mechanism of β-GOS synthesis by transgalactosylation reactions. Figure adapted from Vera *et al.* (2016).

The selectivity of the promotion of microbial growth and fermentation activity by prebiotic NDOs is complex due to temporal variations in the intestinal microbiota and the different segments and conditions of the gastrointestinal tract. Proposed effective daily doses of NDOs in pure form are 4-10 g of FOS and 3.6-10 g of β-GOS per day. Different *in vitro* methodologies based on both static and dynamic digestion systems are performed to simulate the human gastrointestinal conditions with the purpose of testing digestibility or prebiotic effects (Hernandez-Hernandez 2019). Other human studies based on the use of prebiotic FOS and β-GOS have been summarized in **Table 5**. FOS and β-GOS have a safe history of use and have emerged as prebiotic functional food additives of GRAS status (Elferink *et al.* 2019; Gibson *et al.* 2017b; Singh *et al.* 2017).

Table 5. Health end points targeted in human trials of orally administered prebiotics (*i.e.*, inulin, FOS, α- and β-GOS). Table adapted from Gibson *et al.* (2017b).

Health end point	Prebiotic used
Metabolic health: overweight and obesity; type 2 diabetes mellitus; metabolic syndrome and dyslipidaemia; inflammation	Inulin, FOS, α-GOS, β-GOS
Satiety	FOS
Stimulation of neurochemical-producing bacteria in the gut	β-GOS
Improved absorption of calcium and other minerals, bone health	Inulin, FOS
Skin health, improved water retention and reduced erythema	β-GOS
Allergy	α-GOS, β-GOS, FOS
Urogenital health	α-GOS
Bowel habit and general gut health in infants	β-GOS, FOS
Infections and vaccine response	β-GOS, FOS
Necrotizing enterocolitis in preterm infants	β-GOS, FOS
Irritable bowel syndrome	β-GOS
Traveller's diarrhoea	β-GOS
Constipation	Inulin
Immune function in elderly individuals	β-GOS

In addition to the health benefits, NDOs provide several manufacturing advantages, which make their use as food ingredients particularly attractive. They show nutrition-relevant properties like low cariogenicity, low calorimetric value and a low GI. Likewise, NDOs compounds have great potential to improve the quality of many foods, providing modifications to food flavour and improving its physicochemical characteristics such as viscosity, emulsification capacity, gel formation and colour. They are water-soluble and they have typically moderate sweetness (30-60% times as sweet as sucrose). In fact, the sweetness depends on the chemical structure, the DP and the levels of mono- and disaccharides in the mixture, with a decrease in sweetness with the increasing length of the oligosaccharide chain (Bali *et al.* 2013; Crittenden *et al.* 1996; Guine *et al.* 2008; Ruiz-Aceituno *et al.* 2018; Voragen *et al.* 1998). This low sweetness intensity is quite useful in those foods where the use of sucrose is restricted by its high sweetness property. The relatively low sweetness makes the oligosaccharides useful in food production when a bulking agent with reduced sweetness is desirable to enhance other food flavours. They may be used as bulking agents in conjunction with other sweeteners, for example, with the advantage to mask the aftertastes produced by some of these HIS.

In the last few decades, a number of NDOs have been introduced as functional food ingredients, particularly in some European countries and Japan, in beverages, dairy products, prebiotic, probiotic and synbiotic products, desserts, confectionery products, sweeteners, milk products, fruit drinks, bread and pastries, etc (Mussatto *et al.* 2007; Roberfroid *et al.* 2000b).

2 ———

Justification, overview of the research aim and work plan

2 JUSTIFICATION, OVERVIEW OF THE RESEARCH AIM AND WORK PLAN

The incidence of diet-related diseases is progressively increasing while being widely attributed to excessive sugar consumption. In recent years, much effort has been directed towards the development of healthier ingredients to meet increasing consumer demand for food products, not only with a better nutritional profile but also providing a benefit on health.

Sugar has long been part of the human diet and provides many versatile applications in the industry but is also well known for its impact on microbiota composition and health status. There is an increasing concern over the demonstrated relationship between sugar (sucrose) consumption and obesity, cardiovascular diseases, diabetes *mellitus*, etc. Thus, the development of natural occurring high-intensity sweeteners has been moving up a gear in order to provide alternatives for sugars that are preferably noncalorific, noncariogenic and generally safe. To date, many plant-derived compounds have been studied extensively for their sweetness-induced activities, however, relatively few sweet-tasting natural products have been approved by the regulatory authorities as sugar substitutes. Two botanical sweeteners that have enjoyed a prodigious surge in usage in just a few years are Stevia (*Stevia rebaudiana*) and Luo Han Guo (*Siraitia grosvenorii*), despite their previous use in traditional medicine. These sweeteners are natural products with high development potential and have increasingly been the focus of scientific research and commercial attention. Moreover, their several pharmacological properties have been investigated as they might offer potential benefits for health. However, the food industry is not only aiming for healthier possibilities for formulation but also claims these novel alternatives to be able to overcome limitations and to obtain the most possible sensorial appealing products.

Gut microbiota influences many aspects of human health, and strong evidence supports that dietary modulation can bring somehow health benefits. Functional ingredients may represent a novel therapeutic approach to whereby improve the composition of the microbial gut and its metabolic output and prevent or attenuate diet-related diseases. Prebiotics are selectively fermented in the gut, giving rise to positive changes not only in the singular ecosystem that inhabits the colon but also at a systemic level. Furthermore, from an

industrial point of view, prebiotics are additionally considered attractive for their beneficial texture forming properties, low caloric values and adequate flavour profile, and thus could also work as a feasible sugar replacement.

Considering this background, the research aim of this PhD Thesis was to modify the structure of mogrosides and steviol glycosides by using different glycosyl hydrolases to obtain novel low-calorie sweetener ingredients with appealing techno-functional, organoleptic and/or potential prebiotic properties.

To address the aforementioned objective, the following specific objectives were summarized as follows:

- To optimize the enzymatic glucosylation of steviol glycosides and mogrosides by three different microbial cyclodextrin glycosyltransferases (CGTases).
- To optimize the enzymatic parameters to modify steviol glycosides and mogrosides and the simultaneous synthesis of prebiotic oligosaccharides catalyzed by microbial β-galactosidase or β-fructosidase.
- To characterize the structure of the new enzymatic modified steviol glycosides and mogrosides by mass spectrometry.
- To perform a sensorial analysis of the optimal-synthesized samples.
- To investigate the potential role of the oligosaccharide-based sweeteners on the human microbiota by *in vitro* faecal incubation.

To achieve the research aim and objectives, the research project was structured in three different parts: (**Part I**) Enzymatic biotransformation, synthesis and characterization of natural sweeteners, (**Part II**) Sensorial evaluation of the modified natural sweeteners, and (**Part III**) Prebiotic evaluation of the oligosaccharide-based sweeteners.

Initially in **Part I**, the biosynthesis and modification of two natural sweeteners from *Stevia rebaudiana* and *Siraitia grosvenorii*, were performed with the following selected glucosyl-transferases and glycoside hydrolases:

Glucosyl-transferases: CGTases from three different bacteriological sources which were compared regarding the yield of glucosylated products. For this purpose, a design of experiments (DoE) was used taking into account different variables such as acceptor (*i.e.*, sweetener extracts) and donor (*i.e.*, maltodextrins) concentrations, enzymatic activity, temperature, pH and time of reaction. The effect of individual parameters and their interactions on the reaction were studied.

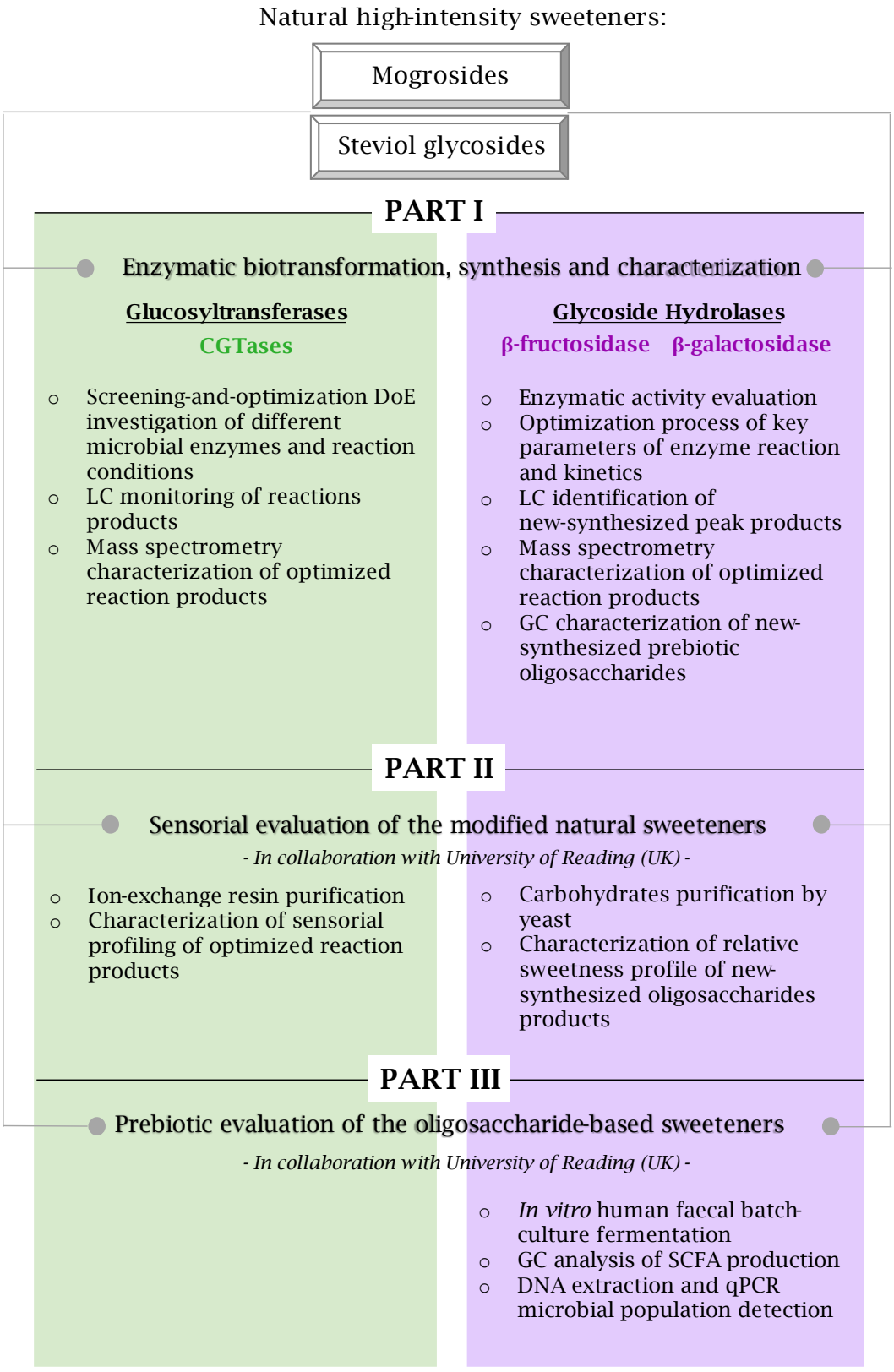
Glycoside hydrolases: The enzymatic reactions were performed with the sweeteners extract as acceptors and with sucrose and lactose as donor substrates for β-fructosidase (*Aspergillus aculeatus*) and β-galactosidase (*Aspergillus oryzae*), respectively. The synthesis optimization was carried out using different parameter ranges such as substrates concentration, time and enzymatic activities. Subsequently, reaction kinetics were carried out in two modalities: with the presence of the disaccharides in the reactions and their absence.

LC-DAD analysis was used to monitor the chemical modifications (the appearance of new hydrophilic and hydrophobic compounds) that occurred along with the optimization methods. Mass spectrometry-based analyses, consisting of LC-ESI-MS and MALDI-TOF, were used to identify the newly synthesized compounds. Likewise, GC-FID analysis was used to examine the oligosaccharides formed with the glycoside hydrolases using their respective carbohydrate donors.

In **Part II**, the assistance of a sensorial analysis was used to determine whether the chemical modification exerted an effect on the sensorial profile. The resultant optimal samples were purified in order to obtain a higher purity grade of the newly synthesized compounds. On one hand, a complete sensorial profiling was accomplished with the reactions performed with glucosyltransferases, in where attributes such as sweet taste, the overall strength of off-taste, liquorice flavour, cooked sugar flavour, metallic taste, sweet aftertaste, and liquorice aftereffect, were determined. On the other hand, a primary sweetness sensorial evaluation was carried out for the optimized oligosaccharide-based sweeteners by the glycosyl-hydrolases. Sweetness value is a critical organoleptic characteristic when assessing the appropriate amount to exert a prebiotic effect.

Lastly in **Part III**, an *in vitro* batch-culture procedure was carried out to simulate the colon conditions and to investigate the potential microbiota modulation of the oligosaccharide-based sweeteners. GC-FID analysis was used to study organic acids (acetate, propionate, butyrate and lactate) production during fermentation. Moreover, the microbiota population was determined by qPCR amplification of different bacterial groups and/or genera.

WORK PLAN:


Natural high-intensity sweeteners:
Mogrosides
Steviol glycosides
PART I
Enzymatic biotransformation, synthesis and characterization
Glucosyltransferases
CGTases
Screening-and-optimization DoE investigation of different microbial enzymes and reaction conditions
LC monitoring of reactions products
Mass spectrometry characterization of optimized reaction products
Glycoside Hydrolases
β-fructosidase β-galactosidase
Enzymatic activity evaluation
Optimization process of key parameters of enzyme reaction and kinetics
LC identification of new-synthesized peak products
Mass spectrometry characterization of optimized reaction products
GC characterization of new-synthesized prebiotic oligosaccharides
PART II
Sensorial evaluation of the modified natural sweeteners
- In collaboration with University of Reading (UK) -
Ion-exchange resin purification
Characterization of sensorial profiling of optimized reaction products
Carbohydrates purification by yeast
Characterization of relative sweetness profile of new-synthesized oligosaccharides products
PART III
Prebiotic evaluation of the oligosaccharide-based sweeteners
- In collaboration with University of Reading (UK) -
In vitro human faecal batch-culture fermentation
GC analysis of SCFA production
DNA extraction and qPCR microbial population detection

3

Materials and Methods

OUTLINE

3 MATERIALS AND METHODS

3.1 Part I Enzymatic biotransformation and synthesis of natural high-intensity sweeteners: *Stevia rebaudiana* (Bertoni) and *Siraitia grosvenorii* (Swingle) extracts

This section presents the enzymatic modifications carried out in different major HIS such as *Stevia rebaudiana* compounds, being a steviol glycosides mixture extract (SVglys) and a single glycoside named rebaudioside A (RebA) (Carbosynth, Berkshire, UK), and *Siraitia grosvenorii* triterpene based on a mixture of mogrosides (MGE; MonkFruit Corp., Libertyville).

3.1.1 Transglucosylation by CGTases using a statistical experimental design

Optimization of transglucosylation reactions with three different CGTase sources (*Geobacillus* sp., *Thermoanaerobacter* sp. and *Paenobacillus macerans*) was carried out by multivariate analysis (Software Design Expert 10.1, StatEase, Minneapolis, MN, USA). The design involved screening the lowest and highest values of the chosen ranges of six experimental variables (**Table 6**): donor substrate concentration (maltodextrin from maize starch, 20 dextrose equivalents, 5-50 mg/mL; Fisher scientific™, Loughborough, UK), acceptor substrate concentration (SVglys/RebA/MGE; 5-50 mg/mL), enzyme activity (5-25 U/g acceptor), temperature (50-70 ºC), reaction time (1-6 h) and pH (5-7). The resultant design consisted of 16 experimental runs to determine the optimal enzyme to proceed with for the optimization. Once the optimal enzyme was chosen, the most influential parameters were established using a fractional factorial design ($2^{(6-2)}$) by representing the responses with the corresponding Pareto chart. The factors significantly affecting the response were then selected for a Central Composite Design (CCD) to obtain the optimal conditions for the glycosylation reactions. Accuracy and precision of the analytical assay method were evaluated using the relative percentage error (RE) and the appropriate relative standard deviation (RSD) from the theoretical concentrations of stevioside, rebaudioside A and mogroside V (Biosynth Carbosynth, Reading, UK) by external calibration.

Table 6. Experimental design by using a fractional factorial design $2^{(6-2)}$.

	Factors					
	1	2	3	4	5	6
Run	Maltodextrin (mg/mL)	Unmodified acceptor mixture (mg/mL)[a]	Enzyme activity (U/g acceptor)[b]	Temperature (ºC)	Time (h)	pH
1	5	50	5	70	6	5
2	5	5	5	70	1	7
3	5	50	25	50	1	5
4	5	50	5	50	6	7
5	50	5	5	50	6	5
6	5	50	25	70	1	7
7	50	50	25	70	6	7
8	50	5	5	70	6	7
9	50	50	25	50	6	5
10	50	50	5	50	1	7
11	5	5	25	50	6	7
12	50	5	25	70	1	5
13	5	5	5	50	1	5
14	50	5	25	50	1	7
15	5	5	25	70	6	5
16	50	50	5	70	1	5

[a]Steviol glycosides (SVglys), rebaudioside A (RebA) and mogrosides (MGE) were used as acceptor substrates.
[b]Enzyme activity (U) was declared by the supplier and determined by methods described elsewhere based on cyclization activity (Tardioli *et al.* 2006).

3.1.2 Enzymatic modifications by other glycoside hydrolases

3.1.2.1 Study of the enzymatic activities for β-galactosidase and β-fructosidase enzymes

The activity of the microbial β-galactosidase (*Aspergillus oryzae*) was assayed by measuring the release of *o*-nitrophenol (*o*NP) after hydrolyzing the chromogenic substrate *o*-nitrophenyl-β-D-galactopyranoside (*o*NPG) Carbosynth (Berkshire, UK). The increase in the absorbance caused by the substrate *o*NP was recorded spectrophotometrically at 420 nm. The reaction mixture contained 11 µL of suitable diluted enzyme solution (β-galactosidase diluted at 1:5000), 220 µL phosphate buffer (0.1M, pH 7.5; Sigma-Aldrich, St Louis, MO, USA) and 66 µL oNPG (4 mg/mL in phosphate buffer 0.1 M, pH 7.5; Sigma-Aldrich, St Louis, MO, USA) and was briefly incubated at 37 ºC. After the incubation for 10 min, the reaction was stopped by adding sodium carbonate (1M) to the enzyme reaction mixture. Enzymatic activity was

expressed as U per mg where one unit is defined as the amount of enzyme required to liberate 1 μmol of *o*NP per min under the above conditions (Liu *et al.* 2017).

The activity of the microbial β-fructosidase (*Aspergillus aculeatus*) was determined by measuring the rate of glucose and fructose released from sucrose hydrolysis. A typical reaction consists of 100 g/L sucrose in 25 mM sodium acetate buffer (pH 5.2) and 0.2 mL of enzyme solution (diluted at 1:10) incubated at 55 ºC (Spohner *et al.* 2016). The released monosaccharides were analysed at 5-minute intervals over 1 h incubation by Liquid Chromatography with Refractive Index Detector (LC-RID) (*section 3.1.3.1*) The enzyme preparation expressed a specific fructosidase activity of 734.2 units per millilitre of enzymatic preparation (U/mL), where one unit is defined as the amount of enzyme needed to convert 1 μmol of sucrose per minute at optimal conditions.

3.1.2.2 Process optimization of the reaction conditions

Considering the respective glycosidases activities, an optimization procedure of the reactions with both enzymes was carried out, in this case, with representative mixtures of both *Stevia rebaudiana* and *Siraitia grosvenorii* extracts.

The optimization considered main parameters such as acceptor concentration, enzymatic activity, and reaction time, which conditions were selected on the basis of the appearance of new chromatographic peaks, identified by RP-LC-DAD (*section 3.1.3.2*), with respect to the initial control samples. Initially, β-fructosidase and β-galactosidase were separately incubated with their respective substrates, sucrose (60%, w:v) and lactose (30%, w:v) with each acceptor at different concentrations (SVglys: 0.2, 1.5, 5, 10 and 20%, w:v; MGE: 0.2, 1.5, 5 and 10%, w:v). Once the acceptors' concentrations were settled, the influence of enzyme activity was determined using different enzyme concentrations, ranging from 5 to 100 U/mL. The experimental conditions for the different reactions consisted of sodium acetate buffer 50 mM at pH 4-5, at 40 ºC for the reactions with β-galactosidase, and sodium acetate buffer 50 mM at pH 5.5 at 60 ºC for the reactions with β-fructosidase.

With the mentioned experimental parameters and both the acceptor concentrations and enzymatic activity selected, the last optimization stage was carried out consisting of an

enzyme kinetics study on the reaction course over time (1, 2, 4, 6, 12, 24, 24, and 48 h). In addition, a parallel set of reactions based on this last optimization stage was also performed. These reactions combined the use of the acceptor with their corresponding enzymes omitting the substrates (sucrose and lactose), and also the absence of the acceptors, incubating only the enzymes with their respective carbohydrates.

3.1.3 Analytical methodologies

3.1.3.1 High-Performance Liquid Chromatographic with Refractive Index Detector (LC-RID)

Liquid Chromatography with Refractive Index Detector (LC-RID) was used in an Agilent Technologies 1220 Infinity CL system-1260 RID (Boeblingen, Germany) equipped with a Kromasil Classic 100-NH_2 column (250 x 4.6 mm; 5 μm particle size, Akzo Nobel, Brewster, NY) and using a mobile phase composed by H2O:ACN at 35:75 (v:v). Samples (50 μL) were eluted at 1.0 mL/min flow rate under isocratic mode.

3.1.3.2 Reversed-Phase High-Performance Liquid Chromatographic with Diode Array Detector (RP-LC-DAD)

The concentration of the enzymatic products at different stages of the optimization was monitored by RP-LC-DAD (Agilent Technologies, 1200 series and 1260 Infinity) equipped with an autosampler, quaternary pump, column oven and DAD detector. The sample injection volume was 20 μL at room temperature and the separation was performed on a reversed-phase C18 column (Poroshell 120 C_{18} column; 250 mm × 4.6 mm, 4 μm particle size, 120 Å pore size; Agilent Technologies, Palo Alto, CA, USA). The wavelength for ultraviolet detection was set at 210 nm. The elution of the samples was carried out at 30 ºC using a binary mobile phase composed of H_2O:ACN.

The initial samples and the products resultant from the reactions carried out with both the glucosyltransferases and the glycoside hydrolases were eluted at 30 ºC with a constant flow rate of 0.7 mL/min, respectively. A binary mobile phase composed of deionized water (eluent A) and acetonitrile (eluent B) was used under the following gradient mode: 90:10 (v:v) for 0-12 min, 88:12 (v:v) for 12-17 min, 84:16 (v:v) for 17-32 min, 50:50 (v:v) for 32-46 min and

90:10 (v:v) for 46-60 min. Data acquisition was performed using Agilent ChemStation software (Wilmington, DE, USA).

3.1.3.3 Gas Chromatography with Flame Ionization Detector (GC-FID)

The synthesized carbohydrates generated during the enzyme kinetics with the β-fructosidase and β-galactosidase enzymes with and without the sweetener acceptor (SVglys and MGE) were determined by GC using an Agilent Technologies gas chromatograph (Mod7890A) equipped with a FID. Samples of soluble fractions of carbohydrates were taken at different reaction times and derivatized as trimethylsilylated oximes (TMSOs) with β-phenyl glucoside as an internal standard following the method developed by Brobst *et al.* (1966). The derivatization process of the samples was initiated by adding hydroxylamine chloride in pyridine (2.5%, w:v) and incubated at 70 ºC for 30 min. Thereafter, the resulting oximes were trimethylsilylated by adding hexamethyldisilazane and trifluoroacetic acid (9:1, v:v) and incubated for 30 min at 50 ºC with occasional shaking. Reaction mixtures were centrifuged at 9,000 × *g* for 2 min, and the resultant supernatants were analyzed by GC-FID.

The TMSOs were separated using a capillary column (15 m × 0.25 mm × 0.10 μm, SGE HT5, North Harrison Road, Bellefont, USA). Nitrogen was used as carrier gas at 1 mL/min flow rate. Injector and detector temperatures were 280 and 385 ˚C, respectively. Samples (1 μL) were injected in split mode (1:20) and the oven temperature was set with a thermal ramp from 150 to 380 ºC at a heating rate of 3 ºC/min. Data were acquired with Agilent ChemStation software (Wilmington, DE, USA) and quantification was performed considering the response factor of different standards previously injected.

3.1.3.4 Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF)

The molecular weight distribution of the samples was determined by MALDI-TOF MS. Samples were diluted 1:100 in water (Milli-Q water, Millipore, Bedford), and 1 μL of the diluted solutions were mixed with 3 μL of the matrix consisting of 2,5-dihydroxybenzoic acid (>98%, Fluka, Buchs, Switzerland) at a concentration of 10 mg/mL in water. Then 1 μL of this mixture was spotted onto a flat stainless-steel sample plate and dried in air before analysis.

A Voyager DE-PRO mass spectrometer (Applied Biosystems, Foster City, USA), equipped with a nitrogen laser emitting at 337 nm and a delayed extraction ion source, was used for MALDI-TOF MS measurements. Ions generated by laser desorption of unmodified and modified samples were introduced into the time-of-flight analyzer (1.3 m flight path) with an acceleration voltage of 25 kV, 94% grid voltage, 0.075% ion guide wire voltage, and a delay time of 400 ns. Mass spectra were obtained over the *m/z* range of 100-5000 in the linear positive ion mode as $[M + Na]^+$. External mass calibration was applied using the average $[M + H]^+$ values of des-Arg1 bradykinin, angiotensin I, Glu1-fibrinopeptide B, ACTH (1-17 clip), ACTH (18-39 clip) and bovine insulin of the Calibration Mixtures 1 and 2 (Sequazyme Peptide Mass Standards Kits, Applied Biosystems).

3.1.3.5 Liquid chromatography-mass spectrometry (LC-MS)

LC-MS analysis was carried out on an Agilent 1100 Series (Agilent Technologies, Palo Alto, USA) LC system equipped with a quaternary pump, an autosampler, and a column oven and coupled to an HTC-Ultra ETD II ion trap mass spectrometer (Bruker 198 Daltonics, Fremont, USA) by electrospray (ESI) interface working in the negative-ion mode. The separation was performed on a C_{18} 180 HyPURITYTM column (100 mm x 2.1 mm, 3 μm particle size, Thermo Fisher Scientific, San José, CA, USA) at 35 ºC and 0.2 mL/min flow rate with a solvent gradient of 0.1% (v:v) formic acid (analytical grade; Merck, Darmstadt, Germany) in water (eluent A), and acetonitrile (OPTIMA®LC/MS grade, ThermoFisher Scientific) containing 0.1% of formic acid (v:v) (eluent B). The elution program was applied as follows: 10% B for 1 min, 10–50% B linear in 45 min, 50% B for 5 min, 50–90% B linear from 50 to 51 min, 90% B isocratic from 51 to 60 min, ramped to original composition in 1 min, and then re-equilibration of the column for 10 min.

Samples were diluted in water (1:100) and 5 μL were injected into the chromatographic system. The electrospray source parameters were adjusted as follows: spray voltage, 3.5 kV; drying gas temperature, 350 ºC; drying gas flow rate, 10 L/min; nebulizer pressure, 40 psi; and skimmer voltage, -40 V. Nitrogen (99.5% purity) was used as a drying and a nebulizer gas. Full scan mass spectra were recorded between *m/z* 100 and 2000. Data acquisition and processing were performed using Bruker Compass 1.2 software.

3.2 Part II Sensorial evaluation of new-synthesized natural sweeteners

3.2.1 Isolation of modified high-intensity acceptors

The samples from the respective optimal reactions were incubated on a larger scale and then purified to eliminate digestible carbohydrates in order to carry out the sensorial analysis.

The reaction mixtures (100 mL) obtained from the DoE with optimal CGTases for SVglys, RebA and MGE, were placed into a Diaion HP-20 column (2.2 × 50 cm^2) and washed with deionized water (1500 mL water). Afterwards, samples were eluted with ethanol (95%) and subsequently dried using a rotary evaporator (40 ˚C). Samples were kept at 4 ºC until analysis. Removal of free digestible carbohydrates was efficiently carried out and monitored by GC-FID.

The reaction product samples belonging to β-fructosidase and β-galactosidase reactions with their respective substrates for both acceptors (SVglys and MGE) were diluted 1:10 (w:w) and incubated at 37 ºC for 24 h with 10 mg/mL *Saccharomyces cerevisiae* yeast (Molino Spadoni, RA, Italia) following the method by Hernández *et al.* (2009). Subsequently, samples were filtered through a 0.22 μm pore size using depth filters (SA-999, Filtrox Southern Europe) and freeze-dried and finally stored at 4 ºC until analysis.

3.2.2 Sensorial test

The sweetness intensity of both unmodified and enzymatically modified samples was evaluated whether the enzymatic reactions affected the sensorial profile. The sensory analysis was carried out using 10 experienced panellists who were trained at the Sensory Science Centre (Department of Food and Nutritional Sciences, University of Reading, UK). The study was approved by the University of Reading Research Ethics Committee (UREC Study Number: 16_19). Sensory analysis was performed in an air-conditioned sensory laboratory (23-24 ºC, room temperature) with individual booths and artificial daylight. Sample order presentation was done in a balanced monadic sequential manner. In order to improve discrimination for sweetness white granulated sugar (Tate and Lyle, London, UK) was used as a reference, the

panel used four sucrose concentrations for the reactions corresponding to the enzyme CGTase which were 0.5, 1.0, 2.0 and 2.6% (w:v), and other four sucrose concentrations for β-fructosidase and galactosidase reactions that were 20, 30, 60 and 80 g/L. The average panel ratings for these standards were 10, 35, 75 and 100, respectively, on a 0-100 line scale, and these four positions were used as anchors to provide a structured scale on which to rate the sweetness of all samples.

The samples resultant from the CGTases reactions were rated in triplicate on separate days and the panel used 16 more attributes to describe the samples (sweet, overall strength of off-taste/flavour, bitter taste, liquorice flavour, sour taste, cooked sugar flavour, cooling sensation. Cardboard/stale, metallic, salty taste, crusty bread flavour, perfume flavour, sweet aftertaste, bitter aftertaste, liquorice aftereffect and cooling aftertaste). All the other attributes tested for CGTase samples were scored as relative values using unstructured line scales (0-100).

Due to the limited sample availability, each panellist was presented with only 0.5 mL of sample for each scoring session. Therefore, training additionally focused on ensuring panellist to sip this small sample volume from a 30 mL transparent polystyrene cup and allow it to flow over the top of their tongue before swallowing and scoring the attributes accurately. Palate cleansing before and between sample scoring was done using filtered water and low salt crackers (Carr´s water crackers, United Biscuits Ltd., Hayes, UK).

Samples were prepared in mineral water (Harrogate Spa mineral water) and labelled with random 3-digital codes. Sample order presentation was done in a balanced monadic sequential manner. Preliminary tests were conducted to define the concentrations of each sweetener to be used. The concentrations were concluded to be 10 g/L for the samples modified with β-fructosidase and β-galactosidase, whilst for the reaction samples corresponding to the study of CGTases were 0.32 g/L for SVglys and 0.24 g/L for RebA and MGE.

The sucrose standards were presented at the start of each panel rating session for refamiliarization to enable the panellists to score the sweetness of the samples accurately

against the standard anchors. The mean sweetness ratings of the four sucrose standards were used to plot a dose–response curve, the linear regression for which was perceived sweetness = (1.55 × sucrose concentration (g/L)) − 22.5 (r^2 = 0.99). The mean sweetness ratings for each sample were converted to equivalent sweetness (ES) values using this equation. Likewise, another characteristic that was considered to gauge the sweetness of the modified samples in relation to sucrose was the sweetness potency (SP). Sweetness potency is the ratio of the concentration of sucrose to that of a sweetener at equivalent sweetness intensities and is represented by the following equation:

SP = (concentration of sucrose) / (concentration of sweetener at equi–sweetness intensity to sucrose).

The sensory profile data were analyzed using a mixed-model ANOVA where panellists were treated as random effects and samples as fixed effects. The main effects were tested against the sample by assessor interaction. Multiple pairwise comparisons were carried out using Fisher's LSD and a significant difference was declared at an alpha risk of 5% ($p \leq 0.05$). Data analysis was carried out using the Senpaq software (Qi Statistics, Reading, UK).

3.3 Part III Prebiotic evaluation of newly synthesized oligosaccharide-based sweeteners with the assistance of glycoside hydrolases

3.3.1 In vitro *batch-culture fermentations*

In vitro fermentations were carried out using human faecal microbiota collected from four health donors (two males and two females, aged 26 – 36 years old) with no preceding history of metabolic or gastrointestinal disorder and without having taken a prebiotic or probiotic supplement or antibiotic within 3 months prior to the study. The faecal slurry inocula were processed in an anaerobic cabinet within 15 min after collection as follows: dilution with sterile phosphate-buffered saline (10% w:v; PBS, 0.1 M, pH 7.4, Oxoid. Basingstoke, UK), and homogenisation for 2 min in a stomacher (Stomacher 400, Seward, UK) at normal speed.

Sterile stirred batch-culture systems were set up in fermenters (20-mL working volume) aseptically filled with 17 mL of sterile, nutrient basal medium containing: 2 g/L peptone water, 2 g/L yeast extract, 0.1 g/L NaCl, 0.04 g/L K_2HPO_4, 0.04 g/L KH_2PO_4, 0.01 g/L $MgSO_4 \cdot 7H_2O$, 0.01 g/L $CaCl_2 \cdot {}_6H_2O$ 0.01 g/L, 1 g/L $NaHCO_3$, 0.5 g/L L-cysteine hydrochloride, 0.5 g/L bile salts, 0.05 g/L haemin, 10 μL/L vitamin K, 2 mL/L Tween 80 and 4 mL/L resazurin (0.025%, w:v). Before incubation, vessels were gassed overnight with oxygen-free N_2 to obtain anaerobic conditions. Carbohydrate substrates were diluted in basal medium (1%, w:v) and filter-sterilized (0.22 μm), and finally added to the corresponding vessels. Non-modified natural sweeteners were also tested (0.2%, w:v; SG Control and MV Control) and short-chain FOS (1%, w:v; ScFOS ≥ 99.7%; FUJIFILM Wako Chemicals, Germany) was used as a positive control. A last vessel with no added prebiotic substrate was also included as the negative control. Briefly, 1 mL of faecal inoculum was added to each vessel. The batch-culture system was continually stirred with a circulating water bath to keep the temperature at 37 ºC and automated pH controllers (Fermac 260; Electrolab UK) kept culture pH at a range of 6.7 and 6.9 by adjusting with NaOH (0.5 M) and HCl (0.5 M) when required. Fermentations were run for a period of 24 h and samples (1 mL) were obtained in duplicate from each vessel after 0, 10, and 24 h fermentation. The samples corresponding to 0 h were only taken from the vessel

corresponding to the negative control. Briefly, the samples were centrifuged at 13,000 x *g* for 10 min and pellets and supernatants were collected for extraction of genomic DNA for the quantitative real-time PCR (qPCR), and the SCFAs analysis.

3.3.2 Bacterial strains and culture conditions

The bacterial strains and the selective media employed in order to elaborate the stock cultures for microbial population detection along the fermentation were: Lysogeny broth (LB) for *Escherichia coli* K12 ATCC 10798 (for *Enterobacteriaceae* and total bacteria), De Man, Rogosa and Sharpe agar (MRS) for *Lactobacillus rhamnosus* ATCC 53103 (for *Lactobacillus*), Glucose Azide Broth for *Enterococcus faecium* DSM 2570, Reinforced clostridial medium for *Bifidobacterium bifidum* DSM 20456 (for *Bifidobacterium*) and modified chopped meat medium for *Blautia coccoides* ATCC 29236 (for *Clostridium coccoides* group), *Bacteroides xylanisolvens* ATCC 18836 (for *Bacteroides* group *Bacteroides – Prevotella – Porphiromonas*) and *Atopobium* ATCC 25986 (for the *Atopobium* cluster). The incubation of the strains listed above was at 37 ºC under either aerobic or anaerobic conditions ($A_{600} \approx 1$). The bacterial count was carried out by ten-fold serial dilutions in duplicate in their respective media-based agar. The estimation of the viable bacteria used for the subsequent quantification process was determined by the colony-forming unit (CFU) of each bacterial strain.

3.3.3 Analytical methodologies

3.3.3.1 DNA extraction

Genomic DNA was extracted from both the cell pellets (1 mL) of the bacterial strain and the collected stool samples using a NZY Tissue gDNA Isolation kit (NZYTech, Portugal) according to an adaption of the manufacturer's instructions. The quality and concentration of the extracted DNA were determined by photometry using a Nanodrop (Thermo Scientific™ NanoDrop ™ OneC). Accordingly, DNA concentrations from faecal samples were normalized by making 10-fold serial dilutions. Purified DNA samples were stored at -20 ºC until analysis.

3.3.3.2 Real-time quantitative PCR assays

The analysis of microbial communities from the faecal batch fermentation was conducted by qPCR using the ViiATM 7 Real-Time PCR System (Applied Biosystems, USA). Fragments of the bacterial 16S rDNA genes were amplified using a commercial set of primers provided by Eurofins Genomics (Ebersberg, Germany) and Invitrogen Thermo Fisher Scientific (Madrid, Spain) (**Table 7**).

The DNA amplification was performed on triplicate using SYBR green methodology. Each master mixture (10 μL final volume) contained 20 μM of each respective primer, molecular grade water at 0.2 μM (NZYTech, Portugal) and NZY qPCR Green Master Mix (2x) at 2 μM (NZYTech, Portugal). Bacterial template DNA (2 μL) was added at 1 to 5 ng/μL in 384-well plates sealed with optical sealing. The bacterial DNA from the stock cultures were plotted with 10-fold serial dilutions with a defined number of CFU/mL (10^0 to 10^8, respectively). The thermal cycling programs together with the specificity of the primers for each standard were previously verified through the melting curves and are shown in **Table 7**. The runs were performed with a default program except for the annealing step: one cycle at 95 ˚C for 3 min; 40 cycles of 95 ºC for 5 s, 60 or 78 ºC (depending on the bacterial species [**Table 7**]) for 30 s; and finally, two cycles of 95 ºC for 15 and 60 ºC for 1 min. The number of the CFU of each sample for the target bacteria was determined by comparing the threshold cycle values 200 (C_T) with the standard curve.

Table 7. Group-specific primer set based on 16S rDNA sequences.

Target bacterial group	Sequence (5' to 3')	Product size (bp)	Annealing temp (ºC)[a]	Reference or source
All bacteria	AAACTCAAAKGAATTGACGG CTCACRRCACGAGCTGAC	180	60	Bacchetti *et al.* 2011
Lactobacillus	AGCAGTAGGGAATCTTCCA CACCGCTACACATGGAG	341	60	Rinttilä *et al.* 2004
Bacteroides group *bacteroides – Prevotella – Porphiromonas*	GAAGGTCCCCCACATTG CGCKACTTGGCTGGTTCAG	238	78	Ramirez-Farias *et al.* 2009
Bifidobacterium	CATCCGGCATTACCACCC CCACCGTTACACCGGGAA	523	78	Kok *et al.* 1996
Clostidrium coccoides group	AAATGACGGTACCTGACTAA CTTTGAGTTTCATTCTTGCGAA	438-441	60	Matsuki *et al.* 2002
Enterobacteria	TCAAGGACCAGTGTTCAGTGTC TGCCGTAACTTCGGGAGAAGGCA	428	60	Matsuda *et al.* 2009
Enterococcus	ACCGCGGGTCCATCCATC CCATCAGAAGGGGATAACACTT	115	78	Matsuda *et al.* 2009
Atopobium cluster	GGGTTGAGAGACCGACC CGGRGCTTCTTCTGCAGG	190	60	Matsuki *et al.* 2004

[a]The PCR programs were modified from reference.

3.3.3.3 Organic acids

The supernatants of the culture samples were used for the analysis of SCFAs and lactic acid. The organic acids were extracted following the method of Richardson *et al.* (1989). To 1 mL of sample, 50 μL of 2-ethylbutyric acid (0.1 M) was added as internal standard (IS) for quantification. The followed extraction of the acids consisted of adding 500 μL of concentrated HCl and 2 mL of diethyl mixed for 1 min and centrifuged at 2000 x *g* for 10 min. The resulting upper layer (400 μL; ether layer) was transferred to a capped vial. The derivatization process was completed once adding 50 μL of N-(ter-butyldimethysilyl)-N-methyltrifluoroacetamine (MTBSTFA) and remaining for 72 h at room temperature to ensure full derivatisation.

The injection of the samples was carried out in split mode (100:1) using helium as carrier gas at 1.7 mL/min flow rate. The gas chromatograph (Agilent/HP 5890) was equipped with an HP-5MS column (30 m x 0.25 mm) with a 0.25 μm coating crosslinked (5%-phenyl)-methylpolysiloxane, Hewlett Packard, UK). The oven temperature was set with a thermal ramp from 63 ºC to 190 ºC at a heating rate of 15 ºC/min, and kept constant for 3 min, and the injector and detector were set at 275 ºC.

Quantification of organic acids was performed using Agilent ChemStation software (Wilmington, DE, USA) based on the retention times of the respective commercial standards

(lactic acid, acetic acid, propionic acid and butyric acid; Sigma-Aldrich, UK) ranging between 0.1 and 10 mM.

The significant differences ($P < 0.05$) of bacterial populations and organic acid concentrations were calculated by applying a non-parametric Kruskal-Wallis test including Bonferroni correction for multiple tests (IBM SPSS, Inc. Illinois USA).

4

Results

OUTLINE

4 RESULTS

4.1 Part I Enzymatic biotransformation and synthesis of natural high-intensity sweeteners: *Stevia rebaudiana* (Bertoni) and *Siraitia grosvenorii* (Swingle) extracts

4.1.1 Characterization of the initial sweetener-based substrates

Before enzymatic treatments, an initial characterization of the three substrates (*i.e.*, steviol glycosides mixture (SVglys), rebaudioside A (RebA), and mogrosides extract (MGE)) was carried out using LC analyses.

- **Steviol glycosides mixture (SVglys)**

Figure 9A shows a representative RP-LC-DAD profile of the control SVglys present in *S. rebaudiana* Bertoni leaves used throughout this study, and whose main components were identified by comparing their relative retention times with commercial standards and by confirming according to their molecular masses determined by MS (**Figure 9B**). Thus, as identified in the chromatogram, the tri-glycoside stevioside (*m/z* 803.3) together with the tetra-glycoside rebaudioside A (*m/z* 965.3) represented the majority of the steviol glycosides with 59.4% and 25.4% of abundance (quantified by RP-LC-DAD), respectively, distantly followed by the tetraglycoside rebaudioside C (*m/z* 949.3; 9.2%), as well as by the minor presence of the diglycoside rubusoside (*m/z* 641.3; 1.4%) and two unknown compounds (marked with an asterisk; 1.1% and 3.5%). Either the elution order or the relative amount data are in good agreement with previous reports on steviol glycosides derived from *S. rebaudiana* herb plant leaves (Espinoza *et al.* 2014; Molina-Calle *et al.* 2015; Ohta *et al.* 2010).

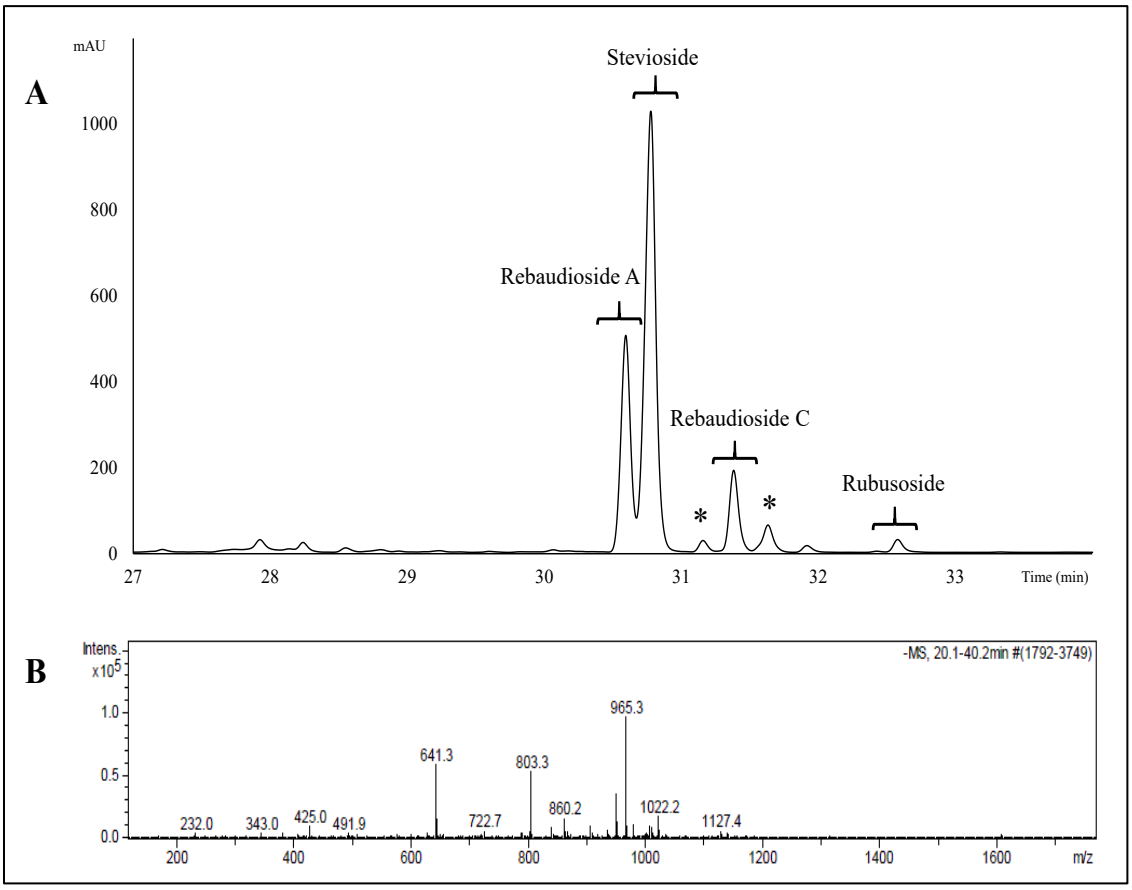


Figure 9. Chromatographic profiling and identification of initial steviol glycosides extract (SVglys) by (A) LC-DAD and (B) LC-ESI-MS analyses: rebaudioside A (*m/z* 965.3), stevioside (*m/z* 803.3), rebaudioside C (*m/z* 949.3), rubusoside (*m/z* 641.3).

- **Mogrosides-based extract (MGE)**

Cucurbitane glycosides contained in the commercial MGE derived from Luo Han Guo fruits were characterized by RP-LC-DAD (**Figure 10A**), with the assistance of LC-ESI-MS (**Figure 10B**). The identities of several mogrosides were identified based on their retention times and mass spectra. RP-LC-DAD determined the abundances of the different mogrosides present in the MGE; mogroside V (MG-V, *m/z* 1285.6) was found to be the major triterpene glycoside with 50.2% of the abundance, as expected on the evidence about the main compounds isolated in ripe fruits from *S. grosvenorii*. Other three quantifiable triterpenes were identified in the extract with the following relative abundance distribution: mogroside VI (MG-VI) and other minor mogrosides, with 31.9% (*m/z* 1447.6), mogroside IV (MG-IV) with 10.2% (*m/z* 1123.6), and mogroside III (MG-III) with 7.7% (*m/z* 965.4) respectively, resembling the content of other previously reported commercial mixtures (Soejarto *et al.* 2019; Xu *et al.* 2015).

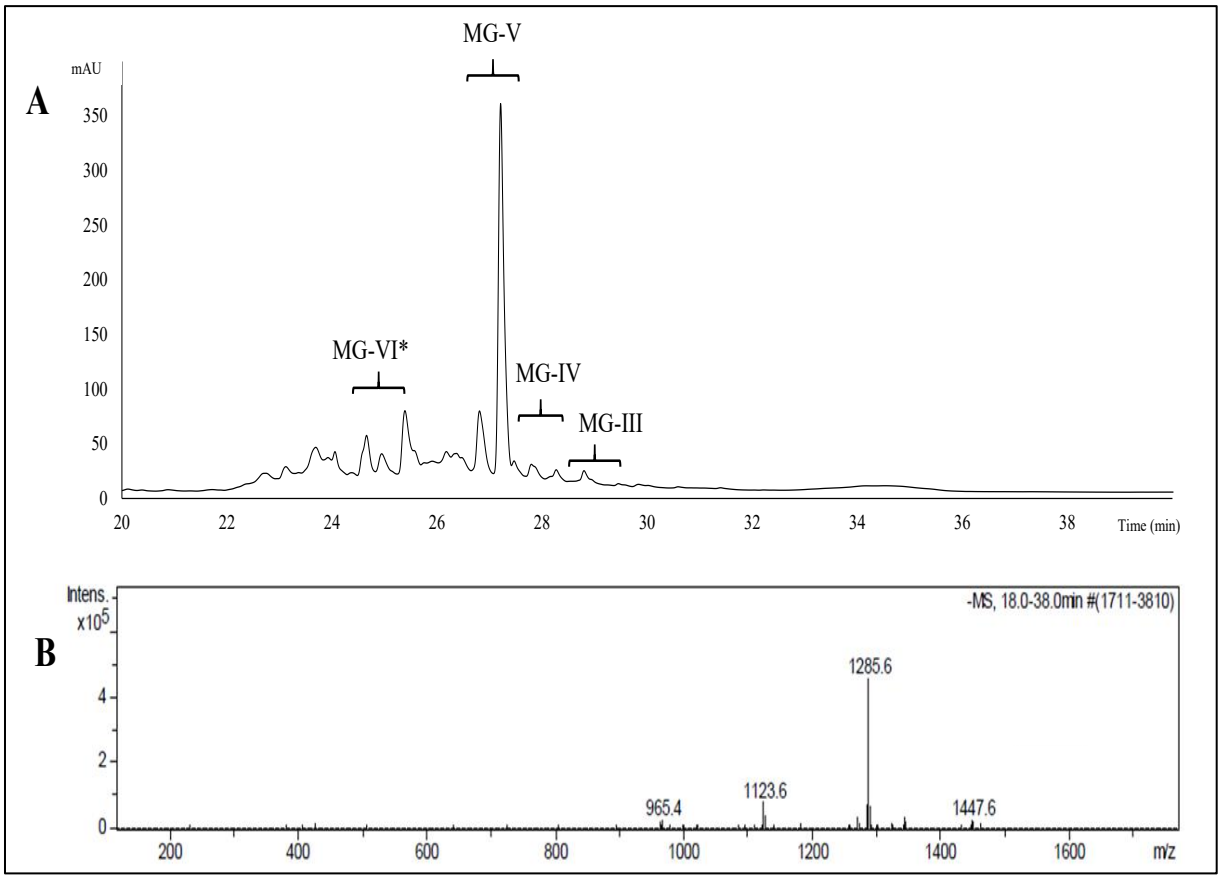


Figure 10. Chromatographic profiling and identification of initial mogrosides extract (MGE) by (A) LC-DAD and (B) LC-MS analyses. Labelled peaks are: MG-III, mogroside III (965.4 *m/z*), MG-IV, mogroside IV (1123.6 *m/z*), MG-V, mogroside V (1285.6 *m/z*), MG-VI*, mogroside VI and other minor non-identified mogrosides (1447.6 *m/z*).

- **Rebaudioside A**

Lastly, the single steviol glycoside extract, performed only for the transglucosylation reactions with CGTases, consisted of rebaudioside A (Carbosynth, Berkshire, UK) in almost its totality. In **Figure 11A** appears an LC-DAD chromatogram where only a peak corresponding to rebaudioside A was eluted and quantified with 96% of the abundance, as well identified by its molecular mass with LC-ESI-MS (*m/z* 965.3; **Figure 11B**).

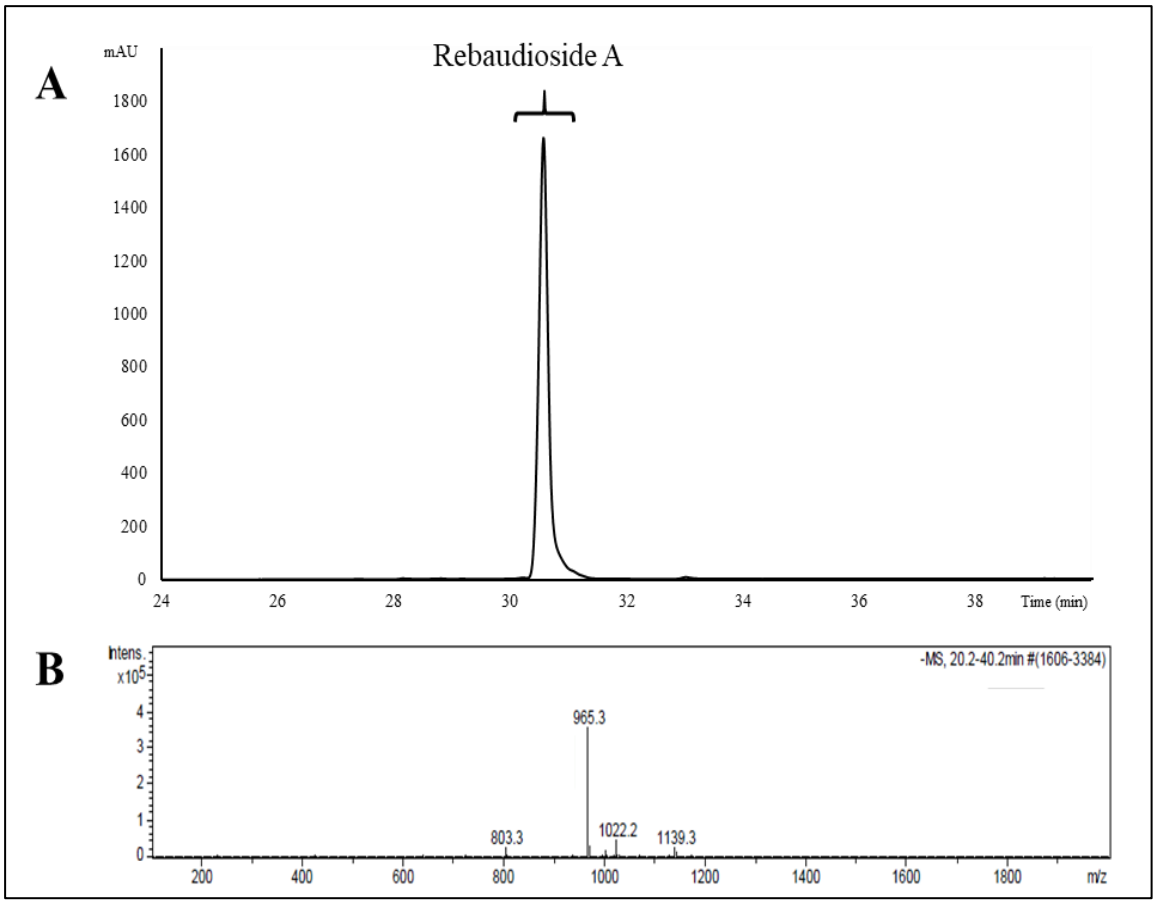


Figure 11. Chromatographic profiling and identification of initial rebaudioside A extract (RebA) by (A) LC-DAD and (B) LC-ESI-MS analyses: rebaudioside A (*m/z* 965.3).

4.1.2 Transglucosylation of natural sweeteners using cyclodextrin glycosyltransferases enzymes

4.1.2.1 Design of Experiment (DoE) techniques

Initially, a screening using a fractional factorial design $2^{(6-2)}$ was used to study the synthesis of both glucosylated SVglys, RebA, and MGE substrates with the three different CGTases employed (*Geobacillus* sp, *Paenibacillus macerans*, *Thermoanaerobacter* sp). This screening allowed us to evaluate the effect of six experimental factors (concentration of maltodextrin, the concentration of unmodified acceptor, enzymatic activity, temperature, time, and pH), using the concentration of glucosylated acceptor (mg/mL) as the response variable. The method performed well concerning accuracy and precision, which were calibrated for each sample and conducted in quadruplicate before sample analysis using LC-

DAD. After the method validation, the optimization procedure was carried out and the corresponding results for the different runs obtained for each enzyme were calculated from LC-DAD analyses and are presented in **Table 8**.

Table 8. Corresponding responses per enzyme and acceptor substrate.

	Responses								
	Synthesized glucosylated SVglys (mg/mL)			Synthesized glucosylated RebA (mg/mL)			Synthesized glucosylated MGE (mg/mL)		
Run	CGTase *Geobacillus* sp	CGTase *Paenibacillus macerans*	CGTase *Thermoanaerobacter* sp	CGTase *Geobacillus* sp	CGTase *Paenibacillus macerans*	CGTase *Thermoanaerobacter* sp	CGTase *Paenibacillus macerans*	CGTase *Geobacillus* sp	CGTase *Thermoanaerobacter* sp
1	0.83	0.66	0.80	1.85	0.06	2.49	2.89	7.82	1.43
2	0.92	0.22	1.50	2.69	0.00	2.04	1.99	1.55	1.55
3	1.07	0.55	1.25	2.78	0.21	2.92	2.89	5.32	8.89
4	9.65	7.85	9.83	28.51	4.07	28.49	5.96	8.21	13.73
5	0.84	0.59	1.13	1.94	0.47	2.28	1.88	1.55	2.60
6	3.10	1.21	4.26	8.97	1.83	12.16	2.54	8.35	13.96
7	0.98	0.77	0.76	2.23	0.48	2.04	4.52	7.45	12.45
8	3.34	8.70	4.49	3.01	0.00	12.16	0.96	1.26	2.10
9	6.19	4.01	9.65	26.04	3.71	25.79	5.27	7.92	13.25
10	8.70	2.12	10.86	22.24	0.41	24.98	7.82	4.81	8.04
11	2.07	2.23	4.02	6.81	1.87	10.32	1.68	1.71	2.85
12	1.55	0.73	0.98	2.68	0.00	3.16	1.43	1.98	3.30
13	3.96	2.96	4.47	11.20	1.83	11.24	1.81	0.17	0.00
14	1.10	0.17	1.02	1.33	0.00	2.92	1.30	0.76	1.28
15	11.33	3.73	11.06	27.71	1.54	26.45	2.30	2.66	4.44
16	1.32	1.09	1.48	2.75	0.00	3.12	7.82	6.60	6.60

The maximum concentrations of glucosylated products for SVglys (11.33 mg/mL) and RebA (28.51 mg/mL) were obtained with the CGTase from *Geobacillus* sp. whilst the highest concentration of glucosylated MGE (14.0 mg/mL) was obtained with the CGTase from *Thermoanaerobacter* sp., such enzymes were used with their respective sweetener acceptor to continue with the next optimization steps. Thereafter, the factors affecting the response were evaluated through a Pareto chart (**Figure 12**) illustrating the ANOVA and *p*-value. Positive values (green bars) denote a directly proportional relationship of the variance with the response, whereas negative values (red bars) reflect an inverse relationship. The horizontal line corresponds to the t-value at a significance level of 5%. The concentration of maltodextrin had a significant effect on the reactions carried out with SVglys and RebA. Similarly, the concentration of the three unmodified acceptors had a significant positive effect on the formation of glycosylated products, whereas the time of reaction only had a significant effect on the formation of glycosylated SVglys and the enzyme activity on the formation of glycosylated MGE.

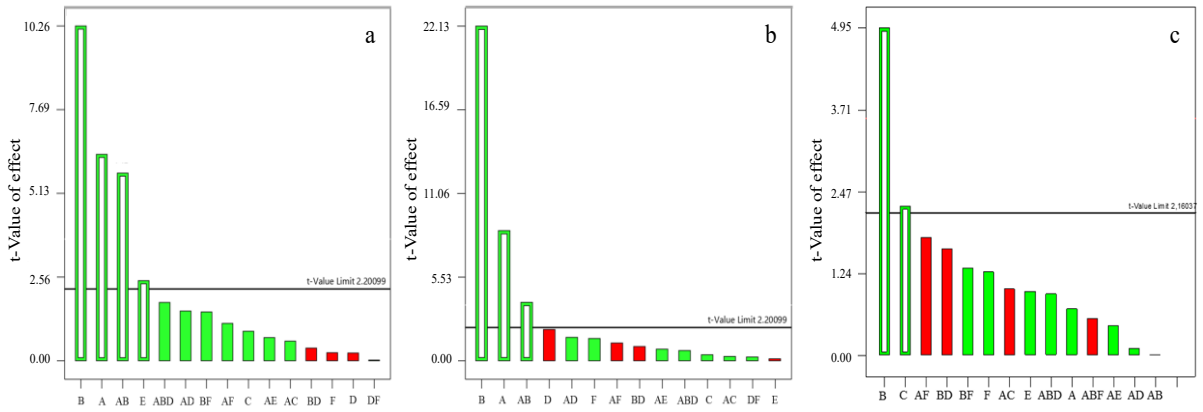


Figure 12. Pareto chart showing individual and interaction effects of the response evaluated: synthesized glucosylated acceptor substrates: steviol glycosides (SVglys) (a) and rebaudioside A (RebA) (b) using cyclodexrin glucosyltransferases (CGTase) from *Geobacillus* sp., and mogrosides (MGE) (c) using CGTase from *Thermoanaerobacter* sp.. (A) Maltodextrin concentration, (B) initial SVglys/RebA concentration, (C) enzyme activity, (D) temperature, (E) time and (F) pH. Using aconfidence value of p = 0.05, based on a null hypothesis test, values exceeding this limit (horizontal line) are considered significant to the response values.

A CCD optimization was performed to study the significant factors obtained for each reaction with an acceptor, setting the resting parameters at the lowest level. The CCD design was composed of 17 runs for SVglys and 11 runs for RebA and MGE, with 3 replicates in the central point. An optimization phase was performed by applying response surface methodology (RSM) to optimize product formation. The relationship between the response evaluated and the variables were fitted into polynomial equations (**Eqs. 1-3**) for each acceptor substrate as follows:

- **Glucosylated SVglys** (**mg/mL**) $= -267.20 + 0.08 \times$ maltodextrin (mg/mL) $+ 0.11 \times$ unmodified SVglys (mg/mL) $+ 102.66 \times$ time (h) /**1**/
- **Glucosylated RebA** (**mg/mL**) $= 0.17 + 0.08 \times$ maltodextrin (mg/mL) $+ 0.38 \times$ unmodified Reb A (mg/mL) /**2**/
- **Glucosylated MGE** (**mg/mL**) $= 1.64 - 0.03 \times$ enzymatic activity (U/g unmodified MGE) $+ 0.43 \times$ unmodified MGE (mg/mL) /**3**/

The ANOVA was carried out to determine the significance and adequacy of the regression model fit. Statistical significance of the model was established at $p \leq 0.05$. The F-values of the obtained model ($F < 0.03$) for the response indicates that the mode was highly adequate and significant. Likewise, the determination coefficients (R^2) of the model are within the range of 0.85-0.98 % for the synthesized glucosylated products. The coefficient of variation (CV, %) had lower values than 10% showing that the variation was acceptable and satisfactory.

Afterwards, the aim was to find the optimum concentration of unmodified SVglys, RebA, and MGE, and the enzymatic activity to maximize the synthesized glucosylated products (mg/mL). The response surface obtained for the global desirability function (D) for each acceptor is presented in **Figure 13**. The coordinates producing the maximum D value (D=1) for SVglys were 60.8 mg/mL for the concentration of maltodextrin, 59.7 mg/mL for the concentration of unmodified SVglys, and 6.8 h for the time of reaction. For RebA, these were 51.9 mg/mL for the concentration of maltodextrin and 57.4 mg/mL for the concentration of unmodified RebA. For MGE, the maximum D values were 56.7 g/L for the concentration of unmodified MGE and 6.5 U/g of MGE for the enzymatic activity.

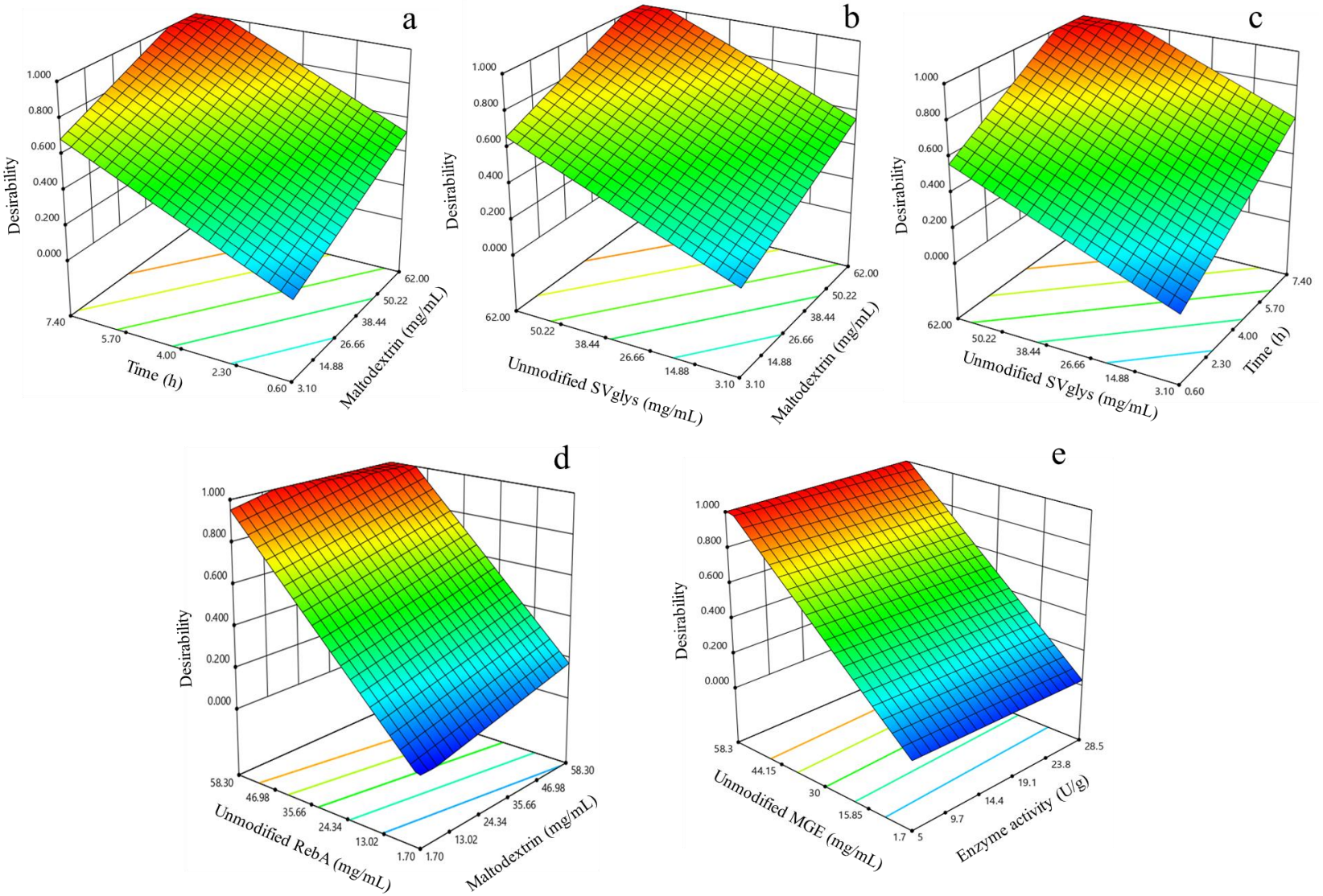


Figure 13. Three-dimensional plots showing the desirability of the maximization of synthesized glucosylated SVglys for the three significant variables (a-c), and synthesized glucosylated RebA (d) and MGE (e) for the two significant variables.

The individual response values and their respective confidence intervals are depicted in **Table 9**. To validate this predictive model, optimal conditions were experimentally assessed through three replicates and these showed no significant differences with the theoretical results. Finally, these conditions were selected to produce higher quantities of synthesized glycosylated products to be structurally characterized and for sensory analysis.

Table 9. Criteria for the optimization obtained from model Equations 1-3 by maximizing the response.

Response	Theoretical Result[a]	Experimental Result[b,c]	Confidence Interval[d]	
			(–)	(+)
Synthesized glucosylated SVglys (mg/mL)	15.8	17.3 ± 1.0 (6.0%)	12.5	19.1
Synthesized glucosylated RebA (mg/mL)	26.2	26.1 ± 2.4 (9.3%)	22.1	30.3
Synthesized glucosylated MGE (mg/mL)	26.2	25.2 ± 1.6 (6.4%)	24.2	28.2

[a]Obtained from model prediction at the optimal settings.
[b]Obtained from an average of additional four runs conducted at the optimal settings.
[c]Standard deviations and relative standard deviations (n = 4) of experimental results are also represented.
[d]Lower (-) and upper (+) confidence interval values calculated to a confidence level of 95%.

4.1.2.2 Structural characterization

At the beginning of the optimization, LC-DAD was used to quantitatively determine the enzyme that generated the highest concentration of synthesized glucosylated substrates. In consequence, according to **Table 8**, the CGTase from *Geobacillus* sp. was selected for SVglys and RebA substrates and CGTase from *Thermoanaerobacter* sp. for MGE.

A comprehensive mass spectrometric approach using LC-ESI-MS and MALDI-TOF MS was carried out to reveal the structural modifications after carrying out the reactions with the optimized conditions (**Table 10**). LC-ESI-MS confirmed the quantitative results obtained with LC-DAD analysis, revealing similar profiles with the same retention times, which chromatographic profiles can be seen in **Figure 14** for SVglys and RebA, and **Figure 15** for MGE, compared with the unmodified substrates. Because the ionization mode was negative, most of the *m/z* data are $[M - H]^-$ ions for the respective glycosides.

Table 10. Optimal reaction conditions for all enzymatic reactions with all the enzymes studied.

Enzyme catalysis	Source	Donor substrate	Donor concentration [g/L]	Acceptor substrate	Acceptor concentration [g/L]	Temperature [ºC]	Buffer [pH]	Enzyme activity [U/mL]	Time [h]
Cyclodextrin glucosyltransferase	*Geobacillus* sp.	Maltodextrin	59.7	Steviol glycosides[a]	60.8	50	5	0.3	6.8
Cyclodextrin glucosyltransferase	*Geobacillus* sp.	Maltodextrin	57.4	Rebaudioside A[c]	51.9	50	5	0.3	1
Cyclodextrin glucosyltransferase	*Thermoanaerobacter* sp.	Maltodextrin	5	Mogroside extract[b]	56.7	50	5	0.3	1
β-fructosidase	*Aspergillus aculeatus*	Sucrose	600	Steviol glycosides[a]	2 – 15	60	5.5	25	24 - 48
β-fructosidase	*Aspergillus aculeatus*	-	-	Steviol glycosides[a]	2 - 15	60	5.5	25	1 - 48
β-fructosidase	*Aspergillus aculeatus*	Sucrose	600	Mogroside extract[b]	2 - 15	60	5.5	25	24 - 48
β-fructosidase	*Aspergillus aculeatus*	-	-	Mogroside extract[b]	2 - 15	60	5.5	25	1 - 48
β-galactosidase	*Aspergillus oryzae*	Lactose	300	Steviol glycosides[a]	2 - 15	40	4.5	5	24 - 48
β-galactosidase	*Aspergillus oryzae*	-	-	Steviol glycosides[a]	2 - 15	40	4.5	5	1 - 48
β-galactosidase	*Aspergillus oryzae*	Lactose	300	Mogroside extract[b]	2 - 15	40	4.5	5	24 - 48
β-galactosidase	*Aspergillus oryzae*	-	-	Mogroside extract[b]	2 - 15	40	4.5	5	1 - 48

[a]Steviol glycosides: 59.4% stevioside, 25.4% rebaudioside A, rebaudioside C 9.2%, rubusoside 1.4%.
[b]Mogroside extract: mogroside V 50.2%, mogroside VI 31.9%, mogroside IV 10.2%, mogroside III 7.7%.
[c]Rebaudiosiode A (96%).

Stevioside and rebaudioside A compounds were not able to be successfully separated in the SVglys substrate analysis, whereas many of the glucosylated derivatives were totally or partially resolved through LC-ESI-MS analysis. As with the initial characterization of the starting substrates (**Figure 14a** and **14c**), some of the glycosides of optimal SVglys and RebA (**Figure 14b** and **14d**) samples were identified by comparison with the retention time and mass spectrum of the commercial standards: rebaudioside A (*m/z* 965.3), stevioside (*m/z* 803.2), rebaudioside C (*m/z* 949.4) and rubusoside (*m/z* 641.3). Other glycosides were tentatively identified by relative retention and molecular masses reported in the literature (Espinoza *et al.* 2014; Pól *et al.* 2007). Chromatographic peaks for higher *m/z* values as 1289.3 and 1451.3 were hypothetically considered as glucosylated glycosides up to seven glucoses in C-13 and/or C-19 positions. **Figure 16** shows the ion traces profiles for the optimal glucosylated SVglys and RebA in which profiles the transglycosylation rate can be depicted.

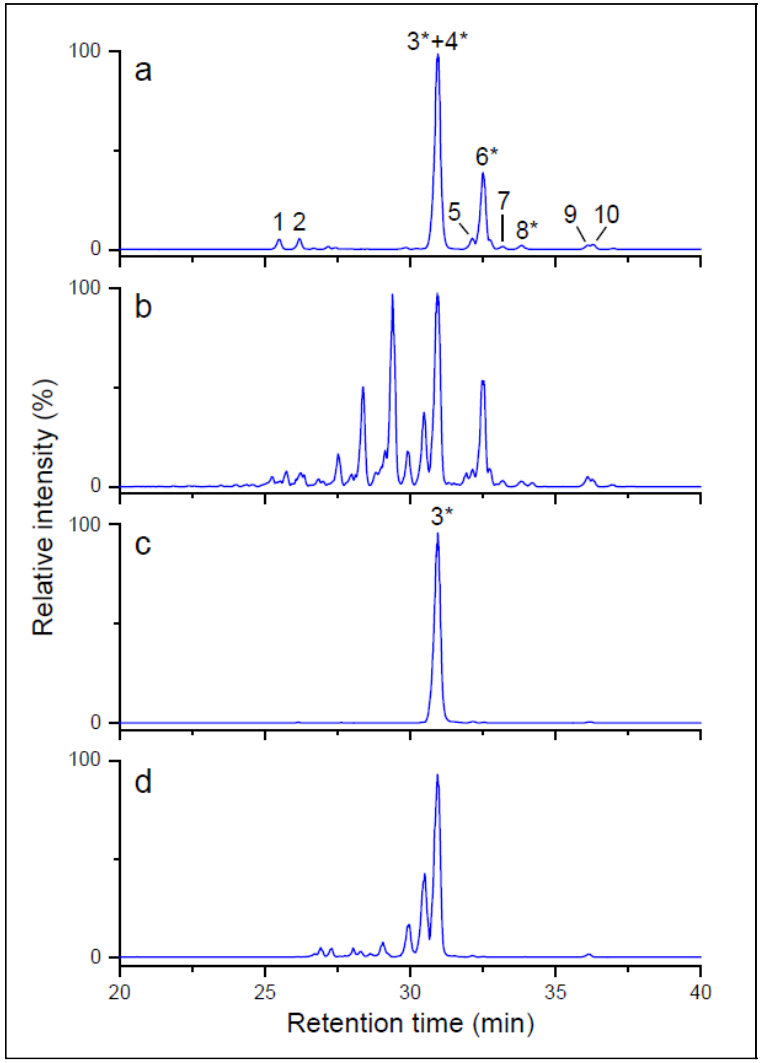


Figure 14. Base peak LC-ESI-MS chromatograms of unmodified SVglys (a), glucosylated SVglys (b), unmodified RebA (c) and glucosylated RebA (d) under optimized conditions using the CGTase from *Geobacillus* sp. Peak identification (peaks marked with asterisks were compared with the respective commercial standards): 1, *m/z* 965.3 (unknown); 2, *m/z* 1127.4 (rebaudioside D); 3, *m/z* 965.3 (rebaudioside A); 4, *m/z* 803.3 (stevioside); 5, *m/z* 935.3 (rebaudioside F); 6, *m/z* 949.6 (rebaudioside C); 7, *m/z* 803.3 (rebaudioside G); 8, *m/z* 641.3 (rubusoside); 9, *m/z* 803.3 (rebaudioside B); 10, *m/z* 641.3 (steviolbioside).

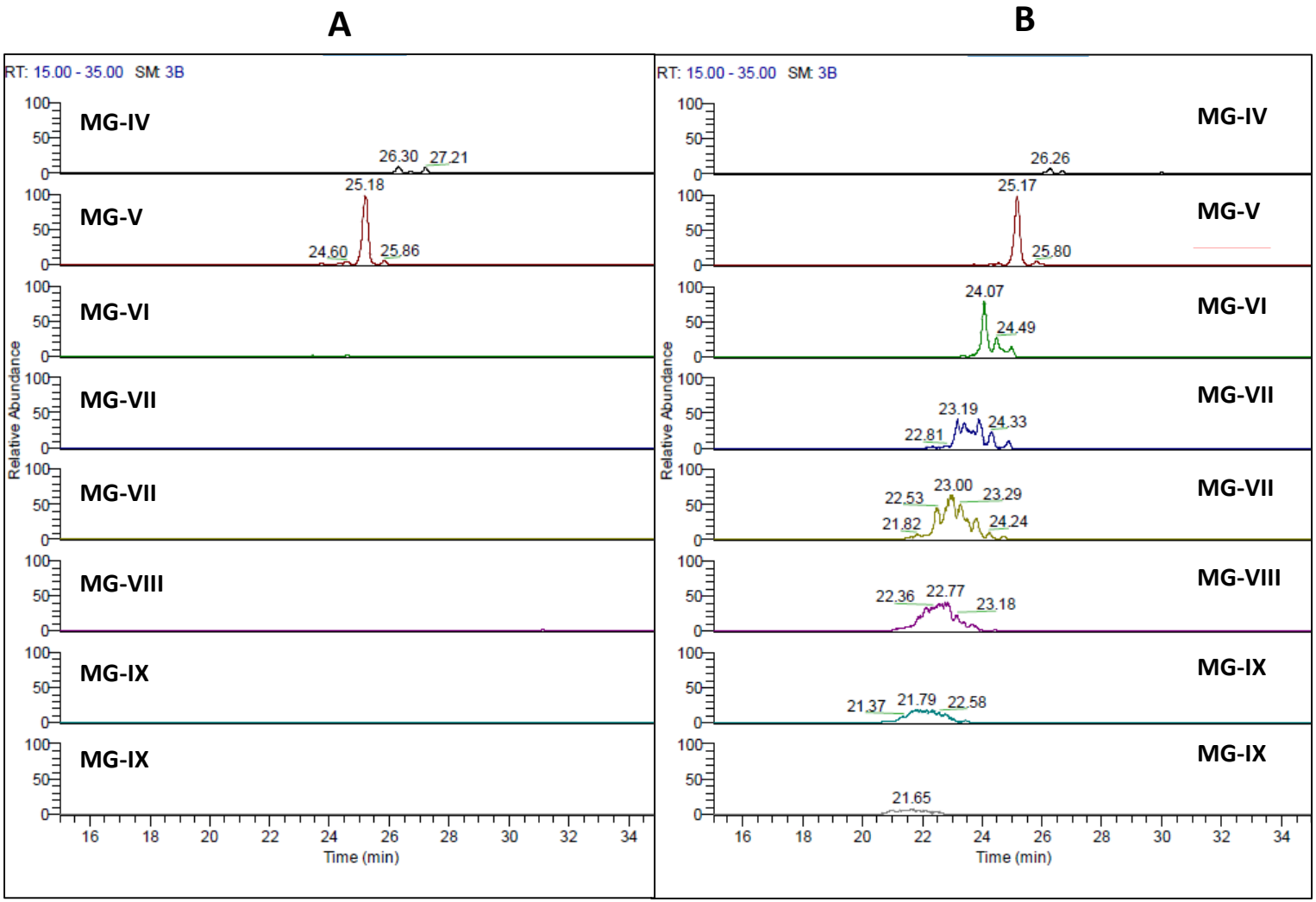


Figure 15. LC–MS ion traces profiles of the unmodified mogroside mixture (A) and the glucosylated mixture obtained under optimized conditions using CGTases from *Thermoanaerobacter* sp. (B).

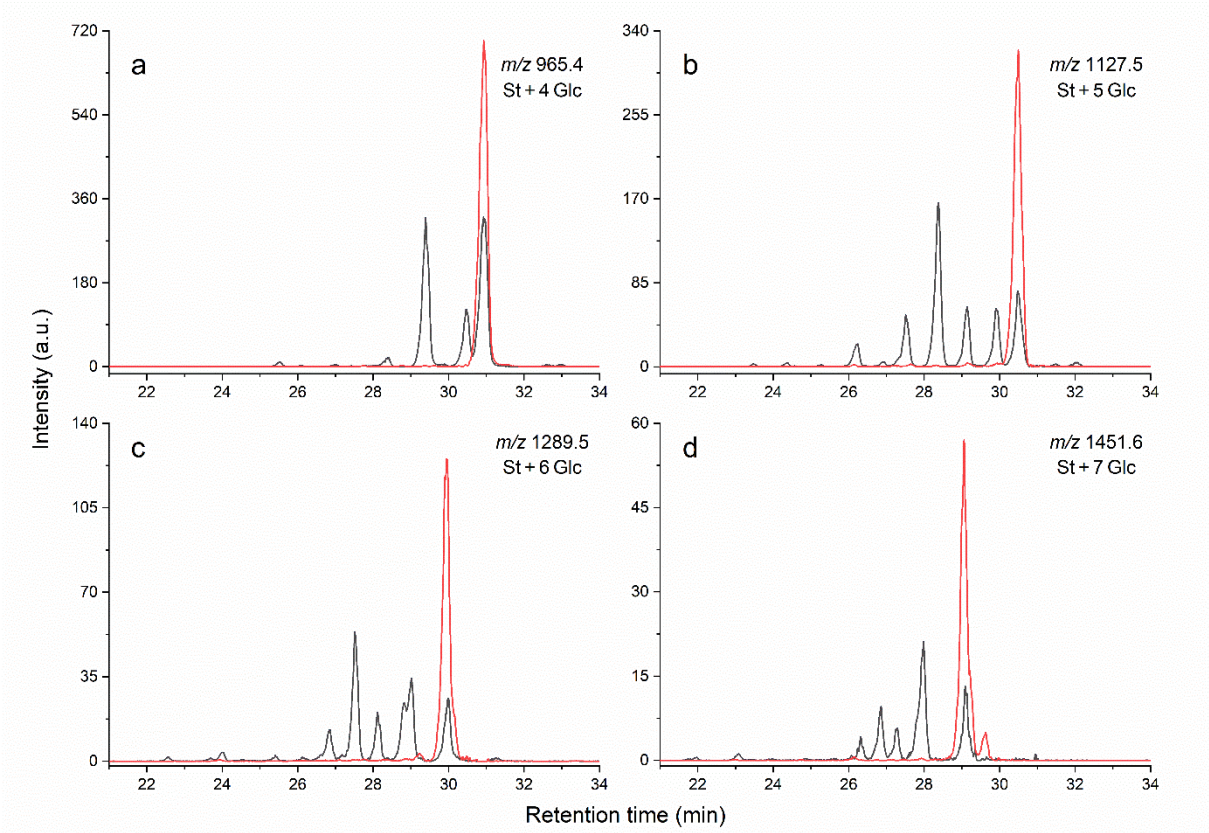


Figure 16. Peaks *m/z* 965.4 (a), *m/z* 1127.5 (b), *m/z* 1289.5 (c), and *m/z* 1451.6 (d): mass spectra of glucosylated products of SVglys (black chromatogram) and RebA (red chromatogram).

Likewise, due to the lack of structural information generated by LC-DAD and the impossibility of identifying if mogroside V and/or other minor mogrosides present in the mixture are glucosylated, an LC-ESI-MS analysis was suitable for this purpose. **Figure 15** confirmed the glucosylation of the mogroside structures by the identification of new peaks with lower retention times (**Figure 15B**) when compared with the initial mixture (**Figure 15A**), and by the $[M - H]^-$ ions corresponding to the newly synthesized mogrosides with up to four new glucose units. Due to the low sensitivity of LC-ESI-MS to higher-molecular-weight compounds, mogrosides with more than nine glycoside units could not be identified by this technique.

However, the LC-ESI-MS profiles also provided information about the different isomers present in the unmodified extract and the transglycosylation rate on specific isomers of the enzymatically modified sample (**Figure 17**). Seven potential mogroside IV isomers (25.8, 26.3, 26.7, 27.0, 27.2, 27.6, and 27.9) were revealed in the unmodified sample (*m/z* 1123.6), when

only two mogroside IV structures have been described: mogroside IVE and mogroside IVA. This is the first time that five additional different potential mogrosides IV have been described. LC–MS data allowed us to determine the ratio of mogroside V and mogroside IV in the unmodified and glucosylated mogroside samples. The most abundant mogroside was mogroside V (*m/z* 1285.5) followed by the mogroside IV isomers (*m/z* 1123.0). The area ratio of mogroside IV/mogroside V was similar in two of the mogroside IV isomers, indicating that the rate of glucosylation is similar between mogroside V and these mogroside IV isomers. However, four of the mogroside IV isomers were more readily glucosylated by the CGTase than mogroside V. Also, after the enzymatic reaction, a new mogroside IV (30.02 min) was synthesized. This new mogroside IV could be a glucosylated form of the minor mogroside III isomers presented in the initial mixture. Interestingly, **Figure 17** also shows similar behaviour in mogroside III compared with mogroside IV. The main two mogroside III isomers decreased and one of mogroside III isomers increased (29.70 min).

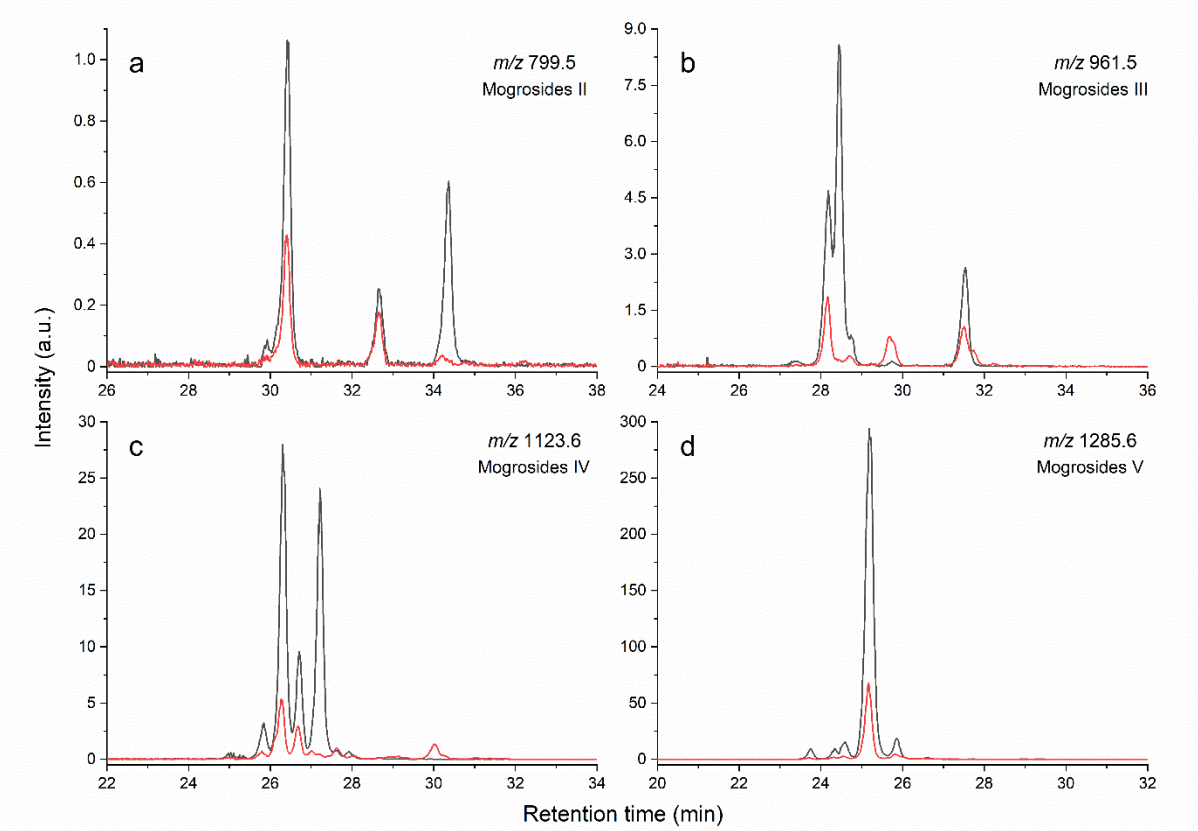


Figure 17. LC–MS ion trace profiles of the initial unmodified mogroside mixture (black line) and the glucosylated mixture (red line) obtained under optimized conditions using CGTases from *Thermoanaerobacter* sp.

In accordance with the results obtained by LC-DAD and LC-ESI-MS, MALDI-TOF MS analysis showed the presence of glucosylated structures as well as that the transglucosylation was equally efficient for SVglys and RebA. However, MALDI-TOF spectra showed a series of $[M + Na]^+$ ions, indicating extension glycoside chains for the three CGTases on each substrate compared to their unmodified structures.

The MALDI-TOF MS profiles of the unmodified SVglys and RebA and their corresponding glucosylated forms catalyzed by the optimal CGTase (*Geobacillus* sp.) can be seen in **Figure 18**. Glucosylation of a mixture of steviol glycosides at both C-13 and C-19 sites by CGTase from *Bacillus subtilis* was previously described. Both enzymatically modified structures contained up to 11 glucose residues, respectively, indicating successful transglucosylation at the C-13 and/or C-19 sites of the steviol aglycone for both samples.

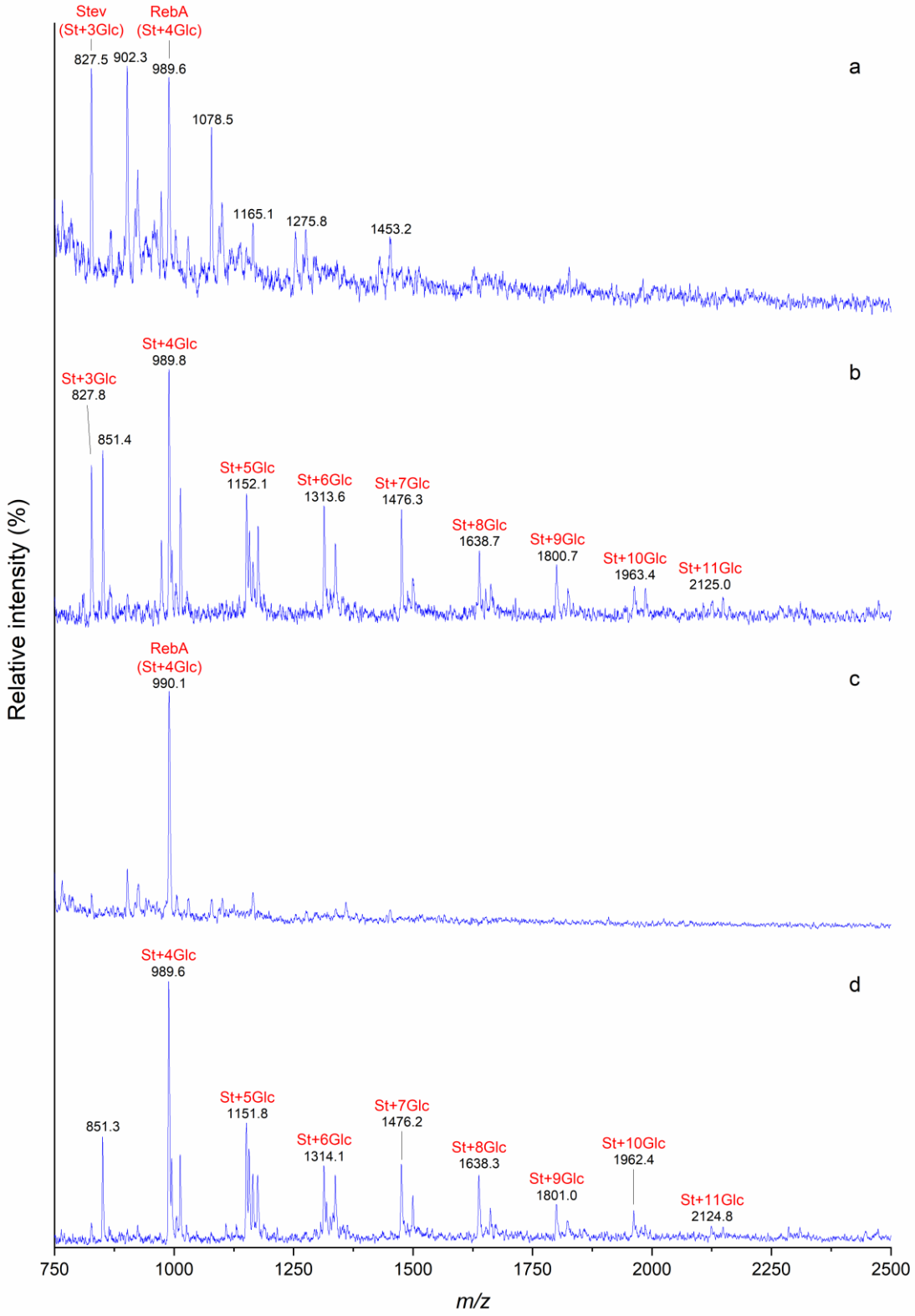


Figure 18. MALDI-TOF MS profile of unmodified SVglys (a), glucosylated SVglys (b), unmodified RebA (c) and glucosylated RebA (d) optimized conditions using the CGTase from *Geobacillus* sp. St: steviol; Stev: stevioside; RebA: rebaudioside A; Glc: glucose.

In **Figure 19**, a comparison between the MALDI-TOF spectra of the unmodified MGE and the MGE modified with the CGTase from *Thermoanaerobacter* sp. can be seen. The MALDI-TOF profile finally revealed that the modified sample (**Figure 19B**) showed a high glucosylation activity of up to 12 units of glucose. Deglucosylation activity was not detected and a significant amount of mogrosides IV and III ([M + Na]$^+$ 1148.3 and 986.2, respectively) were not produced.

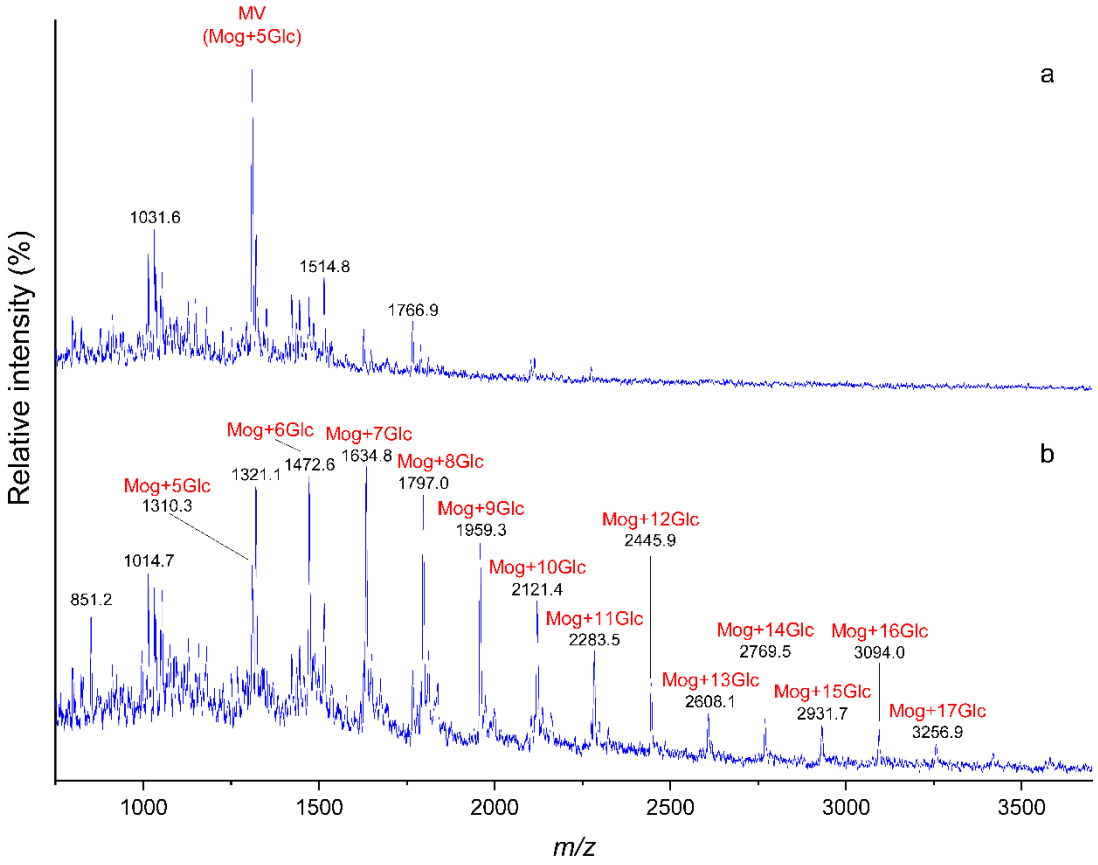


Figure 19. MALDI-TOF MS profiles of unmodified mogrosides (a) and after the glucosylation under optimized conditions, using CGTases from *Thermoanaerobacter* sp. (b). Mog: Mogrol; Glc: glucose.

4.1.3 One-pot enzymatic synthesis of prebiotic oligosaccharides (GOS or FOS) and modified steviol glycosides

4.1.3.1 Optimization conditions for enzymatic synthesis

Two types of binary mixtures consisting of SVglys (at five different concentrations: 0.2, 1.5, 5, 10 and 20%; w:v) and a disaccharide, *i.e.* lactose (30%; w:v) or sucrose (60%; w:v), were incubated with the specific enzyme involved, β-galactosidase or β-fructosidase. In both cases, RP-LC-DAD revealed qualitative and quantitative changes in the chromatographic profiles of only those syntheses performed with SVglys at 0.2 and 1.5% as compared to the control (non-incubated) SVglys, indicating that concentrations of SVglys equal or greater than 5% inhibited both enzymes after 24 and 48 h (data not shown).

Once the two lowest concentrations of SVglys were selected, the next step was to determine the optimal concentration for both enzymes based on the simultaneous effect of (i) modification of the SVglys profile and (ii) the yield of prebiotic oligosaccharides formed. Consequently, 5 U and 25 U of β-galactosidase and β-fructosidase per mL of reaction mixture respectively, were considered as the most appropriate enzyme doses to perform the subsequent reactions over 48 h (**Table 10**).

4.1.3.1.1 <u>Exploring the enzymatic kinetics of the two enzymes on the single substrates</u>

SVglys, as well as lactose and sucrose, were individually incubated with the respective enzymes in order to fully elucidate their main glycosidase activities on either the stevia components and the disaccharides before the following kinetics based on the one-pot synthesis using a combination of SVglys and carbohydrates.

When both enzymes were individually incubated with the SVglys, a notable decrease of the tri-glycoside stevioside together with a concomitant increase of the di-glycoside rubusoside was observed in both cases, whereas the rebaudiosides A and C remained unaltered. This selective mono-deglycosylation of stevioside was much more prominent in the case of β-galactosidase (**Figure 20B**) than that observed following the action of β-fructosidase (**Figure 20D**).

As could be expected, β-galactosidase synthesized GOS from lactose (30%; w:v), which were dominated by the trisaccharide fraction; 6'-galactosyl-lactose being the main carbohydrate, followed by disaccharide whose more abundant structure was 6'-galactosyl-glucose and tetrasaccharide fractions (**Figure 21A**). The incubation of sucrose at 60% (w:w) alone with the β-fructosidase enzyme preparation led to the efficient synthesis of kestose (an inulin-type FOS) (peak 8 in **Figure 21C**), the main oligosaccharide formed during the first six-hour, followed by nystose and fructosyl-nystose, respectively (peaks 9 and 10, respectively, in **Figure 21C**). The β-furanosidase activity of this enzyme preparation has been already described by other authors (Lorenzoni *et al.* 2014; Vega-Paulino *et al.* 2012).

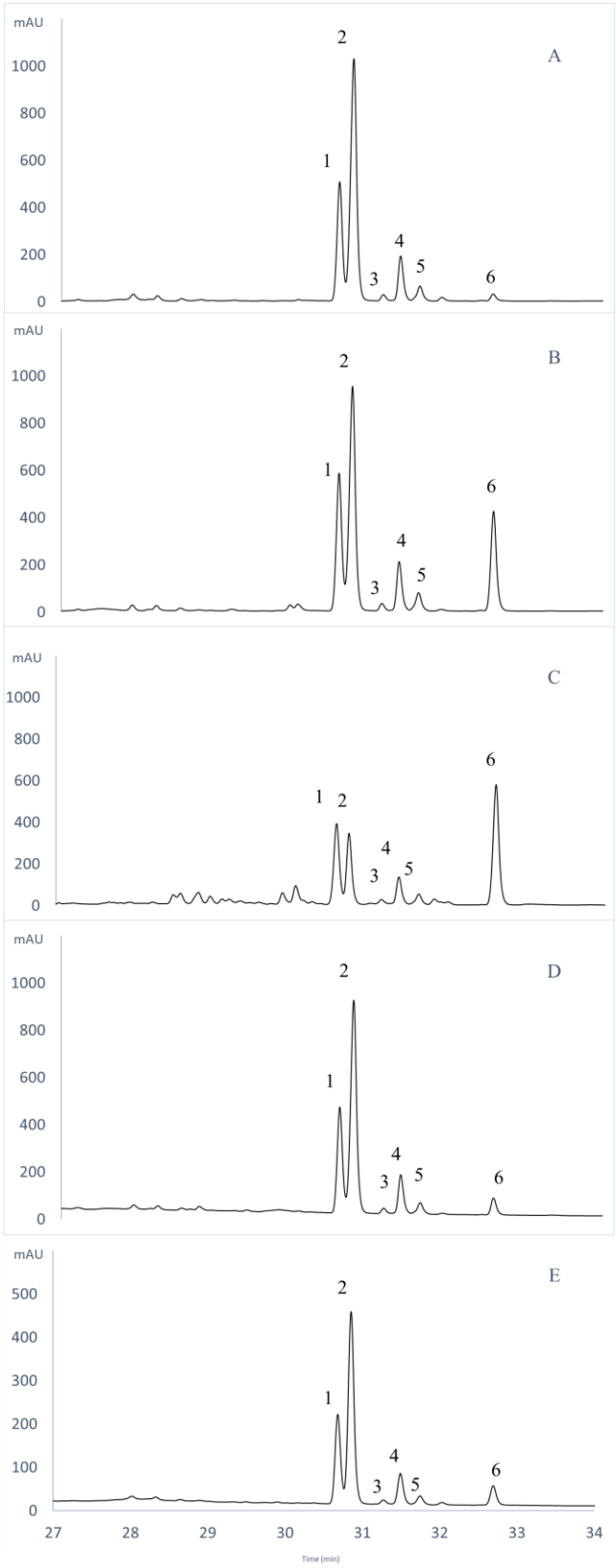


Figure 20. RP-LC-DAD profiles of (A) non-modified SVglys and modified SVglys with (B) β-galactosidase and (C) with the respective carbohydrate, lactose, and (D) with β-fructosidase (E) with the respective carbohydrate sucrose. Labelled peaks are as follows: peak 1, rebaudioside A; peak 2, stevioside; peak 3, unknown steviol glycoside; peak 4, rebaudioside C; peak 5, unknown steviol glycoside; peak 6, rubusoside.

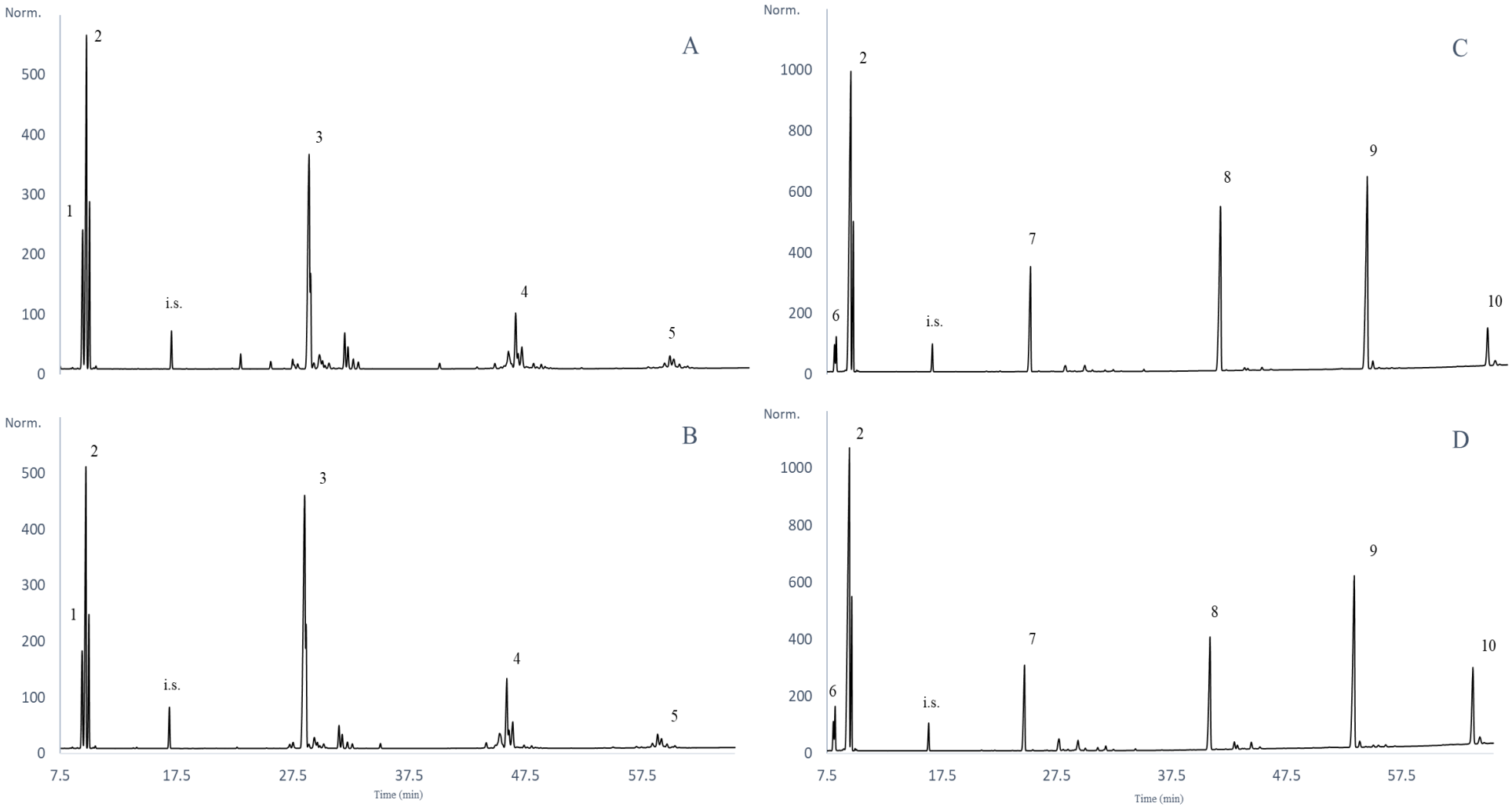


Figure 21. Chromatographic profiles obtained by GC-FID showing: (A) GOS synthesis in the presence of lactose (30%, w:w), (B) GOS synthesis in the presence of lactose (30%, w:w) and steviol glycosides (0.2%, w:w), (C) FOS synthesis in the presence of sucrose (60%, w:w) and (D) FOS synthesis in the presence of sucrose (60%, w:w) and steviol glycosides (0.2%, w:w). Labelled peaks are as follows: peak 1, galactose; peak 2, glucose; peak 3, lactose; peak 4, GOS-trisaccharides; peak 5, GOS-tetrasaccharides; peak 6, fructose; peak 7, sucrose; peak 8, kestose; peak 9, nystose; peak 10, fructosyl-nystose; i.s., internal standard.

4.1.3.1.2 One-pot enzymatic synthesis of modified SVglys with galacto-oligosaccharides (GOS)

Table 11 summarizes the evolution in content of stevia components after 48 h of enzymatic reaction with the two SVglys concentrations chosen by RP-LC-DAD profiles. Two main changes were found in the stevia components originally present: i) mono-deglycosylation of stevioside into rubusoside, which was most pronounced at a starting glycoside concentration of 0.2% (only 5% of initial stevioside remained after 48 h of reaction) versus 1.5% (27.5% of stevioside remained at the end of the reaction); and ii) increase (around 5-fold with both glycoside concentrations) in a series of new hydrophilic compounds (**Figure 22**) that could be due to transglycosylation of glycosides giving rise to elongation of the carbohydrates at C-19 and/or C-13. A maximum formation of the new hydrophilic compounds was quite stable and plateau between 1 and 12 h and 4 and 12 h were achieved using initial glycoside concentrations of 0.2 and 1.5%, respectively (**Table 11**).

With respect to the synthesis of GOS under the presence of SVglys, neither qualitative (**Figure 21B**) nor quantitative (**Figure 23B** and **23C**) were determined by GC-FID as compared to the synthesis in the absence of stevia components (**Figures 21A** and **23A**). The maximum GOS yield (expressed as grams of di-, tri-, and tetrasaccharides per 100 grams of starting lactose) in all reactions was around 31%. Likewise, the maximum concentration of total GOS (~100 g/L) was achieved between 4 and 12 h of reaction in the case of lactose alone. In the mixture of lactose and steviol glycosides at 0.2% and 1.5%, the maximum yield was ~80 g/L (**Figure 23**). Finally, from the first until the sixth hour of reaction, the trisaccharide fraction was the predominant GOS fraction, whereas the disaccharide fraction was the most abundant from the twelfth hour until the end of the reaction (**Figure 23**). This could be attributed to the hydrolysis of the tri- and tetrasaccharide fractions to disaccharides.

Table 11. Concentration (mg/mL ± standard deviation) of the different compounds synthesized during the reaction with (A) SVglys at 0.2% (w:w) and (B) at 1.5% (w:w) using β-galactosidase.

A

		Reaction time (hours)															
		0		1		2		4		6		12		24		48	
Peak number[a]	Compound	Concn ± SD[b] (mg/mL)	R (%)[c]	Concn ± SD[b] (mg/mL)	R (%)[c]	Concn ± SD[b] (mg/mL)	R (%)[c]	Concn ± SD[b] (mg/mL)	R (%)[c]	Concn ± SD[b] (mg/mL)	R (%)[c]	Concn ± SD[b] (mg/mL)	R (%)[c]	Concn ± SD[b] (mg/mL)	R (%)[c]	Concn ± SD[b] (mg/mL)	R (%)[c]
	Total hydrophilic	0.09 ± 0.01[c]	4.32	0.48 ± 0.01	20.68	0.49 ± 0.01	22.33	0.48 ± 0.01	22.85	0.49 ± 0.02	23.77	0.51 ± 0.05	22.58	0.39 ± 0.00	18.48	0.29 ± 0.00	13.19
1	Rebaudioside A	0.51 ± 0.03	24.12	0.48 ± 0.01	20.68	0.45 ± 0.00	20.29	0.42 ± 0.00	20.10	0.41 ± 0.01	19.79	0.45 ± 0.05	19.95	0.44 ± 0.02	20.87	0.50 ± 0.00	22.65
2	Stevioside	1.23 ± 0.07	57.91	0.90 ± 0.02	38.87	0.77 ± 0.00	34.91	0.61 ± 0.01	29.04	0.50 ± 0.01	24.30	0.37 ± 0.04	16.44	0.178 ± 0.00	8.30	0.06 ± 0.00	2.65
3	Unknown 1	0.02 ± 0.00	0.83	0.02 ± 0.00	0.80	0.02 ± 0.00	0.76	0.02 ± 0.00	0.76	0.01 ± 0.00	0.75	0.02 ± 0.01	0.93	0.02 ± 0.00	0.86	0.020 ± 0.00	0.91
4	Rebaudioside C	0.18 ± 0.012	8.37	0.16 ± 0.01	6.87	0.14 ± 0.00	6.55	0.13 ± 0.00	6.31	0.13 ± 0.00	6.18	0.14 ± 0.01	6.27	0.15 ± 0.01	6.90	0.17 ± 0.00	7.51
5	Unknown 2	0.07 ± 0.01	3.15	0.06 ± 0.01	2.57	0.05 ± 0.00	2.49	0.05 ± 0.00	2.46	0.05 ± 0.00	2.43	0.06 ± 0.00	2.56	0.06 ± 0.00	2.80	0.06 ± 0.00	2.66
6	Rubusoside	0.03 ± 0.01	1.30	0.22 ± 0.01	9.52	0.28 ± 0.00	12.67	0.39 ± 0.01	18.49	0.47 ± 0.00	22.80	0.70 ± 0.07	31.27	0.89 ± 0.03	41.78	1.12 ± 0.00	50.42
	Total	2.02		2.36		2.24		2.15		2.10		2.31		2.20		2.28	

B

		Reaction time (hours)															
		0		1		2		4		6		12		24		48	
Peak number[a]	Compound	Concn ± SD[b] (mg/mL)	R (%)[c]	Concn ± SD[b] (mg/mL)	R (%)[c]	Concn ± SD[b] (mg/mL)	R (%)[c]	Concn ± SD[b] (mg/mL)	R (%)[c]	Concn ± SD[b] (mg/mL)	R (%)[c]	Concn ± SD[b] (mg/mL)	R (%)[c]	Concn ± SD[b] (mg/mL)	R (%)[c]	Concn ± SD[b] (mg/mL)	R (%)[c]
	Total hydrophilic	0.73 ± 0.07[c]	6.55	2.28 ± 0.08	17.68	2.55 ± 0.02	20.22	3.15 ±0.34	24.42	3.36 ± 0.05	25.67	3.04 ± 0.14	24.12	2.69 ± 0.01	21.55	2.29 ± 0.04	17.72
1	Rebaudioside A	2.86 ± 0.17	25.86	2.99 ± 0.06	23.15	2.78 ± 0.03	22.09	2.65 ±0.02	20.52	2.61 ± 0.01	19.93	2.49 ± 0.01	19.70	2.50 ± 0.06	20.05	2.75 ± 0.04	21.28
2	Stevioside	5.71 ± 0.28	51.58	5.41 ± 0.06	41.93	4.98 ± 0.05	39.58	4.55 ±0.02	35.19	4.26 ± 0.04	32.51	3.50 ± 0.07	27.76	2.57 ± 0.06	20.60	1.58 ± 0.14	12.18
3	Unknown 1	0.12 ± 0.01	1.05	0.14 ± 0.01	1.07	0.13 ± 0.00	1.02	0.12 ±0.00	0.95	0.12 ± 0.00	0.92	0.12 ± 0.00	0.99	0.13 ± 0.00	1.03	0.14 ± 0.00	1.11
4	Rebaudioside C	1.05 ± 0.07	9.48	1.08 ± 0.02	8.35	0.99 ± 0.01	7.88	0.93 ±0.00	7.18	0.90 ± 0.00	6.85	0.85 ± 0.00	6.73	0.86 ± 0.02	6.88	0.97 ± 0.01	7.47
5	Unknown 2	0.42 ± 0.03	3.80	0.44 ± 0.01	3.39	0.40 ±0.00	3.19	0.38 ±0.00	2.95	0.37 ± 0.00	2.85	0.36 ± 0.00	2.87	0.37 ± 0.01	2.93	0.40 ± 0.00	3.10
6	Rubusoside	0.19 ± 0.01	1.67	0.57 ± 0.02	4.44	0.76 ±0.03	6.02	1.14 ±0.09	8.81	1.48 ± 0.09	11.27	2.25 ± 0.10	17.82	3.36 ± 0.20	26.96	4.80 ± 0.18	37.13
	Total	11.19		13.04		12.72		13.05		13.23		12.71		12.61		13.10	

[a]Labelled peaks as in Figure 20. [b]Standard deviation values (n = 2).[c]Relative % data were calculated considering the total compound values shown above.

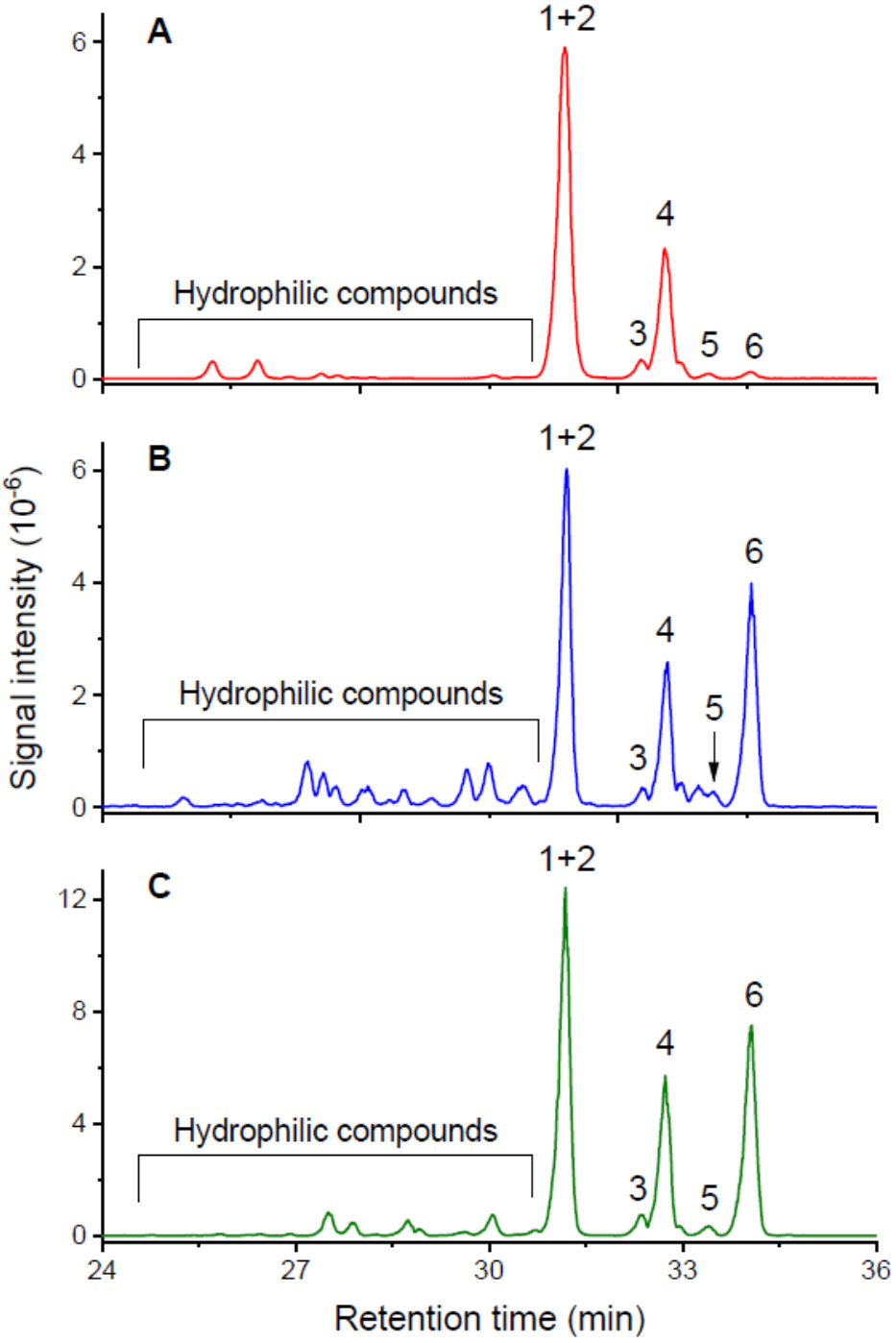


Figure 22. Base peak LC-ESI-MS chromatograms obtained for (A) non-modified steviol glycosides and modified-steviol glycosides (0.2%, w:w) with (B) β-galactosidase in the presence of lactose (30%, w:w), and with (C) β-fructosidase in the presence of sucrose (60%, w:w). Peaks are labelled as in Figure 20.

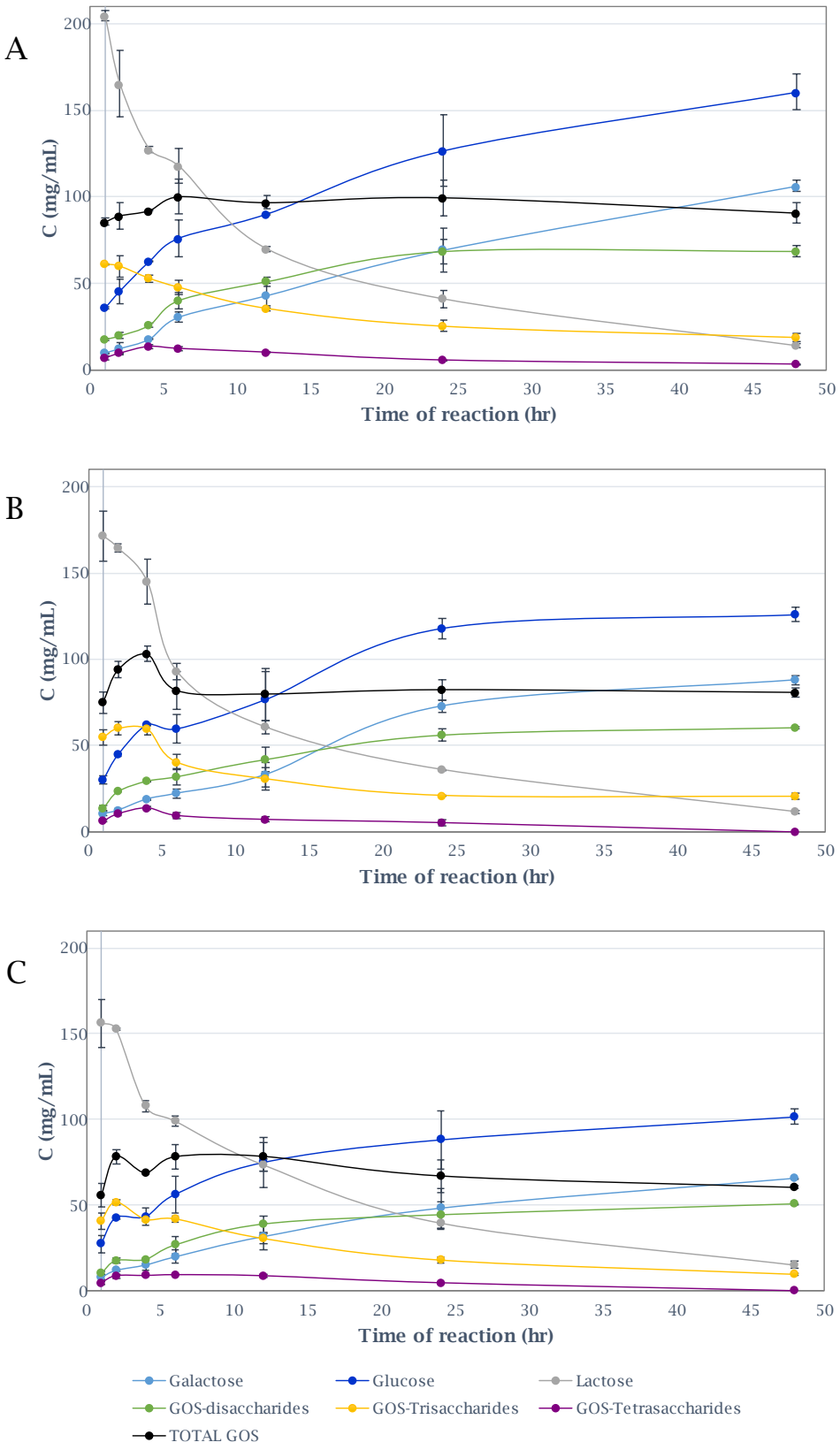


Figure 23. Evolution of carbohydrates concentration as determined by GC-FID throughout the enzymatic reaction of (A) GOS synthesis in the presence of lactose (30%, w:w), (B) GOS synthesis in the presence of lactose (30%, w:w) and steviol glycosides (0.2%, w:w) and (C) GOS synthesis in the presence of lactose (30%, w:w) and steviol glycosides (1.5%, w:w).

4.1.3.1.3 One-pot enzymatic synthesis of modified SVglys with fructo-oligosaccharides (FOS)

Table 12 displays the content of stevia components after the enzymatic reaction at the two SVglys concentrations as determined by RP-LC-DAD. Observed changes were in line with the findings for the synthesis of GOS and modified steviol glycosides. Thus, around 50% of stevioside was deglycosylated, whereas significant increases in rubusoside (a maximum of 6-fold at 24 h of reaction) and hydrophilic compounds (a maximum of 3-fold at 4 h) were also determined at a starting steviol glycosides concentration of 0.2% (**Table 12A**). A 27% of decrease in stevioside together with a maximum 4.8-fold increase in rubusoside were observed at a glycoside concentration of 1.5%, with no significant increase in hydrophilic compounds as determined by RP-LC-DAD (**Table 12B**).

Neither qualitative (**Figure 21**) nor quantitative differences (**Figure 24**) in FOS synthesis were seen in the presence or absence of SVglys. A similar yield of inulin-type FOS (around 80%, expressed as grams of FOS ≥ DP 2 per 100 grams of starting sucrose) was determined in all reactions. Initially, kestose was the main FOS synthesized and the maximum FOS concentration was reached from the sixth hour onwards (~500 g/L) in all reactions. From the sixth hour of reaction, nystose was the most abundant FOS (**Figure 24D**). Lastly, a FOS-hexasaccharide (probably, the difructosyl-nystose) could be detected from the twelfth hour of the enzymatic reaction.

Table 12. Concentration (mg/mL ± standard deviation) of the different compounds synthesized during the reaction with (A) SVglys at 0.2% (w:w) and (B) at 1.5% (w:w) using β-fructosidase.

A

		Reaction time (hours)															
		0		1		2		4		6		12		24		48	
Peak number[a]	Compound	Concn ± SD[b] (mg/mL)	R (%)[c]	Concn ± SD[b] (mg/mL)	R (%)[c]	Concn ± SD[b] (mg/mL)	R (%)[c]	Concn ± SD[b] (mg/mL)	R (%)[c]	Concn ± SD[b] (mg/mL)	R (%)[c]	Concn ± SD[b] (mg/mL)	R (%)[c]	Concn ± SD[b] (mg/mL)	R (%)[c]	Concn ± SD[b] (mg/mL)	R (%)[c]
	Total hydrophilic	0.04 ± 0.01[c]	1.71	0.07 ± 0.01	3.64	0.05 ± 0.01	2.62	0.12 ± 0.01	4.65	0.04 ± 0.01	2.35	0.06 ± 0.00	3.10	0.07 ± 0.01	3.57	0.07 ±0.01	4.53
1	Rebaudioside A	0.61 ± 0.05	23.54	0.49 ± 0.05	24.66	0.47 ± 0.00	23.44	0.62 ± 0.11	23.95	0.45 ± 0.01	25.32	0.52 ± 0.02	25.28	0.54 ± 0.02	27.70	0.54 ±0.01	32.78
2	Stevioside	1.63 ± 0.04	63.12	1.37 ± 0.14	69.43	1.32 ± 0.10	66.61	1.52 ± 0.09	58.71	1.12 ± 0.08	62.46	1.25 ± 0.05	60.29	1.07 ±0.02	54.37	0.81 ±0.04	49.29
3	Unknown 1	0.02 ± 0.00	0.75	0.01 ± 0.00	0.12	0.01 ± 0.00	0.31	0.02 ± 0.00	0.63	0.01 ± 0.00	0.40	0.01 ± 0.00	0.40	0.01 ±0.00	0.38	0.01 ±0.00	0.41
4	Rebaudioside C	0.18 ± 0.02	6.89	0.09 ± 0.01	0.71	0.08 ± 0.00	4.11	0.16 ± 0.01	6.12	0.08 ± 0.01	4.38	0.08 ± 0.01	4.07	0.08 ±0.01	3.95	0.07 ±0.01	4.44
5	Unknown 2	0.07 ± 0.01	2.73	0.03 ± 0.00	0.28	0.02 ± 0.00	1.25	0.05 ± 0.00	1.94	0.02 ± 0.00	1.17	0.02 ± 0.00	0.96	0.01 ±0.00	0.70	0.01 ±0.00	0.61
6	Rubusoside	0.03 ± 0.00	1.27	0.02 ± 0.00	1.16	0.03 ± 0.00	1.66	0.10 ± 0.01	3.99	0.07 ± 0.01	3.92	0.12 ± 0.01	5.91	0.18 ±0.00	9.33	0.13 ±0.18	7.95
	Total	2.41		2.09		2.00		2.59		1.80		2.07		1.97		1.64	

B

		Reaction time (hours)															
		0		1		2		4		6		12		24		48	
Peak number	Compound	Concn ± SD[b] (mg/mL)	R (%)[c]	Concn ± SD[b] (mg/mL)	R (%)[c]	Concn ± SD[b] (mg/mL)	R (%)[c]	Concn ± SD[b] (mg/mL)	R (%)[c]	Concn ± SD[b] (mg/mL)	R (%)[c]	Concn ± SD[b] (mg/mL)	R (%)[c]	Concn ± SD[b] (mg/mL)	R (%)[c]	Concn ± SD[b] (mg/mL)	R (%)[c]
	Total hydrophilic	0.33 ±0.06	2.63	0.26 ± 0.01	2.40	0.25 ± 0.02	2.38	0.26 ± 0.01	2.59	0.27 ± 0.01	2.72	0.27 ± 0.01	2.65	0.30 ± 0.01	3.16	0.35 ± 0.01	3.68
1	Rebaudioside A	3.16 ±0.00	25.16	3.02 ± 0.04	27.61	2.93 ± 0.04	27.44	2.75 ± 0.12	27.05	2.66 ± 0.09	27.03	2.77 ± 0.05	26.76	2.52 ± 0.08	26.77	2.58 ± 0.02	26.93
2	Stevioside	7.02 ±0.06	55.82	6.71 ± 0.07	61.43	6.56 ± 0.05	61.46	6.24 ± 0.33	61.33	5.96 ± 0.38	60.59	6.21 ± 0.00	60.08	5.44 ± 0.27	57.80	5.13 ± 0.02	53.59
3	Unknown 1	0.17 ±0.04	1.36	0.06 ± 0.00	0.52	0.05 ± 0.00	0.51	0.28 ± 0.33	2.77	0.05 ± 0.00	0.51	0.05 ± 0.00	0.49	0.04 ± 0.00	0.48	0.05 ± 0.01	0.48
4	Rebaudioside C	1.21 ±0.01	9.65	0.54 ± 0.01	4.91	0.52 ± 0.01	4.91	0.33 ± 0.21	3.20	0.50 ± 0.00	5.04	0.51 ± 0.00	4.92	0.46 ± 0.02	4.93	0.48 ± 0.01	5.06
5	Unknown 2	0.50±0.00	3.97	0.19 ± 0.00	1.77	0.18 ± 0.00	1.73	0.11 ± 0.08	1.05	0.17 ± 0.00	1.68	0.16 ± 0.00	1.55	0.13 ± 0.01	1.41	0.12 ± 0.01	1.24
6	Rubusoside	0.18 ±0.01	1.41	0.15 ± 0.00	1.36	0.17 ± 0.01	1.58	0.21 ± 0.04	2.02	0.24 ± 0.00	2.43	0.37 ± 0.01	3.55	0.51 ± 0.02	5.46	0.86 ± 0.02	9.02
	Total	11.47		10.93		10.67		10.18		10.20		10.28		9.42		9.57	

[a]Labelled peaks as in Figure 1. [b]Relative % data were calculated considering the total compound values shown above. [c]Standard deviation values (n = 2).

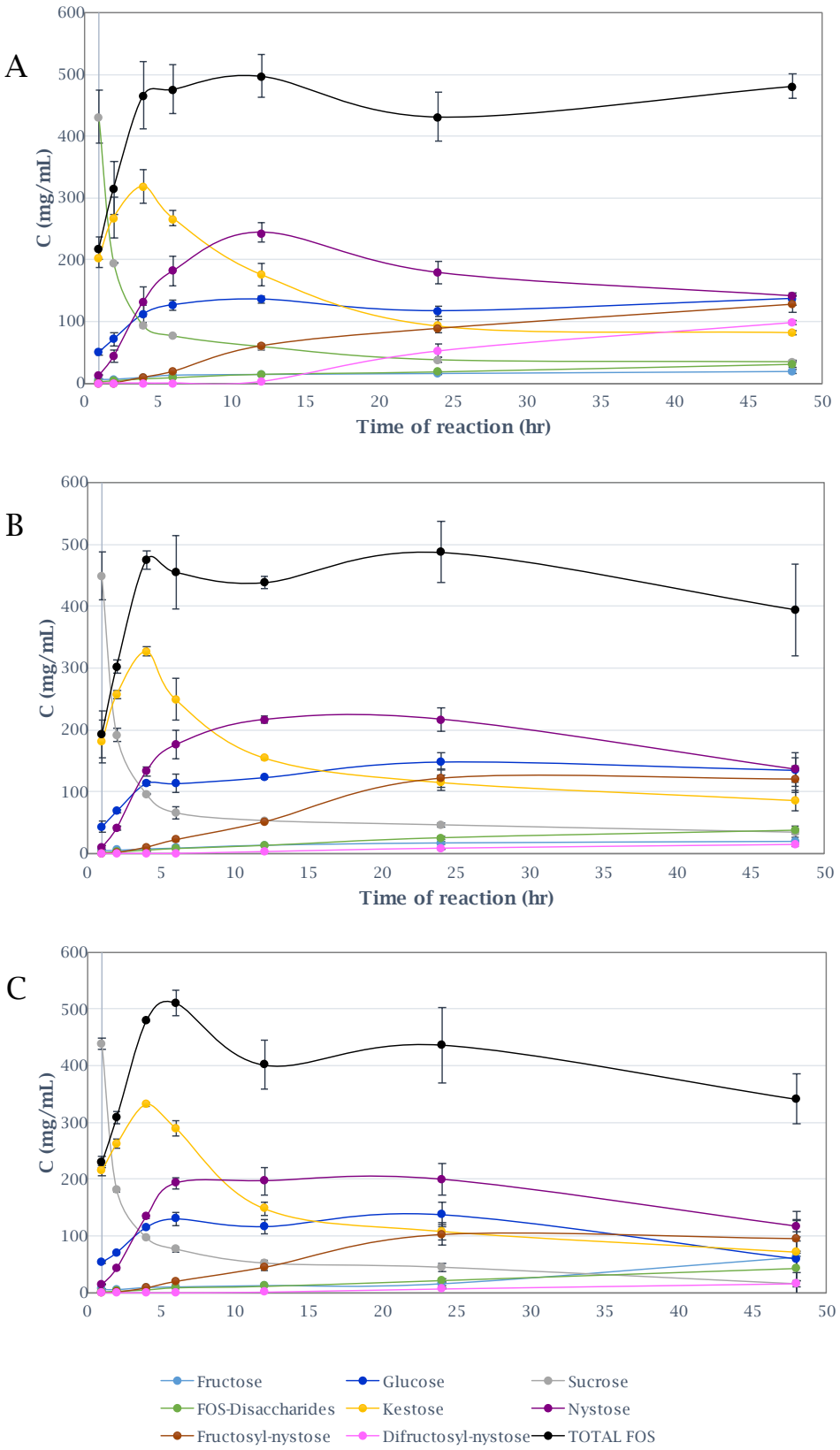


Figure 24. Evolution of carbohydrates concentration as determined by GC-FID throughout the enzymatic reaction of (A) FOS synthesis in the presence of sucrose (60%, w:w), (B) FOS synthesis in the presence of sucrose (60%, w:w) and steviol glycosides (0.2%, w:w) and (C) FOS synthesis in the presence of sucrose (30%, w:w) and steviol glycosides (1.5%, w:w).

4.1.3.2 Characterization of the modified SVglys and prebiotic oligosaccharides by Mass spectrometry

Product structures were characterized by RP-LC-ESI-MS in the negative mode and MALDI-TOF MS. The main stevia components were identified on the basis of the $[M-H]^-$ ions and the comparison of their respective retention times as compared to the individual and commercial standards. Thus, rebaudioside A, stevioside, rebaudioside C, and rubusoside showed *m/z* values of 965.3, 803.2, 949.3, and 641.3 as their respective $[M-H]^-$ ions (**Figure 25**). The MS spectrum of rubusoside formed by deglycosylation showed an *m/z* value of 687.2 (**Figure 25**); this, according to Richman *et al.* (2005), is due to the presence of formate adducts. Furthermore, MS spectra of the unknown peak labelled as 3 in **Figure 22** showed a $[M-H]^-$ ion with an *m/z* value of 935.3 that could be tentatively identified as rebaudioside F or R, whilst that of the unknown peak 5 gave rise to an *m/z* value of 803.2 that could correspond to a wide range of stevia components such as rebaudiosides K, A, B, G or stevioside B (**Figure 25**).

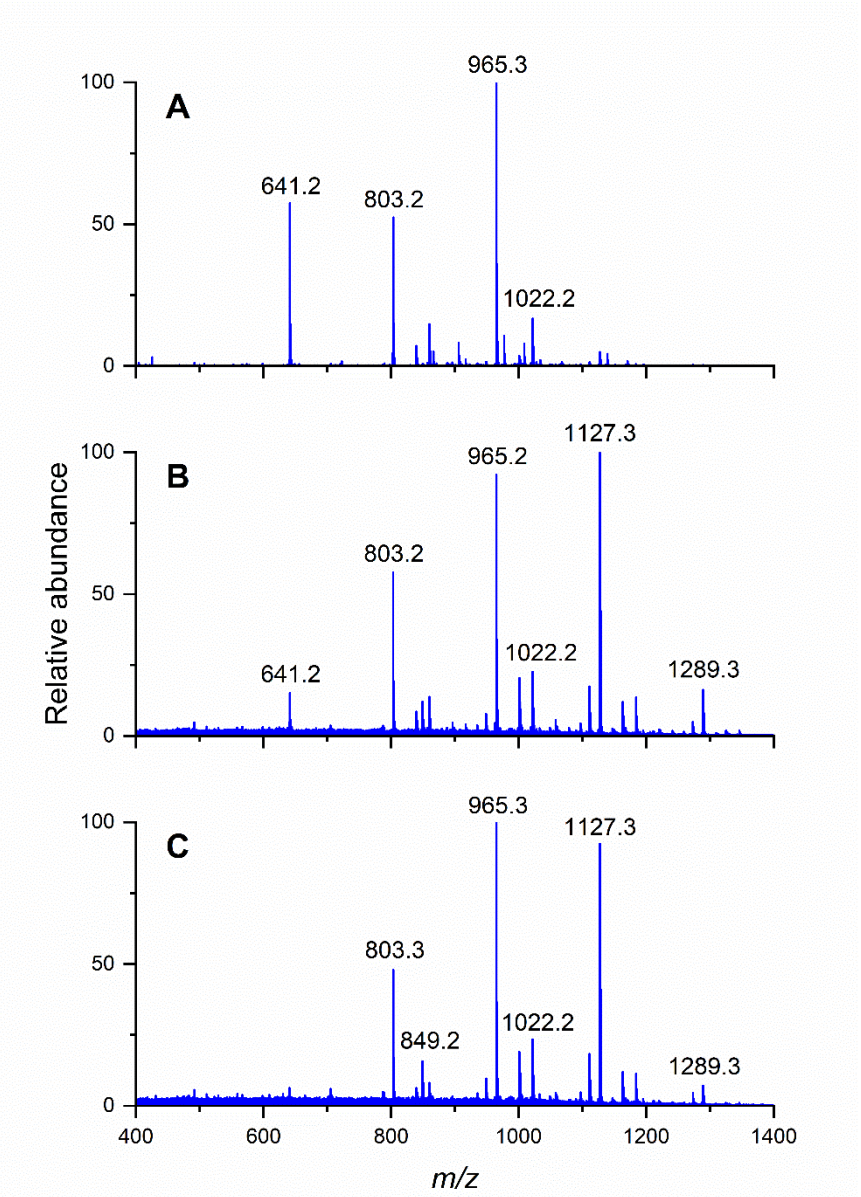


Figure 25. Combined full scan spectra acquired between 24.5 and 30.7 min of the LC-ESI-MS chromatograms from Figure 22.

For the identification of the transglycosylated stevia products that exhibited earlier elution due to their higher polarity (**Figure 22**), LC-ESI(-)-MS analyses showed that their *m/z* values ranged from 641.2 to 1289.3 for mixtures of sucrose plus SVglys and lactose plus SVglys (**Figure 26**). This indicates that these new stevia compounds share the core steviol structure but have up to 6 sugar moieties shared between positions C-13 and C-19. Thus, in

addition to the naturally occurring glucose and rhamnose, the carbohydrate chain of these modified stevia components is likely to be elongated with galactose or fructose residues.

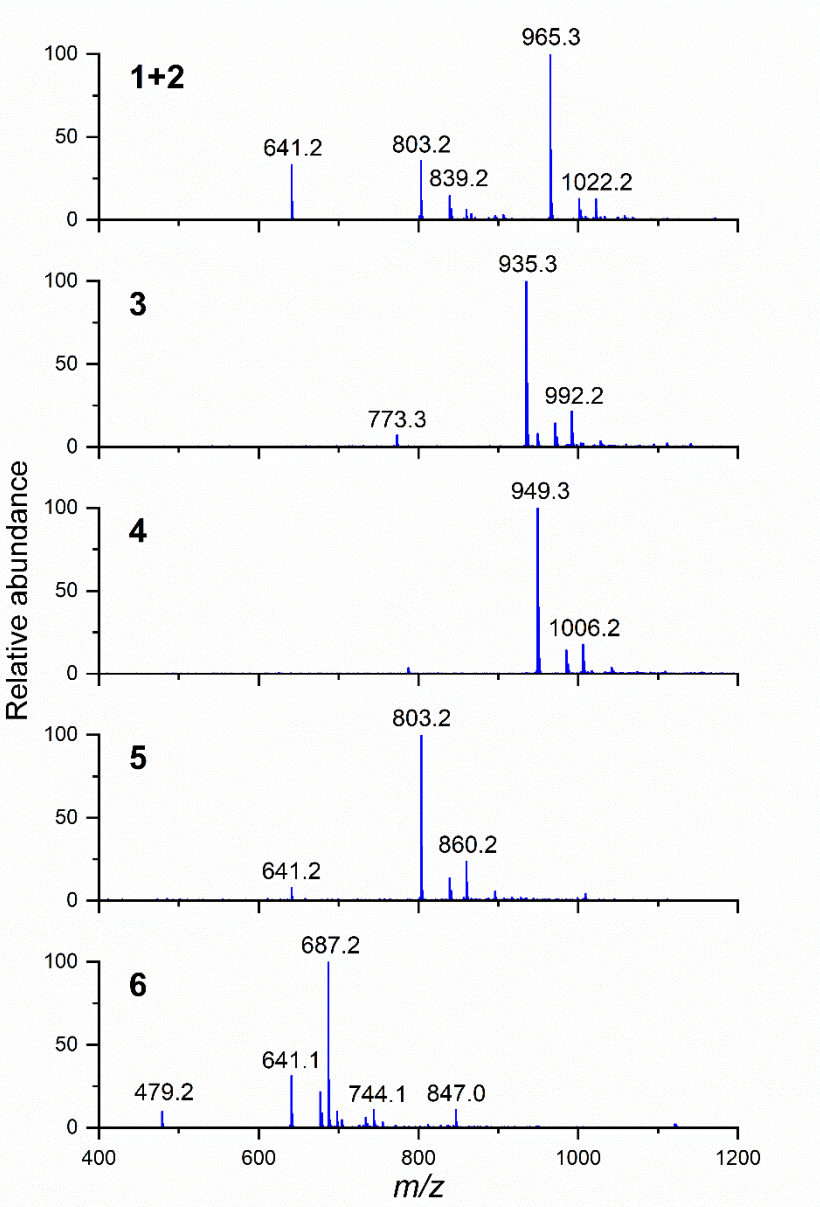


Figure 26. Full scan LC-ESI(–)-MS spectra obtained for the labelled peaks in Figure 20.

Finally, MALDI-TOF MS and LC-ESI(-)-MS spectra also served to complement the GC-FID analyses of the synthesized prebiotics, indicating that both GOS and FOS contained carbohydrates with a DP ranging from 2 to 7, as can be seen in **Figure 27** for the samples carried out with the absence of sweetener substrate. While the reactions carried out with SVglys showed a DP up to 7 for FOS and up to 12 for GOS (**Figure 28**).

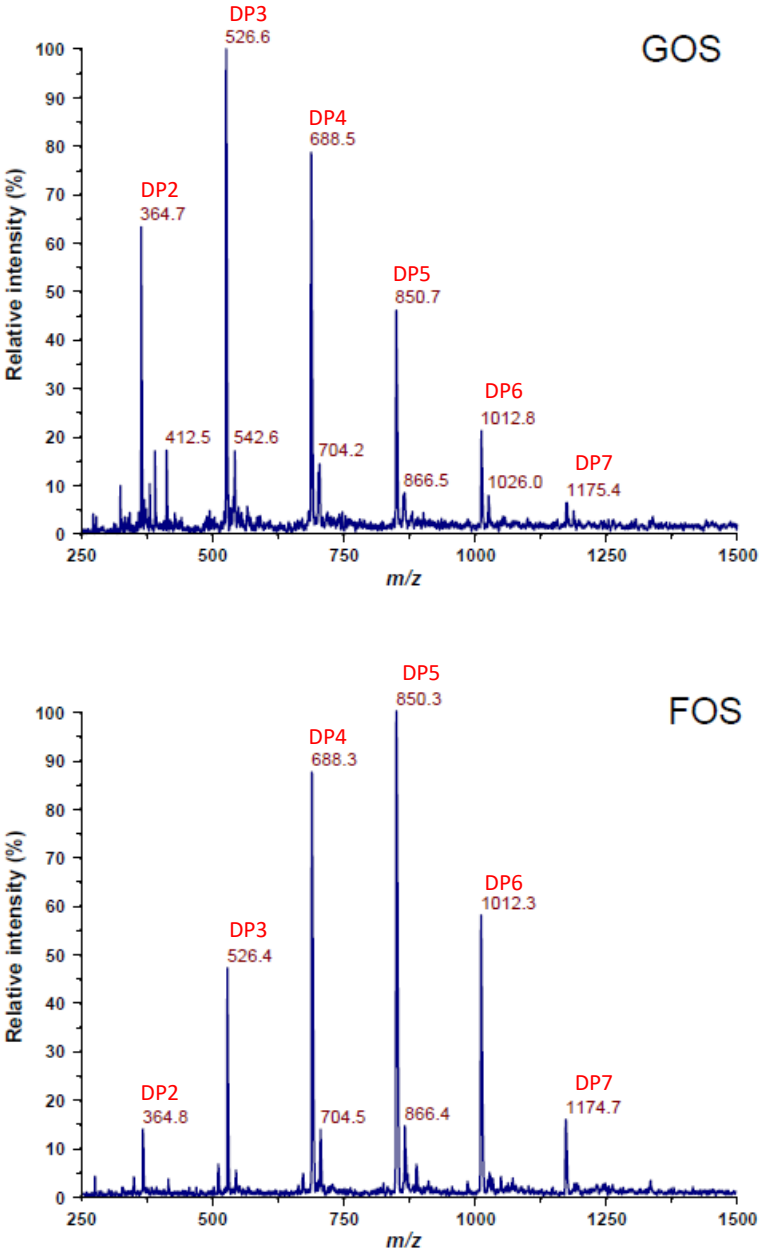


Figure 27. MALDI-TOF MS spectra on linear positive ion mode of prebiotic oligosaccharides from the (A) GOS synthesis in the presence of lactose (30%, w:w) and (B) FOS synthesis in the presence of sucrose (60%, w:w).

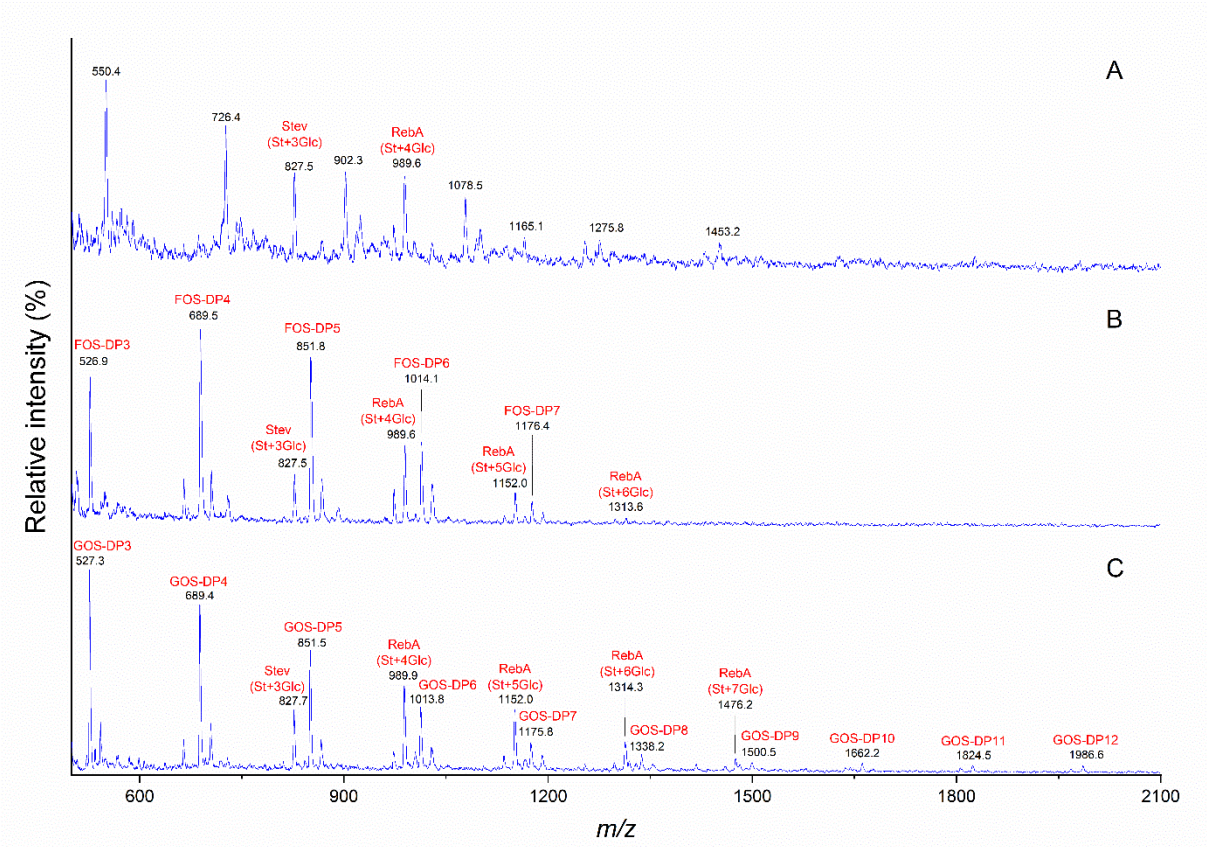


Figure 28. MALDI-TOF profiles of (A) non-modified SVglys with (B) β-fructosidase with sucrose as substrate, and (C) β-galactosidase with lactose as substrate. Labelled peaks are: Stev, stevioside; RebA, rebaudioside A and St, steviol. The different synthesized oligosaccharides are designated as FOS-DPn and GOS-DPn, where n indicates the degree of polymerization (DP).

4.1.4 One-pot enzymatic synthesis of prebiotic oligosaccharides (GOS or FOS) and modified mogrosides

4.1.4.1 Optimization conditions for enzymatic synthesis

The first stage of the optimization consisted of investigating the effect of four different concentrations of MGE (0.2, 1.5, 5, and 10%; w:v) for independent reactions with β-fructosidase and β-galactosidase using sucrose (60%; w:v) and lactose (30%; w:v), respectively, as donor substrates. RP-LC-DAD profiles showed qualitatively and quantitatively differences with respect to the control sample (non-incubated MGE) for both enzymes. However, when using MGE at 5 and 10% of concentrations, the reaction performance was adversely affected, resulting in a lower efficiency for β-fructosidase and a complete enzymatic inhibition for β-galactosidase (data not shown).

Considering the enzymatic activity values elucidated for each enzyme (734.2 U/mL for β-fructosidase and 14 U/mg for β-galactosidase) and the lowest MGE concentration (0.2%, w:v), the subsequent experiments consisted of testing different enzymatic activities during 48 h of incubation. The enzymatic activities were 25, 50, 75, and 100 U/mL for β-fructosidase, and 5, 15, and 50 U/mL for β-galactosidase. In order to select the optimal enzymatic activity, de- and glycosylation modifications associated with hydrophobic and hydrophilic peaks respectively were observed in RP-LC-DAD and LC-ESI-MS analyses and evaluated together with the formation of prebiotic oligosaccharides (that is FOS or GOS) analyses by GC-FID. In this context, β-fructosidase and β-galactosidase showed an optimal enzymatic activity of 100 U/mL and 5 U/mL, respectively.

Since the lowest tested MGE concentrations presented the highest modification yields, 0.2% and 1.5% were the optimal concentration values selected to carry on with the optimization. Similarly, the last stage consisted of developing a kinetic study to evaluate the effect of time of reaction and determine the best conditions leading to the highest concentration of synthesized prebiotic oligosaccharides.

For comparative purposes, MGE (0.2% and 1.5% w:v) were separately incubated with β-fructosidase or β-galactosidase for 48 h. β-Fructosidasetosidase or β-galactosidase were then incubated with MGE together with their respective carbohydrate substrates in order to carry out a single-pot synthesis of modified MGE with the formation of prebiotic oligosaccharides (FOS and GOS). The screening was monitored by RP-LC-DAD and GC-FID analyses.

4.1.4.1.1 Exploring the enzymatic kinetics of the two enzymes on the single substrates

The reactions performed only with the enzymes and the MGE (with the absence of sucrose or lactose) led to structural modifications compared to the initial MGE. Modifications catalyzed by the tested enzymes were observed in RP-LC-DAD profiles due to the formation of new deglycosylated products. The enzymatic kinetics produced different mogroside compositions depending on the enzyme used; however, no differences were found for the type of structures obtained between 0.2 and 1.5% of MGE.

Mogroside glycosylation occurs at C-3 and C-24 positions on the mogrol structure to yield from one to six glycosyl groups (Itkin *et al.* 2016). Certainly, up to six different mogrosides from MGE were detected during incubation (**Figure 29B** and **29D**), which concentrations and relative yields for each mogroside were compared and summarized in **Table 13** for the reactions carried out with MGE at 0.2%. Initial concentrations of MG-V and MG-VI (and other minor non-identified mogrosides coinciding in retention time) are notably decreased with reaction time for both enzymes. Accordingly, it is observed that the longer the reaction time, the greater the deglycosylation rate achieved, where MG-I was mainly formed. Referring to β-fructosidase, the MG-I formation is around 32.6% while for β-galactosidase 39.4% at the end of the reaction.

The reactions performed only with the enzymes and the MGE (with the absence of sucrose or lactose) led to structural modifications compared to the initial MGE. Modifications catalyzed by the tested enzymes were observed in RP-LC-DAD profiles due to the formation of new deglycosylated products. The enzymatic kinetics produced different mogroside compositions depending on the enzyme used; however, no major behavioural differences were found for the type of structures obtained between 0.2 and 1.5% of MGE.

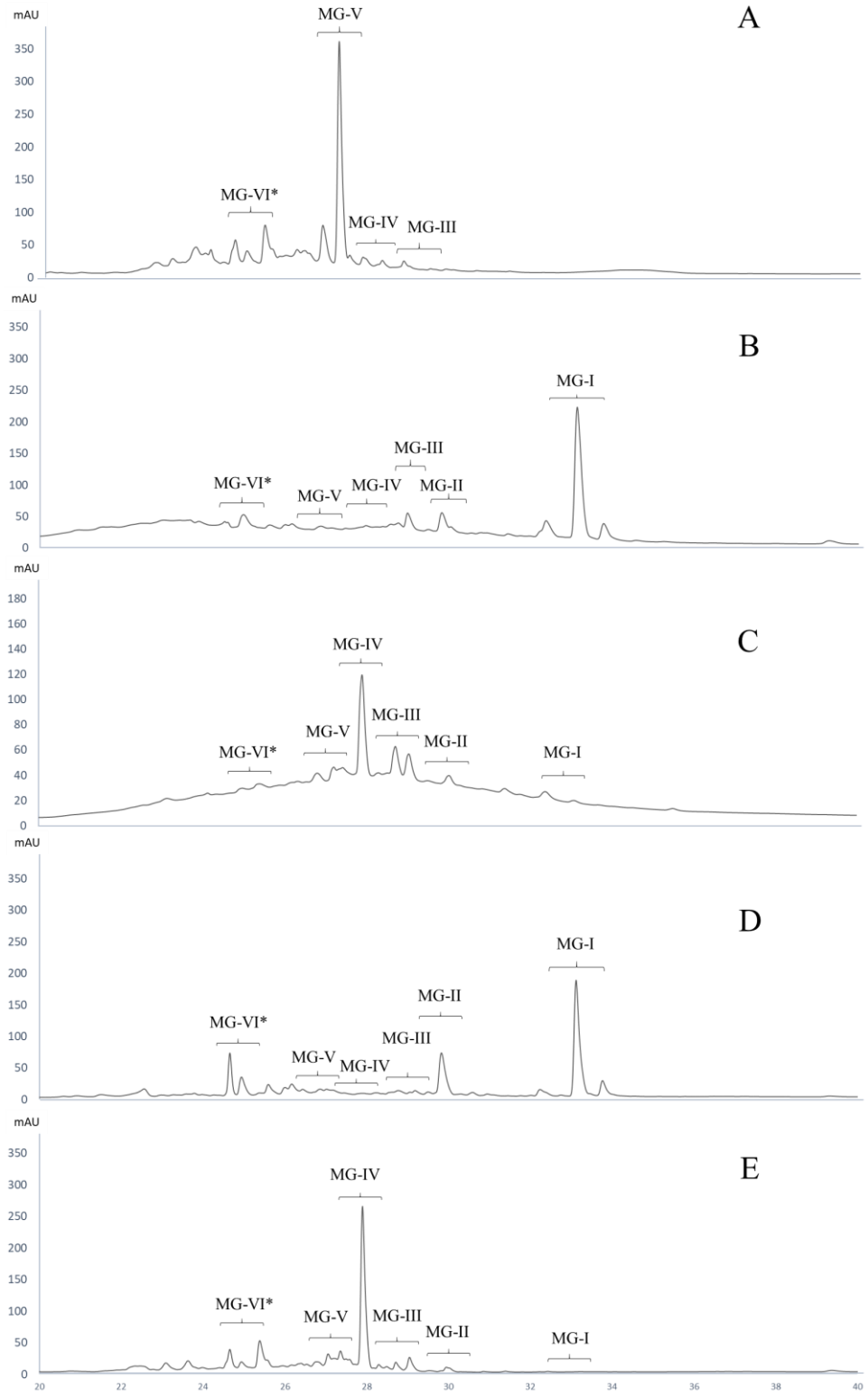


Figure 29. RPLC-DAD profiles of (A) non-modified MGE with (B) β-fructosidase, (C) with β-fructosidase in the presence of sucrose, (D) with β-galactosidase and (E) β-galactosidase with the presence of lactose. Labelled peaks are: MG-I, mogrosides I, MG-II, mogrosides II; MG-III, mogrosides III; MG-IV, mogrosides IV; MG-V, mogrosides V and MG-VI*, mogrosides VI and other minor non-identified mogrosides.

Table 13. Concentration (mg/mL ± standard deviation) of the different compounds synthesized during the time of reaction with MGE at 0.2% using β-fructosidase (A) and β-galactosidase (B).

A	Reaction time (hours)															
	0		1		2		4		6		12		24		48	
Compound	Concn ± SD[a] (mg/mL)	R (%)[b]	Concn ± SD[a] (mg/mL)	R (%)[b]	Concn ± SD[a] (mg/mL)	R (%)[b]	Concn ± SD[a] (mg/mL)	R (%)[b]	Concn ± SD[a] (mg/mL)	R (%)[b]	Concn ± SD[a] (mg/mL)	R (%)[b]	Concn ± SD[a] (mg/mL)	R (%)[b]	Concn ± SD[a] (mg/mL)	R (%)[b]
MG-I	0.00 ± 0.00[c]	0.00	0.41 ± 0.02	16.42	0.65 ± 0.06	35.86	0.57 ± 0.05	37.50	0.82 ± 0.14	34.50	1.14 ± 0.00	43.75	0.98 ± 0.03	37.80	0.84 ± 0.09	32.58
MG-II	0.00 ± 0.00	0.00	0.44 ± 0.01	17.71	0.34 ± 0.02	18.76	0.30 ± 0.01	20.01	0.44 ± 0.09	18.54	0.42 ± 0.05	16.06	0.47 ± 0.07	18.20	0.52 ± 0.07	20.04
MG-III	0.13 ± 0.00	5.90	0.44 ± 0.17	17.77	0.20 ± 0.02	10.80	0.13 ± 0.07	8.79	0.24 ± 0.01	10.04	0.27 ± 0.07	10.20	0.32 ± 0.07	12.48	0.38 ± 0.09	14.61
MG-IV	0.26 ± 0.05	11.82	0.33 ± 0.09	13.43	0.17 ± 0.01	9.35	0.16 ± 0.01	10.55	0.25 ± 0.02	10.41	0.24 ± 0.09	9.21	0.28 ± 0.10	10.89	0.21 ± 0.02	8.07
MG-V	1.03 ± 0.06	46.90	0.38 ± 0.09	15.22	0.18 ± 0.02	9.81	0.14 ± 0.01	9.02	0.22 ± 0.01	9.15	0.27 ± 0.15	10.28	0.31 ± 0.10	11.83	0.31 ± 0.09	12.00
MG-VI*	0.78 ± 0.06	35.38	0.48 ± 0.09	19.45	0.28 ± 0.03	15.43	0.21 ± 0.05	14.12	0.41 ± 0.01	17.37	0.27 ± 0.08	10.50	0.23 ± 0.03	8.80	0.33 ± 0.05	12.69
Total	2.20 ± 0.19		2.47 ± 0.46		1.82 ± 0.10		1.51 ± 0.15		2.37 ± 0.25		2.62 ± 0.44		2.59 ± 0.28		2.59 ± 0.32	
B	Reaction time (hours)															
	0		1		2		4		6		12		24		48	
Compound	Concn ± SD[a] (mg/mL)	R (%)[b]	Concn ± SD[a] (mg/mL)	R (%)[b]	Concn ± SD[a] (mg/mL)	R (%)[b]	Concn ± SD[a] (mg/mL)	R (%)[b]	Concn ± SD[a] (mg/mL)	R (%)[b]	Concn ± SD[a] (mg/mL)	R (%)[b]	Concn ± SD[a] (mg/mL)	R (%)[b]	Concn ± SD[a] (mg/mL)	R (%)[b]
MG-I	0.00 ± 0.00[c]	0.00	0.22 ± 0.04	8.88	0.29 ± 0.01	12.65	0.61 ± 0.06	27.40	0.70 ± 0.01	34.31	0.70 ± 0.01	35.02	0.74 ± 0.00	36.70	0.80 ± 0.01	39.39
MG-II	0.00 ± 0.00	0.00	0.30 ± 0.02	12.27	0.41 ± 0.03	17.88	0.40 ± 0.06	18.23	0.30 ± 0.01	14.55	0.27 ± 0.01	13.60	0.31 ± 0.02	15.25	0.31 ± 0.02	15.26
MG-III	0.10 ± 0.03	5.04	0.46 ± 0.05	18.81	0.43 ± 0.05	18.97	0.21 ± 0.03	9.56	0.14 ± 0.02	6.73	0.13 ± 0.01	6.67	0.10 ± 0.00	4.75	0.08 ± 0.00	3.86
MG-IV	0.19 ± 0.03	10.02	0.50 ± 0.13	20.43	0.30 ± 0.06	13.08	0.18 ± 0.02	7.98	0.15 ± 0.01	7.15	0.17 ± 0.00	8.67	0.21 ± 0.01	10.22	0.20 ± 0.00	9.58
MG-V	1.01 ± 0.09	53.15	0.37 ± 0.10	15.22	0.27 ± 0.05	11.80	0.26 ± 0.04	11.69	0.24 ± 0.02	11.69	0.24 ± 0.02	12.08	0.24 ± 0.01	11.86	0.25 ± 0.03	12.27
MG-VI*	0.60 ± 0.03	31.79	0.60 ± 0.09	24.40	0.58 ± 0.06	25.61	0.56 ± 0.09	25.14	0.52 ± 0.01	25.56	0.48 ± 0.01	23.95	0.43 ± 0.02	21.21	0.40 ± 0.04	19.64
Total	1.90 ± 0.18		2.46 ± 0.43		2.27 ± 0.24		2.22 ± 0.30		2.04 ± 0.06		2.00 ± 0.04		2.02 ± 0.06		2.04 ± 0.07	

[a]Standard deviation values (n = 2). [b]Relative % data were calculated considering the total compound values shown above. *Formed by other minor non-identified mogrosides.

4.1.4.1.2 One-pot enzymatic synthesis of modified MGE with galacto-oligosaccharides (GOS)

The reactions performed with β-galactosidase with MGE and in the presence of lactose as substrate, similarly, formed modified mogrosides, compared to the reactions without lactose, following a uniform tendency throughout the incubation (**Table 14B**). Likewise, MG-V and MG-VI concentrations were found to have dropped by up to 30 and 10% respectively, until the end of the reaction. However, an explicit effect of this enzyme with its respective substrate was observed to exhibits a preference to produce MG-IV, regardless of the time of reaction. Consequently, the maximum yield of MG-IV (34.1%) was obtained after 48 h of reaction. Other minor deglycosylations were formed throughout incubation and can be seen in **Figure 29E**.

The kinetics reaction carried out with MGE at 0.2 and 1.5% concentrations also allowed the synthesis of GOS, and it was found that the product composition did not present differences with respect to the GOS formation without MGE. **Figure 30C** and **30D** compare the GC-FID profiles of the reactions without and with MGE, respectively. The time course of the reactions performed without MGE and the reactions with MGE at 0.2 and 1.5% is shown in **Figure 31.** In both cases, most of the lactose was consumed over the 48 h of reaction due to the formation of transgalactosylated products and the most abundant compounds were trisaccharides reaching a maximum concentration of 80 g/L. The maximum GOS value was reached after 6 h of incubation, with an average concentration of 180 g/L (47%). In terms of the GOS linkage-type synthesized, disaccharides were tentatively identified as 6'galactosyl-glucose, while the trisaccharide fraction was 4'galactosyl-lactose and 6'galactosyl-lactose in agreement with the GOS formed by *Aspergillus oryzae* in a previous study (Urrutia *et al.* 2013). by Lorenzoni *et al.* (2014).

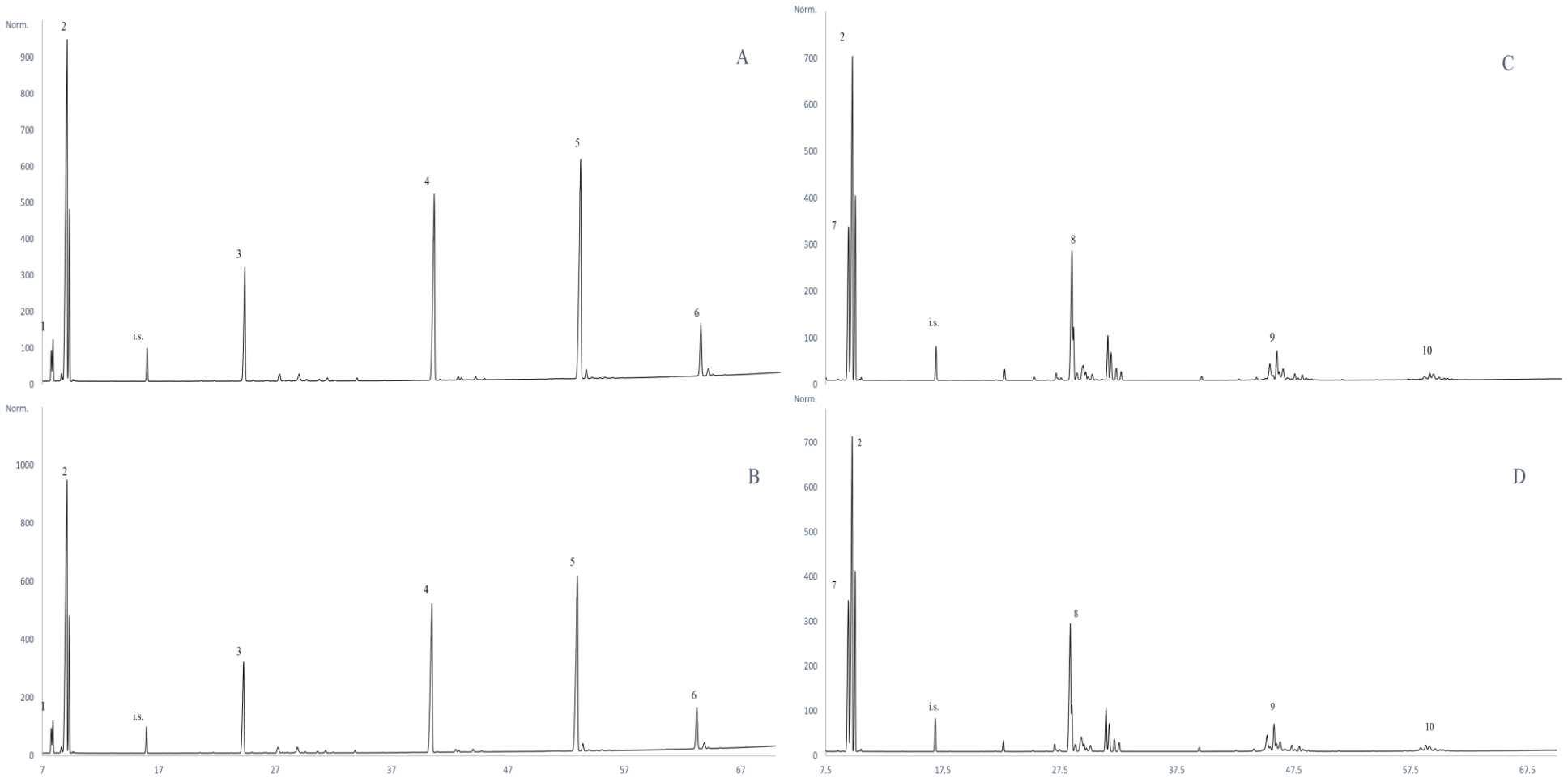


Figure 30. Chromatographic profiles obtained by GC-FID showing: (A) FOS synthesis in the presence of sucrose (60%, w:w), (B) FOS synthesis in the presence of sucrose (60%, w:w) and MGE (0.2%, w:w), (C) GOS synthesis in the presence of lactose (30%, w:w) and (D) GOS synthesis in the presence of lactose (60%, w:w) and MGE (0.2%, w:w). Labelled peaks are as follows: peak 1, fructose; peak 2, glucose; peak 3, sucrose; peak 4, kestose; peak 5, nystose; peak 6, fructosyl-nystose; peak 7, galactose; peak 8, lactose; peak 9, GOS-trisaccharides; peak 10, GOS-tetrasaccharides; i. s., internal standard.

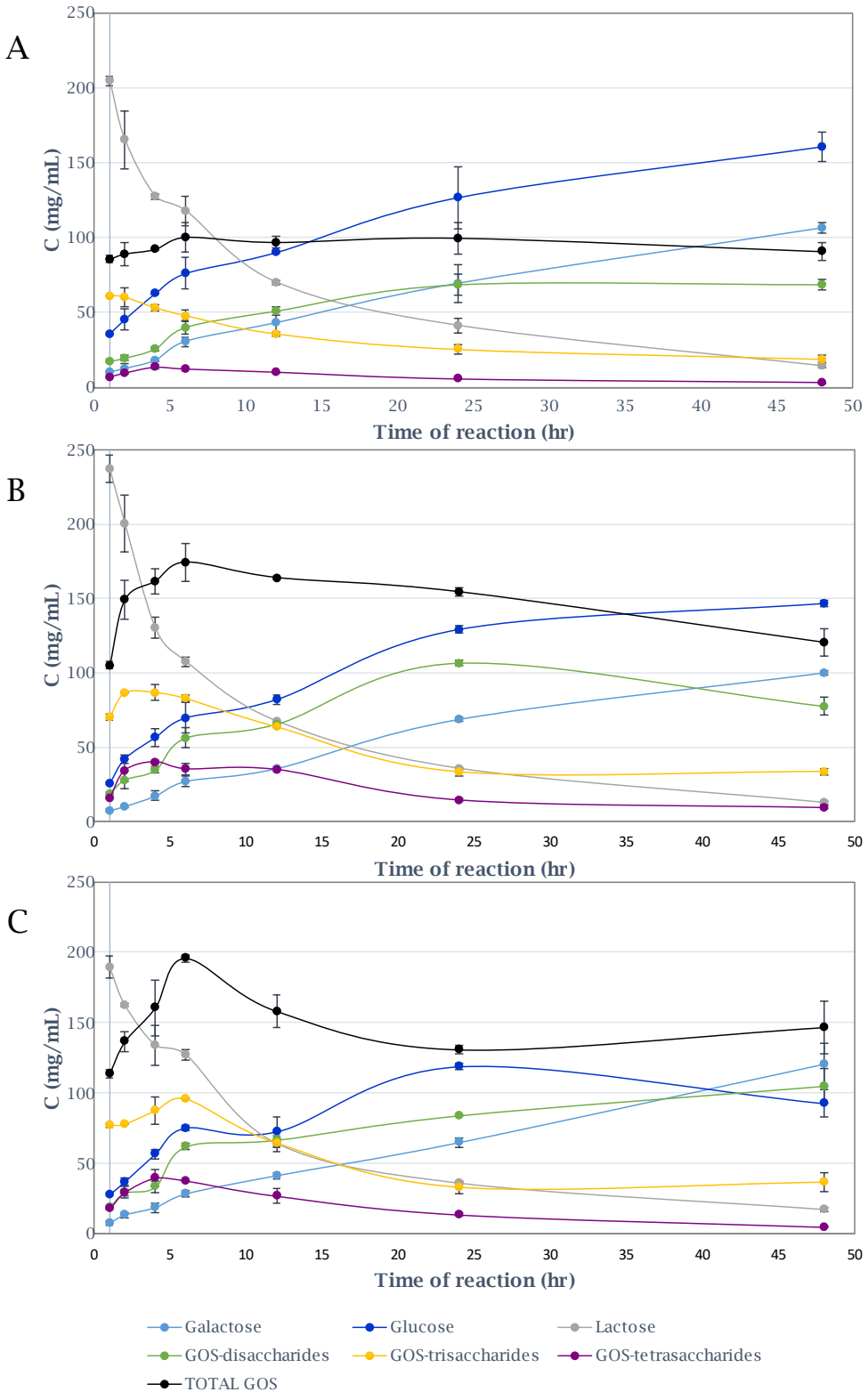


Figure 31. Evolution of carbohydrates concentration as determined by GC-FID throughout the enzymatic reaction of (A) GOS synthesis in the presence of lactose (30%, w:w), (B) GOS synthesis in the presence of lactose (60%, w:w) and MGE (0.2%, w:w) and (C) GOS synthesis in the presence of lactose (30%, w:w) and MGE (1.5%, w:w).

4.1.4.1.3 One-pot enzymatic synthesis of modified MGE with fructo-oligosaccharides (FOS)

Several modifications, mainly deglycosylations, were obtained during the incubation of MGE with β-fructosidase and sucrose (**Table 14A**). These deglycosylations observed in the presence and absence of sucrose occurred from the initial MG-V and MG-VI and other non-identified mogrosides with early retention times of elution, presenting a reduction of 30 and 20% until the end of the reaction. Depending on the reaction time, the production yield of specific deglycosylated mogrosides varied. For instance, 12 h of reaction enabled the production of MG-IV (23.8%), 24 h led to the maximum production of MG-III (20.5%), and 48 h to the production of MG-II (26.9%). Besides the mentioned mogrosides, MG-I was similarly produced during the kinetics, although in lower quantities with respect to the reactions with the absence of sucrose. A prototype profile representing homogeneous quantities for all the mogrosides formed is shown in **Figures 29C** and **32C**, corresponding to 24 h of incubation.

Table 14. Concentration (mg/mL ± standard deviation) of the different compounds synthesized during the time of reaction with MGE at 0.2% using β-fructosidase (A) galactosidase (B).

A	Reaction time (hours)															
	0		1		2		4		6		12		24		48	
Compound	Concn ± SD[a] (mg/mL)	R (%)[b]	Concn ± SD[a] (mg/mL)	R (%)[b]	Concn ± SD[a] (mg/mL)	R (%)[b]	Concn ± SD[a] (mg/mL)	R (%)[b]	Concn ± SD[a] (mg/mL)	R (%)[b]	Concn ± SD[a] (mg/mL)	R (%)[b]	Concn ± SD[a] (mg/mL)	R (%)[b]	Concn ± SD[a] (mg/mL)	R (%)[b]
MG-I	0.00 ± 0.00	0.00	0.41 ± 0.02	16.42	0.65 ± 0.06	35.86	0.57 ± 0.05	37.50	0.82 ± 0.14	34.50	1.14 ± 0.00	43.75	0.98 ± 0.03	37.80	0.84 ± 0.09	32.58
MG-II	0.00 ± 0.00	0.00	0.44 ± 0.01	17.71	0.34 ± 0.02	18.76	0.30 ± 0.01	20.01	0.44 ± 0.09	18.54	0.42 ± 0.05	16.06	0.47 ± 0.07	18.20	0.52 ± 0.07	20.04
MG-III	0.13 ± 0.00	5.90	0.44 ± 0.17	17.77	0.20 ± 0.02	10.80	0.13 ± 0.07	8.79	0.24 ± 0.01	10.04	0.27 ± 0.07	10.20	0.32 ± 0.07	12.48	0.38 ± 0.09	14.61
MG-IV	0.26 ± 0.05	11.82	0.33 ± 0.09	13.43	0.17 ± 0.01	9.35	0.16 ± 0.01	10.55	0.25 ± 0.02	10.41	0.24 ± 0.09	9.21	0.28 ± 0.10	10.89	0.21 ± 0.02	8.07
MG-V	1.03 ± 0.06	46.90	0.38 ± 0.09	15.22	0.18 ± 0.02	9.81	0.14 ± 0.01	9.02	0.22 ± 0.01	9.15	0.27 ± 0.15	10.28	0.31 ± 0.10	11.83	0.31 ± 0.09	12.00
MG-VI*	0.78 ± 0.06	35.38	0.48 ± 0.09	19.45	0.28 ± 0.03	15.43	0.21 ± 0.05	14.12	0.41 ± 0.01	17.37	0.27 ± 0.08	10.50	0.23 ± 0.03	8.80	0.33 ± 0.05	12.69
Total	2.20 ± 0.19		2.47 ± 0.46		1.82 ± 0.10		1.51 ± 0.15		2.37 ± 0.25		2.62 ± 0.44		2.59 ± 0.28		2.59 ± 0.32	
B	Reaction time (hours)															
	0		1		2		4		6		12		24		48	
Compound	Concn ± SD[a] (mg/mL)	R (%)[b]	Concn ± SD[a] (mg/mL)	R (%)[b]	Concn ± SD[a] (mg/mL)	R (%)[b]	Concn ± SD[a] (mg/mL)	R (%)[b]	Concn ± SD[a] (mg/mL)	R (%)[b]	Concn ± SD[a] (mg/mL)	R (%)[b]	Concn ± SD[a] (mg/mL)	R (%)[b]	Concn ± SD[a] (mg/mL)	R (%)[b]
MG-I	0.00 ± 0.00	0.00	0.22 ± 0.04	8.88	0.29 ± 0.01	12.65	0.61 ± 0.06	27.40	0.70 ± 0.01	34.31	0.70 ± 0.01	35.02	0.74 ± 0.00	36.70	0.80 ± 0.01	39.39
MG-II	0.00 ± 0.00	0.00	0.30 ± 0.02	12.27	0.41 ± 0.03	17.88	0.40 ± 0.06	18.23	0.30 ± 0.01	14.55	0.27 ± 0.01	13.60	0.31 ± 0.02	15.25	0.31 ± 0.02	15.26
MG-III	0.10 ± 0.03	5.04	0.46 ± 0.05	18.81	0.43 ± 0.05	18.97	0.21 ± 0.03	9.56	0.14 ± 0.02	6.73	0.13 ± 0.01	6.67	0.10 ± 0.00	4.75	0.08 ± 0.00	3.86
MG-IV	0.19 ± 0.03	10.02	0.50 ± 0.13	20.43	0.30 ± 0.06	13.08	0.18 ± 0.02	7.98	0.15 ± 0.01	7.15	0.17 ± 0.00	8.67	0.21 ± 0.01	10.22	0.20 ± 0.00	9.58
MG-V	1.01 ± 0.09	53.15	0.37 ± 0.10	15.22	0.27 ± 0.05	11.80	0.26 ± 0.04	11.69	0.24 ± 0.02	11.69	0.24 ± 0.02	12.08	0.24 ± 0.01	11.86	0.25 ± 0.03	12.27
MG-VI*	0.60 ± 0.03	31.79	0.60 ± 0.09	24.40	0.58 ± 0.06	25.61	0.56 ± 0.09	25.14	0.52 ± 0.01	25.56	0.48 ± 0.01	23.95	0.43 ± 0.02	21.21	0.40 ± 0.04	19.64
Total	1.90 ± 0.18		2.46 ± 0.43		2.27 ± 0.24		2.22 ± 0.30		2.04 ± 0.06		2.00 ± 0.04		2.02 ± 0.06		2.04 ± 0.07	

[a]Standard deviation values (n = 2). [b]Relative % data were calculated considering the total compound values shown above. *Formed by other minor non-identified mogrosides

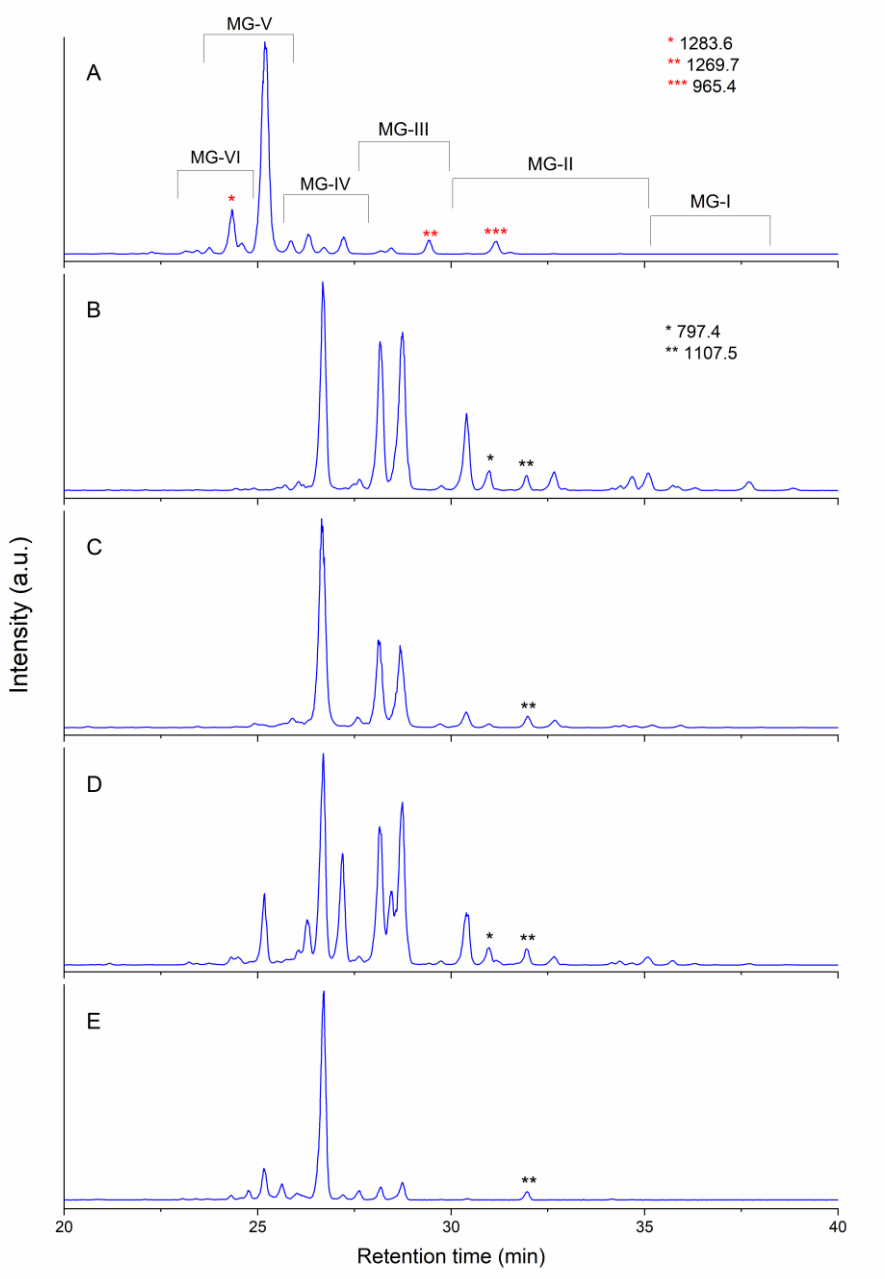


Figure 32. Combined full scan spectra of the LC-MS chromatogram acquired between 22 and 39 min of (A) non-modified MGE with (B) β-fructosidase and (C) with sucrose as substrate, and (D) β-galactosidase with (E) lactose as substrate.

These chemical modifications carried out in the presence of sucrose, gave rise to synthesizing other compounds, fructo-oligosaccharides (FOS), as determined by GC-FID. As shown in **Figure 30B**, no structural differences can be appreciated with respect to the FOS produced in absence of MGE (**Figure 30A**). Similarly, MGE concentration did not negatively interfere with the formation of prebiotic oligosaccharides. This evidence can be confirmed

when comparing the FOS formation between 0.2 and 1.5% (**Figure 33B** and **33C**), which results do not present significant differences as well as for the LC-DAD analyses. The corresponding components for both reactions are: monosaccharides (fructose and glucose), disaccharides (sucrose), minor FOS-disaccharides, trisaccharide 1-kestose, tetrasaccharide nystose, pentasaccharide fructosyl-1-nystose, and hexasaccharide difructosyl-nystose (**Figure 30B**). This structural composition was fully characterized by using standards with known structures. **Figure 33** shows the evolution of product relative composition throughout the synthesis. Sucrose concentration decays along with the reaction, inversely to the formation of total FOS (84%), respectively. Different FOS are produced during the reaction, as identified by GC-FID: kestose was the most abundant compound (300 g/L) up to the sixth hour of reaction, followed by nystose (200 g/L) up to the twenty-fourth hour of reaction. Afterwards, fructosylnystose (150 g/L) was the major compound until the end of the reaction. Difructosylnystose (20 g/L) was also synthesized from the twelfth hour of reaction. These results are in line with the data reported for the same enzyme, from *Aspergillus aculeatus* by Lorenzoni *et al.* (2014).

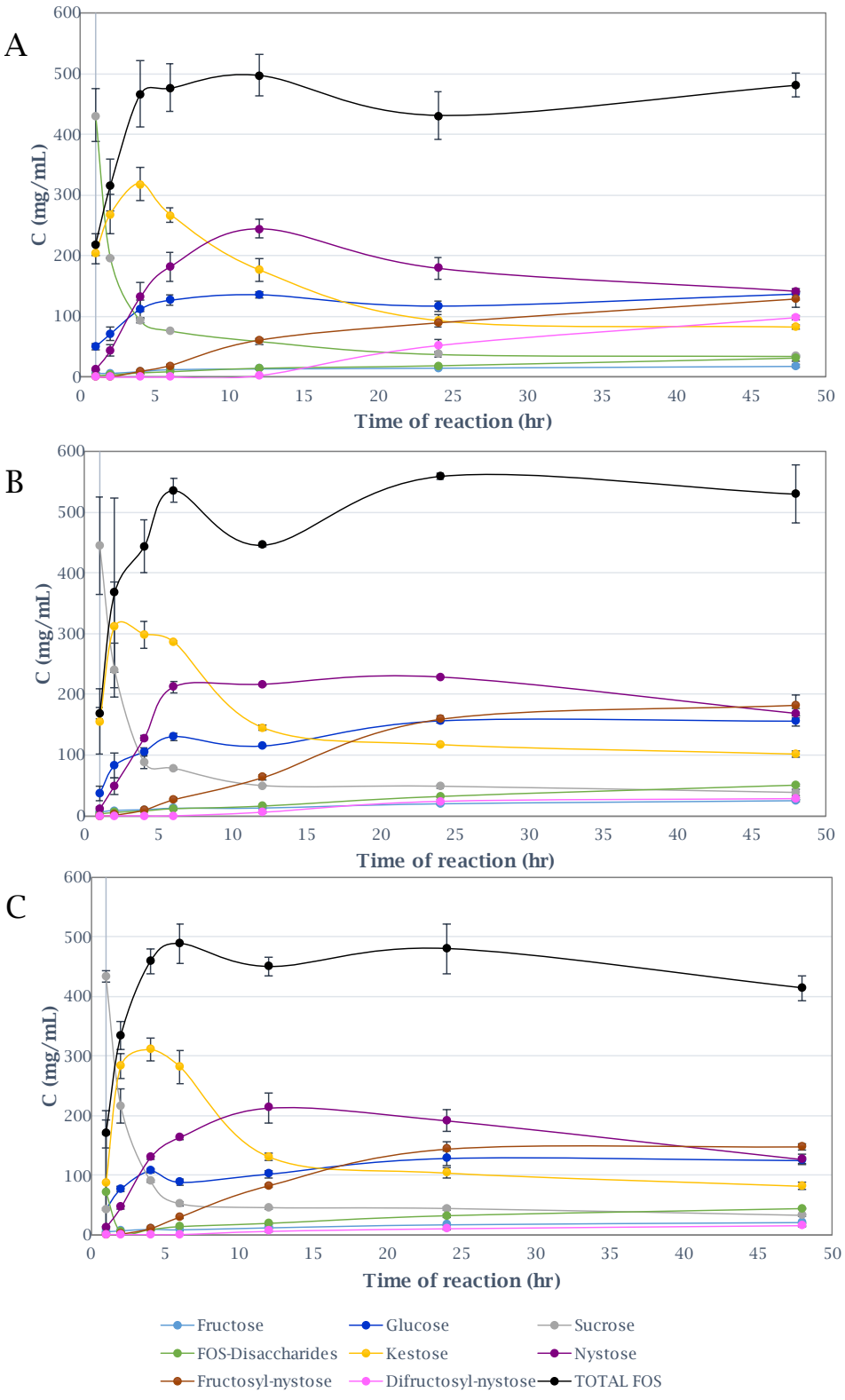


Figure 33. Evolution of carbohydrates concentration as determined by GC-FID throughout the enzymatic reaction of (A) FOS synthesis in the presence of sucrose (60%, w:w), (B) FOS synthesis in the presence of sucrose (60%, w:w) and MGE (0.2%, w:w) and (C) FOS synthesis in the presence of sucrose (60%, w:w) and MGE (1.5%, w:w).

4.1.4.2 Characterization of the modified mogrosides and prebiotic oligosaccharides by Mass spectrometry

To better understand the possible structural modifications, a comprehensive mass spectrometric approach was conducted by combining LC-MS and MALDI-TOF MS analyses. **Figure 34** shows the combined full scan spectra for the samples obtained from the four target incubations (with and without their respective substrates) in ESI negative mode corresponding to the LC-MS chromatograms shown in **Figure 32**. Different mogrosides were identified, in agreement with those recently reported (Jia *et al.* 2019; Luo *et al.* 2016; Shen *et al.* 2014; G. Zhou *et al.* 2016). In the samples treated with both tested enzymes, a decrease of MG-V and MG-VI (which identified peaks are also composed by other minor non-identified mogrosides) mass signals and an increment of deglycosylated mogrosides can be appreciated. Additionally, new mogrosides masses were identified as for MG-II at *m/z* 799.4 and MG-I at *m/z* 683.3, respectively. **Figure 34C** and **34E** show the *m/z* of the products obtained with β-fructosidase and sucrose and, β-galactosidase and lactose, respectively. **Figure 34B** and **34D** show the ions corresponding to the reactions carried out without the presence of sucrose and lactose substrates, respectively. The identified mogrosides are found in accordance with the previously exposed in **Tables 13** and **14**. However, additional features have been particularly detected. On the contrary, MG-VI (*m/z* 1447.6) was not detected in any of the incubations carried out with β-fructosidase (**Figure 34B** and **34C**), while for β-galactosidase (**Figure 34D** and **34E**), MG-VI was identified with a slight signal, whereas RP-LC-DAD results (**Tables 13** and **14**) present quantitative results of MG-VI for β-fructosidase and β-galactosidase with the peculiarity that other minor compounds eluting at same retention times with MG-VI. Furthermore, apart from the observed de-glycosylations, MALDI-TOF MS spectra (**Figure 35**) showed new glycosylations respectively to the unmodified MGE (**Figure 35A**) for the samples treated with β-fructosidase and sucrose (**Figure 35B**) and with β-galactosidase and lactose (**Figure 35C**) corresponding to *m/z* 1633.5/1634.8 and *m/z* 1797.4.

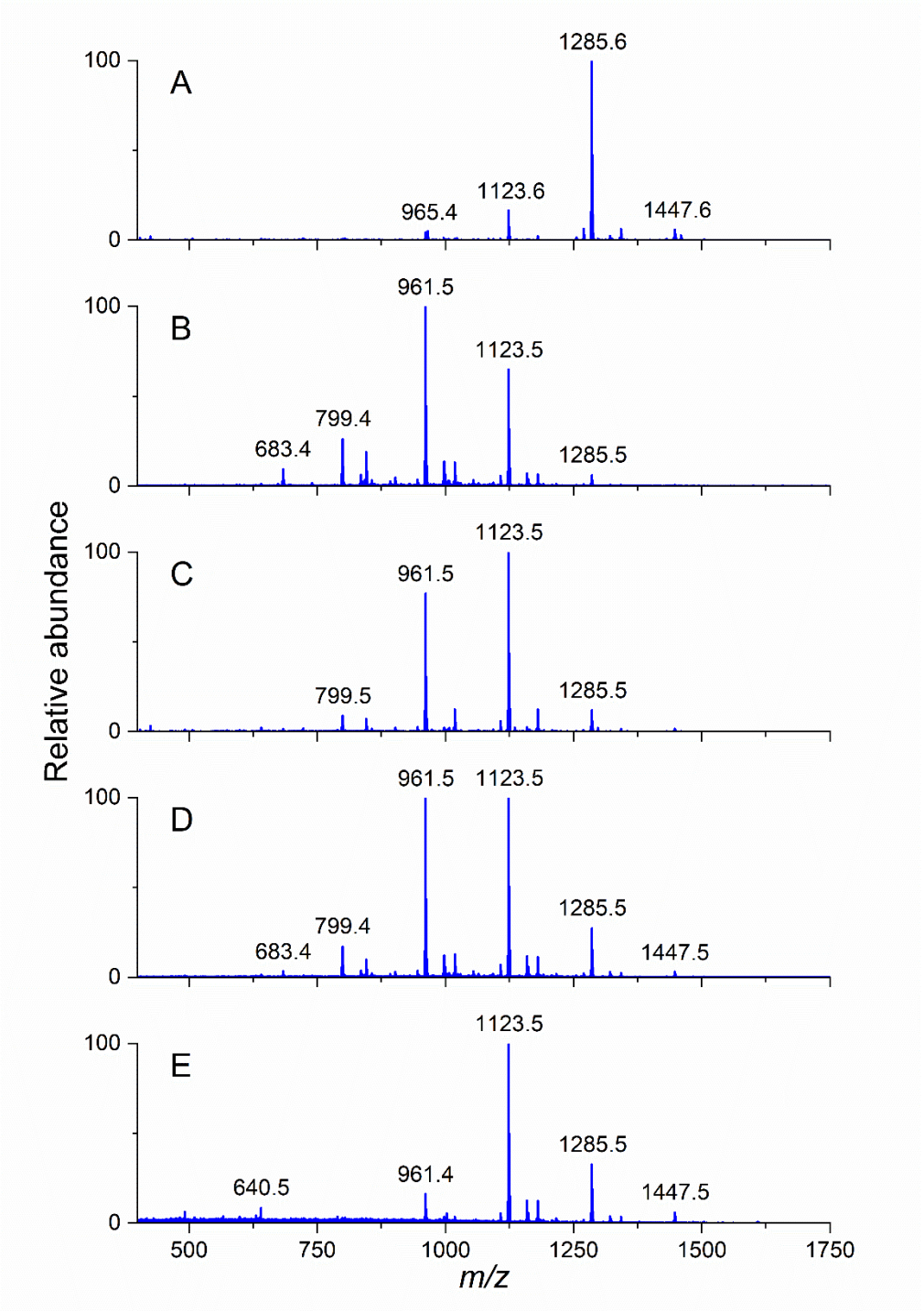


Figure 34. Full LC-ESI-MS spectra of the (A) non-modified MGE with (B) β-fructosidase with (C) sucrose as substrate, and (D) with β-galactosidase with (E) lactose as substrate.

MALDI-TOF MS analyses on the oligosaccharides formed, also supported the GC-FID analyses previously described. The oligomers identified showed a mass difference of 162 u, which corresponds to hexose residues. **Figure 35B** and **35C** show the chain length distribution of FOS (DP up to 7) and GOS (DP up to 8) (Borromei *et al.* 2011; S. E. Lee *et al.* 2011; Oh *et al.* 2017; S. Wang *et al.* 2020).

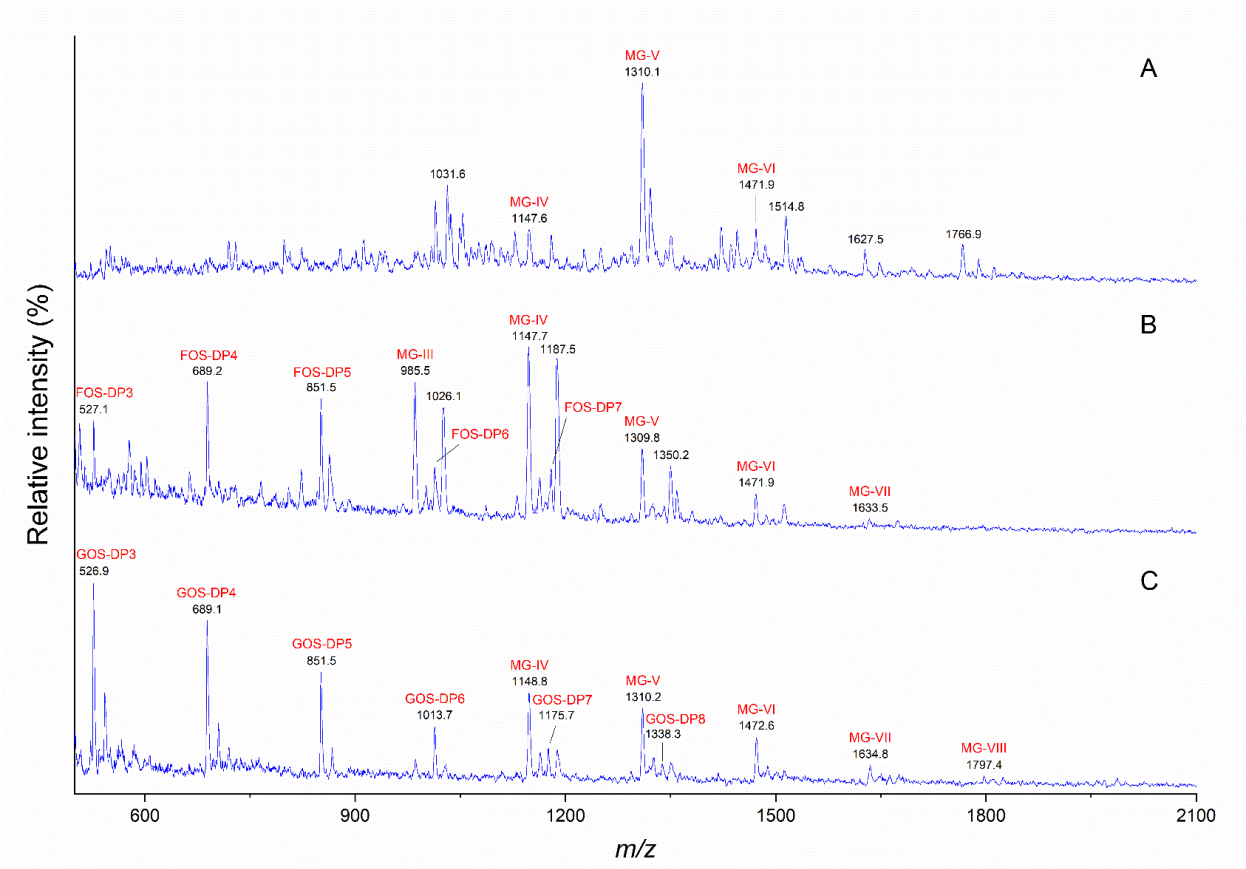


Figure 35. MALDI-TOF profiles of (A) non-modified MGE with (B) β-fructosidase with sucrose as substrate, and (C) β-galactosidase with lactose as substrate. Labelled peaks are: MG-I, mogrosides I, MG-II, mogrosides II; MG-III, mogrosides III; MG-IV, mogrosides IV; MG-V, mogrosides V and MG-VI, mogrosides VI. The different synthesized oligosaccharides are designated as FOS-DPn and GOS-DPn, where n indicates the degree of polymerization (DP).

4.2 Part II Sensorial evaluation of new-synthesized natural sweeteners

4.2.1 Sensory profiling of new transglucosylated sweeteners by CGTases

4.2.1.1 Steviol glycosides: SVglys and RebA

Steviol glycosides such as stevioside and rebaudioside A have been described to exhibit bitter-taste and liquorice flavour characters (Pawar *et al.* 2013). In this context, the enzymatically modified SVglys and RebA samples resulting from the DoE optimization in **Part I** (*section 3.1.1*) were subjected to a sensorial analysis in order to study whether the transglucosylation had an effect on the flavour profile. The trained panellists performed a sensorial analysis based on the taste evaluation of 16 attributes in the original samples and the samples modified with the respective optimal CGTases. The optimal reactions carried out with the SVgls and RebA with the CGTase *Geobacillus* sp. were formerly purified as indicated in **Part II** (*section 3.2.1*) for an accurate sensorial application.

As shown in **Figure 36**, of the 16 attributes rated, 7 were significantly different between the samples. It was reported for natural steviol glycosides with β-D-glucopyranosyl units as constituents that the ratio of the glucose units at C-13 to C-19 of the steviol core has a relationship with the sweetness as well as with the quality of taste of the steviol glycosides (Gerwig *et al.* 2016). The possible glucosidic linkages formed at C-19 of rebaudioside A could also have an impact on the bitter aftertaste; however, there was no significant effect on bitter taste by modification of the RebA in this. Additionally, the bitter taste was significantly and substantially higher for unmodified SVglys, mainly consisting of a mixture of stevioside and rebaudioside A than for unmodified RebA, whose results were in accordance with previous reports which stated that rebaudioside A is preferred for its sweetness and for being devoid of aftertaste bitterness over stevioside (Lindley, 2012; Singla *et al.* 2016; Wang *et al.* 2016). Likewise, regarding the sweetness, the initial RebA was significantly sweeter than the initial SVglys due to the quantity of stevioside present in this mixture, whose values are in agreement with the literature where is reported that rebaudioside A is 250-450 and stevioside 250-300 times greater than the sweetness of sucrose (Kochikyan *et al.* 2006). However, the sweetness of the RebA (**Figure 36**) was significantly reduced after the treatment with the CGTases compared to the unmodified RebA. The modified SVglys and modified RebA did not differ in

sweetness (mean ratings 47.1 and 49.6, respectively). Likewise, sucrose equivalent (%) and the sweetness potency for SVglys and RebA, and their corresponding modified samples, respectively, did not show significant changes (**Table 15**). All the other sensorial attributes measured were rated at low levels, except liquorice flavour which did not differ between the RebA and SVglys samples, with or without modification. Importantly, metallic taste, bitter taste and the bitter aftertaste of SVglys were significantly reduced following their glucosylation by CGTase from *Geobacillus* sp (**Figure 36**).

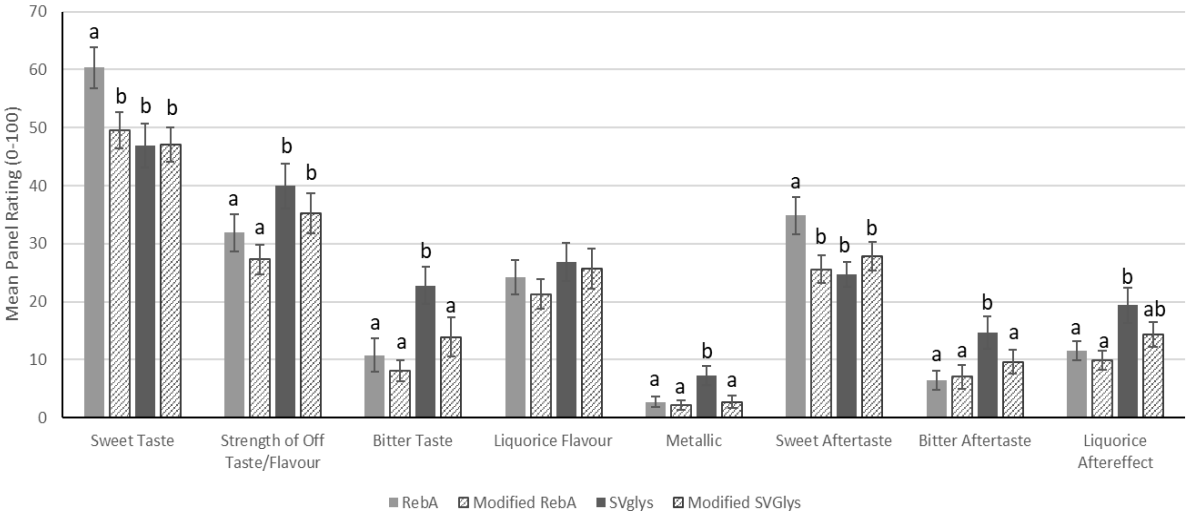


Figure 36. Mean panel ratings of attributes that either differed significantly between samples (* indicates $p<0.05$ from Fishers LSD) or where mean rating > 10. Attributes that were not significantly different between samples or rated < 10 were: Sour taste, Salty taste, Cooked sugar flavour, Cooling sensation, Stale flavour, Crusty bread flavour, Perfume and Cooling aftereffect.

Table 15. Sucrose equivalent and potency for unmodified and modified SVglys and RebA by CGTase from *Geobacillus* sp.

	Sucrose equivalent (%)	Sweetness potency
SVglys	4.5	140
Modified SVglys	4.5	140
RebA	5.3	223
Modified RebA	4.7	194

4.2.1.2 Mogrosides: MGE sample

Mogroside V is reported to have a sweetness potency approximately 250 times that of sucrose (Lindley, 2012). Previous literature has shown that bitterness and other off-flavours exist in MGE (50% mogroside V) (Tan *et al.* 2019). The transglucosylation reaction carried out with the CGTase from *Thermoanaerobacter* sp. under optimal conditions (see *section 4.1.2*), could lead to an improvement of taste qualities. In this study, we decided to test solutions at equivalent sweetness levels above 2% sucrose to ensure that we were able to evaluate both sweetness and bitterness in levels suitable for potential food application. The glucosylated MGE sample had a purity of >98% w/w respectively after the purification previously seen in **Part II** (*section 3.2.1*). This purification technique allowed to eliminate free carbohydrates, *i.e.* glucose, maltose and maltodextrins, present after the synthesis (as checked by GC-FID) and the resulting product was only based on mogrosides (unmodified and enzymatically glucosylated forms).

Of the 16 attributes rated, 5 differed significantly from sample to sample (**Figure 37**). In general, liquorice and metallic flavours were significantly decreased by the enzymatic modification, although so was the sweet taste. All other specifically defined taste and flavour attributes were rated at low levels. The lowering power of glucosylation on sweetness has been observed previously (Yoshikawa *et al.* 2005). This decrease is in inverse proportion to the amount of glucose monomer attached to the aglycone. However, in the case of the MGE obtained after the glucosylation reaction, the sweetness potency is only 1.2 times lower than in the unmodified mixture (**Table 16**). Consequently, the glucosylated mogroside resulted from modified MGE may still be considered as a HIS.

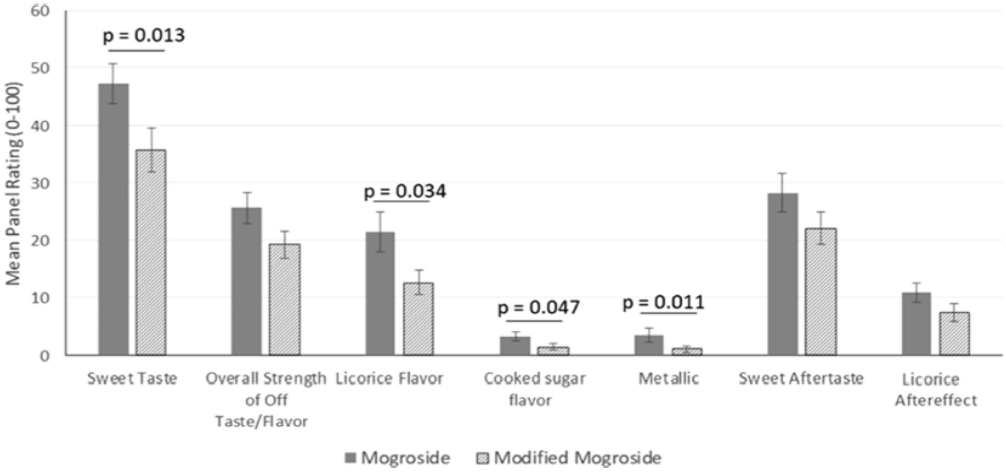


Figure 37. Mean panel ratings of attributes that either differed significantly between samples or where mean rating >10. Attributes that were not significantly different between samples or rated <10 were bitter taste, sour taste, salty taste, cooling sensation, stale flavour, crusty bread flavour, perfume, bitter aftertaste, and cooling aftereffect.

Table 16. Equivalent Sucrose and Potency for Unmodified and Modified MGE by CGTase from *Thermoanaerobacter* sp.

	Sucrose equivalent (%)	Sweetness potency
MGE	4.5	188
Modified MGE	3.7	156

4.2.2 Sweetness Potency of the modified steviol glycosides with prebiotic oligosaccharides

The sweet-tasting components of *S. rebaudiana* can be up from 250 to 400 times sweeter than sucrose (Ashwell, 2015). The maximum equivalent sucrose (% w/v) scores for the enzymatically modified steviol glycosides and GOS (mSVglys-GOS) and FOS (mSVglys-FOS) were 1.2% and 2.6%, respectively (**Table 17**). Devlamynck *et al.* (2019) efficiently trans-α-glucosylated stevioside and observed a decrease in sweetness due to the relatively large proportion of multi-α-glucosylated products of stevioside. Our results seem to support this hypothesis, as we observed a higher sweetness decrease on the one-pot synthesized GOS and modified SVglys which were, indeed, associated with a higher level of transglycosylation than on the modified SVglys on the one-pot synthesis of FOS. A possible explanation for the decrease in the sweetness potency of the modified SVglys could be the increased carbohydrate

length rendering them too large to interact with the taste receptor, as well as the fact that the prebiotic carbohydrates are considerably less sweet than sucrose (Crittenden *et al.* 1996; Ruiz-Aceituno *et al.* 2018).

Table 17. Concentrations matching for equivalent sweetness and sweetness potency of the modified SVglys sweeteners in comparison to sucrose.

	ES (% w/v)			SP % w/v)		
Sweetener (1% w/v)	Mean	Min	Max	Mean	Min	Max
Modified SVglys + FOS	2.4	2.2	2.6	240	220	260
Modified SVglys + GOS	0.8	0.5	1.2	80	50	120

4.2.3 Sweetness Potency of the modified mogrosides with prebiotic oligosaccharides

The profile of the different mogrosides from *S. grosvenorii* and consequently their flavour quality varies depending on the fruit maturity and the harvest period (Pawar *et al.* 2013; Tan *et al.* 2019). Certain mogrosides are known for presenting undesirable tastes as well as exerting different sweetness intensities which would compromise their uses (Muñoz-Labrador *et al.* 2021).

A primary sensory evaluation was carried out to evaluate the equivalent sucrose and the sweetness potency to 1% of enzymatically modified mogrosides and prebiotic oligosaccharides (FOS and GOS). The evaluation was performed to find out whether enzymatic modifications could lead to a reduction of the sweetness intensity and make them quantitatively more suitable in order to be efficiently used as a prebiotic. The assessment of the sensorial characteristic was carried out by a trained panel that used sucrose as a reference. The maximum equivalent sucrose (% w/v) scores for the enzymatically modified mogroside and GOS (mMV-GOS) and FOS (mMV-FOS) were 2.3% and 1.4%, respectively (**Table 18**).

Table 18. Concentrations matching for equivalent sweetness and sweetness potency of the modified MGE sweeteners in comparison to sucrose.

	ES (% w/v)			SP % w/v)		
Sweetener (1% w/v)	Mean	Min	Max	Mean	Min	Max
Modified MGE + FOS	0.9	0.4	1.4	90	40	140
Modified MGE + GOS	1.8	1.3	2.3	180	130	230

4.3 Part III Prebiotic evaluation of newly synthesized oligosaccharide-based sweeteners with the assistance of glycoside hydrolases

In **Part I** of Materials and Methods (*section 3.1.2*), two different fungal glycoside hydrolases (*i.e.,* β-fructosidase and β-galactosidase) were utilized to carry on optimal reactions of transglycosylation. Prebiotic structures (from DP 3 to 8) resulted from these reactions and previously characterized (*sections 4.1.3.1* and *4.1.4.1*), consisted in GOS, mainly formed by 6'-galactosyl-lactose and inulin-type FOS mainly formed by kestose, nystose and fructosyl-nystose. The synthesis of these prebiotic oligosaccharides in conjunction with the natural sweeteners studied (steviol glycosides and mogrosides) achieved similar yields compared to the reactions carried out only with the donor substrates as seen in **Part I** of Results (*section 4.1.3.1*). Simultaneously with the synthesis of the prebiotic oligosaccharides, the steviol glycosides and mogrosides were also enzymatically modified as explained in previous sections. In consequence, the evaluation of the prebiotic potential of these newly synthesized oligosaccharide-based sweeteners (labelled as mSG-FOS, mMV-FOS, mSG-GOS and mMV-GOS) together with their respective control samples will be addressed in the following sections.

4.3.1 Quantification of human faecal microbiota from in vitro *fermentation*

The composition of the faecal microbiota in the samples was evaluated by the analysis of the bacterial 16S rDNA genes. Seven bacterial groups, in addition to the total bacteria population, were analysed by the quantitative PCR procedure. The numbers and the significant differences of the individual bacterial groups obtained for the two sets of reactions (one-pot synthesis of GOS and FOS sweeteners), were expressed as the mean log CFU/mL of the four donors. Besides the negative control, in each set of reaction, SG and MV control samples were tested to optimally evaluate the modulatory effect on the microbiota of the hypothetical prebiotic synthesized substrates (mSG-FOS, mMV-FOS, and mSG-GOS, mMV-GOS). In order to evaluate the effect on the faecal bacterial population, the sample-based only in GOS parallelly synthesized without substrate was included in the fermentation analysis as an appropriate control. Positive control (scFOS) was also used as baseline control. The Kruskal-Wallis test showed some correlations between the samples.

4.3.1.1 Modulatory effect of new synthesized GOS-based sweeteners

4.3.1.1.1 Steviol glycosides group

Changes in bacterial population after 10 and 24 h of fermentation, using steviol glycosides, are shown in **Figure 38**. In the course of the fermentation, positive control, mSG-GOS and GOS control generally presented an increase in the bacterial count with respect to the negative control. As the significant effect ($p < 0.05$) seen for these mentioned bacteria in the total bacterial group compared to the negative at different hours.

The most remarkable effects were found for mSG-GOS at 10 h of fermentation, which values were significantly higher ($p < 0.05$) than the negative control at the start of the study in total bacteria, *Bacteroides-Prevotella Porphiromonas*, *Bifidobacterium*, *Clostridium coccoides*, *Enterobacteria* and *Enterococcus*, which values were finding also significant differences in the mentioned first three groups with the negative control after 10 h of fermentation. These behaviours agree with the significant differences found for either 10 or 24 h for GOS control and positive control samples. No major effects were found for *Atopobium* group, and no significant differences were stated for any of the samples for the *Lactobacillus* group probably due to the typical inter-individual variability found among the donors. In spite of this, positive control, mSG-GOS and GOS control samples increased with respect to the negative control during fermentation.

Accordingly, except for *Enterobacteria* group, SG control did not present significant differences with the negative control, otherwise showing in some cases significant differences with the other tested samples bringing out the potential prebiotic effect of them.

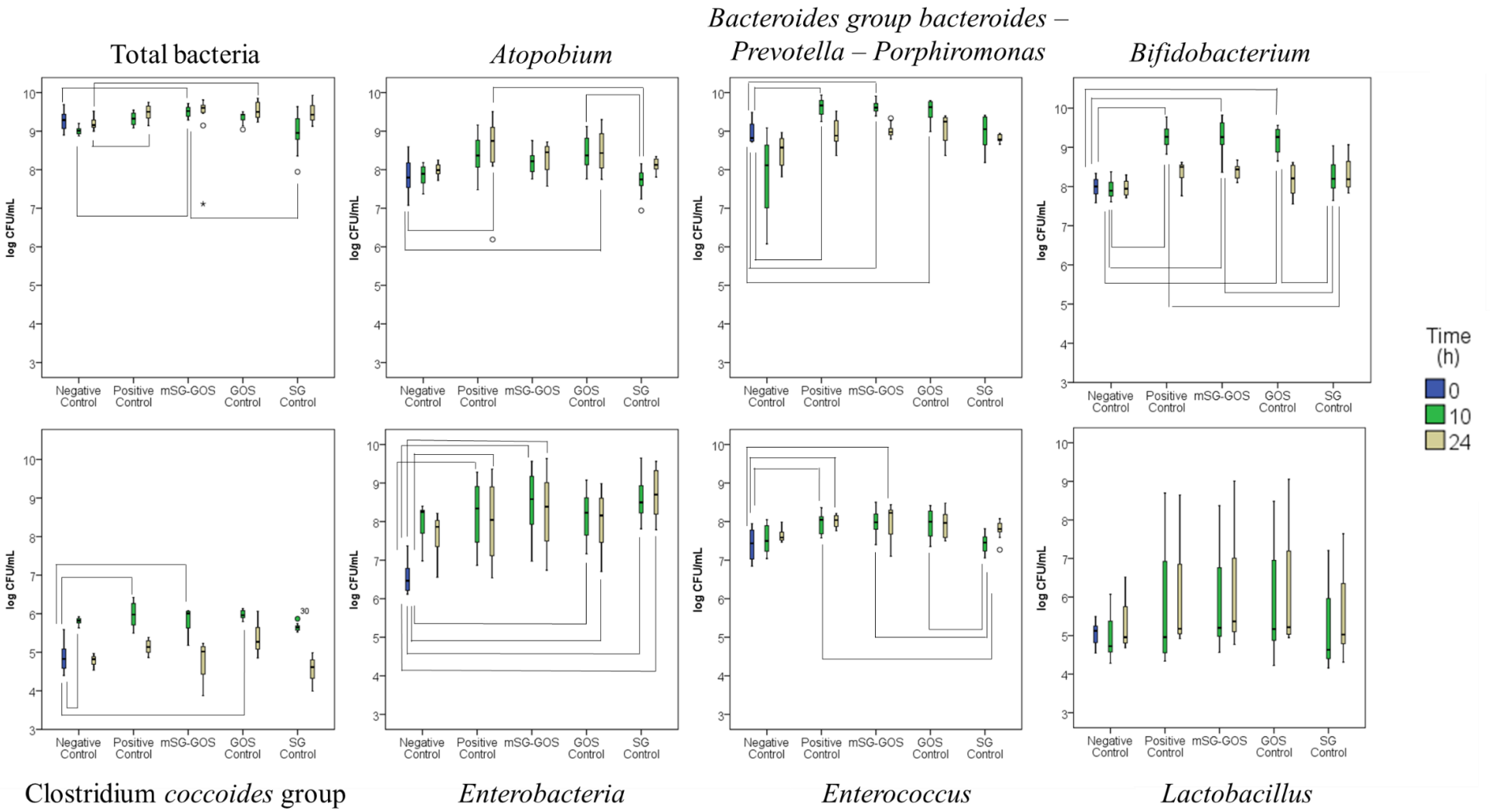


Figure 38. Mean quantitative real-time PCR results for steviol glycosides group samples, obtained from the faecal slurry cultures from four donors after 0 (corresponds to the sample taken from negative control vessel at the beginning of incubation), 10 and 24 h for each bacteriological group. The linked samples present significant differences ($p < 0.05$). SG control: unmodified steviol glycosides; mSG-GOS: enzymatically modified steviol glycosides.

4.3.1.1.2 Mogrosides

The boxplots in **Figure 39** show the summed data for mogroside samples with all bacterial groups at which samples were taken at 10 and 24 h after the start of the fermentation.

The sample mMV-GOS showed the maximum value for total bacteria at 24 h which were the only one significantly different compared to the negative sample at 0 h (9.6 ± 0.1 log CFU/mL versus 9.3 ± 0.2 log CFU/mL). Likewise, mMV-GOS sample showed an increase over time in the abundance of specific bacterial strains compared to the negative control values at different fermentation points. These changes are significant ($p < 0.05$) in all the bacterial groups but *Atopobium*, where mMV-GOS value is still higher than the negative control and MV control. Like in the steviol glycosides group of samples, GOS control and positive control served to clarify the prebiotic effect of mMV-GOS samples since the trends were similar. However, in contrast to steviol glycosides, mMV-GOS showed an effect on *Lactobacillus* by significantly increasing during 24 h of fermentation in comparison to negative control at different times of fermentation. Interestingly, mMV-GOS values for this bacterial group were equally significantly higher than MV control unlike positive control and GOS control that did not present any statistical variations.

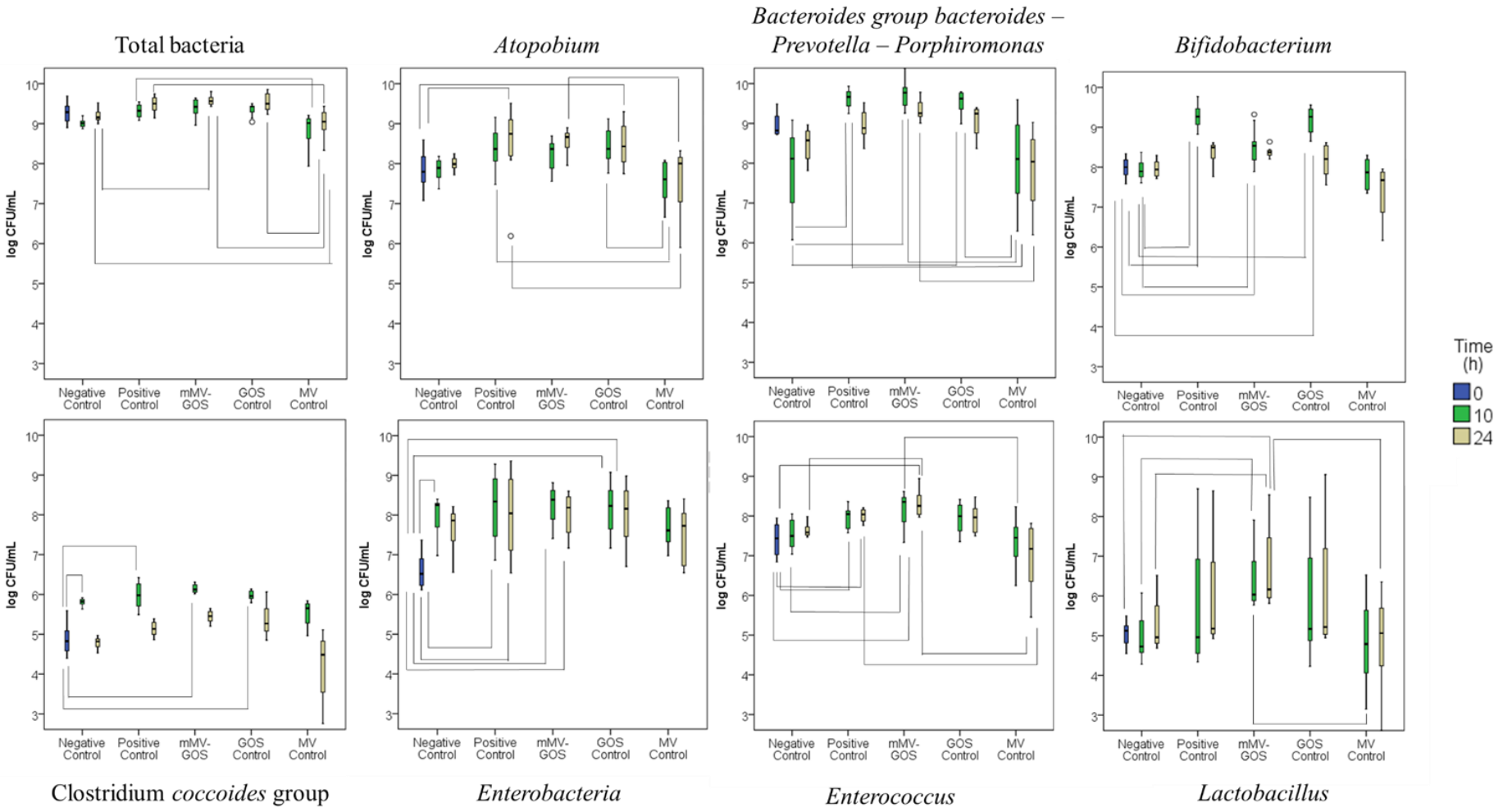


Figure 39. Mean quantitative real-time PCR results for mogrosides group samples, obtained from the faecal slurry cultures from four donors after 0 (corresponds to the sample taken from negative control vessel at the beginning of incubation), 10 and 24 h for each bacteriological group. The linked samples present significant differences ($p < 0.05$). MV control: unmodified mogrosides; mMV-GOS: enzymatically modified mogrosides.

4.3.1.2 Modulatory effect of new synthesized FOS-based sweeteners

4.3.1.2.1 Steviol glycosides

Figure 40 shows the results referring to the steviol glycosides group combined with FOS formation. Total bacteria population obtained values of 8.8 – 9.5 log CFU/mL, being lower in negative control and unmodified steviol glycosides (SG control) samples at 10 h.

With regard to the mSG-FOS sample, 10 h of incubation for four bacterial groups such as *Bacteroides-Prevotella Porphiromonas*, *Clostridium coccoides*, *Enterobacteria* and *Lactobacillus* were the best-performing ones for being significantly different ($p < 0.05$) with negative control at either the beginning or after 10 h of fermentation. Moreover, significant differences at some points of the fermentation were also noted with respect to SG control.

Although no other significant contributions were seen for the other faecal microbiota groups analysed, positive control and mSG-FOS samples generally presented similar values during fermentation, like for *Bifidobacterium* at 24 h of fermentation, and *Atopobium*, *Enterococcus* and total bacteria at 10 and 24 h. Similarly, while the results for mSG-FOS present a close tendency to positive control no relevant counts were depicted.

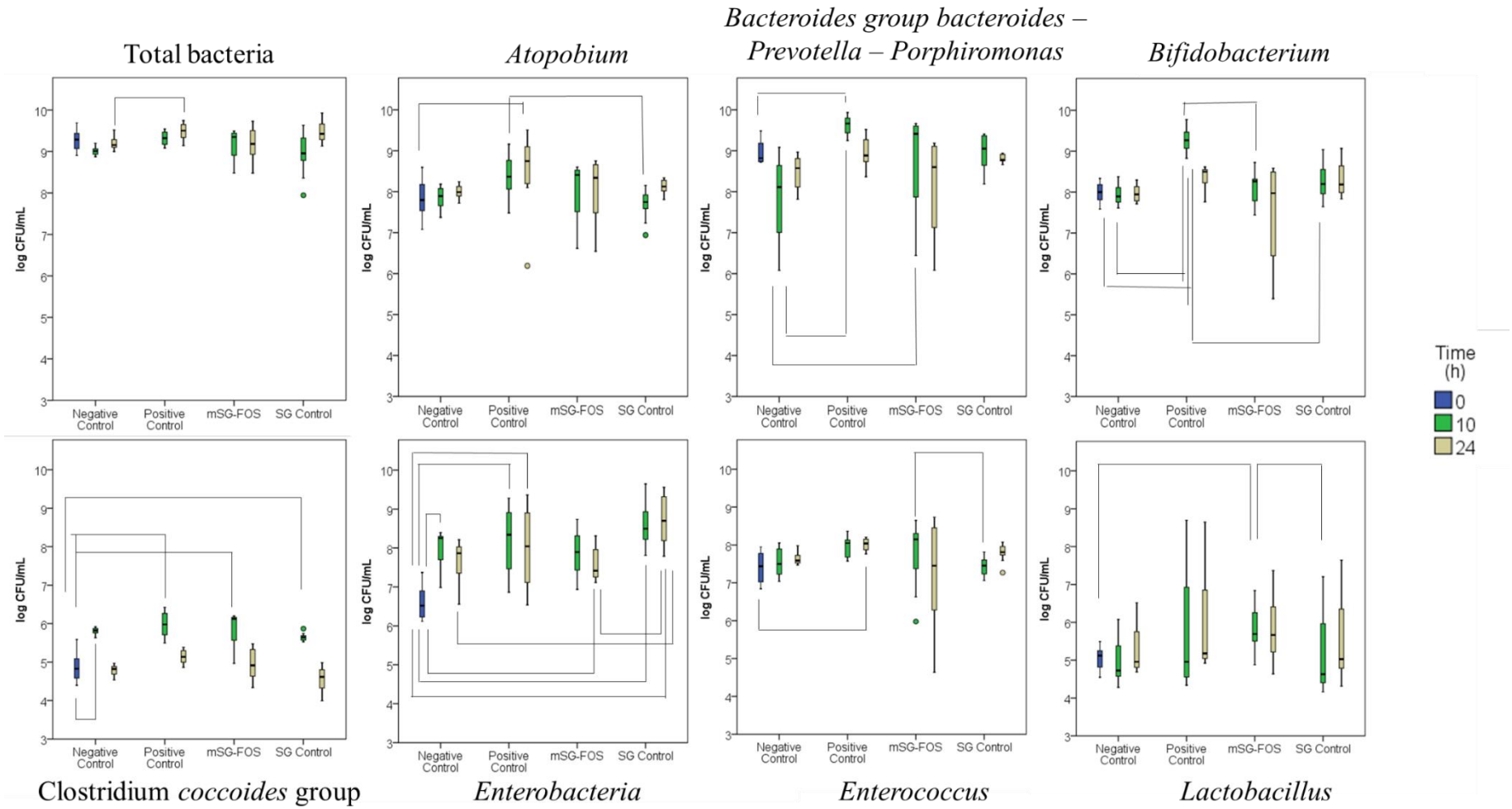


Figure 40. Mean quantitative real-time PCR results for steviol glycosides group samples, obtained from the faecal slurry cultures from four donors after 0 (corresponds to the sample taken from negative control vessel at the beginning of incubation), 10 and 24 h for each bacteriological group. The linked samples present significant differences ($p < 0.05$). SG control: unmodified steviol glycosides; mSG-FOS: enzymatically modified steviol glycosides.

4.3.1.2.2 Mogrosides

The bacterial population of the set of experiments based on the synergic formation of mogrosides and FOS is given in **Figure 41**. The concentration of total bacteria at 10 and 24 h, for both the positive control and mMV-FOS samples, were 9.4 ± 0.1 log CFU/mL and 9.5 ± 0.2 log CFU/mL, respectively.

Notable effects were seen for mMV-FOS samples at 10 and 24 h compared to the negative control at either 0, 10 and 24 h, to significantly promote the growth ($p < 0.05$) of total bacteria count and other five bacterial groups such as *Atopobium*, *Bacteroides-Prevotella Porphiromonas*, *Clostridium coccoides*, *Enterobacteria*, and *Enterococcu*s.

A slight bifidogenic effect was observed for the mMV-FOS sample reaching significant values compared to the MV control sample at the same time of fermentation (24 h). And the levels of *Lactobacillus* group were enhanced for mMV-FOS together with the positive control sample, but no further significances were noted.

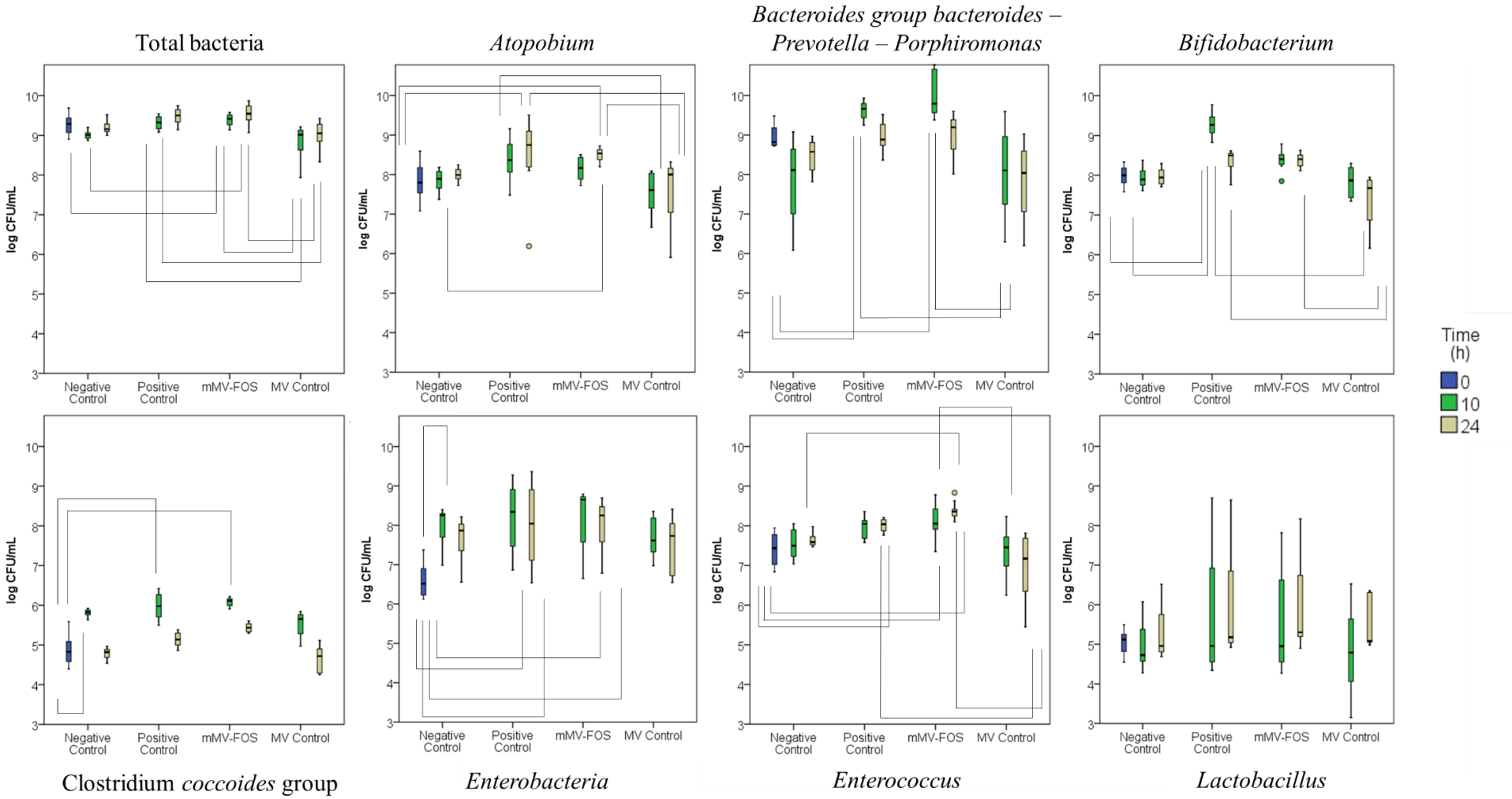


Figure 41. Mean quantitative real-time PCR results for mogrosides group samples, obtained from the faecal slurry cultures from four donors after 0 (corresponds to the sample taken from negative control vessel at the beginning of incubation), 10 and 24 h for each bacteriological group. The linked samples present significant differences ($p < 0.05$). MV control: unmodified mogrosides; mMV-FOS: enzymatically modified mogrosides.

4.3.2 Evolution of microbial metabolites lactate and SCFAs in faecal batch-cultures

Different organic acids were monitored during the *in vitro* fermentation cultures. Lactate, acetate, propionate, and butyrate amounts were found in close molar proportions to those reported by other authors testing other prebiotics (Gibson *et al.* 1995b; Kleessen *et al.* 1997). **Tables 19** and **20** expressed the concentration of the organic acids as the mean data obtained from the duplicate samples of the four volunteers (n=8), for comparative purposes. As expected, the most abundant organic acid was acetate followed by propionate and butyrate. The production of the organic acids varied between the substrates studied during the incubation. Generally, all the SCFAs experimented a concentration increment along with fermentation in contrast to the lactate organic acid. A lack of fermentation was observed for the negative control vessel for all the measured organic acids compared to the carbohydrates-based substrates.

4.3.2.1 Analysed organic acids in new synthesized GOS-based sweeteners

Prebiotic-synthesized compounds either with or without sweeteners showed higher cumulative SCFA production than the SG and MV control samples, behaving closely as the positive control tested (**Table 19**). In particular, mSG-GOS and mMV-GOS were positively correlated with acetate and propionate production, whose values obtained were significantly different from the negative control at the initial point and along with the fermentation (at 10 and 24 h). Butyrate production instead, presented significant differences for mMV-GOS in agreement with the positive control sample, which is significantly different at the same fermentation times compared to the negative control sample (at 24 h). The highest production of lactate for all well-influenced samples was at 10 h. Opposite responses were taken by the SG and MV control samples, presenting lower organic acid concentrations at 10 and 24 h of fermentation, similar to negative control results. Moreover, the results for both SG and MV control samples were interestingly different with respect to positive and GOS control samples, with statistical significances.

Table 19. Mean organic acid concentrations after *in vitro* fermentation at 0, 10 and 24 h for the synthesis of GOS-based samples.

		Acid concentration (mM)						
	Time (h)	**Negative Control**	**Positive Control**	**GOS Control**	**mSG-GOS**	**SG Control**	**mMV-GOS**	**MV Control**
Lactate	0	0.11±0.00	0.11±0.00	0.11±0.00	0.11±0.00	0.11±0.00	0.11±0.00	0.11±0.00
	10	0.03±0.00	20.90±0.19* **	15.35±0.34 * **	13.09±0.36* **	0.09±0.02# ##	3.35±0.03* **	0.00±0.00# ##
	24	0.00±0.00	3.75±5.30	5.93±0.10	0.26±0.37	0.00±0.00	0.00±0.00	0.00±0.00
Acetate	0	0.73±0.01	0.73±0.01	0.73±0.01	0.73±0.01	0.73±0.01	0.73±0.01	0.73±0.01
	10	8.15±0.35	43.89±0.10 * **	43.09±0.30* **	37.97±0.11* **	4.57±0.14# ##	20.32±0.15* **	6.44±1.16# ##
	24	12.99±0.16	43.60±0.17 * **	56.11±0.24* **	40.03±2.46* **	13.89±5.86# ##	30.78±0.12* **	9.25±0.12# ##
Propionate	0	0.15±0.00	0.15±0.00	0.15±0.00	0.15±0.00	0.15±0.00	0.15±0.00	0.15±0.00
	10	1.86±0.12	5.84±0.01* **	4.85±0.07* **	4.31±0.02* **	1.35±0.06# ##	9.49±0.17* **	2.34±1.19# ##
	24	2.53±0.10	22.15±10.94* **	11.89±0.27* **	13.12±0.57* **	3.70±2.20# ##	14.19±0.08* **	1.82±0.05# ##
Butyrate	0	0.11±0.00	0.11±0.00	0.11±0.00	0.11±0.00	0.11±0.00	0.11±0.00	0.11±0.00
	10	1.36±0.01	2.89±0.01*	4.74±0.03* **	2.49±0.03*	0.68±0.01# ##	2.43±0.02*	1.66±1.15# ##
	24	2.45±0.00	6.56±0.69* **	13.10±0.05 * **	5.41±1.20*	2.18±0.97# ##	7.27±0.00* **	1.60±0.01# ##

Negative control vessel corresponds only to the inoculum, no carbohydrate was included. Time point 0 h refers to the aliquot taken from the negative control vessel. Experiments were carried out with faecal microbiota from four donors. Results are shown as mean (n = 5) with the corresponding standard deviation.
*Significantly different ($p<0.05$) at 0 h
**Significantly different ($p<0.05$) from negative control at the same fermentation time
Significantly different ($p<0.05$) from GOS control at the same fermentation time
##Significantly different ($p<0.05$) from positive control at the same fermentation time

4.3.2.2 Analysed organic acids in new synthesized FOS-based sweeteners

The production pattern for lactate consisted of an increment during fermentation up to the tenth hour followed by a decrease until the end of the incubation (**Table 20**). The lactate was substantially higher for positive control. Higher quantities were also observed for the mSG-FOS and mMV-FOS samples. However, significant values were observed only for the positive control and mSG-FOS compared to the negative control at 10 h. Likewise, acetate concentrations resulted in an increase throughout fermentation with significant differences found for mSG-FOS and MV-FOS together with the positive control in comparison to the negative control at 0 h. Nonetheless, the acetate concentrations remained stable from 10 to 24 h of fermentation. Despite the increase in lactate and acetate productions for SG and MV control samples, their values were significantly different with respect to the positive control at the same time of fermentation.

Conversely, propionate and butyrate values were moderate giving rise to an increase over the 24 h of fermentation at which time the maximum values were obtained. Hypothetical prebiotic substrates presented similar behaviour obtaining the higher values. This behaviour can be seen in either the positive control, mSG-FOS and mMV-FOS samples at 10 and 24 h of fermentation, which were significantly different in respect to the negative control at 0 h.

Table 20. Mean organic acid concentrations after *in vitro* fermentation at 0, 10 and 24 h for the synthesis of FOS-based samples.

	Acid concentration (mM)						
SFCAs	Time point (h)	Negative control	Positive Control (sc-FOS)	mSG-FOS	SG control	mMV-FOS	MV control
Lactate	0	0.11±0.00	0.11±0.00	0.11±0.00	0.11±0.00	0.11±0.00	0.11±0.00
	10	0.03±0.00	20.90±0.19[b]	1.52±0.01[b]	0.09±0.02[c]	0.94±0.09	0.00±0.00[c]
	24	0.00±0.00	3.75±5.30	0.20±0.01	0.00±0.00	0.00±0.00	0.00±0.00
Acetate	0	0.73±0.01	0.73±0.01	0.73±0.01	0.73±0.01	0.73±0.01	0.73±0.01
	10	8.15±0.35	43.89±0.10[a,b]	15.59±4.28[a]	4.57±0.14[c]	21.53±0.07[a]	6.44±1.16[c]
	24	12.99±0.16	43.60±0.17[a]	21.93±0.15[a]	13.89±5.86[c]	29.56±0.28[a]	9.25±0.12[c]
Propionate	0	0.15±0.00	0.15±0.00	0.15±0.00	0.15±0.00	0.15±0.00	0.15±0.00
	10	1.86±0.12	5.84±0.01[a]	6.94±1.20[a]	1.35±0.06	9.71±0.09[a]	2.34±1.19
	24	2.53±0.10	22.15±10.94[a]	8.66±0.04[a]	3.70±2.20	13.08±0.16[a]	1.82±0.05
Butyrate	0	0.11±0.00	0.11±0.00	0.11±0.00	0.11±0.00	0.11±0.00	0.11±0.00
	10	1.36±0.01	2.89±0.01[a]	2.59±0.80[a]	0.68±0.01	3.02±0.14[a]	1.66±1.15
	24	2.45±0.00	6.56±0.69[a]	6.14±0.04[a]	2.18±0.97	6.89±0.03[a]	1.60±0.01

Negative control vessel corresponds only to the inoculum, no carbohydrate was included. Time point 0 h refers to the aliquot taken from the negative control vessel. Experiments were carried out with faecal microbiota from four donors. Results are shown as mean (n = 5) with the corresponding standard deviation.
[a]Significantly different ($p < 0.05$) from the 0 h value.
[b]Significantly different ($p < 0.05$) from the negative control value at the same fermentation time.
[c]Significantly different ($p < 0.05$) from the positive control value at the same fermentation time.

5

Discussion

OUTLINE

5 DISCUSSION

Many metabolic disorders and other severe comorbidities are becoming global issues reaching epidemic proportions. This transversal health concern is widely attributed to excessive sugar consumption through many forms of processed foods, including sugary foods and beverages, with serious consequences to human health (Miele *et al.* 2017; Van Dam *et al.* 2020; Yang *et al.* 2014). Thus, the public perception of the impact of diet on public health has established the need for alternative low-calorie sweeteners as a way to offer healthier alternative formulations by significantly reducing the use of sucrose. These abridge a diversity of compounds, from high-intensity sweeteners to oligosaccharides with low sweetening power but with a prebiotic role. For this reason, among natural sweeteners, the extracts obtained from *Stevia rebaudiana* and *Siraitia grosvenorii* are gaining special attention due to their sweet quality, low-calorie characteristics and also their pharmacological properties (Gong *et al.* 2019; Prakash, 2016).

However, certain terpene glycosides as mogrosides and steviol glycosides are known to present undesirable taste characteristics such as bitterness due to liquorice and cooling elements consequently limiting their uses (Lindley, 2012). A significant relationship between the structure and flavour has been described and the flavour profile is mostly determined by the glycosyl moieties with linkage- and regiospecificity (Adari *et al.* 2016; Xia *et al.* 2008). As a consequence, some biosynthetic procedures based on enzymes from different bacteriological sources have been carried out, focused on either producing specific glycosides or improving their taste profile (Muñoz-Labrador *et al.* 2020; Muñoz-Labrador *et al.* 2021; Chaturvedula *et al.* 2011; Gerwig, *et al.* 2016; Zhao *et al.* 2018). The application of enzymatic approaches for low caloric natural sweetener extraction and/or modification is a relatively new area and significant developments toward improving the enzyme-assisted processes still require more research.

Throughout the dissertation, it had been seen the influence of specific enzymes on the natural sweeteners steviol glycosides and mogrosides extracts. The enzymatic modifications not only vary regarding the native producer strains and the functionality expressed but on the

performance conditions. The new conformation changes on the original structures of the studied natural sweeteners displayed either deglycosylation, transglycosylation and/or transglucosylation activities that resulted in the formation of diverse compounds that were already present in different concentrations or were newly synthesized.

With the aim to provide a global overview of how the enzymatic modifications might affect their edulcorating qualities and/or have a positive repercussion to health, this section is structured according to the enzymes utilized in the following points:

- the products resulted from the enzymatic modifications by Cyclodextrin Glycosyl Transferases, and
- the products resulted from the enzymatic modifications by β-Fructosidase and β-Galactosidase.

5.1 Enzymatic modification of *Stevia* glycosides and mogroside V by Cyclodextrin Glycosyl Transferases

CGTases are enzymes mainly used in the food industry to catalyze the conversion of starch in cyclodextrins and are a promising approach to modify natural products (Gerwig *et al.* 2016). In **Part I** (*section 3.1.1*), the use of the response surface methodology approach allowed to obtain optimal rates of transglucosylation of a *Stevia* extract; composed of a mixture of steviol glycosides (SVglys) and the single rebaudioside A (RebA) compound, and a cucurbitane triterpenoid extract formed mainly by mogroside V (≥ 50% [MGE]). Due to the nature of the catalytic activity of the CGTases, the reactions could yield up to multiple-(α1-4)-glucosylated products. More specifically, the elongations could hypothetically occur on the C-13-β-sophorosyl unit and the C-19-ester-linked Glc(β1-residue of the aglycone of steviol glycosides, and either on the C-3 or the C-24 chain of the aglycone of mogrosides. However, it has been also identified in steviol glycosides transglucosylation reactions 1,6- and 1,2-linkage products in α-orientation (Charan *et al.* 2019; Gerwig *et al.* 2016; Wang *et al.* 2014).

The reaction products of the enzymatic modifications carried out in this thesis were subjected to a complete structural analysis using LC-DAD, LC-ESI-MS and MALDI-TOF-MS techniques. The transglucosylation resulted in 11 and 17 glucose units on the respective aglycones for the modified SVglys/RebA and MGE, reaching for all substrates the highest glucosylation rate ever reported, to the best of our knowledge. Prakash *et al.* (2014) carried out a maximum transglucosylation of up to 8 glucose units for steviol glycosides samples using CGTases from *Bacillus stearothermophilus*, while Yoshikawa *et al.* (2005) obtained transglucosylated products with only 1 to 3 additional glucose residues on the mogroside V structure.

In addition, in **Part II** (*section 3.2*) the impact of structural modification on the sensorial profile was evaluated by a tasting panel. These modifications significantly improved some flavour attributes such as liquorice taste, bitter taste, metallic taste or bitter aftertaste, however, the sweetness potency remained practically unaltered from the original starting extracts. These interesting sensorial characteristics of the newly synthesized products make

them more suitable compounds to be applied as alternative high-intense sweeteners for both consumptions or within food formulations.

5.2 Enzymatic modification of Stevia glycosides and mogroside V by β-Fructosidase and β-Galactosidase

In a similar way, other enzymatic conversions of these natural sweetening mixtures (SVglys and MGE) comprised the use of two fungal glycoside hydrolases: a β-galactosidase from *Aspergillus oryzae* and a β-fructosidase from *Aspergillus aculeatus*.

Health-promoting properties have been described for either mogrosides from *Siraitia grosvenorii* or steviol glycosides from *Stevia rebaudiana*, such as antioxidative, anti-inflammatory, immunomodulatory, and anticancer properties, among others (Gong *et al.* 2019; Kurek *et al.* 2019). In this context, these reactions enabled the production of suitable amounts of specific compounds for further investigations into their sensorial characteristics, biological activities and therapeutic uses (Liu *et al.* 2015; Harada *et al.* 2016; Mizushina *et al.* 2006). Moreover, it is worth noting that the concentrations of the different compounds of the extracts, including those from *Stevia rebaudiana* and *Siraitia grosvenorii*, are also influenced by different factors such as geographical location, climate conditions, nutrition status, ripening process, botanical varieties, etc (Lu *et al.* 2012; Muanda *et al.* 2011; Wang *et al.* 2018).

For steviol glycosides extract, SVglys, both enzymes catalyzed the highly selective conversion of stevioside to rubusoside through the cleavage of the β-1,2-glucosidic linkage of the β-sophorosyl moiety at the C-13 site of the steviol backbone, whilst the β-glucosidic linkage at C-19 was unaffected by the hydrolytic activities for any of the enzymes. Similar enzymatic hydrolysis of stevioside into rubusoside has been reported using fungal β-galactosidase from *Aspergillus* sp. and from *Penicillium* sp. (Wan *et al.* 2012; Ko *et al.* 2013) or bacterial enzymes such as a lactase from *Thermus thermophilus* (Nguyen *et al.* 2014). This dual functional activity has been previously reported for other microbial β-galactosidases (Fan *et al.* 2011; Hansson *et al.* 2002; Ishikawa *et al.* 2005; Nakkharat *et al.* 2006). Interestingly, the use of a representative mixture of steviol glycosides rather than an isolated glycoside has put in evidence the

substrate specificity of both enzymes since no hydrolytic activity on rebaudioside A or rebaudioside C was observed (**Figure 22, Tables 11** and **12**), in agreement with Wan *et al.* (2012) using a β-galactosidase from *Aspergillus* sp. This is especially remarkable in the case of rebaudioside A as it is considered to be the best steviol glycoside sweetener with respect to the intensity of sweetness as well as the quality of taste with no bitterness or after-taste (Fukunaga *et al.* 1989).

In the case of the mogroside V-based extract, MGE, the resulting mogrosides varied on the glucose units linked at the C-3 and C-24 hydroxyl groups of the mogrol aglycone (Dai *et al.* 2014; Itkin *et al.* 2016). With this extract, interesting behaviours were observed for either the reactions based only on the substrates and the same reactions but including the disaccharide acceptors. The disaccharide-free reactions promoted the formation of higher mono-glycosylated compounds as MG-I (either MG-1A1 or MG-IE1), which can be easily reconverted into its aglycone mogrol. These conversions suppose a revalued methodology to obtained specific compounds, such as MG-I and mogrol, which are minor compounds identified in LHG extract, whilst the reactions carried out in the presence of the respective disaccharides led to the formation of larger quantities of MG-II and MG-IV, respectively. Also, further chemical modifications corresponding to ion peaks identified as glycosylations were revealed. Several authors have also studied the bioconversion of mogrosides through crude enzymes extracts from the human intestine (Yang *et al.* 2007), extracellular β-glucosidases from fungi (Chiu *et al.* 2020), β-glucosidases from yeast cultures (Chiu *et al.* 2013) and enzymes from different bacteriological sources (Zhou, 2014).

In addition to the development of NCDs and other chronic diseases associated with diet, sugar consumption can also lead to a disruption of the gut bacterial populations (*i.e.*, dysbiosis) resulting in gastrointestinal chronic diseases, including ulcerative colitis, Crohn's disease and irritable bowel syndrome, and in the same manner, this dysbiosis *per se* can influence to more systemic diseases such as obesity and types 1 and 2 diabetes (Brown *et al.* 2012; Carding *et al.* 2015; Di Rienzi *et al.* 2020). Hence, there is a growing interest calling to formulate products with not only low-calorie and reduced-sugar content, but also functional ingredients that confer added health benefits (Farzanmehr *et al.* 2009; Sangeetha *et al.* 2005).

In this sense, the reactions performed with β-galactosidase and β-fructosidase using disaccharides donors, lactose and sucrose, specific to each enzyme, supposed an innovative proceeding, via one-pot enzymatic synthesis of a combination of modified steviol glycosides and modified mogrosides together with high-value prebiotic oligosaccharides (*i.e.* GOS or FOS). The methodology established optimal values for different parameters, and among the set conditions, the final reactions were performed with the lowest sweetener concentrations (0.2 and 1.5%; w:v). The concentrations for the samples obtained with steviol glycosides obey the ADI established by the FAO/WHO specifications (4 mg/kg body weight per day). Based on the reviewed toxicological data, it is concluded a "not specified" ADI is appropriate for LHG extract, however, several studies such as the recent study published by Bhusari *et al.* (2021) have evaluated the exposure through *in vitro* metabolism of purified monk fruit extracts in order to provide support for an approach to assess the ADI for mogrosides (2 mg/kg body weight per day) although additional well designed *in vivo* studies would be needed to validate it. The results revealed that both enzymes catalyzed the deglycosylation and transglycosylation of both steviol glycosides and mogrosides. In order to make our data more easily extrapolated to an industrial context, a representative primary extract of the sweeteners was used (SVglys and MGE commercial extracts). However, the complexity of such mixtures impaired the isolation of the new individual transglycosylated compounds, restricting the unambiguous identification of the elongation of the carbohydrate chains on the different carboxyl groups of each aglycone. Previous studies on the transglycosylation of steviol glycosides have been mainly focused on individual stevia components rather than on a representative steviol glycosides mixture. For instance, both isolated rubusoside and stevioside were transgalactosylated, with lactose as a donor, by β-galactosidases from *Bacillus circulans, Escherichia coli, A. oryzae, Penicillium multicolor, Kluyveromyces lactis* (Kitahata *et al.* 1989) and *Sulfolobus* sp. (Wan *et al.* 2015), respectively. Rubusoside was transglycosylated preferentially on the glucosyl residue at the 13-hydroxyl group whereas the stevioside yielded mono-, di-, and tri-glycosylated steviosides although their structures were not determined. No data on the potential simultaneous formation of GOS was provided by any of these studies. Likewise, transfructosylated derivatives of the individual stevioside and rubusoside were synthesized by β-fructofuranosidase from *Arthrobacter* sp. K-1 following the transfer of a β-

2,6-linked fructose residue to the glucosyl moiety at the 19-carboxyl group (Ishikawa *et al.* 1990). Similarly, Xu *et al.* (2009) described the introduction of a fructose molecule at the 19-carboxyl group on both the stevioside and rebaudioside A catalyzed by a β-fructofuranosidase from *Arthrobacter* sp. 10137.

The prominent deglycosylation obtained for the β-galactosidase and β-fructosidase with each substrate and the respective acceptor seemed to prompt a decrease of the relative sweetness. This advantageous qualification enables applying similar doses to sucrose and makes these newly synthesized products more suitable to be used as potential sweetener-based ingredients, but also to be capable of exerting the nutritional and functional effects proper of both the dietary fibre and the prebiotic oligosaccharides formed. In certain formulations, HIS such as *Stevia rebaudiana* and *Siraitia grosvenorii* are commonly blended with other sweeteners in order to mask undesired flavours and other off-note characteristics and to provide the bulk needed to be incorporated as ingredients (Konar *et al.* 2016; O'Donnell *et al.* 2012; Pawar *et al.* 2013). Prebiotic oligosaccharides are described to be less sweet than sucrose but lacking undesirable attributes (Ruiz-aceituno *et al.* 2018; Spohner *et al.* 2016). Thus, in a more in-depth sensory analysis, a synergistic improvement of the sensorial attributes of the sweeteners could even be observed due to the sensorial profiles that prebiotic oligosaccharides have together with the structural modification carried out in the reaction itself. So, these very last synthesized compounds would not only be more suitable to be consumed but would also suppose the formation of fibre-rich dietary products which have both physicochemical and physiological advantageous properties.

Thus, we aimed to study the prebiotic potential of the synthesized oligosaccharide-based sweeteners by an *in vitro* evaluation assessed by the human faecal microbiota and end-products detection (**Part III**; *section 4.3*). The batch-culture system consisted of a fast and cost-effective procedure that permitted pH control (6.7 – 6.9) to simulate the colon conditions (Reichardt *et al.* 2018). The examination of the targeted bacterial population was permitted by the 16S rDNA species-specific qPCR primers, a technique characterized by its rapidity and high reproducibility (>99 %), aside from being proven as one of the most accurate methods for bacterial population identification (Kurakawa *et al.* 2015; Matsuda *et al.* 2009). SCFAs are the

main products arisen from microbial fermentation. In combination with the mentioned biomolecular techniques, organic acids were quantified through analytical techniques, allowing a comprehensive approach of the major human faecal microbiota produced and the metabolic response pathways.

The large intestine consists of a dynamic microbial ecosystem with a population of 10^{10} to 10^{11} CFU/g of living bacteria, comprising around 50 genera (Gibson *et al.* 1995; Topping *et al.* 2001) such as *Lactobacillus*, *Bacillus*, *Clostridium*, *Enterococcus*, *Ruminococcus*, *Bacteroides* and *Bifidobacterium* genera representing 90% of the gut microbiota (Rinninella *et al.* 2019). The colonic bacteria are responsible for metabolic interactions ensuing from the fermentation process which course produces the synthesis and absorption of metabolites and nutrients (Roberfroid *et al.* 2010). It is evidenced how nondigestible components as the well-known prebiotics promote the growth of beneficial bacteria that perform metabolic mechanisms, mainly by enhancing the SCFAs productions (Guarner *et al.* 2003; Martins *et al.* 2019). The main SCFAs produced in the anaerobic fermentation are acetate, propionate, and butyrate (60:20:20) in an estimated range from 20 mM to 140 mM depending on whether it is produced in the proximal or distal colon (Wong *et al.* 2011).

As previously seen, the consumption of sweeteners has an impact on the gut microbiota and, therefore, on health (Suez *et al.* 2014; Vamanu *et al.* 2019). However, low calories sweeteners as the herein studied steviol glycosides and mogrosides have barely been investigated regarding their influence on the microbial community (Lobach *et al.* 2019). Only several *ex vivo, in vivo* and *in vitro* studies — performed with humans, mice, hamsters, rats and pigs — using these sweeteners as substrates, acknowledged the hydrolysis by digestive enzymes and intestinal microbiota, mainly by *Bacteroidaceae*, and their metabolization into their respective aglycones, steviol and mogrol (Geuns, 2003; Hutapea *et al.* 1997; Koyama *et al.* 2003; Purkayastha *et al.* 2016; Xu *et al.* 2015). Additionally, although there is a minimal influence on determining bacterial strains due to the glucose-conjugated groups linked to the core structure of the mentioned sweeteners, no substantial selective growth of beneficial bacteria occurred, suggesting a lack of effect on the colonic microbiota (Mahalak *et al.* 2020).

Hence, both unmodified steviol glycosides and mogrosides studied in this work, did not appreciably interfered with the growth of the bacteria studied, nor on the SCFAs or lactate production when compared to the corresponding negative controls. It can be also explained by the low quantity of each sweetener employed in the culture system, whose amounts are equivalent to that used in the modified substrates while in an adequate quantity to still act as a sweetener. Gardana *et al.* (2003) reported the fermentation profile using human faecal inoculum with stevioside and rebaudioside A, and any steviol glycosides showed an increase in the bacterial population. However, to the best of our knowledge, no reports have addressed the effect of single mogrosides on human gut microbiota so far.

In numerous *in vitro* and *in vivo* models, prebiotic oligosaccharides such as fructooligosaccharides (FOS) or β-galactooligosaccharides (GOS) have been shown to confer beneficial functions to the host health by selectively stimulating the growth of beneficial bacteria in the colon (Cummings *et al.* 2002; Ferreira-Lazarte *et al.* 2017; Hernandez-Hernandez, 2019; Poeker *et al.* 2018; Tzortzis *et al.* 2004). The fermentation of FOS and GOS and their conversion to lactic, acetic, butyric and propionic acids, take place in the proximal colon resulting in an easy digestion (Kumar *et al.* 2018; Nie *et al.* 2020; Wang *et al.* 2020). The molecular weight, monomer composition and the glycosidic linkages play an important role in the biological processes (Gibson *et al.* 2004; Li *et al.* 2015). Moreover, colonic microbiota and consequently the metabolites greatly vary among hosts due to inter-individual variations throughout life (Gill *et al.* 2008; Ursell *et al.* 2012).

A strong positive correlation was found between SCFAs production and bacterial abundance following the fermentation of the newly synthesized oligosaccharide-based sweeteners, whose overall results can be seen in **Table 21**. All carbohydrates seemed to act as fermentable substrates enhancing the SCFAs and bacterial production during fermentation. Quantitatively, the molar ratio distribution in response to the synthesized sweeteners was greater than to those values corresponding to the negative control. However, it is important to point out the existence of cross-feeding between members of the colon microbiota towards the organic acids production (Falony *et al.* 2006). *Bifidobacterium* species are mostly acetate and lactate producers (Salazar *et al.* 2008); positive control and GOS-based sweeteners resulted

in great quantities of *Bifidobacterium*, being significantly higher ($p < 0.05$) than negative and/or unmodified substrates (SG control and MV control). Nevertheless, the significant increase in *Bifidobacterium* species was not observed in the FOS-based sweeteners regardless of the HIS (*i.e.*, steviol glycosides or mogrosides) used as acceptor (**Table 21**). This effect is in concordance with the lactate values, which were also significant in the positive control, mSG-GOS and mMV-GOS, and both abundances decreased at 24 h of fermentation. Beneficial effects, many of which attributed to the SCFAs production, are described for *Bifidobacterium* such as the inhibition of pathogens, vitamins synthesis, restoration of the intestinal flora, immunomodulatory, among others (Gibson *et al.* 2004). However, it must be noted that acetate production is significantly higher in all prebiotic samples experiencing an increase upon the time of fermentation. *Bacteroides-prevotella-porphiromonas* group also interferes in the acetate production and converts propionate from lactate via succinate pathway, and butyrate from lactate and acetate according to the encoded enzymes in the genes they express, whose SCFAs increased after 24 h. For this bacteriological group, a significant increase has also been observed for all the prebiotic structured samples, which together with the *Bifidobacterium* results are in agreement with the reported results of other authors for commercial FOS (Ho *et al.* 2018; Likotrafiti *et al.* 2014; Moniz *et al.* 2016).

Table 21. Main population significantly stimulated by the newly synthesized oligosaccharide-based sweeteners and their millimolar (mM) SCFAs concentrations at different times of incubation.

Modified sweeteners	Bacterial population	SCFA* (Propionate/Acetate/Butyrate, mM)	
		10 h	24 h
mSG-GOS	*Bacteroides-Prevotella Porphiromonas* ↑[10ab] *Bifidobacterium* ↑[10ab] *Clostridium coccoides*↑[10a] *Enterobacteria* ↑[10a,24a] *Enterococcus* ↑[24a]	4/37/2	13/40/5
mSG-FOS	*Bacteroides-Prevotella Porphiromonas* ↑[10b] *Clostridium coccoides*↑[10a] *Enterobacteria* ↑[24a] *Lactobacillus*↑[10b]	7/16/2	9/22/6
mMV-GOS	*Bacteroides-Prevotella Porphiromonas* ↑[10b] *Bifidobacterium* ↑[10ab] *Clostridium coccoides*↑[10a] *Enterobacteria* ↑[10a,24a] *Enterococcus* ↑[10ab,24ab] *Lactobacillus*↑[10b,24ab]	9/20/2	14/30/7
mMV-FOS	*Atopobium* ↑[24ab] *Bacteroides-Prevotella Porphiromonas* ↑[10b] *Clostridium coccoides*↑[10a] *Enterobacteria* ↑[10a,24a] *Enterococcus* ↑[10a,24ab]	10/21/3	13/30/7

*Millimolar (mM) concentrations for negative control are 2/8/1 (10 h) and 2/13/2 (24 h). Abbreviations: ↑ , increase respect to negative control. Significant differences ($p<0.05$) of the samples at 10 h ([10]) and 24 h ([24]) are compared to the negative control at 0 h ([a]) or at the same time of fermentation ([b]).

A first study reported that FOS also appears to influence a clostridium-related species (Kleessen *et al.* 2001). Therefore, the results evidenced a significant increase of *Clostridium coccoides* group in all the samples corresponding to the prebiotic group at 10 h, compared to the negative value at 0 h ($p < 0.05$) (**Table 21**), which mid-range values agreed well with the results obtained by Wang *et al.* (2019) for other prebiotic carbohydrates. Additionally, butyrate and propionate productions, which are known to exert a beneficial role in gut health, showed similar results in the previously mentioned samples when compared to the negative control at the beginning of the fermentation, as they are based upon the presence of *Clostridium* (Gerasimidis *et al.* 2019; Scott *et al.* 2008). This could explain the increase of butyrate and propionate after 10 h of fermentation. Barrier function, immune system modulation and drug and toxin metabolism are only some of the essential functions provided by *Clostridium* (Lopetuso *et al.* 2013).

Among all newly synthesized oligosaccharide-based sweeteners, only mMV-FOS achieved a significant increase ($p < 0.05$) for *Atopobium* (**Table 21**). No precedent studies focused on the effect of FOS on this particular bacterium, which despite the uncertain role on

the human colon, Altonsy *et al.* (2010) indicated a possible induction of apoptosis of colonic cancer cells.

Enterococcus was detected in all the samples. However, the abundances were relatively higher in prebiotic samples, denoting significant differences between this group and all the other control substrates (negative, SG and MV control samples). The qPCR detection rates were in accordance with those obtained by Matsuda *et al.* (2009). This genus has attracted great interest due to its potential role as a natural antimicrobial agent amongst other approved potential functions (Food & Authority, 2012; Hanchi *et al.* 2018). Besides *Enterococcus*, other lactic-acid bacteria tested as *Lactobacillus* are related to lactate production. *Lactobacillus* together with *Bifidobacterium* are important strains not only sharing numerous health benefits such as the contribution to improved nutrition or microbial balance but also being commercially used in probiotic compounds and fermented food formulations(Lee *et al.* 2009; Valdés *et al.* 2013). Remarkably, mSG-FOS after 10h of incubation and mMV-GOS after 10 and 24h of incubation promoted a significant increase of *Lactobacillus* (**Table 21**).

Despite the favourable batch-cultures fermentations together with the optimized qPCR assays provided advantageous methodologies for bacterial populations quantification, it must be noted the potential precision bias due to the slightly lower purity of the synthesized sweetener substrates (≥ 82.9%) compared to the commercial scFOS (positive control; ≥ 99.7%), and the utilization of the SCFAs by other bacteria as the precursor to produce others such as acetate, butyrate and propionate due to the slow fermentation (Moniz *et al.* 2016; Venema *et al.* 2020; Wang *et al.* 2019).

To summarize, this work provides optimal procedures to give rise to the production of a wide variety of specific compounds and flavour enhanced extracts. The findings of this thesis provide an innovative technology to produce a new generation of sweeteners that may meet the demands of the consumers regarding the search for alternatives to sugar, being entirely appropriate under the WHO recommendations of reducing sugar intake to 10% of the total daily energy need due to requiring a minimum amount to exert an equivalent sweetness, in addition to being considered low-calorie compounds (Pia *et al.* 2017). Furthermore, this PhD Thesis also brings ingredients that are nutritionally suitable and beneficial to be composed of

a high quantity of dietary fibre and to potentially provide prebiotic properties typical of the oligosaccharides that take part in the biosynthesis formulation of the sweeteners. Future prospects would require further sensorial, biological investigations, and human trials to establish consumer acceptance and health-related properties of these new synthesized prebiotic sweeteners prior to being commercialized.

6 Conclusions

6 CONCLUSIONS

In the present PhD Thesis, diverse enzymatic reactions were performed in order to modify two natural sweeteners extracts based on *Stevia rebaudiana* and *Siraitia grosvenorii*. The major glycoside compounds present in the extracts were: stevioside, rebaudioside A and mogroside V. The study of the conditions of the enzymatic reactions and the effect of the enzymatic modifications on the structural characterization, sensorial profile and human faecal microbiota composition and activity of the newly synthesized compounds have driven into the following concluding points:

1. A CGTase from *Geobacillus* sp. yielded the maximum transglucosylation ever described which probably occur in C-13 and/or C-19 sites of the steviol aglycone, consisting of up to 11 glucose units for both steviol glycosides mixture and rebaudioside A samples and an improvement of bitter and metallic attributes ensued.
2. A CGTase from *Thermoanaerobacter* sp. was the optimal enzyme to provide a higher rate of glucosylated products for the mogrosides mixture sample resulting in up to 17 glucose units attached in either the C-3 or C-24 of the mogrol core, and producing a flavour enhancement by lowering liquorice and metallic flavours.
3. The use of a β-galactosidase from *Aspergillus oryzae* with the two major steviol glycosides and mogrosides, either with or without a donor substrate based on lactose, resulted in the production of different di- and triterpene glycosides according to different reaction conditions. Reactions of the sweetener samples together with lactose gave rise to a one-pot synthesis of new sweetener compounds and galactooligosaccharides (up to DP 8 and 12), where the main transgalactosylation products were structurally characterized as 6'-galactosyl-glucose (disaccharide) and 6'-galactosyl-lactose (trisaccharide).
4. The use of a β-fructosidase from *Aspergillus aculeatus* with the two major steviol glycosides and mogrosides, either with or without a donor substrate based on sucrose, resulted in the production of different di- and triterpene glycosides according to different reaction conditions. Reactions of the sweetener samples together with sucrose gave rise to a one-pot synthesis of new sweetener compounds and inulin-type

fructooligosaccharides (up to DP 7), where the main transfructosylation products were structurally characterized as kestose, nystose, fructosyl-nystose and difructosyl-nystose.

5. The reactions catalyzed by the fungal glycoside hydrolase enzymes (β-galactosidase and β-fructosidase) with their respective donor substrates (lactose and sucrose) led to experimental conditions where the minimal concentration (0.2 and 1.5%) of the natural sweeteners was the optimal set. These optimal values are inside either the established ADI for *Stevia* or the indicated values by pharmacokinetic studies for mogrosides, however, the ADI values for the latter compounds are still to be determined.
6. The newly synthesized oligosaccharide-based sweeteners, properly purified, led to a reduction of the sensory sweetness perception allowing them to be used in the proper quantities to exercise their bioactivity as a functional ingredient.
7. *In vitro* human faecal fermentation studies of the purified newly synthesized oligosaccharide-based sweeteners showed, in comparison with reference control samples, a general increase of the specific bacterial strains studied, mainly for *Bifidobacterium*, *Bacteroides*, *Clostridium coccoides* and *Enterococcus*, that were well correlated with the quantified organic acids products (acetate, propionate, butyrate and lactate), potentially granting benefits for human health.

The proposed methodologies in this PhD Thesis provide feasible enzyme-assisted production, modification, and synthesis pathways of specific natural high-intensity sweeteners, with enhanced sensorial profile and with the possibility of seizing a prebiotic character.

CONCLUSIONES

En la presente tesis doctoral se han llevado a cabo diversas reacciones enzimáticas con el fin de modificar dos extractos edulcorantes de las especies *Stevia rebaudiana* y *Siraitia grosvenorii*. Los principales glucósidos presentes en estos extractos son: el esteviósido, el rebaudiósido A y el mogrósido V. El estudio de las condiciones de las reacciones enzimáticas y el efecto de las modificaciones enzimáticas en la caracterización estructural, el perfil sensorial y en la composición y actividad de la microbiota fecal humana han permitido alcanzar las siguientes conclusiones:

1. La enzima CGTase de *Geobacillus* sp. obtuvo el mayor rendimiento de transglucosilación descrito hasta el momento en los glucósidos de esteviol y rebaudiósido A. De este modo, se identificó hasta un máximo de 11 unidades de glucosa unidas tanto en las posiciones C-13 como C-19 del esteviol. Este nivel de transglucosilación permitió una mejora de atributos sensoriales como son el amargor y el sabor metálico.
2. La CGTase de *Thermoanaerobacter* sp. fue la enzima óptima en conseguir la mayor tasa de productos glucosilados para la muestra basada en la mezcla de mogrósidos. De este modo, se identificó hasta un máximo de 17 unidades de glucosa unidas tanto en las posiciones C-3 como C-24 del mogrol. Este nivel de transglucosilación permitió una mejora del sabor mediante la disminución del regusto a regaliz y del sabor metálico.
3. El uso de la enzima β-galactosidasa de *Aspergillus oryzae* con las muestras representativas de los extractos empleados, glucósidos de esteviol y mogrósidos, en presencia y ausencia de lactosa (como sustrato donante) dio lugar a la producción de diferentes glicósidos di- y triterpénicos según las condiciones de reacción empleadas. Las reacciones de las muestras de edulcorantes junto con la lactosa dieron lugar a la síntesis simultánea de nuevos compuestos edulcorantes y galacto-oligosacáridos (hasta un grado de polimerización de 8 y 12), donde los productos de transgalactosilación principales fueron 6'-galactosil-glucosa y 6'-galactosil-lactosa.

4. El uso de la enzima β-fructosidasa de *Aspergillus aculeatus* con las muestras representativas de los extractos empleados, glucósidos de esteviol y mogrósidos, en presencia y ausencia de sacarosa (como sustrato donante) resultó en la producción de diferentes glicósidos di- y triterpénicos según las condiciones de reacción empleadas. Las reacciones de las muestras de edulcorantes junto con la sacarosa dieron lugar a una síntesis simultánea de nuevos compuestos edulcorantes y fructo-oligosacáridos de tipo inulina (hasta un grado de polimerización de 7), donde los principales productos de transfructosilación fueron kestosa, nistosa, fructosil-nistosa y difructosil-nistosa.
5. Las concentraciones óptimas de los edulcorantes naturales en las reacciones catalizadas por las enzimas fúngicas glicósido hidrolasas (β-galactosidasa y β-fructosidasa) con sus respectivos sustratos donantes (lactosa y sacarosa) fueron las mínimas estudiadas (0.2% y 1.5%). Estos valores óptimos entran dentro tanto de la ingesta diaria admisible (IDA) establecida para la *Stevia* como de los valores indicados en estudios farmacocinéticos para los mogrósidos; sin embargo, los valores IDA para estos últimos compuestos están aún por determinar.
6. Los edulcorantes sintetizados a base de oligosacáridos, tras ser purificados adecuadamente, generaron una disminución de la percepción del dulzor, lo cual les permite ser utilizados en cantidades adecuadas como para ejercer su bioactividad como ingrediente funcional.
7. Los estudios *in vitro* de la fermentación de las heces humanas con los edulcorantes sintetizados a base de oligosacáridos mostraron, en comparación con las muestras control de referencia, un aumento general de las cepas bacterianas investigadas, siendo estas principalmente *Bifidobacterium*, *Bacteroides*, *Clostridium coccoides* y *Enterococcus*, y cuyos valores se correlacionaron con los valores cuantitativos obtenidos de ácidos orgánicos (acetato, propionato, butirato y lactato). El incremento de estos grupos bacterianos y metabolitos han sido relacionados con diferentes beneficios para la salud.

Las metodologías propuestas en esta tesis doctoral han permitido el desarrollo de diferentes procedimientos basados en la producción, modificación y síntesis enzimática de edulcorantes naturales de alta intensidad con un perfil sensorial mejorado y con la posibilidad de adquirir un carácter prebiótico.

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