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SYNTHESIS AND CHARACTERIZATION OF NEW OLIGOSACCHARIDES WITH PREBIOTIC ACTIVITY

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Maria Inês Pereira Montenegro

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Obtenção e Caracterização de Novos Oligossacáridos com Atividade Prebiótica

Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfil the requirements of Master of Science degree in Applied Microbiology

by

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Place: CBQF/Escola Superior de Biotecnologia da Universidade Católica Portuguesa

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[December 2013]

Resumo

A relevância da microbiota intestinal na manutenção da saúde do hospedeiro é bem conhecida e, nas últimas décadas, a consciencialização dos consumidores para a escolha de alimentos saudáveis tem vindo a aumentar. Existem diversas estratégias para estimular a proliferação de bactérias intestinais benéficas, incluindo o consumo de prebióticos. Atualmente, existe uma vasta gama de hidratos de carbono prebióticos no mercado, a maior parte isolados de polissacarídeos de plantas, de que são exemplo a inulina e frutooligossacarídeos (FOS). No entanto, existe um interesse crescente no desenvolvimento de novos prebióticos com funcionalidade adicional. Nesse sentido, o quitosano, sendo um polissacarídeo composto por unidades de glucosamina (GlcN) e N-acetil glucosamina (GlcNAc) unidas por ligações β $(1 \rightarrow 4)$, apresenta uma estrutura muito semelhante à dos atuais prebióticos glucooligossacarídeos. A diferença principal consiste na presença de grupos amina na sua estrutura, que lhe confere uma importante atividade antimicrobiana. A modificação química do quitosano por substituição dos seus grupos amina poderia eliminar este efeito antimicrobiano e converter o quitosano num novo e interessante ingrediente prebiótico. Assim, este trabalho teve por objetivo modificar quimicamente o quitosano para ser usado como ingrediente prebiótico na indústria alimentar.

Por forma a atingir este objetivo, foi levada a cabo a otimização da síntese dos derivados do quitosano por reacção de Maillard e hidrólise enzimática. Os derivados obtidos foram analisados quanto à distribuição de massa molar e caracterizados estruturalmente por Cromatografia de Exclusão Molecular (SEC), Espectroscopia de Infravermeho por Transformada de Fourier (FT-IR), Ressonância Magnética Nuclear (^{1}H-NMR) e titulação coloidal. O potencial prebiótico do produto purificado foi avaliado em ensaios de fermentação *in vitro* realizados em culturas puras e em inóculos humanos fecais.

Os resultados obtidos demostraram que os quitooligossacarídeos (COS) sintetizados, possuem potenciais efeitos prebióticos, que incluem alterações no padrão de produtos metabólicos gerados e nas contagens de *Bifidobacterium*, podendo, assim, contribuir para um ambiente intestinal saudável. Por fim, a avaliação da citotoxicidade dos derivados de COS foi realizada *in vitro* por citometria de fluxo. Os resultados obtidos demonstraram que os derivados sintetizados são moléculas biocompatíveis e que a substituição dos grupos amina diminuiu a citotoxicidade dos derivados quando comparados com COS não modificados. No entanto, estudos *in vivo* são recomendados para confirmar estes resultados *in vitro*.

Abstract

The importance of human intestinal microbiota in maintaining host health is well-known and in the past few decades, the consumer's awareness for healthier foods has increased. There are several strategies to stimulate the proliferation of beneficial intestinal bacteria, including the consumption of prebiotics. Currently, there is a range of prebiotic carbohydrates on the market, most of them isolated from plant polysaccharides such as inulin and fructooligosaccharides (FOS) but there is an increasing interest in the development of new prebiotics, with added functionality. In this sense, chitosan, which is a polysaccharide composed of glucosamine (GlcN) and N-acetyl glucosamine (GlcNAc) units, linked by β (1→4) bonds, presents a structure very similar to prebiotic glucooligosaccharides. The main difference is the presence of amino groups, which are the cause of antimicrobial effect of chitosan. Chemical modification of chitosan by substitution of its amino groups could eliminate the antimicrobial effect and convert chitosan in a new interesting prebiotic ingredient. Thus, the objective of the present work is to chemically modify chitosan to be used in the food industry as prebiotic ingredient.

In order to achieve this objective, the optimization of the synthesis of chitosan derivatives with glucose by the Maillard reaction and enzymatic hydrolysis, was conducted and the obtained purified derivatives were assayed for molar mass distribution and structural characterization by Size Exclusion Chromatography (SEC), Fourier Transform Infrared Spectroscopy (FT-IR), Proton Nuclear Magnetic Resonance (^{1}H-NMR) and colloid titration. The purified product was assayed for its prebiotic potential by means of *in vitro* fermentability assays performed with individual microbial strains and human faecal inocula.

The experimental data showed that the refined chitooligosaccharide (COS) derivatives obtained in this work had potential prebiotic effects, inducing changes in both the pattern of generated metabolic products and the count of *Bifidobacterium*, which might contribute to a healthy intestinal environment. Finally, the *in vitro* cytotoxicity of the COS derivatives synthesized was performed by flow cytometry. The results obtained demonstrated that the COS derivatives are biocompatible molecules. Also, the assay showed that the substitution of the amino groups decreased the cytotoxicity of the COS derivatives when compared to unmodified COS. Nevertheless, further studies are recommended, mainly *in vivo* tests, to eventually confirm these *in vitro* results.

Acknowledgments

I would like to thank Prof. Manuela Pintado for conceding me the opportunity to develop this thesis under her supervision. Thank you very much for your kindness and support.

I would also like to express my profound gratitude to Alejandra Cardelle-Cobas and Beatriz Gullón for their unconditional support during all the stages of this thesis. Thank you for your patience and for the many things you have taught me.

My gratitude is extended to all my laboratory colleagues who were always willing to offer their help and made the days spent in the laboratory much more pleasant.

I would also like to thank the Department of Chemical Engineering, Faculty of Science, University of Vigo (Campus Ourense), specially to Dr. José Luis Alonso for giving me the opportunity to develop the *in vitro* fermentation assays with human faecal microbiota and the study of dynamics of the *Bifidobacterium* population by FISH in his laboratory. Thank you very much for your kindness.

I would not forget to thank the Department of Chemistry and Functionality of Carbohydrates and Derivatives, at the Institute of Food Research in Madrid (Centro de Investigación en Ciencias de la Alimentación, CIAL), specially to Dr. Nieves Corzo and Dr. Ana Ruiz. Thank you for your support and for providing all the facilities to carry out the spectroscopic analysis of the samples.

Moreover, I would like to express my gratitude to Prof. Alice Santos-Silva for conceding me the opportunity to develop the cytotoxicity assays in the Faculty of Pharmacy, University of Porto. In particular, I would like to thank João Fernandes for his help during the experiments and for all the hours dedicated to this work.

I must acknowledge with deep thanks my parents and all my family and friends for giving me the strength and advice I needed in the most difficult moments.

Finally and above all, I would like to express my infinite gratitude to my boyfriend, Carlos. Only through your love, patience, support and unwavering belief in me, I have been able to complete this journey. Thank you with all my heart and soul.

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Abbreviations

CFU	Colony Forming Unit
COS	Chitooligosaccharides
COS-Glc1	Hydrolyzed LMWC-Glc derivative
COS-Glc2	Hydrolyzed LMWC subjected to the Maillard reaction
DA	Degree of Acetylation
DD	Degree of Deacetylation
DP	Degree of Polymerization
DS	Degree of Substitution
DSC	Differential Scanning Calorimetry
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FI	Faecal Inoculum
FISH	Fluorescent *In Situ* Hybridization
FOS	Fructooligosaccharides
FT-IR	Fourier Transform Infrared Spectroscopy
GC	Gas Chromatography
Glc	Glucose
GlcN	Glucosamine
GlcNAc	N-acetyl glucosamine
GPC-UV	Gel Permeation Chromatography-Ultraviolet
GRAS	Generally Recognized As Safe
HMWC	High Molecular Weight Chitosan

HPLC	High Performance Liquid Chromatography
^{1}H-NMR	Proton Nuclear Magnetic Resonance
IR	Infrared
LMWC	Low Molecular Weight Chitosan
LMWC-Glc	LMWC subjected to the Maillard reaction
LS	Light-Scattering
MRS	Man-Rogosa-Sharpe
Mw	Molecular Weight
NDOs	Non-Digestible Oligosaccharides
NIR	Near-Infrared
NMR	Nuclear Magnetic Resonance
OD	Optical Density
PCR	Polymerase Chain Reaction
PVSK	Potassium Polyvinyl Sulfate
PS	Phosphatidylserine
RI	Refractive Index
RPS	Reduced Physiological Salt Solution
SCFAs	Short Chain Fatty Acids
SEC	Size Exclusion Chromatography
TSB	Trypticase Soya Broth
UV	Ultraviolet
YNB	Yeast Nitrogen Base

1. Introduction

Chitin is a linear polymer of N-acetyl glucosamine (GlcNAc) units linked by β (1→4) bonds and is mainly obtained as waste biomass from the seafood processing industry, although other sources such as fungi are also increasing. It is the second most abundant polymer in nature after cellulose and is the primary structural component of the shells of crustaceans, insects and fungal cell walls (Aranaz *et al*., 2009; Luo and Wang, 2013; Ruiz-Matute *et al.*, 2013).

The deacetylated form of chitin is chitosan which is composed of units of glucosamine (GlcN) and GlcNAc linked by β (1→4) bonds. It is the only natural polysaccharide that presents cationic character due to the presence of free amino groups, usually responsible for biological activity. At low pH these groups are protonated and can interact with negatively charged compounds such as proteins, anionic polysaccharides (alginates, carragenates, pectins, among others), fatty acids, bile acids and phospholipids (Agulló *et al.*, 2003; Guo *et al.*, 2005). This fact combined with its biocompatibility, biodegradability and non-toxicity has made it prone to multiple applications namely in fields such as technology, food, cosmetics, medicine, biotechnology, agriculture and the paper industry (see Figure 1.1) (Prashanth and Tharanathan, 2007; Aranaz *et al.*, 2009).

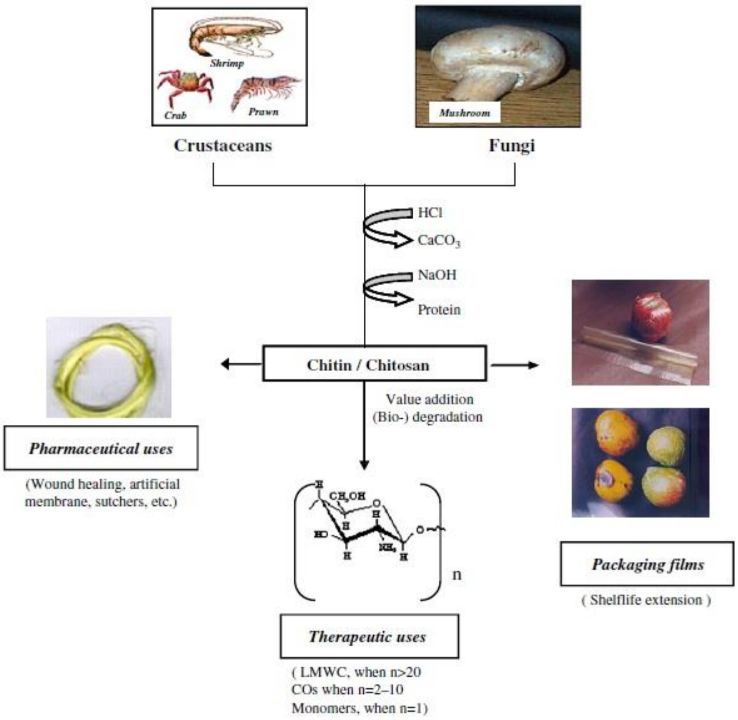

Figure 1.1. Production and value use of chitin/chitosan (Prashanth and Tharanathan, 2007).

Despite this broad spectrum of applications, it is already recognized that the physicochemical properties of chitosan, such as solubility, molecular weight (Mw) and viscosity, can restrict its applicability in certain fields. In particular, chitosan is only soluble in acid aqueous solutions with a pH below its pKa (6.0-6.5) being insoluble in water and most organic solvents (Ruiz-Matute *et al.*, 2013). The poor solubility of chitosan is the major limiting factor to its use and consequently, the interest in developing new strategies to modify the structure of chitosan in order to obtain novel derivatives with different physicochemical properties has increased in the last years (Luo and Wang, 2013; Ruiz-Matute *et al.*, 2013). Moreover, the Mw and, consequently, the viscosity of chitosan, is considered a limiting factor in its application in certain fields. In fact, chitosan with high Mw, due to its high viscosity, pose important problems in terms of manipulation, which restricts its applicability in a commercial context (Chung *et al.*, 2005; Aranaz *et al.*, 2009).

Due to the presence of hydroxyl and amino groups on its backbones (see Figure 1.2), chitosan offers scope for manipulation enabling the production of a vast range of derivatives with application in different fields (Prashanth and Tharanathan, 2007; Luo and Wang, 2013).

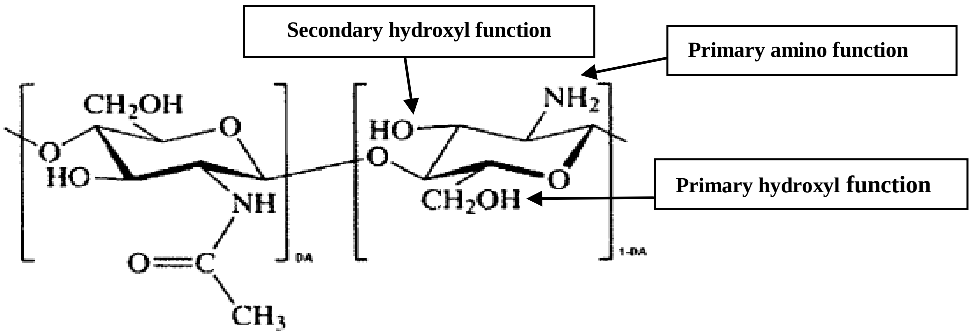

Figure 1.2. Chemical structure of partially acetylated chitosan and its main reactive functional groups; DA=Degree of Acetylation (Rinaudo, 2006).

1.1. Chitosan derivatives

Chemical derivatives of chitosan have received increasing interest over the past decades due to their associated chemical, biological, and functional advantages over unmodified chitosan. These include, but are not limited to, improved biocompatibility, better solubility in aqueous solutions over a wider range of pH, gelling properties, possibility to revert the net charge from polycationic to polyanionic, design of hydrophobic derivatives with amphiphilic character and capacity to harness self-assembling nanostructures and chemical conjugates with an assortment of bioactive and therapeutic molecules (Sarmento *et al.*, 2011). Several chemical modifications such as oligomerization, alkylation, acylation, quaternization, hydroxyalkylation, carboxyalkylation, thiolation, sulfation, phosphorylation, enzymatic modifications and graft copolymerization along with many assorted modifications have been carried out. Figure 1.3 summarizes the different methods that have been used to modify the structure of chitosan as well as some examples of derivatives already synthesized. The chemical modification affords a wide range of derivatives with modified properties for specific applications in diversified areas mainly pharmaceutical, biomedical and biotechnological.

The chemical modification of chitosan is often applied to obtain low molecular weight chitosan (LMWC), oligosaccharides and/or monomers. Among the various methods already described, the enzymatic methods are gaining importance because they allow a regioselective depolymerization under mild conditions (Prashanth and Tharanathan, 2007). In the case of enzymatic degradation of chitosan, LMWC with high water solubility was produced using chitinase, chitosanase, glucanase, lipase and some proteases (Pantaleone *et al.*, 1992; Kumar *et al.*, 2004). There are also non-specific enzymes (Muzzarelli, 1997), including lysozyme, cellulase, lipase, amylase, papain and pectinase (Nordtveit *et al.*, 1996; Grigolon *et al.*, 2001; Ruiz-Matute *et al.*, 2013) that are capable of depolymerizing chitosan.

In recent years, assorted modifications including chitosan hybrids with sugars, cyclodextrins, dendrimers, and crown ethers have also emerged as interesting multifunctional macromolecules. The versatility in possible modifications and applications of chitosan derivatives presents a great challenge to scientific community and to industry and open opportunities for new research studies (Mourya and Inamdar, 2008).

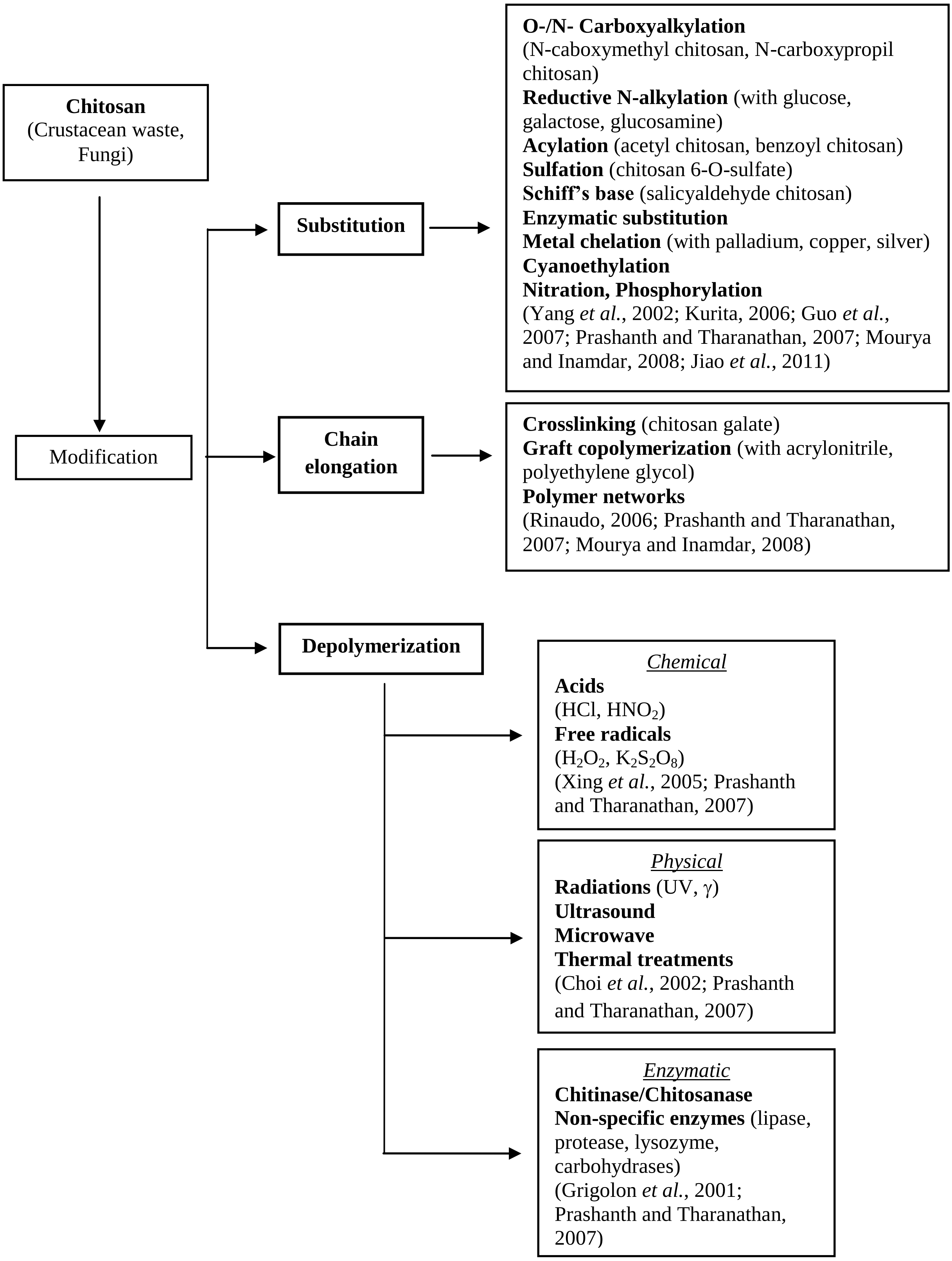

Figure 1.3. Methods of chitosan modification described in the literature.

1.1.1. Modification of chitosan via sugar introduction. The Maillard reaction

The introduction of hydrophilic residues in the chitosan molecule, through the formation of covalent bonds with the reactive amino groups, is one of the most described strategies to increase the solubility of chitosan. Because of its hydrophilic nature, carbohydrates and particularly mono- and disaccharides, have been the most preferred compounds to carry out these modifications. It has been established that substitutions of 3-20% of the amino groups leads to a drastic increase in solubility, whereas the remaining free amino groups ensure the biological activity of the derivatives (Il'ina and Varlamov, 2007). The preparation of glycosylated derivatives of chitosan, involves, among other reactions, the Maillard reaction, reductive alkylation (the Maillard reaction in the presence of sodium borohydride) and amide formation.

Amide formation. Derivatives of chitosan with lactobionic acid through amide formation generate branched derivatives with modified characteristics. These derivatives have been widely used as drug delivery systems for low molecular drugs, peptides and genes (Gao *et al.*, 2003; Li *et al.*, 2011a; Zhang *et al.*, 2011), as effective synthetic extracellular matrices for the attachment of hepatocytes (Chung *et al.*, 2002; Park *et al.*, 2003; Mi *et al.*, 2007) and as stabilizer for obtaining iron oxide nanoparticles for the preparation of multifunction nanoprobes (Bahadur *et al.*, 2009). Recently, Ruiz-Matute *et al.* (2013) showed that these derivatives are very promising compounds to be applied as an additive in the food industry (for example to bind fat and cholesterol or avoid hardening of foods).

Reductive alkylation. By reductive alkylation it is possible to obtain branched chitosan with modified functional properties or even to induce some new chemical and biological properties. To expand the range of solubility, derivatives of chitosan have been prepared with mono-, di-, tri- and polysaccharides (using cianoborohydride for the reduction), which presented an excellent solubility in water (Yalpani and Hall, 1984; Yang *et al.*, 2002). Using sodium borohydride, N-alkyl derivatives of chitosan have been prepared with better fungicidal and insecticidal properties than native chitosan (Rabea *et al.*, 2006). Cianoborohydride has been used for other applications of chitosan than the food sector, in which the presence of trace amounts of cyanide does not represent a problem. The use of sodium borohydride allows the formation of derivatives in which the presence of boron in trace amounts can be admitted in food ingredients at similar levels to those present in several foods and not exceeding the maximum recommended amounts (Li and Zhang, 2007).

Maillard reaction. The **Maillard reaction** takes place by condensation of the carbonyl group of reducing sugars, aldehydes or ketones with amino groups of amino acids, proteins or nitrogenous compounds (Chang *et al.*, 2011). It is well known that this reaction occurs during the heating, storage, and processing of foods. It influences the food quality by affecting factors such as colour, flavour, taste, and nutrition (Kanatt *et al.*, 2008). It is characterized by the mildness of the reaction, easy operation and controllability (Chung *et al.*, 2005).

Owing to the presence of a large number of amino groups in the chitosan molecule, it can be involved in the Maillard reaction. In fact, several authors have carried out this modification with the main objective of improving chitosan solubility and increase its applicability at neutral pH values. Indeed, Tanaka *et al.* (1993) obtained chitosan-glucose derivatives by Maillard reaction with improved functional properties. Depending on the stage of the Maillard reaction, changes in certain properties were observed. In particular, water binding capacity and antimicrobial activity decreased to some extent in the initial stages, while acidic dye binding capacity increased considerably as the reaction progressed. Other properties such as fat binding capacity and iron chelating were not affected by the reaction (Tanaka *et al.*, 1993). In addition, Chung *et al.* (2005, 2006) prepared derivatives of chitosan with different carbohydrates (glucose, fructose, maltose and GlcN) by the Maillard reaction. In these studies, the derivatives formed with GlcN were those showing the higher solubility and the best metal chelating and antibacterial properties. Derivatives obtained by Maillard reaction from chitosan and xylose showed a bactericidal effect higher than native chitosan confirming their application to preserve refrigerated fresh pasta (Huang *et al.*, 2007). More recently, Kanatt *et al.* (2008), obtained derivatives of chitosan with glucose by Maillard reaction that presented excellent antioxidant and antimicrobial properties and could be considered as promising food preservatives.

1.1.2. Applications of chitosan derivatives. Food applications

As it was mentioned before, chitosan is the only natural polysaccharide that presents cationic character due to the presence of free amino groups. Indeed, these groups are responsible for various unique properties such as biocompatibility, biodegradability, non-toxicity, and characteristic physicochemical and biological activities, which open opportunity for multiple applications. The broad fields of application of chitosan include medicine, biotechnology, pharmaceutical, cosmetics, foods and agriculture (Kurita, 2006; Prashanth and Tharanathan, 2007; Aranaz *et al.*, 2009).

Regarding the food industry, conversion of processing waste and by-products into valuable products and alternative specialty materials has been identified as a timely challenge for food research and development associated with numerous applications of chitosan polymers. In that sense, these biopolymers offer a wide range of unique applications including production of value-added food products (Rinaudo, 2006; Aranaz *et al.*, 2009), preservation of foods from microbial deterioration assuring extension of shelf-life (No *et al.*, 2002; Bautista-Baños *et al.*, 2006; Vargas and González-Martínez, 2010; Jiang *et al.*, 2012), biodegradable edible films and coatings (Hernández-Muñoz *et al.*, 2006; Santos *et al.*, 2006; Ribeiro *et al.*, 2007; Aranaz *et al.*, 2009), recovery of valuable compounds from food processing waste and by-products (Shahidi *et al.*, 1999; Casal *et al.*, 2006), purification of water (No and Meyers, 2000; Crini, 2005; Guibal *et al.*, 2006), separation and purification of proteins (Chen and Chuang, 2013) and clarification and deacidification of fruit juices (Soto-Perlata *et al.*, 1999; Chatterjee *et al.*, 2004; Rungsardthong *et al.*, 2006).

Another important property attributed to chitosan and its derivatives is their scavenging capacity against different radical species, conferring them antioxidant properties (Prashanth and Tharanathan, 2007; Aranaz *et al.*, 2009; Vargas and González-Martínez, 2010). The origin of the scavenging ability of chitosan and derivatives is related with the presence of active hydroxyl and amino groups in the polymer chains. The hydroxyl groups in the polysaccharide units can react with free radicals and the amino groups can react with free radicals to form additional stable macroradicals (Yen *et al.*, 2008). Regarding the effect of the Mw of chitosan on its antioxidant properties, Xing *et al.* (2007) showed through an *in vitro* study that LMWC had stronger scavenging activity effect on oxygen and hydroxyl groups than high molecular weight chitosan (HMWC). The same effect was observed by Feng *et al.* (2007) by reducing chitosan Mw by means of irradiation treatments.

Associated to the antioxidant property, chitosan and chitosan derivatives can be used to decrease oxidation process, in particular to prevent oxidative reaction in food or beverage products (Vargas and González-Martínez, 2010). The antioxidant activity of chitosan solutions was tested in a cooked cod fish model system, demonstrating a reduction in peroxides index and thiobarbituric reactive substances, with increasing efficiency according chitosan concentration and decrease in the Mw (Shahidi *et al.*, 2002). Table 1.1 shows the main applications of chitin, chitosan and their derivatives in the food industry.

Table 1.1. Applications of chitin, chitosan and their derivatives in the food industry.

Area of Application	Examples
Antimicrobial agent	Bactericidal Fungicidal Control of mold contamination in agricultural commodities (Aranaz *et al.* 2009; Martinez *et al.*, 2010; Luo and Wang, 2013)
Edible film	Controlled moisture transfer between food and surrounding environment Controlled released of antimicrobial substances Controlled release of antioxidants Controlled release of nutrients, flavors and drugs Reduction of oxygen partial pressure Controlled rate of respiration Temperature control Controlled enzymatic browning in fruits Reverse osmosis membranes (Aider, 2010; Xiao *et al.*, 2011; Siripatrawan and Noipha, 2012)
Additive	Clarification and deacidification of fruits and beverages Natural flavour extender Texture controlling agent Emulsifying agent Antioxidant agent Food mimetic Thickening and stabilizing agent Colour stabilization (Shahidi *et al.*, 2002; Luo and Wang, 2013; Torrico *et al.*, 2013)
Nutritional and functional	Dietary fibre Hypocholesterolemic effect Reduction of lipid absorption Antigastritis agent Infant feed ingredients Production of single cell protein Livestock and fish feed additive (Zhou *et al.*, 2006; Liu *et al.*, 2008; Aranaz *et al.*, 2009)
Recovery of solids materials from food processing wastes	Affinity flocculation (*e.g.* proteins) Fractionation of agar (Shahidi *et al.*, 1999)
Purification of water	Recovery of metal ions, pesticides, phenols and polychlorinated biphenyls Removal of dyes (Aranaz *et al.*, 2009; Vargas and González-Martínez, 2010)
Other applications	Enzyme immobilization Encapsulation of nutraceuticals Chromatography Analytical reagents (Aranaz *et al.*, 2009; Vargas and González-Martínez, 2010)

1.2. Structural characterization of chitosan and its derivatives

The content and sequence of GlcN and GlcNAc units in the chitosan molecule will determine the physicochemical and the biological properties of the polymer. It is known that heterogeneous conditions during deacetylation of chitin to obtain chitosan, provide a block-wise distribution, whereas under homogeneous conditions a random distribution of acetyl groups appears in chitosan. Thus, the degree of acetylation (DA) and the Mw (specifically the weight-average molecular weight) are the two major parameters that affect the properties and the applications of chitosan (Rinaudo, 2006; Aranaz *et al.*, 2009). In the case of the derivatives obtained by substitution, the degree of substitution (DS) is also an important parameter to take into account (Ruiz-Matute *et al.*, 2013). Additionally, due to its rigid and specific crystalline structures, possible through intra- and intermolecular hydrogen bonding, chitosan has the ability to exist in nature in different polymorphic forms, whose properties vary considerably.

1.2.1. Determination of the degree of acetylation and degree of substitution

The DA corresponds to the ratio between the acetylated and not acetylated GlcN units into the molecule of chitosan (Kasaai, 2008; 2009). For the chitosan derivatives, the DS can be obtained by the difference between DA before and after substitution reaction (Ruiz-Matute *et al.*, 2013). Several methods have been reported to determine the DA of chitosan. These methods can be classified in three groups: (a) conventional, (b) spectroscopic and (c) destructive (Rinaudo, 2006; Kasaai, 2009). Table 1.2 shows the main advantages and disadvantages of these methods, which will be briefly explained below.

Table 1.2. Summary of the advantages and disadvantages of the different methods for the determination of DA of chitosan and its derivatives (Kasaai, 2009).

Method	Advantages	Disadvantages
Conventional	Availability of the instruments is not a problem Easy to use the instrument and easy to perform the method Humidity does not interfere	Applicable only for soluble chitosan samples Proteins and mineral ions may induce interference peak(s) and result in unreliable results
Spectroscopic		
Ultraviolet	Simple, easy to use apparatus, high accuracy for the DA results High sensitive method Humidity and mineral ions do not induce interference Can be used for routine analysis with high accuracy	Requires sample preparation Applicable for a limited range of DA The accuracy is reduced with an increase in solvent concentration
Infrared	More suitable for the DA determination of crystalline samples Several possibilities exist to choose absorption band ratios and determine the DA	Drawing baseline is a general difficulty Wet samples may result in less accurate data Some absorption ratios result in less accurate data
Near-Infrared	Accurate results for DA ≤ 60	Possible error from adsorbed humidity
^{1}H-Nuclear Magnetic Resonance	Gives accurate results and some information on the distribution of the co-units	Requires sample preparation
^{13}C-Nuclear Magnetic Resonance	No sample preparation Applicable for entire range of DA Gives information on sequential distribution No need to dry the sample Applicable for soluble and insoluble chitosan samples Some information on chemical structure and sequence of co-monomer units may be obtained from the spectra	Low sensitivity (for low values of the DA) High cost Impurities in the sample (moisture, protein, pigments, and metal ions) may create interference peaks
Destructive	Entire range of the DA	Time consuming: two steps are required for analysis (decomposition and analysis of the decomposed species)

a. <u>Conventional Methods</u>. Conventional methods are applicable to soluble chitosan samples as well as those with low acetyl contents. These methods include *titration with an acid or alkali*, *colloid titration*, *conductometry*, *potenciometric titration*, *the ninhydrin assay* and the *adsorption of free amino groups of chitosan by picric acid* (Kasaai, 2009). The use of these different methods by diverse research groups has given contradictory data and conclusions for low acetyl contents of chitosans. This is because the amino groups of chitosan (with low acetyl contents) are accessible for titration in the conventional methods (Nanjo *et al.,* 1991; Varum *et al.,* 1995).

b. *Spectroscopic Methods*. The spectroscopic methods not only give information about the DA of chitosan and its derivatives but also on their chemical structure, sequence and morphology. These methods include Nuclear Magnetic Resonance (NMR) spectroscopy (^{1}H-NMR, ^{13}C-NMR and ^{15}N-NMR), Infrared (IR) spectroscopy, Ultraviolet (UV) spectrometry and Gel Permeation Chromatography with Ultraviolet detection (GPC-UV) (Kasaai, 2009).

A chitosan sample produces a larger absorbance in UV technique in comparison with near-infrared (NIR), IR and NMR methods. In the latter methods, in order to obtain sufficient absorbance, more amounts of the sample or greater concentrations are needed. Thus, determination of the DA by UV spectrophotometry results in greater accuracy when compared to NIR, IR and NMR methods. In fact, the IR technique is mainly used for qualitative analysis and comparison studies. It has been used for quantitative analysis of crystalline samples, since these samples created sharper signals and higher resolution compared to amorphous samples (Kasaai, 2009). ^{1}H-NMR technique has usually been employed as a standard method to calibrate other methods (Varum *et al*., 1991a; Shigemasa *et al*., 1996; Brugnerotto *et al*., 2001). Among various conditions proposed for determining the DA of chitosan by ^{1}H-NMR (Brugnerotto *et al*., 2001; Terry and Joyce, 2004; Bautista-Baños *et al.*, 2006), the procedures proposed by Hirai *et al*. (1991) and Varum *et al*. (1991a) have been widely accepted. ^{15}N-NMR and ^{13}C-NMR do not need a dried sample. However, these two techniques are not appropriate for chitin/chitosan having low DA values since they resulted in underestimated values (Domard, 1987; Raymond *et al*., 1993; Deserieres *et al*., 1996; Heux *et al*., 2000).^{1}H-NMR and ^{13}C-NMR spectroscopy may also provide information on the sequential distribution of free amino and N-acetyl groups (Varum *et al*., 1991a; Varum *et al*., 1991b).

c. *Destructive Methods*. Destructive methods (Elemental analysis, Thermal analysis by Differential Scanning Calorimetry (DSC), acid or enzymatic hydrolysis followed by High Performance Liquid Chromatography (HPLC) or spectrophotometry analysis, and pyrolysis-Gas Chromatography (GC) analysis) can be used for the entire range of the DA. In the HPLC and GC analyses of chitin/chitosan samples in the presence of other carbohydrates and polysaccharides, overestimated DA values may be obtained. This is due to the formation of additional acetic acid from the impurities. Excess amounts of oxalic acid had an adverse effect on the experimental results obtained from the pyrolysis-GC method (Sato *et al.*, 1998).The impurities do not create any difficulties for the DA analysis by HPLC and GC if the impurities are clearly separated in different peaks from those corresponding to the sample. The error variation in the elemental analysis method is relatively large (Roberts, 1992). This is because the presence of organic materials or polysaccharides other than chitin/chitosan (as impurities) significantly changes the ratio of Nitrogen (N)/Carbon (C).

1.2.2. Molecular weight determination

The physicochemical, biological and rheological properties of chitosan vary significantly as a function of its weight-average Mw and Mw distribution (Beri *et al.*, 1993; Denuzière *et al.*, 1995). It is therefore important to have precise and accurate values of the Mw of chitosan (Nguyen *et al.*, 2009).

It is well known that the determination of the Mw of polyelectrolytes is complex (Terbojevich *et al.*, 1993). In the case of chitosan, this situation is exacerbated due to the marked tendency of this polymer to form resilient aggregates in solution (Anthonsen *et al.*, 1994; Philippova *et al.*, 2001; Liu and Yao, 2002; Schatz *et al.*, 2003). In order to determine the Mw of chitosan and its derivatives, viscosimetry (Knaul *et al.*, 1998; Kumar, 2000; Aranaz *et al.*, 2009), Size Exclusion Chromatography (SEC) (also called gel filtration chromatography) (Aranaz *et al.*, 2009; Nguyen *et al.*, 2009) and Light-Scattering (LS) (Kumar, 2000; Aranaz *et al.*, 2009) have been used. Table 1.3 describes the principles of each method as well as their advantages and disadvantages.

Table 1.3. Principal techniques used to establish the Mw of chitosan and its derivatives.

Method	Description	Advantages/Disadvantages
Viscosimetry	Based on the use of the Mark–Houwink equation: $[\eta] = K \times M^{a}$, where $[\eta]$ is the intrinsic viscosity, M is the Mw, K and a are constants depending on the polymer and the solvent system used as well as the temperature (Rinaudo, 2006)	Simple and rapid method Not greatly affected by the presence of negligible amounts of very high Mw polymer Requires calibration curves (Terbojevich and Cosani, 1997)
Size Exclusion Chromatography (SEC)	A liquid mobile phase is passed through a column (stationary phase) at a fixed flow rate. The molecules with different molecular sizes are separated into distinct chromatographic bands (Wu, 1995)	Provides the number-average Mw and the weight-average Mw in a single measurement Requires calibration curves The ionic strength of the SEC mobile phase can affect precision and variability of chitosan SEC analyses (Nguyen *et al.*, 2009)
Light-Scattering (LS)	Based on the interaction of electromagnetic waves with matter by measuring the changes in the number (intensity), the direction (momentum) and the frequency (energy) of each type of photon in the incident and the emerging light beam (Chu, 1970)	LS measurements are difficult to perform Sometimes the data are not easy to interpret, in the presence of aggregation and/or association (Terbojevich and Cosani, 1997)

1.3. Functional foods

In the last decades, consumer demands in the field of food production have changed considerably. In fact, nowadays foods are not intended to only satisfy hunger and to provide necessary nutrients to the normal body function, but also to prevent nutrition-related diseases and improve physical and mental well-being (Roberfroid, 2000b; Menrad, 2003). In this view, functional foods play an outstanding role (Roberfroid, 2000a, 2000b; Kotilainen *et al.*, 2006).

The term "functional food" was first used in Japan, in the 1980s, for food products fortified with special constituents that possess advantageous physiological effects (Hardy, 2000; Stanton *et al.*, 2005; Kaur and Das, 2011). Presently, there is no universally accepted definition for functional foods, which are more accurately viewed as a concept than a well-defined group of food products (Siró *et al.*, 2008; Kaur and Das, 2011). Indeed, a large number of definitions exist worldwide for functional foods (Doyon and Labrecque, 2008; Siró *et al.*, 2008).

In a review paper published in 2008 (Doyon and Labrecque, 2008) a study of over a hundred definitions for functional foods was carried out. In this study, the following working definition for functional foods was proposed:"***A functional food is, or appears similar to, a conventional food. It is part of a standard diet and is consumed on a regular basis, in***

normal quantities. It has proven health benefits that reduce the risk of specific chronic diseases or beneficially affect target functions beyond its basic nutritional functions".

According to Spence (2006), Siró *et al.* (2008) and Kaur and Das (2011), it is possible to classify the functional foods in different classes, as it is shown in Table 1.4.

Currently, the market of functional foods is dominated by gut health products (Siró *et al.*, 2008). Indeed, it is now well established that the gut microbiota has a profound influence on health. In this view, there is presently a great deal of interest in the use of functional foods to manipulate the composition of the gut microbiota in order to improve health (Wang, 2009; Roberfroid *et al.*, 2010).

Table 1.4. Main classes of functional foods (Spence, 2006; Siró *et al.*, 2008; Kaur and Das, 2011).

Type of Functional Food	Definition	Example
Fortified product	A food fortified with additional nutrients	Fruit juices fortified with vitamin C
Enriched products	A food with added new nutrients or components not normally found in a particular food	Margarine with plant sterol esters Products with probiotics and prebiotics
Altered products	A food from which a deleterious component has been removed, reduced or replaced with another substance with beneficial effects	Fibres as fat releasers in meat or ice cream products
Enhanced commodities	A food in which one of the components has been naturally enhanced for example through special growing conditions, new feed composition or genetic manipulation	Eggs with increased omega-3 content achieved by altered chicken feed

1.3.1. Gut microbiota

The human gastrointestinal tract is the natural habitat for a complex bacterial community (Roberfroid *et al.*, 2010). It is recognized that its colonization starts immediately after birth (Guarner and Malagelada, 2003) and then a pattern that resembles the adult microbiota is established (Gibson and Roberfroid, 1995).

Moreover, it is known that factors such as pH, nutrient availability, host health, bacterial adhesion, transit time, among others, influence the numbers and diversity of bacteria present in the different regions of the gastrointestinal tract (Kerckhoffs *et al.*, 2006). Figure 1.4 illustrates the basic gut anatomy and shows that different types of microbial community in terms of both species diversity and numbers colonize the different regions within the gut. The conditions of the stomach reduce the microbial load to approximately 10^2 colony forming unit

(CFU) (per mL) while in the small intestine it reaches 10^2-10^4 CFU/mL. The colon is the region with the highest microbial load namely 10^{10}-10^{12} CFU/g. Furthermore, within the colon the distal region is the area of highest colonization with more than 500 different species and up to 100 billion microbial inhabitants per gram of contents (Gibson and Roberfroid, 2008; Roberfroid *et al.*, 2010).

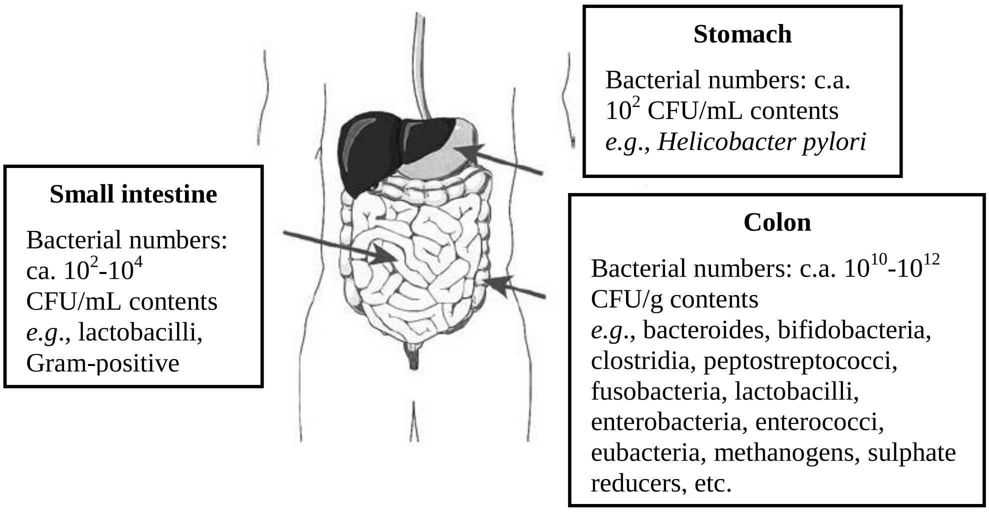

Figure 1.4. Basic gut anatomy (Gibson and Roberfroid, 2008; Roberfroid *et al.*, 2010).

Apart from the knowledge on the complexity of the gut microbiota, it is also known that certain bacteria are associated with toxin formation and even pathogenicity when they become dominant. These potentially harmful bacteria belong to species within groups such as clostridia and bacteroides. On the other hand, potentially healthy bacterial groups are characterized, besides absence of toxin production, by a beneficial metabolism towards the host through the formation of short chain fatty acids (SCFAs) or vitamin synthesis. They may also inhibit pathogens through a multiplicity of mechanisms (competition for colonization sites and nutrients, production of bacteriocins and acids and consequently reduction of *in situ* pH). Acknowledged examples of beneficial bacteria present in the gut microbiota are bifidobacteria and lactobacilli (Roberfroid *et al.*, 2010). Other groups like streptococci, enterococci, eubacteria and bacteroides have also been considered as potentially beneficial to health, however some of them include potentially harmful species, in particular enterococcus. With regard to some of the most recently identified genera in the major phyla (*Firmicutes, Actinobacteria* and *Bacteroidetes*), classification as potentially beneficial or potentially harmful to health still remains to be ascertained (Roberfroid *et al.*, 2010). Figure 1.5 illustrates the main bacteria present in the adult gut.

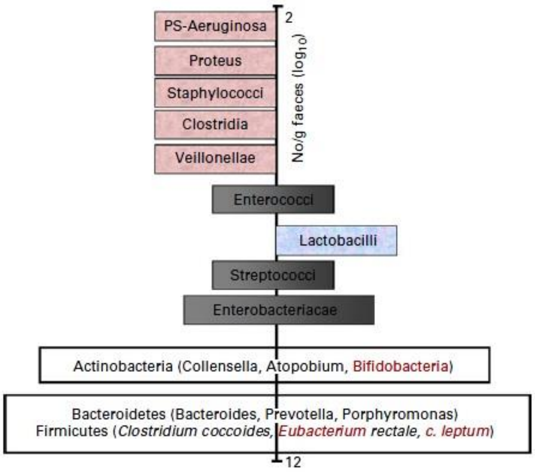

Figure 1.5. Schematic representation of an adult microbiota (Roberfroid *et al.*, 2010). The major phyla and genera are located on a logarithmic scale as number of CFU/g of faeces. Genera on the left site (pink) are likely to be potentially harmful whereas those on the right site (blue) are potentially beneficial to health. Those that are located both on the left site and the right site either (black) contain species that are potentially harmful and species that are potentially beneficial to health or contain genera/species that still need to be classified (white). Indeed many of these have only recently been identified in the gut microbiota and their roles are still largely unknown.

It has become clear that the gut microbiota plays important nutritional and physiopathological roles which include: prevention of gut colonization by potentially pathogenic microorganisms by competing with invading pathogens for ecological niches and metabolic substrates; important sources of energy for the cells of the gut wall through the fermentation of carbohydrates to SCFAs; modulation of the immune system; modulation of gene expression and cell differentiation in the gut wall (Gibson and Roberfroid, 2008; Roberfroid *et al.*, 2010).

It is already recognized that products causing a selective modification in the composition and/or activity of gut microbiota can induce beneficial effects in human health and well-being. In this view, there is currently a great deal of interest in the use of food ingredients to manipulate the composition of the microbiota, particularly in the colon, in order to improve health (Wang, 2009).

There are three methods of manipulating the composition of the colonic microbiota, namely by the use of probiotics, prebiotics or the combination of both (symbiotic) (Roberfroid *et al.*, 2010).

The concept of probiotics was introduced long before that of prebiotics. A probiotic has been defined as "Live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2002). In the case of probiotics, the microorganism is introduced into the host intestinal microbiota and causes a selective modification of its composition. It is the probiotic by itself that, by implanting into the gut microbiota, is responsible for the resulting effects (Gibson and Roberfroid, 2008). The most common probiotics currently used belong to the genera *Bifidobacterium* and *Lactobacillus*. The intake of probiotics has been associated with a considerable number of health benefits, namely reduction of diarrhoea, reduction of gastrointestinal tract symptoms (constipation, bloating, etc.), stimulation of the immune system, prevention of cancer, among others (Simmering and Blaut, 2001; Parvez *et al.*, 2006).

Another approach consists in the use of prebiotics. In this case, the effect is essentially indirect because the prebiotic selectively feeds one or a limited number of microorganisms thus causing a selective modification of the host's colonic microbiota. Therefore, it is not the prebiotic by itself but rather the changes induced in the composition of gut microbiota that are responsible for its effects (Gibson and Roberfroid, 2008).

1.3.2. Prebiotics

The concept of prebiotic was first defined by Gibson and Roberfroid in 1995 as "A non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improving host health". The most recent definition of prebiotic is the following: "**A dietary prebiotic is a selectively fermented ingredient that results in specific changes, in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health**" (ISAPP, 2008).

To be classified as a prebiotic, a food ingredient must fulfil three requirements: 1) resistance to gastric acidity; 2) fermentation by intestinal microbiota; and 3) selective stimulation of the growth and/or activity of those intestinal bacteria that contribute to health and well-being (Roberfroid, 2007).

In this view, any dietary component that reaches the colon intact (or partly so) is a potential candidate for prebiotic attribute. However, it is the latter of the three mentioned criteria which is crucial but still the most difficult to fulfil (Roberfroid *et al.*, 2010). Several dietary carbohydrates (polydextrose, glucooligosaccharides, lactose, hemicellulose, resistant starch, resistant dextrins, β-glucans, sugar alcohols such as lactitol) and also other compounds (for example peptides and yeast extract) showed some fermentation selectivity when tested in laboratory systems. Nevertheless, the ultimate test for prebiotic activity (i.e. human trials) is lacking for the majority of these compounds (Roberfroid *et al.*, 2010).

Therefore, there is much interest in the development of novel prebiotics with validated bioactivity, with well-established relationship structure-activity, addressed to reach the distal regions of the colon unaltered and promote the growth of specific bacteria. These may conceivably possess desired attributes not present in the current generation of molecules (Rastall and Maitin, 2002). In particular, prebiotic oligosaccharides have been a target of intensive research as ingredients with potential health-promoting properties (Korhonen, 2002).

1.3.2.1. Prebiotic oligosaccharides

Oligosaccharides are carbohydrates with a low degree of polymerization (DP) and consequently low Mw. They have been defined as including from 2 to 20 monosaccharide units (Roberfroid and Slavin, 2000). However, according to IUB-IUPAC (Joint Commission on Biochemical Nomenclature, 1982) the dividing point between oligo- and polysaccharides is 10. Moreover, oligosaccharides have been classified according to their physiological properties as digestible and non-digestible (Cummings *et al.*, 1997). The concept of non-digestible oligosaccharides (NDOs) originates from the observation that the anomeric C atom (C1 or C2) of the monosaccharide units of some dietary oligosaccharides has a configuration that makes their osidic bounds non-digestible to the hydrolytic activity of the human digestive enzymes (Roberfroid and Slavin, 2000).

NDOs can be found as natural components in milk, honey, fruits and vegetables. Generally, the concentration of NDOs in these types of foods range between 0.3 and 6% of fresh weight (Mussatto and Mancilha, 2007). In what concerns the caloric value, it has been estimated to be 1.5-2.0 Kcal/g, which is approximately 40-50% of those of digestible carbohydrates such as sucrose (Mussatto and Mancilha, 2007).

Among all the food ingredients, NDOs are the most relevant prebiotic candidates. Due to their chemical structure, they are resistant to gastrointestinal absorption and hydrolysis by digestive enzymes being considered as colonic ingredients. Moreover, some oligosaccharides can selectively stimulate the growth and/or activity of certain intestinal bacteria with positive health outcomes which make them prebiotic ingredients. Among all the recognized NDOs there are currently three that fulfil the criteria mentioned before and are thus considered as prebiotics: inulin-type fructans, galactooligosaccharides and lactulose (Gibson and Roberfroid, 2008). In fact, these are the only compounds that have, until now, proved their prebiotic effects by their ability to change the gut microbiota composition after a short feeding period at reasonably low doses (Roberfroid *et al.*, 2010).

Oligosaccharides obtained from chitosan by partial hydrolysis - i.e. chitooligosaccharides (COS), as well as LMWC have been claimed to exhibit prebiotic effects (Lee *et al*., 2002; Fernandes *et al*., 2012). In fact, these compounds possess a structure similar to prebiotic glucooligosacharides. However, the presence of the amino groups in their molecule confers an important antimicrobial activity (Kong *et al*., 2010; Hafdani and Sadeghinia, 2011), which can cause a decrease in the bacterial host population (Vernazza *et al*., 2005) with negative health outcomes. Chemical modification of chitosan and COS by substitution of their amino groups could eliminate this antimicrobial effect and convert them into new prebiotic ingredients.

Another important aspect to consider is related to the biocompatibility and toxicity of these potential prebiotic ingredients. In fact, any substance that is intentionally added to food (food additive), must be subjected to premarket review and approval by Food and Drug Administration (FDA). Indeed, it must be demonstrated that the substance is safe under the conditions of its intended use, so the ingredient must be Generally Recognized As Safe (GRAS). Some studies carried out with COS reported toxic effects upon human cells (Xu *et al.,* 2008; Fernandes *et al.,* 2011) associated to its amino groups. In this view, the modification of COS through the substitution of the amino groups may reduce their cytotoxicity allowing their safe use as prebiotic compounds. However, prior to its use, it is important to evaluate the cytotoxicity of the modified COS when compared with the unmodified COS to guarantee its safety as prebiotic ingredients. Most of the studies on COS cytotoxicity use the MTT colorimetric assay (Fernandes *et al.*, 2011) which is based on the capacity of mitochondrial enzymes of viable cells to transform the MTT tetrazolium salt into MTT formazan (Mosmann, 1983). Flow cytometry is another method widely used to evaluate the cytotoxicity. This method can provide rapid, quantitative and objective evaluation of cell viability, and may further provide enumeration of apoptotic or necrotic cells. It has become the method of choice to assay for apoptosis and necrosis in a variety of cell systems. The double-staining Annexin V/7-AAD assay discriminates cells that are undergoing early or late apoptosis and necrosis (Baudouin *et al*., 2007).

Prebiotics have received much attention in the last years and intensive research has been conducted in this field. In particular, prebiotic oligosaccharides derived from food industry by-products have been a target of great interest as ingredients with potential health-promoting properties (Korhonen, 2002). This type of products are regarded as an alternative to synthetic polymers combining the production of manufactured products with the protection of environment, cost reductions and waste material recycling (Ruiz-Matute *et al.*, 2013). Since chitosan and COS are by-products of the seafood industry, they represent an alternative with great potential in the development of novel prebiotic ingredients with health-promoting properties.

1.3.2.1.1. Health benefits associated to prebiotics

The fermentation of prebiotic oligosaccharides by colonic bacteria may result in several health benefits (see Table 1.5).

Table 1.5. Main health benefits of prebiotic oligosaccharides (Mussato and Mancilha, 2007).

Health Benefit	Description
Modification of the colonic microbiota	Stimulation of the growth and proliferation of beneficial bacteria (*Lactobacillus* and *Bifidobacterium*). The production of SCFAs by these microorganisms results in a pH decrease and thus inhibition of the growth of pathogenic bacteria
Nutrient production	Production of vitamins of the B complex (B1, B2, B6 and B12, nicotinic and folic acid)
Constipation relief and effects on intestinal motility	The ingestion of prebiotic oligosaccharides has demonstrated to prevent constipation. The end products of the fermentation, the SCFAs, are efficiently absorbed and used by the human colonic epithelial cells, stimulating their growth as well as salt and water absorption. The increased humidity of the faecal bolus improves the intestinal motility
Protective effect against infection in the gastrointestinal, respiratory and urogenital tracts	These effects are related to the pH decrease due to the production of SCFAs, the production of bacteriocins by several species of *Lactobacillus* and *Bifidobacterium* and the competition for nutrients and adhesion sites in the epithelial surface
Increase in absorption of minerals	The increased absorption of iron, calcium, and magnesium is related to the binding/sequestering capacity of the prebiotic oligosaccharides. Thus, these minerals are not absorbed in the small intestine and reach the colon, where they are released from the carbohydrate matrix and absorbed
Beneficial effect on carbohydrates and lipids metabolism	The ingestion of prebiotic oligosaccharides decreases the blood concentration of cholesterol, triglycerides and phospholipids, reducing the risk of diabetes and obesity. Some strains of *Lactobacillus acidophilus* assimilate the cholesterol present in the medium, while others appear to inhibit the absorption of cholesterol through the intestinal wall. Also, changes in lipid metabolism were suggested to be a consequence of a metabolic adaptation of the liver induced by SCFAs
Reduction of cancer risk	The anticarcinogenic effect appears to be related to the increase of beneficial bacteria and SCFAs production during the fermentation. It has been demonstrated that the intake of certain prebiotic disaccharides reduces faecal physiological parameters (pH, ammonia, p-cresol and indole) considered to be risk factors for cancer development

When evaluating the potential benefits associated with the ingestion of prebiotics it must be kept in mind that there is a dose-effect relationship, however a minimum effective dose is in general difficult to establish. Indeed, the major determinant that quantitatively controls the prebiotic effect is the number of targeted bacteria genus/species per gram of faeces that the volunteers have before the supplementation with the target compound presumed to show a prebiotic effect (Roberfroid *et al.*, 2010).

1.3.2.1.2. In vitro and in vivo evaluation of prebiotic properties

As it was mentioned before, there are three criteria that a food ingredient has to fulfil in order to be classified as a prebiotic, and the selective stimulation of growth and/or activity of bacteria is the most difficult to achieve (see section 1.3.2).

The increasing interest on prebiotics benefits has led to the improvement of the methodology for studying its functionality and in particular, the microbiota compositional changes as a response to selective fermentation has improved (Gibson *et al.*, 2004; Roberfroid, 2007). The main methods used in the study of the prebiotic properties will be next discussed:

(a) Pure cultures. The first studies on prebiotics describe the use of pure cultures. Typically, it involves the selection of a range of strains of *Bifidobacterium* spp., *Lactobacillus* spp. and other bacteria such as *Bacteroides* spp., *Clostridium* spp., *Eubacterium* spp. and *Escherichia coli*. This is the simplest method for the study of the prebiotic properties of a substrate and consists in the addition of the substrate to a basal medium (without carbohydrate source) where the bacterial growth takes place, during a fixed incubation period. The most common way of monitoring the bacterial growth is by measuring the optical density (OD) or via enumeration of viable microorganisms (CFU/mL). This type of studies is very useful for an initial screening, since it gives an idea of how the fermentation occurs. However, it does not consider the possible interactions between bacteria and is not representative of the colonic microbiota. Thus, it is not able by itself to establish if the test carbohydrate is metabolised selectively (Gibson *et al.*, 2004; Roberfroid, 2007).

(b) Mixed cultures. The studies performed with mixed cultures include studies on mixed pure cultures and studies using faecal samples.

(b.1.) <u>*Mixed pure cultures*</u>. The studies on mixed pure cultures are characterized by the use of a certain number of selected species (beneficial and harmful) of the gastrointestinal

tract. Although this approach does not reflect all the interactions that take place in the colonic microbiota in physiological conditions, it introduces a certain grade of competition between the microorganisms. These types of studies are very useful to predict the influence of the prebiotic substrate in the growth of pathogenic species.

(b.2.) Human faecal inocula. A more meaningful method is the use of faecal inocula. In fact, it ensures that a representative range of bacterial species is exposed to the test carbohydrate and by the study of changes in populations of selected species it can be established if the fermentation is selective or not. The problem with the use of faeces is that it only gives an accurate representation of events in the distal colon. It is known that both the composition and activities of the microbiota indigenous to the colon depend upon the region being sampled. To overcome this, complex gut models, which replicate different anatomical areas, should be used in concert with human trials (Gibson *et al.*, 2004; Roberfroid, 2007).

(c) ***Models of the gastrointestinal tract***. As it was mentioned before, the human gastrointestinal tract is a highly dynamic ecosystem being the natural habitat for a complex bacterial community (Roberfroid *et al.*, 2010). The stomach and small intestine contain only few species of bacteria adhering to the epithelia and some other bacteria in transit. The scarcity of bacteria in the upper tract seems to result from the composition of the luminal medium (acid, bile, pancreatic secretion), which kills most ingested microorganisms. By contrast, the large intestine contains a complex and dynamic microbial ecosystem with high densities of living bacteria. Moreover, the different regions of the colon present different physiological conditions. In the proximal region, the fermentation is very intense with high production of SCFAs, an acidic pH (5-6) and rapid bacterial growth. By contrast, the substrate in the distal colon is less available, the pH is close to neutral, putrefactive processes become quantitatively more important and bacterial populations are close to static (Guarner and Malagelada, 2003).

In this view, multistage chemostats have been developed and are used as efficient gut models in that each vessel represents a different physicochemical region of the intestine (Macfarlane *et al.*, 1998, Gibson and Fuller, 2000).

(d) ***Animal models and human trials***. The final demonstration of a prebiotic effect must be carried out *in vivo* through appropriate nutritional trials using validated methodologies (Roberfroid, 2007). Animal models, typically rats or mice, have been used to evaluate the effect of substrate on the faecal microbiota. Nevertheless, differences exist between animal and human microbiota and, for this reason, comparative results are likely to be compromised. Human microbiota-associated rats, or mice, give a more accurate representation of the situation in the human colon, although the intestinal physiology is not the same (Gibson and Fuller, 2000). Obviously, the best test of efficiency is a human volunteer trial with placebo control and blind coded samples. Nonetheless, drawbacks still exist since such tests may be difficult and expensive to set up. In addition, unlike trials using animals, different regions of the gut are inaccessible, since only faecal material is readily available (Gibson and Fuller, 2000).

1.3.2.1.3. Molecular methods of bacterial identification

The molecular methods of bacterial identification have been developed to circumvent biases inherent to bacterial culture (Charalampopoulos and Rastall, 2009). In fact, these methods have advantages over culture-based technologies since they have improved reliability and can encompass the full microbiota diversity (Roberfroid, 2007). Table 1.6 summarizes the principal molecular methodologies used in bacterial identification.

Table 1.6. Principal molecular methodologies of bacterial identification (Gibson *et al.*, 2004; Roberfroid, 2007).

Method	Description	Advantages	Disadvantages
Fluorescence *in situ* hybridization (FISH)	Group-specific oligonucleotide probes target discrete discriminatory regions of the rRNA molecule. By targeting highly conserved areas of the rRNA, specific groups of bacteria can be distinguished from others in mixed culture	Applicable to both unculturable and culturable bacteria Highly specific	Availability of probes limited to known bacteria Time consuming
Polymerase chain reaction (PCR)	The genes that code for the 16S subunits of the bacterial ribosomes are comprised of both conserved and variable regions. Sequencing of the 16S rRNA gene enables bacterial identifications to be made. By using PCR, segments of this gene can be amplified to a level whereby their sequence can be determined	Applicable to both unculturable and culturable bacteria High reliability, allows placement of previously unidentified bacteria	Expensive and time consuming Subject to bias in the PCR process
Direct community analysis	Characterizes the 16S rRNA diversity of the sample. The total bacterial DNA is extracted and partial 16S rDNA genes are amplified via PCR. The purified amplification products are subsequently cloned. Clones containing the 16S rDNA inserts are sequenced and identified by comparison with database 16S rDNA sequences	Culture-independent Applicable to elucidate the diversity of entire samples	Some loss of bacterial diversity due to the bias introduced by PCR
Denaturating and temperature-gradient gel electroforesis	A whole community PCR is carried out and partial 16S rDNA sequences are amplified from the different bacterial species present. Separation occurs due to the decreased electrophoretic mobility of the partially melted, double-stranded DNA molecule in polyacrylamide gels containing either a temperature or chemical denaturant gradient. Identification can be made either by excising fragments from the gel and sequencing them or by comparing their motility with that of known control sequences	Rapid Applicable to both culturable and unculturable bacteria	Qualitative rather than quantitative Subject to bias in the PCR process

1.4. Objectives

The aim of the present thesis was to produce novel derivatives of chitosan and study their prebiotic properties for a potential application as a functional ingredient in the food industry. To reach this general objective several specific objectives were established:

1. Optimization of the synthesis and hydrolysis of chitosan derivatives with glucose by the Maillard reaction.
2. Chemical characterization of chitosan derivatives by different analytical and spectroscopic methods.
3. Demonstration of the *in vitro* fermentability and selectivity of chitosan derivatives using pure cultures and mixed cultures (human inoculum).
4. Determination of the *in vitro* cytotoxicity of the chitosan derivatives.

2. Materials and Methods

2.1. Reagents

Low molecular weight chitosan (LMWC), high molecular weight chitosan (HMWC) and D-glucose (Glc) were purchased from Sigma-Aldrich Co. (Steinheim, Germany). The commercial enzymatic preparation of β-galactosidase from *Aspergillus aculeatus* (Pectinex Ultra SP-L) was kindly provided by Novozymes (Bagsvaerd, Denmark). Potassium polyvinyl sulfate titration solution (PVSK, N/400) was acquired from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All the reagents used were of analytical grade.

2.2. Microorganisms

The bacterial strains used in this study were: *Lactobacillus brevis* L24, *Lactobacillus acidophilus* LA10, *Lactobacillus paracasei* L26 and *Bifidobacterium lactis* B94 that were kindly provided by DELVO PRO LAFTI (DSM, Netherlands); *Lactobacillus casei* L01, *Lactobacillus acidophilus* LA5 and *Bifidobacterium lactis* Bb12 that were supplied by CHR HANSEN (Denmark); *Lactobacillus plantarum* 299 that was obtained from Probi AB (Lund, Sweden).

Strains were stored at -80 ºC in Man-Rogosa-Sharpe (MRS) broth (Biokar Diagnostics, France) with 30% (v/v) glycerol. Before the assays, the strains were grown in a static culture with MRS broth at 37 ºC for 16 h.

2.3. Optimization of the synthesis of chitosan derivatives by Maillard reaction

Synthesis of chitosan derivatives. The optimization of the synthesis of chitosan derivatives by Maillard reaction was carried out according to the method of Chung *et al.* (2006) slightly modified. Thus, LMWC and HMWC were dissolved in 1% (v/v) acetic acid to give a final concentration of 2% (w/v). Subsequently, 0.5, 1 and 2% (w/v) of Glc were added under stirring to each chitosan solution. Reactions were performed at 40, 60 or 80 ºC under shaking at 100 rpm during 72, 52 and 24 h, respectively. Samples were withdrawn every 4 h to evaluate the extent of the Maillard reaction. Reactions were stopped by keeping the solution in an ice bath during almost 10 min. Then, the reaction product was purified by dialysis (cut-off of Mw of 3500 Da, Thermo Scientific Inc., USA) against distilled water for 2 days and frozen for subsequent freeze-drying. The freeze-drying process was performed using a Vacuum Freeze Drier (Model FT33, Armefield, UK), under a vacuum pressure of 100 millitorr; the temperature in the freezing chamber was -46 ºC and the temperature in the sample chamber was 15 ºC.

Synthesis of COS derivatives. The production of COS derivatives was performed by using the COS obtained from the hydrolysis of LMWC (see section 2.4). COS were dissolved in 1% (v/v) acetic acid to give a final concentration of 2% (w/v). Subsequently, Glc was dissolved in the solution to a final concentration of 2% (w/v) and reaction was then performed at 60 ºC with shaking (100 rpm) during 32 h. Samples were withdrawn every 2 h to evaluate the extent of the Maillard reaction. After 32 h, the reaction was stopped by keeping the solution in an ice bath for 10 min. With the objective to eliminate the unreacted Glc and other monosaccharides present in the enzymatic mixture, the reaction product was treated with yeast according to the method of Sanz *et al.* (2005) with some modifications. Briefly, the reaction product was treated with fresh *Saccharomyces cerevisiae* in a concentration of 1% (w/v) at 35 ºC for 15 h in an orbital shake incubator (Wiggen Hauser) (100 rpm). Then, the reaction product was subjected to centrifugation (3858 x g for 15 min) and vacuum filtration to remove the yeast. The product obtained was then, frozen for subsequent freeze-drying. The COS derivative obtained was named COS-Glc2.

2.4. Enzymatic hydrolysis of chitosan and chitosan derivatives

The enzymatic hydrolysis of chitosan and chitosan derivatives was performed using the commercial preparation Pectinex Ultra SP-L produced by *Aspergillus aculeatus* following the method described by Ruiz-Matute *et al.* (2013) with some modifications. Briefly, a 2% (w/v) LMWC/chitosan derivatives in 1% (v/v) acetic acid was prepared and the pH adjusted to 5.5. The solution (1 L) was incubated with 200 mL (62 UE/mL) of the enzymatic preparation Pectinex Ultra SP-L and placed in a water bath at 40 ^{o}C for 16 h under shaking (100 rpm). Reaction was stopped by heating at 100^{o}C for 5 min, and after that samples were centrifuged at 3858 x g for 15 min at 20 ºC. The supernatant was filtered and treated with 50% ethanol solution to separate the fraction of high Mw and then centrifuged at 3858 x g for 15 min. The supernatant was recovered, and the ethanol was then evaporated by rotary evaporator (Buchi, Switzerland) at 45 ºC and finally frozen for subsequent freeze-drying. The product obtained from the hydrolysis of LMWC were chitooligosaccharides, namely COS and the product obtained from the hydrolysis of the chitosan-Glc derivative was named COS-Glc1.

2.5. Analytical determinations

2.5.1. Characterization of chitosan, COS and derivatives

2.5.1.1. Determination of weight-average molecular weight

The weight-average Mw of LMWC, HMWC, COS and their Glc derivatives was determined by SEC-HPLC. Two ultrahydrogel columns (Ultrahaydrogel 250 x Ultrahydrogel 2000) along with a Ultrahydrogel guard column (Waters, Massachusetts, U.S.A.) were combined and coupled to a RID-10A Shimadzu refractive index (RI) detector. Analyses were performed at 30 ºC using 0.25 M acetic acid/0.1 M sodium acetate as mobile phase at a flow rate of 0.8 mL min^{-1}. Mobile phase was filtered before use through a HVLP filter with 0.45 µm pore size (Millipore, Irland). 100 µL of chitosan solution were injected into the chromatographic system. Commercial pullulan samples (Waters, Massachusetts, U.S.A.) of different Mw (0.3-800 KDa) were used for the preparation of the calibration curve.

2.5.1.2. Determination of the extent of the Maillard reaction

To assess the reactive extent of the Maillard reaction, all the samples collected during the reaction were analyzed by measuring the absorbance at 294 nm for the intermediate stages and 420 nm for the final stage of the reaction. The equipment used was an UV mini 1240 spectrophotometer (Shimadzu, Tokyo, Japan).

The fluorescence of the samples was also measured (at an excitation wavelength of 350 nm and an emission wavelength of 420 nm) using an automated microplate reader (Fluostar Optima, BMG Labtech, Germany).

2.5.1.3. Determination of degree of acetylation (DA) and the degree of substitution (DS)

2.5.1.3.1. Colloid titration method

The DA of chitosan, COS and their derivatives was determined following the method described by Ying *et al.* (2011) with minor modification. Briefly, 20 mg of sample were dissolved in 10 mL of acetic acid (0.1 mol/L) and completely dissolved for 1 h at room temperature. Then, the mixture was diluted with 40 mL distilled water. 5 mL of the diluted solution was withdrawn and one drop of 1% (w/v) toluidine blue (Sigma-Aldrich Co., Steinheim, Germany) was added as an indicator. PVSK (N/400) was successively added until the titration end point was reached (a pronounced flocculation in the solution could be observed).

Since the consumption of PVSK (N/400) (A mL, Eq. 1) might correspond to the GlcN unit in chitosan, COS and their derivatives, the total weight of a GlcN unit (X g, Eq.1) in the solution was obtained by (Eq. 2.1):

$$X = (1/400) \text{ x } (1/1000) \text{ x } F \text{x } 161 \text{ x } A \tag{2.1}$$

where F is the factor of PVSK (N/400), 161 is the Mw of the GlcN and 203 is the Mw of the N-acetyl-D-glucosamine. The chitosan, COS and their derivatives unit (Y g) is expressed as (Eq. 2.2):

$$Y = [0.2 \text{ x } (1/100)] - X \tag{2.2}$$

Thus, degree of deacetylation (DD) is calculated by (Eq. 2.3):

$$\text{DD } (\%) = [(X/161)/(X/161+Y/203)] \text{ x } 100 \tag{2.3}$$

The DA is then calculated by (Eq. 2.4):

$$\text{DA } (\%) = 100 - \text{DD } (\%) \tag{2.4}$$

The DS of primary amino groups with Glc residues in the LMWC and COS samples was calculated as the difference of the DD before and after reaction.

2.5.1.4. Fourier Transform Infrared Spectroscopy (FT-IR)

FT-IR spectra were recorded in the middle infrared (4000 cm^{-1} to 400 cm^{-1}) with a resolution of 4 cm^{-1} for 15 accumulations at room temperature in a Perkin-Elmer spectrometer (Spectrum one). Samples were prepared by grinding the dry LMWC or COS samples with KBr in a ratio 1:100 (1 mg sample: 100 mg KBr) and then compressed to form discs.

2.5.1.5. Proton Nuclear Magnetic Resonance (^{1}H-NMR)

^{1}H-NMR analyses were performed following the method described by Hirai *et al.* (1991) on a Bruker DRX-500 spectrometer (Bruker BioSpin GmBH, Rheinstetten, Germany) which operated at 500 MHz. Samples were dissolved in 2% DCl/D_2O solution to give a polymer concentration of 5 mg/mL. Measurements were carried out at 70 ºC.

2.6. *In vitro* fermentation of COS derivatives

2.6.1. Growth with pure cultures

The COS derivatives (COS-Glc1 and COS-Glc2) were dissolved in MRS broth without citrate and Glc to give a final concentration of 0.5% (w/v). Also, to test the possible inhibition of the bacterial growth, the derivatives were dissolved in the same broth with Glc in the same concentration referred above. All the solutions were sterilized by heating at 100 ºC for 5-10 min. In the case of anaerobic strains, MRS was supplemented with filter-sterilized 0.5 g/L of L-cysteine-HCl (Fluka, Switzerland) as reducing agent. Subsequently, aliquots of the solutions were pipetted into 300 µL wells of sterile 96-wellmicroplates (Thermo Scientific, Denmark) and 2% of the inoculum of each strain was added to each well. Each inoculum contained about $1x10^6$ CFU/mL of the considered strain. Culture controls were also pipetted into the microplates, consisting of MRS broth, without carbohydrate, and with each strain tested. All the strains were grown in duplicate.

The aerobic strains were grown at 37 ºC for 48 h in an automated microplate reader (Fluostar Optima, BMG Labtech, Germany) and the optical densities at 620 nm (OD_{620}) were recorded at 60 min intervals. In the case of the anaerobic strains, the microplates were kept in an incubator at 37 °C for 48 h under anaerobic conditions (GasPak^{TM}EZ, Anaerobe Container System, BD, USA). The OD_{620} was recorded punctually at the microplate reader at 0, 15, 24 and 48 h. At 15 and 24 h, after the OD_{620} measurement, the anaerobic conditions of the microplates were restored and they returned to the incubator.

2.6.2. Growth with mixed cultures (human faecal inocula)

2.6.2.1. Faecal inocula

Faecal samples were obtained from three healthy human volunteers, who usually ingested a normal diet, presented no digestive diseases and did not receive antibiotics for, at least, 3 months. Faeces were collected into sterile vials, kept in an anaerobic cabinet and used within a maximum of 2 h after collection. Faecal inoculum (FI) was prepared by dilution in a reduced physiological salt solution (RPS; cysteine-HCl 0.5 g/L and NaCl 8.5 g/L) at a ratio of 10% (w/v). Before use, and during preparation of FI, anaerobiosis and pH (6.8) were maintained by continuous bubbling of CO_2 and N_2. The slurry was mixed and homogenized for 2 min under a CO_2 and N_2 stream. Blended, diluted faeces were filtered through four layers of surgical gauze to remove non-digested materials and transferred to serum bottles (Hartemink and Rombouts, 1999; Gullón *et al.*, 2010).

2.6.2.2. Fermentation media

The nutrient base medium used in fermentation experiments was prepared as described previously (Rivas *et al.*, 2012). Thus, a solution containing 5.0 g/L trypticase soya broth (TSB) without dextrose (BBL, Lockeysville, MD), 5.0 g/L bactopeptone (Amersham, Buckinghamshire, U.K.), 5.0 g/L yeast nitrogen base (YNB; Difco, Detroit, MI), 0.5 g/L cysteine hydrochloride (Merck, Darmstadt, Germany), 1.0% (v/v) salt solution A (100.0 g/L NH_4Cl, 10.0 g/L $MgCl_2{\cdot}6H_2O$, 10.0 g/L $CaCl_2{\cdot}2H_2O$), trace mineral solution, 0.2% (v/v) salt solution B (200.0 g/L $K_2HPO_4{\cdot}3H_2O$), and 0.2% (v/v) 0.5 g/L resazurin solution was prepared in distilled water. After deoxygenation with CO_2 and N_2 and pH adjustment to 6.8, aliquots of 7 mL were distributed into airtight anaerobic culture tubes, which were capped with butyl rubber stoppers and sealed with aluminium caps before autoclave sterilization.

Stock solutions of YNB, COS and COS derivatives (COS-Glc1 and COS-Glc2) were sterilized through 0.2 μm Chromafils filters (MACHEREY-NAGEL GmbH & Co. KG**,** Duren, Germany) into sterile airtight serum bottles.

Before inoculation, all the solutions of COS and COS derivatives were aseptically added to the anaerobic culture tubes with nutrient base medium, to achieve a final concentration of 10 g/L.

The tubes with the fermentation media were inoculated with 0.2 mL of faecal slurry dilution (2% v/v) and incubated at 37 °C for 48 h without shaking. At each sampling time (0, 5, 10, 24 and 48 h), tubes were withdrawn and cultures were centrifuged (8973 x g for 10 min), and pellets and supernatants were collected for further analysis. All additions and inoculations were carried out under anaerobic conditions, in a cabinet flushed with a gas stream containing 5% H_2, 10% CO_2 and 85% N_2.

2.6.2.3. Determination of fermentation products in batch cultures

Supernatants from the faecal batch cultures were filtered through 0.20 µm cellulose acetate membranes. Aliquots of the filtered samples were assayed for organic acids (acetic, propionic and butyric acids) using an Agilent 1200 series HPLC system with a RI detector (Agilent, Germany) operated at 50 ºC. Other analysis conditions were as follows: Aminex HPX-87H column (BioRad, Hercules, CA); mobile phase, 0.003 mol/L H_2SO_4; flow, 0.6 mL min^{-1}.

2.6.2.4. Fluorescent *in situ* hybridisation (FISH) assays

The quantification of bifidobacteria in faecal batch fermentations was performed by FISH (Gullón *et al.*, 2011a), using synthetic oligonucleotide probes (targeting specific regions of the 16S rRNA gene) labelled with fluorescent dye Cy3 (Sigma).

At fixed fermentation times, samples were centrifuged at 15000 x g for 2 min to separate particulate matter, and fixed overnight in paraformaldehyde (4 g paraformaldehyde/100 g solution) at 4 ºC (volume ratio of sample/paraformaldehyde solution, 1:3). Cells were washed with phosphate-buffered saline (0.1 M, pH 7.0), resuspended in 150 µL phosphate buffered saline plus 150 µL ethanol, and stored at -20ºC for at least 1 h before further processing. In 1.5 mL microcentrifuge tubes, 200 µL of filtered hybridization buffer (40 mmol/L Tris-HCl pH 7.2, 1.8 mol/L NaCl and 20 mL/L of a solution of 100 g sodium dodecyl sulphate/L), 64 µL of deionized distilled water and 16 µL of the fixed cells were added. In a 0.5 mL microcentrifuge tubes, 5 µL of each probe (50 ng/µL) were mixed with 45 µL of the above hybridization mixture, and the mixture was shaken and incubated overnight at 50 ºC (Rivas *et al.,* 2012).

In a 10 mL centrifuge tube, 5.0 mL of pre-warmed hybridization buffer (20 mmol/L Tris-HCl pH 7.2, 0.9 mol/L NaCl) and 20 µL of nucleic acid stain 4',6-diamidino-2-phenylindole (DAPI, 500 ng/mL) were added. Hybridization mixture was added and shaken. The mixture was returned to the oven at the adequate temperature for 30 min. The washing mixture was filtered through a 0.20 µm pore size filters (Whatman, Nuclepore Policarbonate) under vacuum. The filters were then placed on slides and covered with a coverslip, after addition of 5 µL of antifade reagent (polyvinyl alcohol mounting medium with $DABCO^{TM}$ antifading, Sigma). Slides were stored in the dark at 4ºC for a maximum of 3 days, and during this period were examined using an epifluorescence microscope (Olympus BX41, Tokyo, Japan) equipped with Fluor 100 lents.

2.7. Evaluation of the cytotoxic effect of COS derivatives by flow cytometry

The evaluation of the cytotoxic effect of COS derivatives (COS-Glc1 and COS-Glc2) was performed following the method described by Fernandes *et al.* (2011) with minor modifications. For comparative purposes, the cytotoxicity of COS was also evaluated.

Fresh peripheral blood samples were collected from healthy volunteers, into heparinized vacutainers. Lymphocytes were then isolated by density gradient separation (Histopaque-1077 and-1119). Three extra washes with a cold saline solution (pH 7.4) containing 3% (w/v) of fetal bovine serum (FBS), were performed. The viability of the lymphocytes was evaluated by the trypan blue exclusion test using a Neubauer counting chamber. Lymphocytes were then resuspended at a concentration of $1x10^6$ viable cells/mL, in RPMI 1640 culture medium, supplemented with 10% (w/v) FBS and penicillin. In all sets of experiments, a negative control (with saline solution) was used, as well as a positive control with staurosporine at 2 µM – a strong cytotoxic alkaloid added 20 h before the end of incubation. COS derivatives and COS were added to the lymphocyte suspensions at three concentrations (0.5; 0.05 and 0.005 mg/mL) and incubated for 24 h at 37 ºC. The assay was performed in a 96-well plate and each tested condition was performed in triplicate. After incubation, the cells were washed twice with cold saline solution with FBS and then stained according to the general Annexin V staining procedure by BD Biosciences (Annexin V-PE Apoptosis detection kit I, BD Biosciences, San Diego, US): the cells were resuspended in 1xbinding buffer to obtain a cell density of ca. 105 cells; Annexin V and 7-Aminoactinomycin D (7AAD) were then added, and the samples incubated for 15 min at room temperature, in the dark; 400 µL of 1x binding buffer was finally added to each tube. The treated samples and controls were analyzed by flow cytometry within a 1 h period. Flow cytometric analysis was carried out in a FACS Calibur (San Jose, CA, USA) based on the acquisition of 20.000 events. Detectors for forward (FSC) and side (SSC) light scatter were set on a linear scale, whereas logarithmic detectors were used for all three fluorescence channels (FL-1, FL-2 and FL-3). Compensation for spectral overlap between FL channels was performed for each experiment using single-colour-stained cell populations of positive control. All data were collected ungated to disk and were analyzed using CELLQuest Pro software. Lymphocytes were then analyzed for their expression of Annexin and 7AAD to determine: the number of viable cells Annexin V and 7AAD negative (Annexin V^{-}/$7AAD^{-}$); cells in early apoptosis, Annexin V positive and

7AAD negative (Annexin V^+/$7AAD^-$); and dead cells or cells that were in late apoptosis, Annexin and 7-AAD positive (Annexin V^+/$7AAD^+$).

2.8. Statistical analysis

Statistical analysis was performed using SPSS for Windows version 21.0 (IBM SPSS, Chicago, IL). Univariate analysis of variance (ANOVA) was used to determine the significance of the effect of COS derivatives on *Bifidobacterium* population. Pair wise comparisons were done using the posthoc Tukey test. Differences were considered significant at the 5% level of significance.

3. Results and Discussion

3.1. Optimization of the synthesis of chitosan/COS derivatives by Maillard reaction

It is well known that the Maillard reaction is characterized by the development of brown colour in advanced stages of the reaction. In fact, highly UV-absorbing and colourless compounds are formed at intermediate stages, whereas brown compounds are formed at final stages of the reaction (Ajandouz *et al*., 2001). Several studies suggest that intermediate stages of the reaction can be detected by recording the UV-absorbance at 294 nm, while the absorbance at 420 nm allows the detection of the final stages (Ajandouz *et al*., 2001; Kanatt *et al*., 2008; Mahae *et al*., 2011). Moreover, it is described in literature that fluorescent products are formed during the Maillard reaction. Some authors reported that these compounds can be detected in early stages of the reaction being considered precursors of the brown products in the final stages of the Maillard reaction (Jing and Kitts, 2002; Kanatt *et al*., 2008; Luo *et al*., 2013).

In this view, the optimization of the reaction conditions in the synthesis of chitosan with Glc by Maillard reaction was assessed by combining the analysis of the absorbance at 420 and 294 nm with the analysis of fluorescence as useful tools to determine the extent of the reaction. The effect of chitosan Mw, Glc concentration, temperature and reaction time on the Maillard reaction was evaluated.

3.1.1. Effect of the molecular weight of chitosan, glucose concentration, temperature and reaction time on the Maillard reaction

Effect of molecular weight

In order to study the effect of the chitosan Mw on the synthesis of its derivatives with Glc by the Maillard reaction, LMWC and HMWC were tested. Analysis by SEC-HPLC allowed determining, before the reaction, the weight-average Mw of the samples. Figure 3.1 shows the chromatograms obtained from the analysis of LMWC (A) and HMWC (B). As it can be observed, similar profiles for the two samples were obtained. Two peaks, one corresponding to chitosan (peak 1 and 1’) and other corresponding to the solvent (peak 2 and 2’) appears in both chromatograms. In the case of LMWC, the retention time obtained for the chitosan was **19.62 min**, which corresponded to a Mw of **123.01 ± 5.32 KDa**. The SEC-HPLC profile of

HMWC was similar with the exception that the retention time of the sample was lower, due to its higher size. Thus, the retention time of HMWC was **18.40 min** corresponding to a Mw of **202.93 ± 6.41 KDa**.

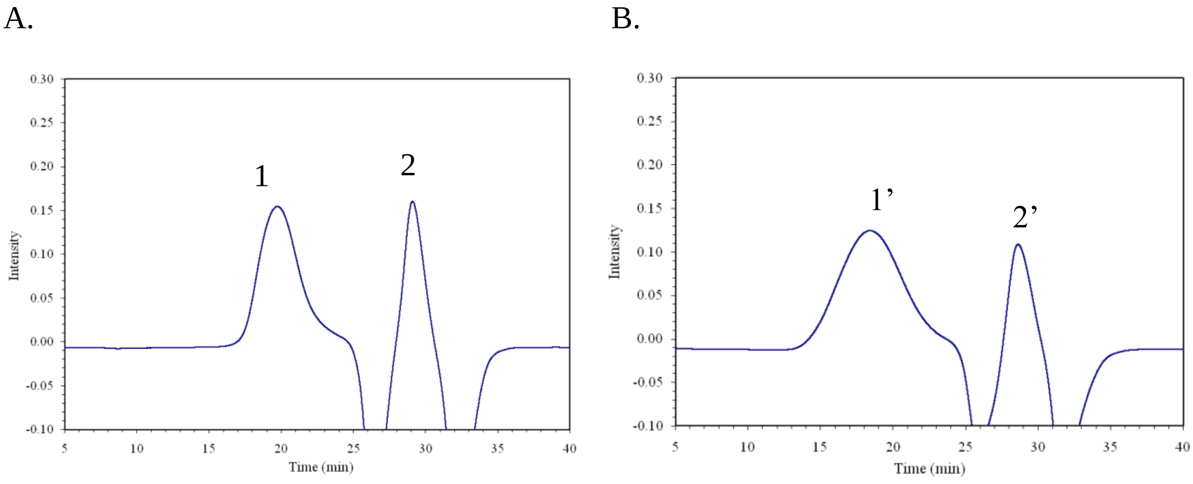

Figure 3.1. SEC-HPLC profiles of LMWC (A) and HMWC (B).

The DA of each sample was also determined by the colloid titration method. The DD obtained for LMWC and HMWC was 90.10 ± 5.79 % and 84.14 ± 4.55 %, respectively.

Once the Mw and the DD of the chitosan samples were determined, the samples were submitted to reaction with Glc through Maillard reaction. Thus, samples were prepared at 2% (w/v) in 1% (v/v) acetic acid and mixed with 0.5% (w/v) of Glc. Then, the reaction was conducted at a temperature of 40 ºC during 72 h. Aliquots were taken at different times. For each one, absorbance at 294 and 420 nm as well as fluorescence were measured as presented in Figure 3.2.

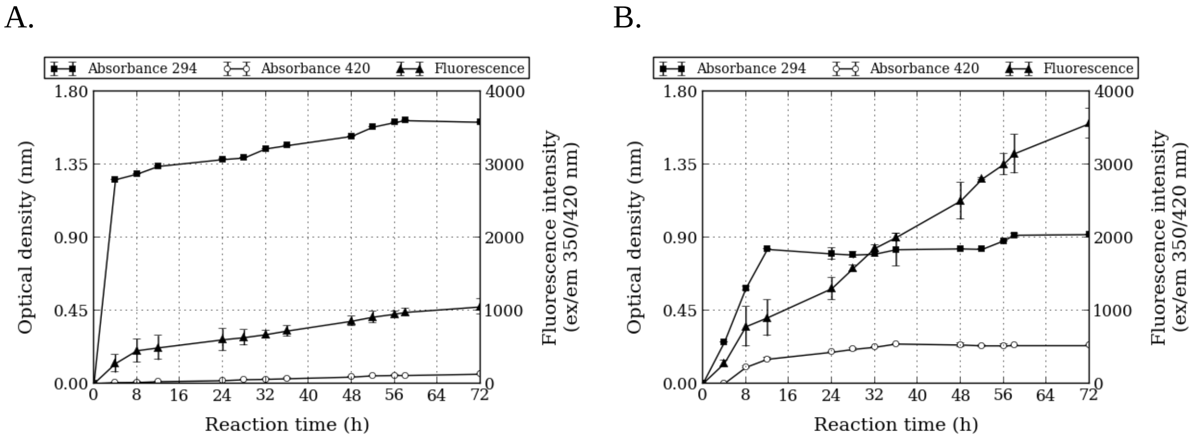

Figure 3.2. Absorbance (294 and 420 nm) and fluorescence measurements (excitation wavelength of 350/emission wavelength of 420 nm) for the samples withdrawn during the reaction of LMWC (A) and HMWC (B) with 0.5% (w/v) of Glc at 40 ºC during 72 h. Error bars indicate standard deviations.

Reactions carried out with both LMWC and HMWC showed an increase in the absorbance at 294 nm, which demonstrates that intermediate products were formed. However, the production of intermediate compounds in the reaction with LMWC showed a sharp increase in the first 4 h of reaction and reached higher values when compared with HMWC, demonstrating that the amount of intermediate compounds produced was higher. This can be explained by the accessibility of the Glc to the amino groups of chitosan. The higher Mw of chitosan could be blocking the access of the Glc units to the amino groups, since they are less exposed in HMWC than in LMWC. Thus, in the case of HMWC, Glc was not able to react with more NH_2- groups, which caused a stabilization in the formation of intermediate products. In this case, the advanced products were produced to a larger extent than in the reaction with LMWC, where the better accessibility of the NH_2- groups promoted the higher formation of intermediate products.

The increase in fluorescence is also a demonstration that the reaction was carried out under favourable conditions. The higher values obtained in the reaction with HMWC are a result of the greater formation of brown products originated from the intermediate compounds, which are formed to a lesser extent due to the Mw of the chitosan, as mentioned before.

Since it is important to select the conditions in which intermediate products are formed preferentially, we decided to choose the LMWC as the more adequate substrate to obtain the Maillard derivatives. Additionally, there were difficulties associated to the manipulation and analysis of HMWC, due to its high viscosity.

Effect of glucose concentration

In order to study the effect of Glc concentration in the formation of chitosan derivatives by the Maillard reaction, three concentrations were tested, 0.5, 1 and 2% (w/v). The chitosan (LMWC) concentration used was 2% (w/v) and the reaction was conducted at a temperature of 40 ºC during 72 h. Figure 3.3 shows the values of absorbance obtained, as well as the fluorescence, for the aliquots collected during the reaction. As it can be observed, the use of 0.5 and 1% (w/v) of Glc did not show important differences in the values of absorbance obtained at 420 and 294 nm. During the first 12 h, the absorbance at 294 nm for the reaction between LMWC and 0.5% (w/v) of Glc was higher, indicating a higher formation of intermediate products of the Maillard reaction. For the results obtained with 1% (w/v) of Glc, the absorbance values were much lower and values obtained for fluorescence at the same reaction time were higher. This indicates that precursors of advanced products of the Maillard reaction were formed to a major extent than in the reaction with 0.5% (w/v) of Glc. However, the curve obtained for absorbance at 294 nm showed a continuous increase until 52 h, indicating that intermediate products continued to be formed.

The data obtained for the reaction between LMWC and 2% (w/v) of Glc showed similar values of absorbance at 420 nm when compared with the reactions with 0.5 and 1% (w/v) of Glc. This indicates that the formation of the advanced products in the Maillard reaction was similar for all cases. However, lower values of absorbance at 294 nm were obtained during the 72 h of the reaction, indicating that lower levels of intermediate products of the Maillard reaction were present in the medium. The higher values obtained for fluorescence, in comparison with the other two reactions confirmed that the intermediate products were being degraded giving rise to precursor products of the final stages of the Maillard reaction.

Since the results obtained with 0.5% and 1% (w/v) of Glc were similar and no differences were shown, the concentration of 0.5% (w/v) was discontinued and concentrations of 1 and 2% (w/v) were assayed with different temperatures.

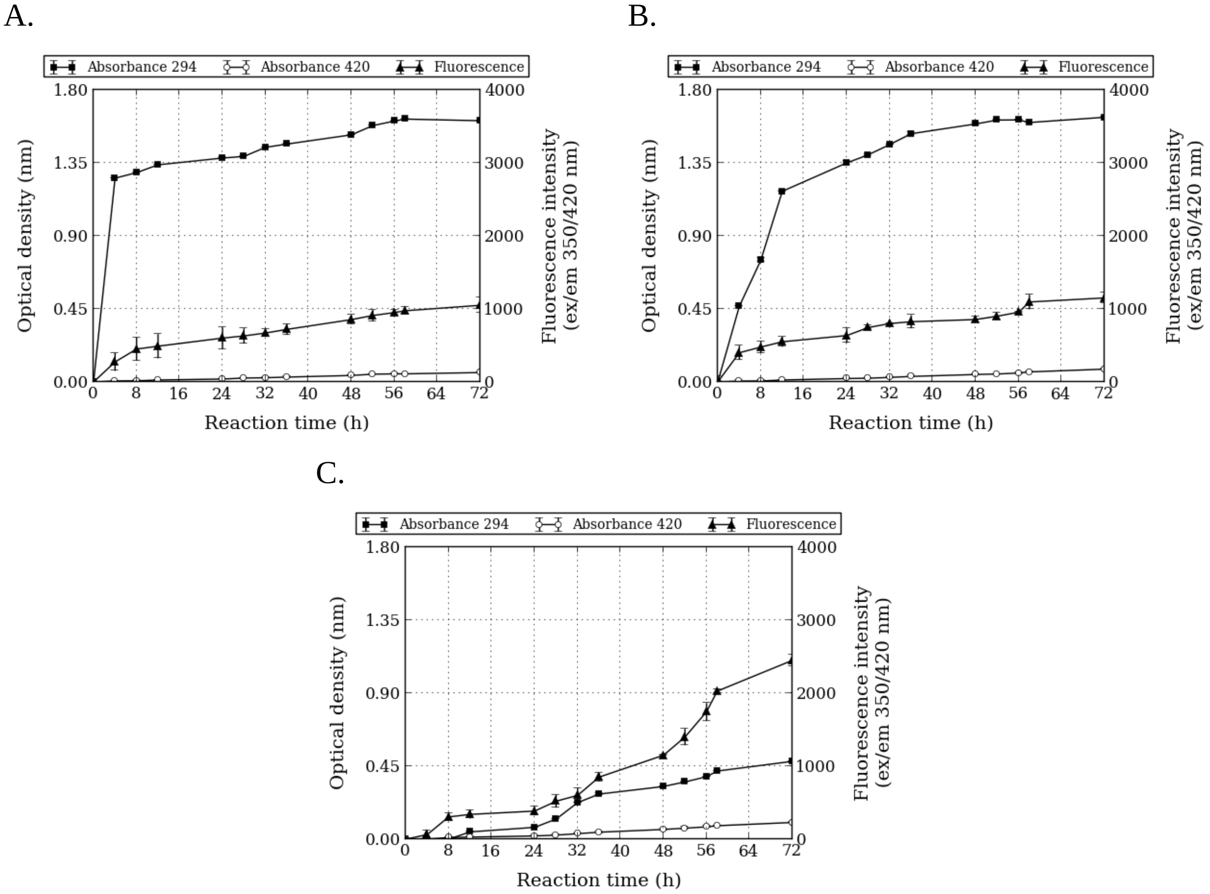

Figure 3.3. Absorbance (294 and 420 nm) and fluorescence measurements (excitation wavelength of 350/emission wavelength of 420 nm) for the samples withdrawn during the reaction of LMWC with 0.5% (w/v) (A), 1% (w/v) (B) and 2% (w/v) (C) of Glc at 40 ºC during 72 h. Error bars indicate standard deviations.

Effect of temperature

The following step was to test the effect of the temperature on the Maillard reaction between LMWC and Glc. To carry out these assays, 2% (w/v) of LMWC was prepared in 1% (v/v) acetic acid and 1% and 2% (w/v) of Glc was added. The reaction was conducted at three different temperatures, namely 40, 60 and 80 ºC during 72, 52 and 24 h, respectively. For each sample collected during the reaction, absorbance at 294 and 420 nm as well as, fluorescence were measured and results are depicted in Figure 3.4.

As it was previously observed, at 40 ºC the differences found for 1 and 2% (w/v) of Glc showed that, with 1% (w/v) of Glc, the intermediate products of the Maillard reaction were the major products, whereas few brown precursor products of the advanced products and advanced products themselves, were present in the reaction mixture. However, when 2% (w/v) of Glc was used, the noticeable increase in fluorescence occurred as the brown precursors increased in the medium, giving rise to the formation of the advanced products. For this reason, a slight increase could be observed in the data obtained for the measurements of absorbance at 420 nm. The formation of the advanced products of the Maillard reaction was practically absent at 40 ºC whereas at temperatures of 60 and 80 ºC the formation was pronounced. At 60 ºC and 2% (w/v) of Glc, the higher values of fluorescence and absorbance at 420 nm indicates that the intermediate products were rapidly degraded to brown intermediates of advanced products of the Maillard reaction and at the same time, these intermediates gave rise to advanced products of the Maillard reaction. The values of absorbance at 294 nm are similar to the reaction carried out with 1% (w/v) and there was an increase with time because of the presence of a higher amount of Glc, which allowed continuing this formation.

In the assays carried out at 80 ºC and 1 and 2% (w/v) Glc concentrations, the absorbance values were similar to the ones obtained for the assay at 60 ºC and 2% (w/v) of Glc. However, important differences could be observed for fluorescence. Fluorescence values during the first 4 h of reaction at 80 ºC were very high, indicating an important formation of brown precursors of the Maillard reaction. However, after 4 h, the values of fluorescence showed an important decrease indicating that no formation of brown intermediates of the advanced products were being formed and reaching values next to 0 after 24 h of reaction, when LMWC was reacting with 1% (w/v) of Glc and after 12 h when 2% (w/v) was used. This decrease of fluorescence was coincident with the formation of advanced products of the

Maillard reaction (increase of absorbance values at 420 nm), especially in the reaction carried out with 2% (w/v) of Glc.

In both reactions at 80 ºC, an increase in absorbance at 294 nm was observed throughout reaction time. The amount of Glc allowed that intermediate products of the Maillard reaction continued to be formed.

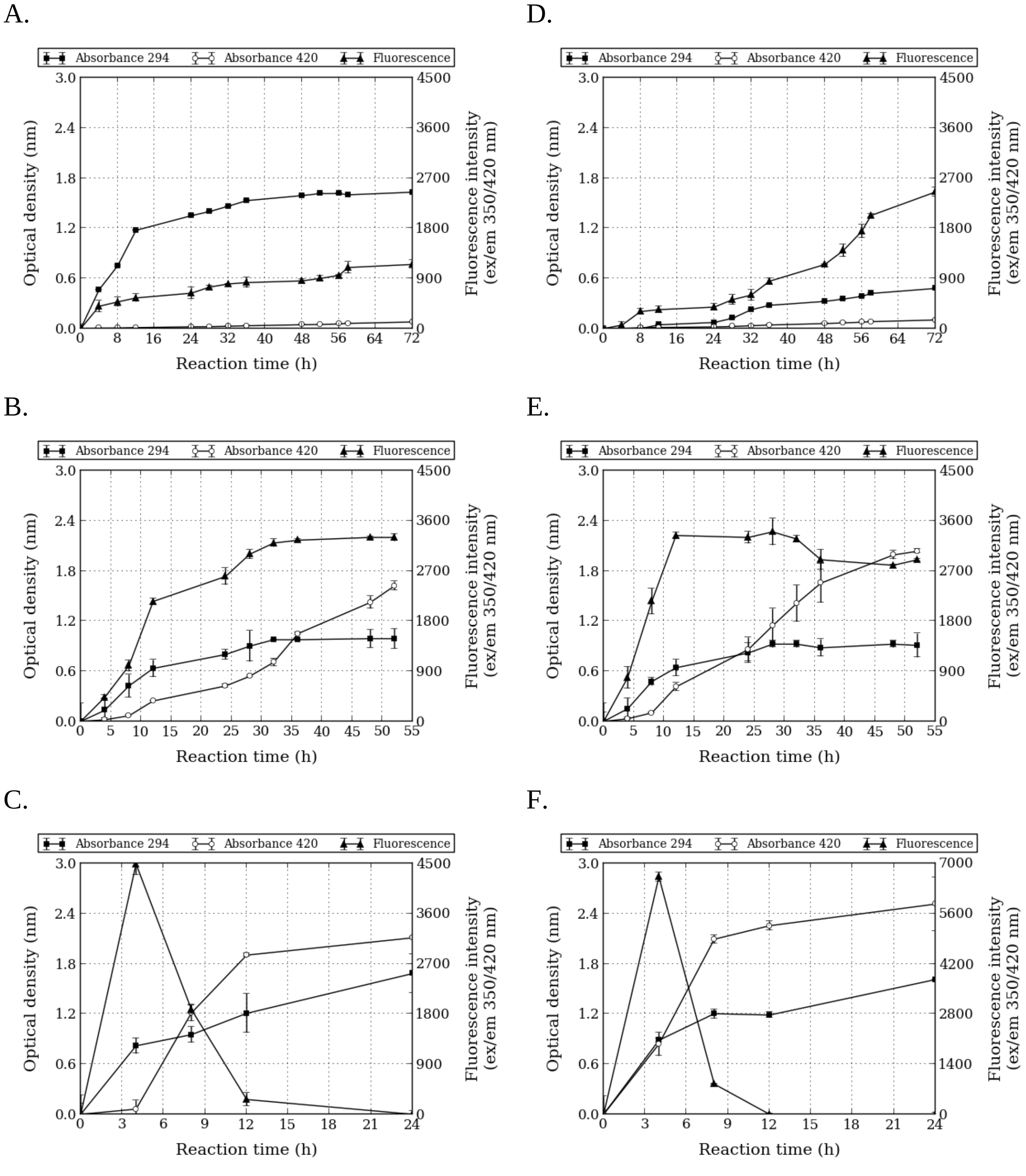

Figure 3.4. Absorbance (294 and 420 nm) and fluorescence measurements (excitation wavelength of 350/emission wavelength of 420 nm) for the samples withdrawn during the reaction of LMWC with 1% and 2% (w/v) of Glc at 40 ºC (A, D), 60 ºC (B, E) and 80 ºC (C, F) during 72, 52 and 24 h respectively. Error bars indicate standard deviations.

The profile obtained for fluorescence was slightly different, since the values corresponding to the formation of intermediate brown products of the advanced products of the Maillard reaction were higher when 1% (w/v) of Glc was used than for 2% (w/v). On the other hand, values of absorbance at 420 nm indicated a higher presence of advanced products of the Maillard reaction when 2% (w/v) of Glc was used. Combining these results, it is evident a higher reaction rate towards the formation of advanced products of the Maillard reaction when 2% (w/v) of Glc was used. The decrease of the fluorescence values after 4 h and the stabilization of the formation of the advanced products after 12 and 8 h for reactions at 80 ºC and 1 and 2% (w/v) of Glc, respectively, indicate that this temperature could conduct to degradation of the advanced products and produce caramelization reactions.

Based on these results, 40 ºC was not selected as an optimal temperature for the synthesis of the LMWC-Glc derivative, since only intermediate products of the Maillard reaction were formed and it is well known that for an optimal attachment of the Glc to the chitosan molecule, presence of the advanced products must be attained (Kosaraju *et al.*, 2010).

At 80 ºC, the presence of advanced products is noticeable but due to the pronounced decrease of fluorescence and the stabilization in the formation of the advanced products, which indicates the possibility of caramelization reactions, we decided to select 60 ºC as the optimal reaction temperature for the production of the LMWC-Glc derivatives. When comparing 1 and 2% (w/v) of Glc, it was possible to observe that a higher amount of advanced products was formed with 2% (w/v), whereas the presence of intermediate products was similar. For that reason, we decided to use this concentration as the optimal to obtain the derivative.

Effect of reaction time

Once the optimal conditions to obtain the LMWC-Glc derivative were selected, it was important to decide the reaction time in which the reaction should be stopped. Figure 3.5 shows the results obtained for the analysis of absorbance at 420 and 294 nm, as well as fluorescence, for samples corresponding to the reaction carried out with 2% (w/v) of LMWC in 1% (v/v) of acetic acid with 2% (w/v) of Glc at 60 ºC during 52 h of reaction.

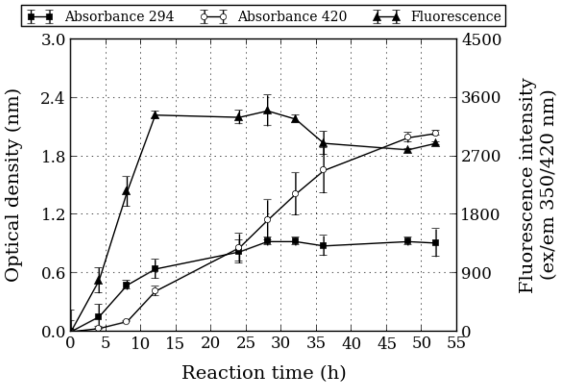

Figure 3.5. Absorbance (294 and 420 nm) and fluorescence measurements (excitation wavelength of 350/emission wavelength of 420 nm) for the samples analyzed during the reaction of 2% (w/v) of LMWC with 2% (w/v) of Glc at 60 ºC during 52 h. Error bars indicate standard deviations.

As the figure shows, the values obtained from the measurements of absorbance at 294 nm (indicative of the formation of intermediate products of the Maillard reaction) increased during approximately 32 h of reaction. After this time, the formation of these products started to stabilize, indicating that, after 32 h, formation of intermediate products did not occur. This is confirmed by the values of fluorescence obtained, since the formation of brown products from intermediate Maillard reaction products increased during the first 12 h, stabilized until 32 h and then started to decrease. Data obtained for the measurements of absorbance at 420 nm indicated a progressive increase in the values, and stabilization after 48 h. Since the intermediate products were not produced after 32 h of reaction and fluorescence values start to decrease at this time, 32 h was selected as the optimal time to obtain the LMWC-Glc derivatives. After this time, caramelization products may be formed causing a degradation of the chitosan derivatives. Thus, after testing all the conditions discussed above, **the conditions selected as optimal** were:

2% (w/v) of LMWC in 1% (v/v) of acetic acid, 2% (w/v) of Glc, 60ºC and 32 h of reaction

3.1.2. Synthesis of COS-Glc derivative under optimal conditions

The conditions selected as optimal for the synthesis of the LMWC derivatives with Glc through the Maillard reaction were applied to obtain a COS-Glc derivative. This COS-Glc derivative was obtained by reacting COS, previously produced by hydrolysis of chitosan, and Glc (see section 2.3). Figure 3.6 shows the absorbance values at 294 and 420 nm, as well as fluorescence results, obtained during the reaction (reaction mixture: 2% (w/v) of COS in 1% (v/v) of acetic acid and 2% (w/v) of Glc at 60 ºC during 32 h).

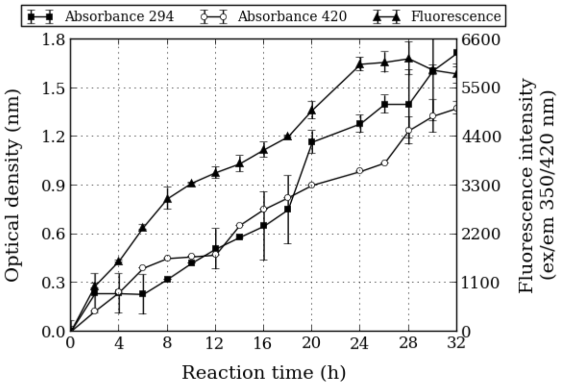

Figure 3.6. Absorbance (294 and 420 nm) and fluorescence measurements (excitation wavelength of 350/emission wavelength of 420 nm) for the samples analyzed during the reaction of 2% (w/v) of COS with 2% (w/v) of Glc at 60 ºC and 32 h. Error bars indicate standard deviations.

As Figure 3.6 shows, the formation of intermediate products (absorbance at 294 nm) and advanced products (absorbance at 420 nm) of the Maillard reaction, followed the same tendency. Thus, an increase in the formation of both products was observed during the 32 h. Measurements at 294 nm reached approximately the same values than those reached by the LMWC reacting with Glc under the same conditions. Fluorescence measurements showed that the formation of brown products was higher than for the reaction carried out with LMWC. This is expected because due to the lower Mw of COS when comparing to LMWC, COS are more reactive and more intermediate products are formed. As it can be seen, there was a decrease in the values of fluorescence after 24 h of reaction, which is indicative that there was a decline in the formation of brown products from intermediate Maillard reaction products. For this reason, this was the selected time to conduct the reaction. Thus, **the conditions selected as optimal** to produce COS-Glc derivative were:

2% (w/v) of COS in 1% (v/v) of acetic acid, 2% (w/v) of Glc, 60 ºC and 24 h of reaction

3.2. Characterization of LMWC-Glc derivative obtained by Maillard reaction

To confirm the production of the LMWC derivative with Glc by the Maillard reaction, a complete characterization of the LMWC was conducted before submitting the sample to enzymatic hydrolysis to obtain derivatives with a lower Mw. Thus, different analytical determinations were performed in order to confirm the successful introduction of Glc chains in the LMWC molecules.

3.2.1. SEC-HPLC analysis

The SEC-HPLC analysis of the LMWC-Glc derivative was carried out aiming to analyse if differences before and after the reaction were noticeable. Figure 3.7 shows the chromatograms obtained from the analysis of the chitosan sample before and after reaction.

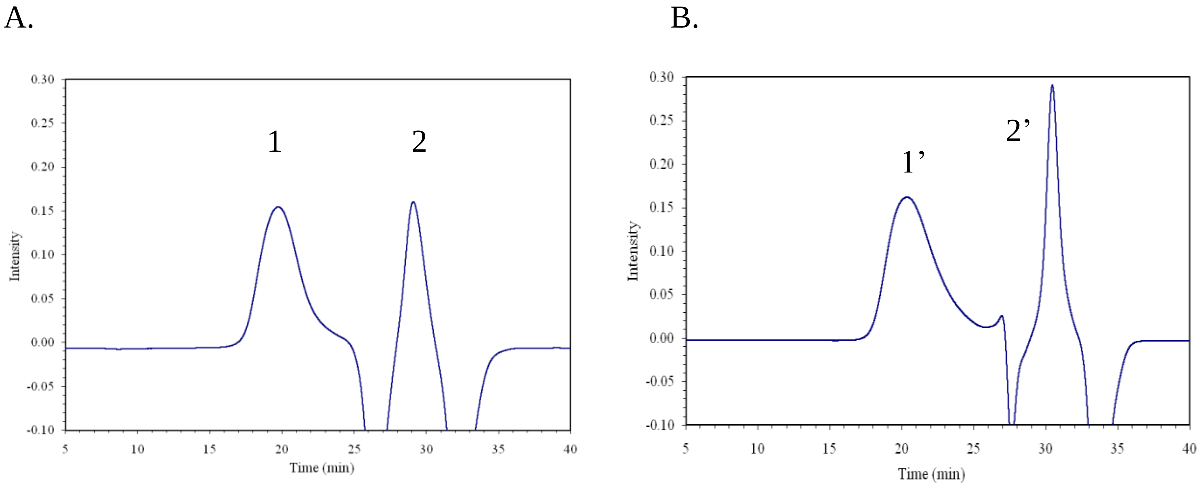

Figure 3.7. SEC-HPLC chromatograms obtained from the analysis of chitosan samples before (A) and after (B) reaction carried out with 2% (w/v) of LMWC, 2% (w/v) of Glc at 60ºC and 32h of reaction.

Similar chromatographic (HPLC-SEC) profiles were obtained for LMWC (A) and its LMWC-Glc (B) derivative, however a displacement of the elution time was observed for the derivative. Thus, the peak 1 (retention time =19.62 min) corresponding to a Mw of 123.01 ± 5.32 KDa for native chitosan (LMWC) showed after reaction with Glc (peak 1’), a shift towards a higher retention time (20.30 min) giving a Mw of 76.00 ± 4.25 KDa. The unexpected decrease observed in the Mw (since an increase due to the introduction of Glc was expected), is due to the fact that during the synthesis of the derivatives, the positive charges of the amino groups of chitosan are reduced by the introduction of Glc, which implies lower electrostatics repulsions between the chains, and consequently the compaction of the molecules and thus increasing their density. This suggests that they interact less with other surrounding molecules producing a less viscous solution and indicating a more compact structure for LMWC-Glc derivative. The same effect was observed by Il’ina and Varlamov (2007) and Ruiz-Matute *et al*. (2013) for galactosylated chitosans with different DS obtained from lactobionic acid through amide formation.

Additionally, in Figure 3.7 it is possible to observe that the peak corresponding to the LMWC-Glc (B) is wider than that observed for the peak of LMWC before reaction (A) indicating a change in the distribution of the Mw. This fact is also indicative that changes in the LMWC were produced due to its reaction with Glc.

Analysis to confirm the formation of complexes between LMWC and Glc, as well as their mass distribution, should be performed with HPLC-SEC analyses using a triple detection (refractive index, light scattering and intrinsic viscosity). These detectors provide information of Mw, polydispersity, intrinsic viscosity and hydrodynamic volume of chitosan samples, which affords a convenient and rapid way to characterize chitosan, since the intrinsic viscosity can be used in combination with Mw to predict the structure of the molecule. However, this equipment is very expensive and only specialized research groups incorporate them in their laboratories. Therefore, other complementary techniques should be used to complete the characterization of chitosan and chitosan derivatives.

3.2.2. Colloid titration analysis

The analysis of LMWC-Glc derivative by the colloid titration method described in section 2.5.1.3.1 allowed determining the DD of the chitosan derivative, and in consequence the DS. The results obtained indicate that after reaction the DD of the LMWC-Glc derivative was 25.34 ± 4.40%, when the native LMWC had a DD of 90.10 ± 5.79% (see section 3.1.1). As it can be deducted, the introduction of the Glc units in the chitosan molecule was produced and the DS obtained was 64.76 ± 4.40%.

3.2.3. FT-IR analysis

FT-IR spectrometry was also used for the analysis of the derivative formed by Maillard reaction. As shown in Figure 3.8, the characteristic absorption bands of chitosan observed at around 1697 and 1560 cm^{-1} were assigned to amide I and primary amino group, respectively. In the enlarged part of the figure, it can be observed the characteristic bands corresponding to the region 800-1400 cm^{-1} of the spectra obtained for native LMWC and LMWC-Glc derivative obtained under the optimal conditions of reaction. Most of the changes in the chitosan molecule are registered in this region of the spectra. Thus, after reaction, it can be observed that the absorption bands of chitosan corresponding to amide I and the primary amino group showed changes. An inversion in the intensity of the bands corresponding to the LMWC molecule at 1697 and 1560cm^{-1} is observed, giving rise, the first one, to a new band at 1595 cm^{-1}. These changes suggest that Schiff base (C=N double bond) was formed between the reducing termination of Glc and the amino groups of chitosan (Liu *et al.*, 2001; Umemura and Kawai, 2007). These results also corroborate data reported by Li *et al.* (2011b) when a xylan chitosan conjugate obtained by Maillard reaction was analyzed by the same technique. They additionally observed a decrease in the band at 1560 cm^{-1} with the time of reaction. These results were also consistent with the study of Umemura and Kawai (2007) in which it was suggested that the secondary amines were converted into tertiary amines with few changes of the polymer structures.

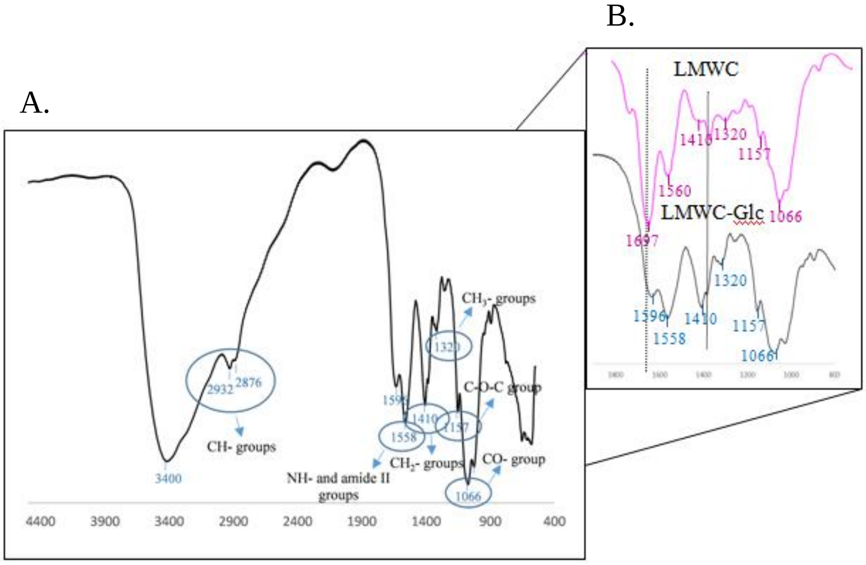

Figure 3.8. FT-IR spectra obtained for the LMWC-Glc derivative obtained by Maillard reaction under the optimal conditions (A) and comparison between LMWC and LMWC-Glc IR spectra (B) (region 800-1400 cm^{-1}).

An increase in the band at 1410 cm^{-1} in the spectra obtained for LMWC-Glc could also be observed. This increase was produced by the increase of the number of CH$_2$- groups due to the introduction of Glc chains in the chitosan molecule. The profile of the bands between 1200 and 100 cm^{-1} also exhibited variations, being more pronounced in the case of the LMWC-Glc derivative than in the case of native chitosan. This region is specific of the saccharide structure and this modification is also a signal that modified substitution was produced.

3.2.4. ^{1}H-NMR analysis

With the objective to confirm the introduction of the Glc units in the chitosan molecule, ^{1}H-NMR analyses were carried out. A typical ^{1}H-NMR spectrum of LMWC is shown in Figure 3.9.

The signal observed at δ 2.0 ppm was assigned to the hydrogens of the methyl moieties of the acetamide groups while the signal at δ 3.2 ppm was attributed to hydrogen bonded to the C2 of the GlcN ring. The multiplet at δ 3.3-4.0 ppm was assigned to the hydrogens on the carbon atoms C3, C4, C5 and C6 and the signal at δ 4.9 ppm to the hydrogen on the C1 (Hirai *et al*., 1991; Park *et al*., 2003; Mi *et al*., 2007; Zhang *et al*., 2010).

Comparing the spectra, differences were observed between the native LMWC (Figure 3.9A) and its Glc derivative (Figure 3.9B). As it can be observed in the Figure 3.9B, the ^{1}H-NMR spectra showed that, modification by Maillard reaction, was carried out. Although a signal should appear for the linkage –N=CH-, the NMR analysis did not show it. However, one of the most important changes observed in the spectra were referred to new signals appearing at 2.09 ppm, corresponding to CH_2 linked to the amino group of chitosan (-NH-CH_2-), indicating a displacement of the –N=CH- linkage. In the region around 4.5 ppm, some signal also appeared indicating the N-substitution of the NH_2- groups of chitosan and partially overlapping with the signal due to the solvent. Signals due to the OH- groups of the carbohydrate overlapped also with the OH- groups of the chitosan molecule and no differences could be observed in the spectra. These results were in coincidence with the results obtained by other authors.

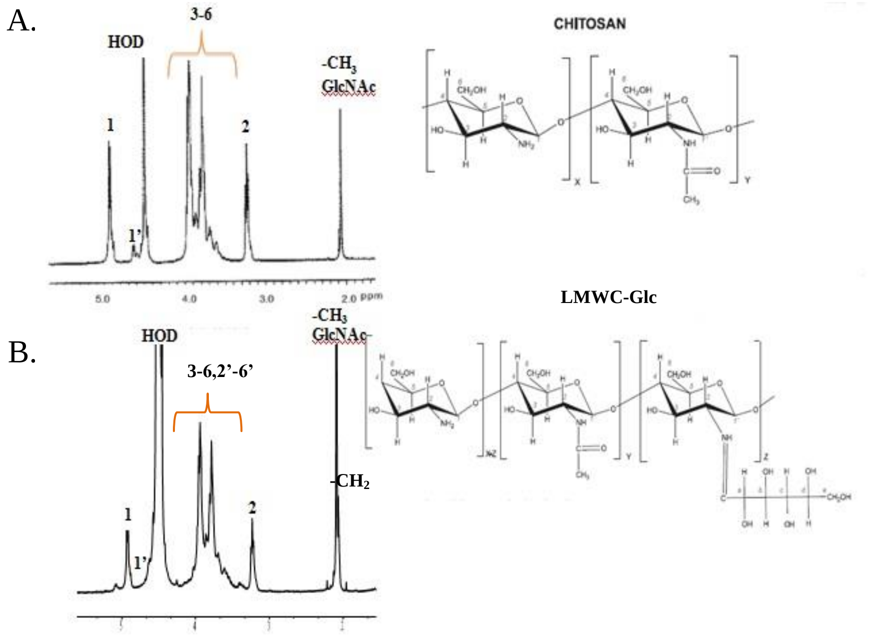

Figure 3.9. ^{1}H-NMR spectrum of LMWC (A) and LMWC-Glc derivative (B). HOD: Signal corresponding to solvent.

3.3. Enzymatic hydrolysis of chitosan/chitosan derivatives

It is already known that the chemical structure of oligosaccharides may affect their fermentation and prebiotic properties (Djouzi and Andrieux, 1997; Rycroft *et al.*, 2001; Cardelle-Cobas *et al.*, 2009; Cardelle-Cobas *et al.*, 2011). Thus, the available results show generally, that disaccharides with β (1→2), β (1→4) and β (1→6) linkages are more suitable as prebiotics than disaccharides with other different linkages (Sanz *et al*. 2005), while no information has been reported in relation to β (1→1) glycosidic linkages. Moreover, in general, carbohydrates with DP of 3 showed the highest selectivity towards bifidobacteria (Kaneko *et al.*, 1995; Kaplan and Hutkins, 2000). In this view, with the aim of producing derivatives with lower DP, the enzymatic hydrolysis of LMWC was performed and the COS obtained were then used to produce the Glc derivative. In order to accomplish the same purpose through another approach, the enzymatic hydrolysis of LMWC-Glc derivative was also conducted.

Enzymatic reactions were carried out following the methodology employed by Ruiz-Matute *et al*. (2013) slightly modified (see section 2.4). Previous screening assays were carried out to select the amount of enzyme to obtain a chitosan fraction with the lowest DP (results not shown). Figure 3.10 shows the chromatograms profiles obtained under the optimal hydrolysis conditions for LMWC and LMWC-Glc before and after enzymatic hydrolysis.

As it can be observed in the chromatograms, an increase in the retention time took place in the samples analyzed after enzymatic reaction. That means that LMWC and LMWC-Glc were converted into compounds with a lower Mw. Table 3.1 shows the Mw calculated for the samples before and after reaction.

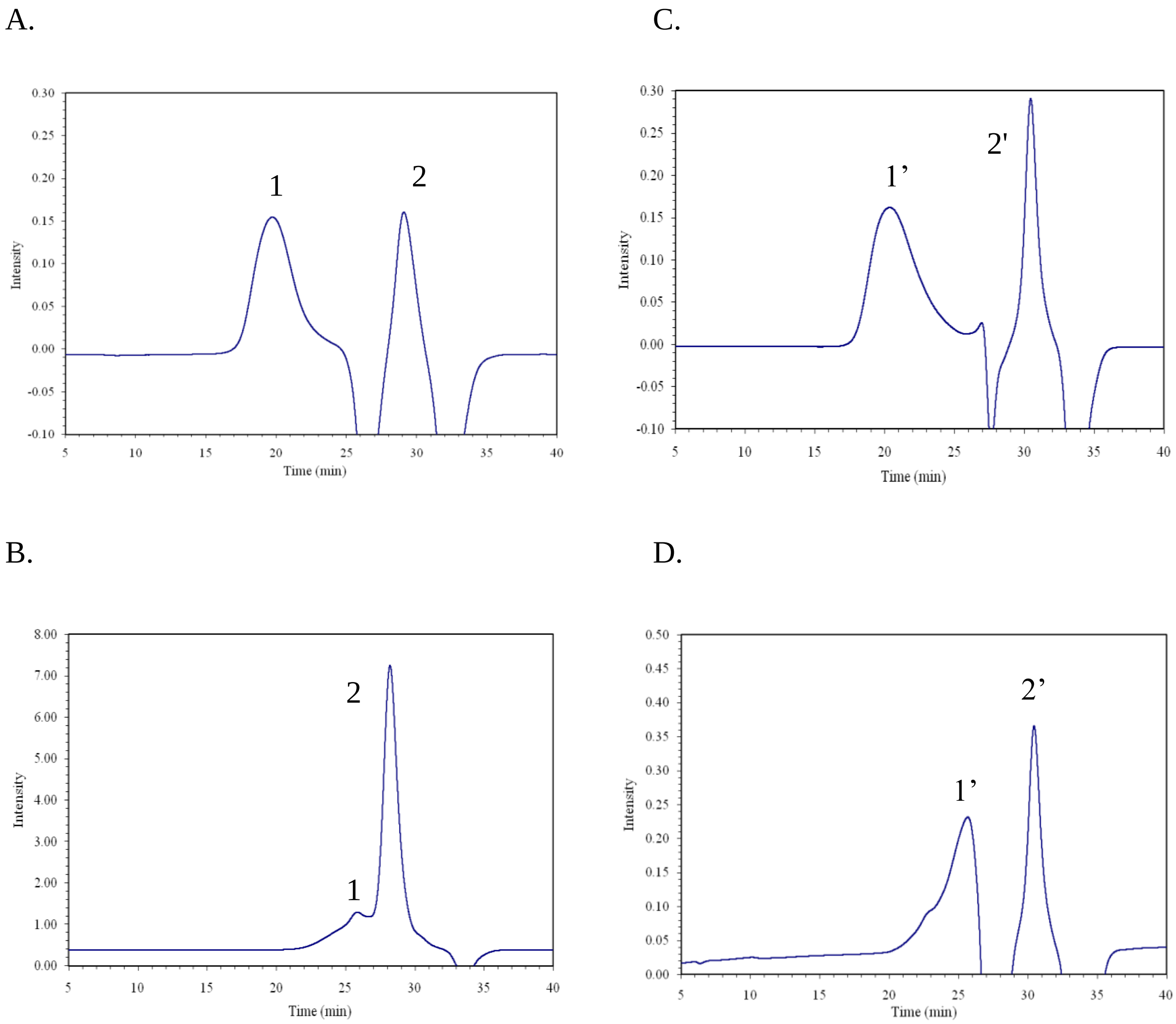

Figure 3.10. SEC-HPLC profiles obtained from the analysis of LMWC and LMWC-Glc before (A and C, respectively) and after enzymatic reaction (B and D) at a concentration of 2% (w/v) with Pectinex Ultra SP-L (120 UE) at 40 °C during 16 h of reaction.

Table 3.1. Average molecular weights (Mw ± SD) obtained for the analysis of the samples of LMWC and LMWC-Glc by SEC-HPLC before and after reaction of hydrolysis with Pectinex Ultra SP-L.

Sample	**Mw (KDa)**
LMWC	123 ± 5.32
Hydrolyzed LMWC	1.8 ± 0.12
LMWC-Glc	76 ± 4.25
Hydrolyzed LMWC-Glc	1.9 ± 0.20

Once optimized the conditions for the synthesis of LMWC/COS derivatives by Maillard reaction, the substrates selected for the determination of their prebiotic activity were:

-The hydrolyzed LMWC-Glc derivative previously obtained by Maillard reaction, named COS-Glc1.

-The hydrolyzed LMWC subjected subsequently to the Maillard reaction under the optimal conditions established in the section 3.1.2. This derivative was named as COS-Glc2.

Previously to bioactivity characterization, a complete characterization of both substrates was carried out.

3.4. Characterization of COS-Glc derivatives

Similarly to the characterization performed for LMWC-Glc derivative, analysis of SEC-HPLC, FT-IR and ^{1}H-NMR, were also carried out for COS-Glc1 and COS-Glc2.

3.4.1. SEC-HPLC analysis

The SEC-HPLC analysis of both COS-Glc derivatives was carried out with the objective to establish if some differences could be evident. Figure 3.11 shows the chromatograms obtained from the analysis of the COS-Glc1 (A) and COS-Glc2 (B).

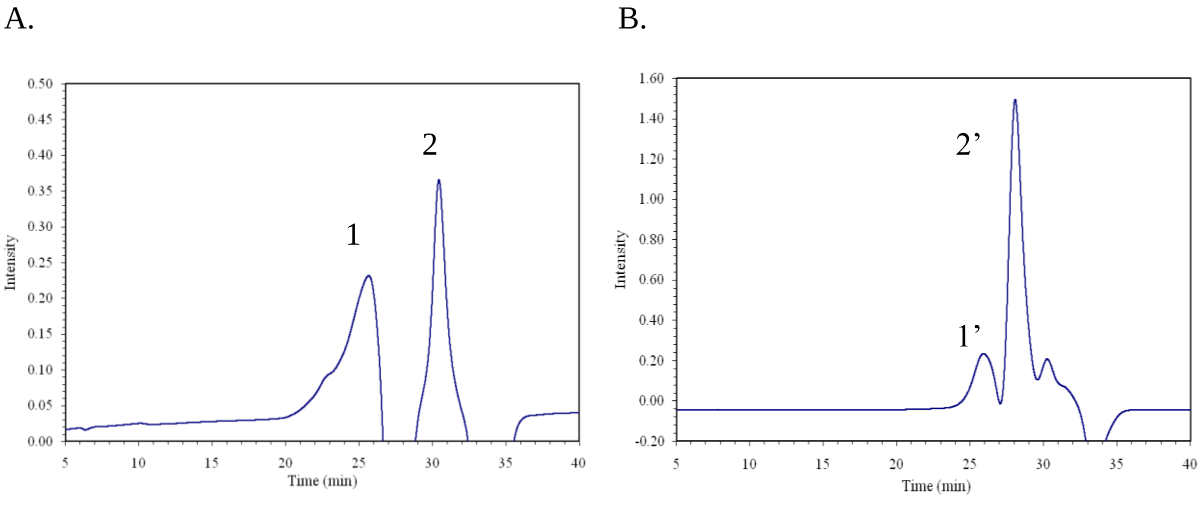

Figure 3.11. SEC-HPLC profiles obtained from the analysis of COS-Glc1 (A) and COS-Glc2 (B).

As in all the chromatographic profiles, two peaks were observed in each sample analyzed. One peak (1 and 1’) corresponding to the COS derivative (COS-Glc1 and COS-Glc2, respectively) and other peak corresponding to the solvent (2 and 2'). Figure 3.11A shows a retention time at 25.75 min for the COS-Glc1 corresponding to a Mw of 1.90 ± 0.20 KDa.

Figure 3.11B shows a retention time at 25.70 min for the derivative COS-Glc2, corresponding to a Mw 2.10 ± 0.05 KDa. In the chromatograms, it is also possible to observe that the Mw distribution of COS-Glc1 towards higher retention time is more pronounced than in the case of COS-Glc2. That means that the COS-Glc1 possess derivative fractions with higher Mw.

3.4.2. Colloid titration analysis

The analysis of COS-Glc derivatives by the colloid titration method described in section 2.5.1.3.1 allowed determining their DS. Thus, the results obtained indicated that the DS of the COS-Glc1 derivative was 42.04 ± 5.37%, and the DS for the COS-Glc2 derivative was 49.62 ± 0.48%.

3.4.3. FT-IR analysis

Characteristic absorption bands at 3480-3440, 3260-3270 and 2960-2878 cm^{-1} corresponding to OH, NH and CH stretching regions of chitosan/COS were observed in all samples (data not shown). Figure 3.12 shows the FT-IR spectra obtained in the region between 2000 and 400 cm^{-1} for COS and COS-Glc1 and COS-Glc2. As in the case of LMWC-Glc derivative (see section 3.2.3), the characteristic absorption bands of chitosan observed at around 1643 cm^{-1} and 1550 cm^{-1} were assigned to amide I and primary amino group, respectively.

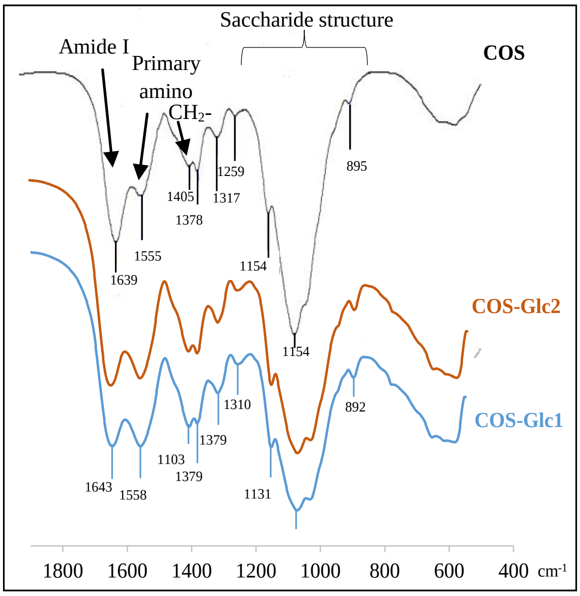

Figure 3.12. FT-IR profiles obtained for unmodified chitooligosaccharides (COS) and modified chitooligosaccharides (COS-Glc1 and COS-Glc2).

After reaction, it can be observed that certain absorption bands of COS were changed. Thus, an inversion in the intensity bands corresponding to the amide I (1639 cm^{-1}) and the amino group (1560 cm^{-1}) could be observed, being more noticeable in the derivative COS-Glc1. These results are also coincident with data found by Li *et al.* (2011b), who obtained the same data for the FT-IR analyses of a xylan chitosan conjugate synthesized by Maillard reaction. When the LMWC-Glc derivative was analyzed by FT-IR (section 3.2.3) the results suggested that Schiff base (C=N double bond) was formed between the reducing end of Glc and the amino groups of chitosan (Liu *et al*., 2001; Umemura and Kawai, 2007). However, in COS-Glc1 a shift was not observed. That could mean that the Schiff base conjugate could be displaced from the C=N bound towards the bound C=C. An increase could also be observed in the absorption band at 1405 cm^{-1}, corresponding to the CH_2- groups, which increased due to the introduction of saccharide groups into the COS molecule, especially for the derivative COS-Glc1. A thickening in the bands corresponding to the saccharide structure was also observed in the COS-Glc1 and COS-Glc2 derivatives.

3.4.4. ^{1}H-NMR analysis

A typical ^{1}H-NMR spectrum of COS is shown in Figure 3.13A. The signal observed at δ 2.0 ppm was assigned to the hydrogens of the methyl moieties of the acetamide groups while the signal at δ 3.2 ppm was attributed to hydrogen bonded to the C2 of the GlcN ring. The multiplet at δ 3.3-4.0 ppm was assigned to the hydrogens on the carbon atoms C3, C4, C5 and C6 and the signal at δ 4.5 ppm to the hydrogen on the C1 (Hirai *et al*., 1991; Park *et al*., 2003; Zhang *et al*., 2005; Mi *et al*., 2007). Comparing the spectra, differences were observed between the native COS and their derivatives. Signals were assigned following that reported in the bibliography (Hirai *et al*., 1991; Park *et al*., 2003; Zhang *et al*., 2005; Mi *et al*., 2007). As it can be observed in the spectra obtained for COS-Glc1 and COS-Glc2, a new signal appeared in the range of 4.1-4.3 ppm (Signal *a* in Figure 3.13B and 3.13C) due to the hydrogens bonded to the introduced Glc residues, especially for the derivative COS-Glc2. The most important changes observed in the spectra were referred to new signals appearing around 4.5 ppm indicating the N-substitution of the NH_2- groups of chitosan and partially overlapping with the signal due to the solvent. Signals due to the OH- groups of the carbohydrate (signals 2’-6’ in all figures) overlapped also with the hydrogen atoms of the OH-groups of the chitosan molecule (signals 3-6 in all figures) and no differences could be observed in the spectra. These results were in coincidence with the results obtained by other authors.

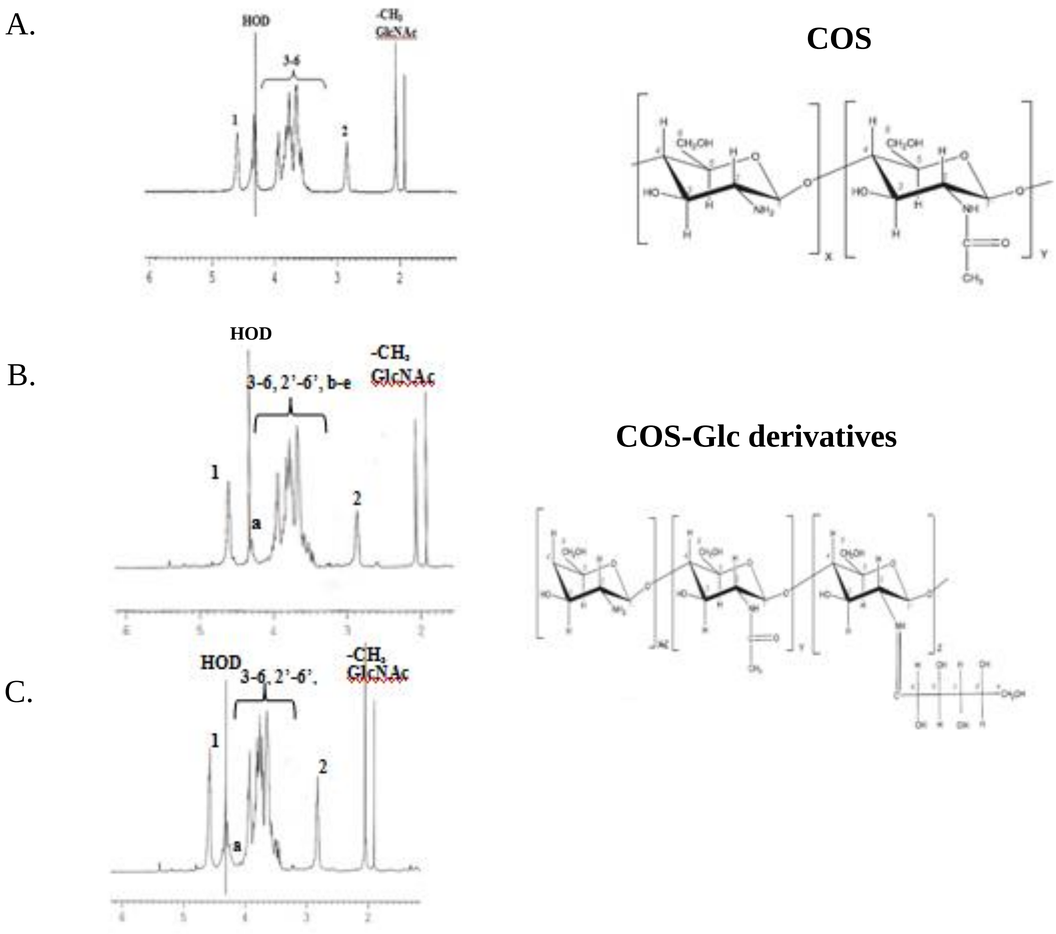

Figure 3.13. ^{1}H-NMR spectrum of COS (A) and COS derivatives, COS-Glc1 (B) and COS-Glc2 (C). HOD: Signal corresponding to solvent.

3.5. *In vitro* fermentation studies of chitosan derivatives

Over the past decades, many food products have been launched with prebiotic compounds and probiotic microorganisms added - which are known for their beneficial effect on the balance of intestinal microbiota. Inulin and such oligosaccharides, as fructooligosaccharides (FOS), are known examples of prebiotics (Roberfroid *et al.*, 2010; Fernandes *et al.*, 2012). Currently, there is a great interest in obtaining new prebiotic carbohydrates with improved properties, addressed to reach the distal regions of the colon unaltered to promote the growth of specific beneficial bacteria.

Oligosaccharides obtained from chitosan - i.e. COS, have been claimed to exhibit prebiotic effects (Lee *et al.*, 2002). However, recent studies (Fernandes *et al.*, 2012) showed that this prebiotic effect is slight and highly dependent on the concentration, since for higher concentrations the effect of amino groups improve its antimicrobial effect and decrease its prebiotic activity. In this view, our research group has recently developed enzymatic and chemical approaches for the synthesis of new chitosan-derived oligosaccharides, which may also be bioactive compounds and whose beneficial properties should be investigated.

3.5.1. Effect of COS derivatives on bacterial growth in pure cultures

In order to study the effect of COS and COS derivatives on the growth of lactic acid bacteria and bifidobacteria, these microorganisms were cultured in MRS broth (with and without Glc as carbon source), supplemented with 0.5% (w/v) of COS derivatives and incubated for 48 h at 37 ºC. Results obtained for COS showed that they produced inhibition in all the strains tested (results not shown). Results obtained for COS derivatives are shown below.

Growth curves of representative strains grown aerobically on the COS-Glc1 are depicted on Figure 3.14. Overall, these COS derivatives did not have a stimulatory effect upon none of the bacteria tested. Furthermore, none of these bacteria could use these compounds as a Glc substitute, or as a secondary source of carbon. Indeed, the COS-Glc1 showed inhibitory effects upon all the tested strains. In particular, *L. acidophilus* LA5, *L. acidophilus* LA10 and *L. paracasei* L26 appeared to be the most sensitive bacteria, since no growth was observed in the presence of the COS derivatives and Glc. *L. brevis* L24, *L. casei* L01 and *L. plantarum* appeared to be less susceptible to inhibition. In fact, it can be noticed that although inhibition was observed in the first 20 h of growth, after that period these strains were able to grow in the presence of Glc.

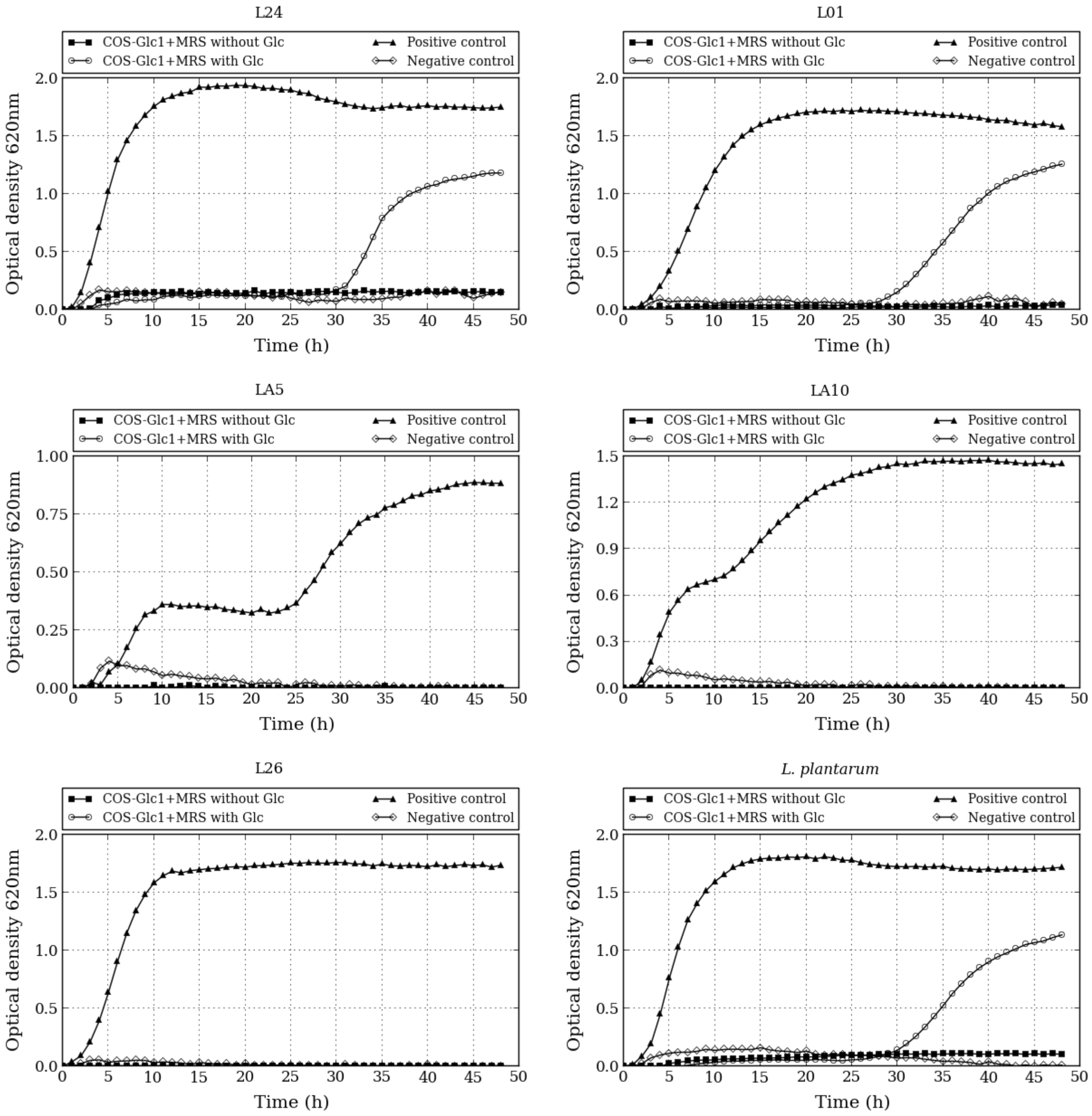

Figure 3.14. Growth curves of *L. brevis* L24, *L. casei* L01, *L. acidophilus* LA5, *L. acidophilus* LA10, *L. paracasei* L26 and *L. plantarum*, in media containing MRS broth (with and without Glc as carbon source), supplemented with 0.5% (w/v) of COS-Glc1.

Figure 3.15 shows the curves for the strains tested with the COS-Glc2 derivative. As it can be observed, in this case, inhibition of the growth of the tested strains was not produced. Moreover, these COS derivatives showed a stimulatory effect on *L. plantarum*, *L. casei* L01 and *L. acidophilus* LA10. The inhibition caused by the COS-Glc1 derivative can be related with the Mw. In fact, the chromatographic profile obtained by SEC-HPLC showed that COS-Glc1 and COS-Glc2 derivatives present different Mw distribution. In particular, the COS-Glc1 derivative possess fractions with higher Mw. It is interesting to notice that most of the lactic acid bacteria tested were able to use these COS derivatives (COS-Glc2) as a primary source of carbon (see MRS without Glc and supplemented with 0.5% (w/v) of COS derivatives). In the tests performed with *L. casei* L01, *L. acidophilus* LA10 and *L. acidophilus* LA5 in MRS broth without Glc supplemented with COS derivatives, similar OD_{620} values were reached when compared to those of the positive control. These results indicate that COS-Glc2 were excellent growth stimulators for some strains of *Lactobacillus* sp. tested.

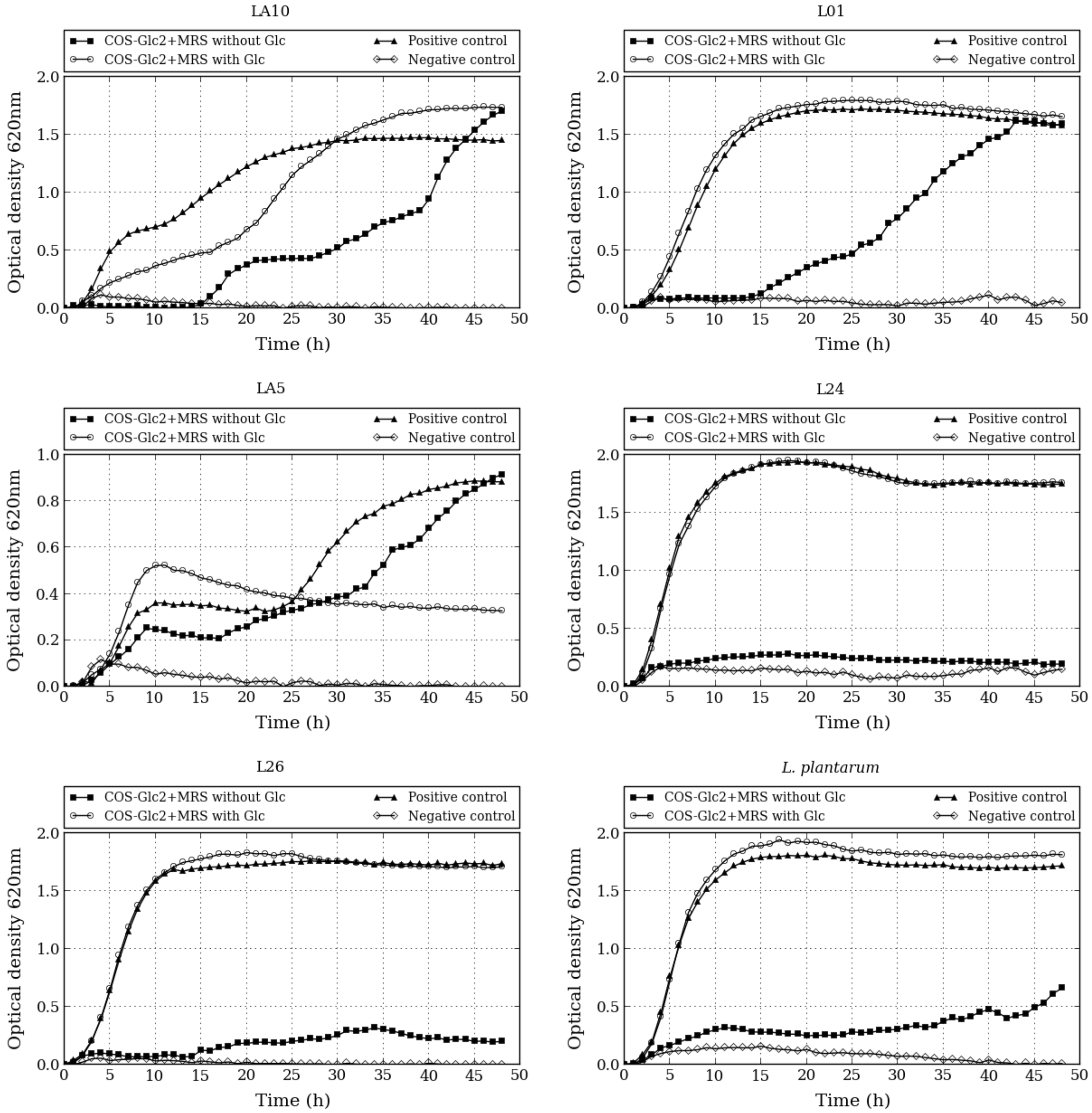

Figure 3.15. Growth curves of *L. brevis* L24, *L. casei* L01, *L. acidophilus* LA5, *L. acidophilus* LA10, *L. paracasei* L26 and *L. plantarum*, in media containing MRS broth (with and without Glc as carbon source), supplemented with 0.5% (w/v) of COS-Glc2.

Literature reports on prebiotic potential of COS are few and show different conclusions. Lee *et al*. (2002) reported that COS prepared by enzymatic hydrolysis stimulated the growth of *L. casei* and *L. brevis*. However, Fernandes *et al*. (2012) observed that commercial COS mixtures did not stimulate the growth of *L. acidophilus* Ki. Recently, Liang *et al*. (2013) studied the prebiotic potential of COS mixtures obtained by enzymatic hydrolysis. In this study, the LMWC oligomers synthesized stimulated the growth of *L. paracasei* and *L. kefir*, showing that these oligomers have potential use as prebiotic ingredients. The different results obtained in these studies may be due to the use of different *Lactobacillus* strains and different types of COS.

It has been considered that the biological activities of chitosan derivatives, and in particular the antimicrobial activity, are correlated with their DD and Mw (Mengíbar *et al*., 2011; Xia *et al*., 2011). Indeed, it has been reported in several studies that the bacterial inhibition tends to increase with the increase of the DD and the Mw of the chitosan derivatives (Choi *et al.*, 2001; Jeon *et al*., 2001; Mengíbar *et al*., 2011; Xia *et al*., 2011).

Additionally, the results of the DA and DS showed that COS-Glc2 derivatives present a higher DS (49.62%) than the COS-Glc1 derivatives (42.04%). That is, the COS-Glc2 derivative has less free amino groups that could be causing certain antimicrobial activity. Up to now, few studies reported the effects of COS derivatives on lactic acid bacteria in terms of DA (Liang *et al*., 2013).

In what concerns the strains grown anaerobically, growth curves for COS-Glc1 and COS-Glc2 derivatives are depicted on Figure 3.16. The results showed that bifidobacteria appears to be less susceptible to COS derivatives than the lactic acid bacteria. As it can be observed, a slight inhibitory effect was obtained, since the OD_{620} values were lower than that of the positive control; however, both bacteria continued to grow until they reached OD_{620} values similar to those of MRS control at 48 h. These results suggest that such COS derivatives do not cause growth inhibition of *B. animalis* Bb12 and *B. lactis* B94, although they are ineffective as main source of carbon.

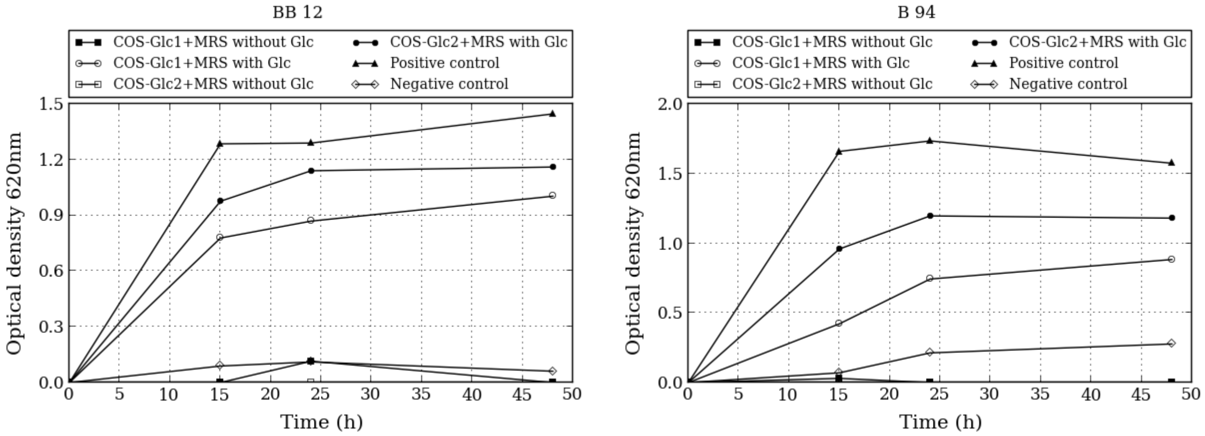

Figure 3.16. Growth curves of *B. animalis* Bb12 and *B. lactis* B94 in media containing MRS broth (with and without Glc as carbon source), supplemented with 0.5% (w/v) of COS-Glc1 or 0.5% (w/v) of COS-Glc2.

The results obtained in this work are not in agreement with the data reported in related studies. So, Lee *et al.* (2002) studied the effect of COS with DP 2-8, and confirmed that COS stimulated the growth of *B. bifidum* KCTC 3440. Recently, Fernandes *et al*. (2012) also observed that commercial COS mixtures and in the presence of Glc as the main source of carbon, COS led to a small increase in the growth of the bifidobacteria, although, these authors also reported that COS cannot be used as main source of carbon by any bifidobacteria tested.

In general, the results obtained in pure cultures showed some variation according to the strain used. However, it is possible to conclude that the COS-Glc2 was the derivative with more promising results. At a concentration of 0.5% (w/v) these compounds did not cause the inhibition of the growth of any of the strains tested. Moreover, most of the lactic acid bacteria tested were able to use these COS derivatives as a primary source of carbon. As far as we know, this is the first study on the effect of different types of COS derivatives on pure cultures growth. These results indicate that COS-Glc2 emerge as candidate prebiotic ingredients, although further research on their functionally and safety is required.

3.5.2. Modulation of the intestinal microbiota by COS-Glc derivatives

In vitro fermentation of prebiotic oligosaccharides provides an easy, fast and cheap alternative to assess the fermentation of different substrates at the laboratory scale on a comparative basis (Madhukumar and Muralikrishna, 2010; Gullón *et al*., 2013). In this work, the prebiotic potential of the freeze-dried COS derivatives (COS-Glc1 and COS-Glc2) were evaluated by *in vitro* fermentation with human faecal microbiota following methods reported in literature (Barry *et al*., 1995). For comparative purposes, assays with COS were also carried out. In addition, a control without substrate was carried out (negative control). Fermentations were followed by evolution of the *Bifidobacterium* populations and measuring the accumulation of SCFAs (acetate, propionate and butyrate) in the media.

3.5.2.1. Dynamics of the *Bifidobacterium* population

Monitoring the populations of selected species provides information suitable for assessing colon health (Woodmansey, 2007). In this work, FISH was used to evaluate the bifidogenic effects caused by COS-Glc1 and COS-Glc2, and COS on a comparative basis.

The use of the different substrates by colonic microbiota resulted in increased bifidobacteria populations with respect to the negative control cultures confirming a stimulatory effect (see Figure 3.17). After 48 h of incubation all carbon sources promoted similar shifts in bifidobacteria and did not show significant changes (p> 0.05).

The bifidogenic *in vitro* response of the colonic microbiota to COS and derivatives of chitosan has not been well reported. To our knowledge, the only study that investigated the evaluation of the prebiotic effect of COS and chitosans of different Mw was performed by Vernazza *et al*. (2005). In this work, only the COS and the LMWC stimulated bifidobacteria population in similar proportion to that observed in our work. Our results are in agreement with those reported by other studies using different source of carbohydrates. So, Salazar *et al*. (2009) and Hongpattarakere *et al*. (2012) reported similar increases in the levels of bifidobacteria (increase of Log cells/mL of 0.3) using exopolysaccharides as carbon sources.

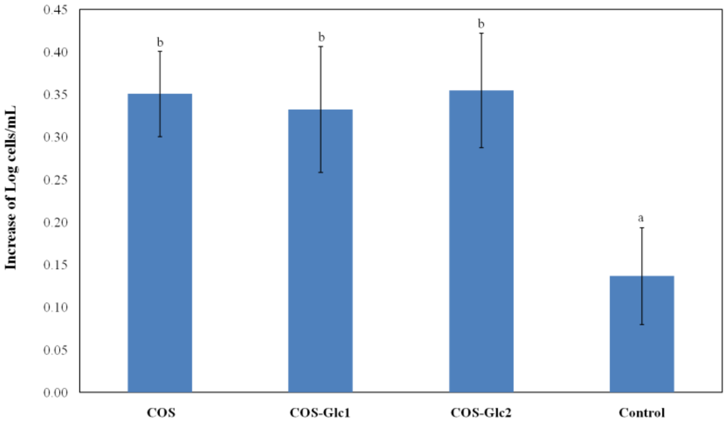

Figure 3.17. Increment in total bacteria and *Bifidobacterium* counts determined by FISH in faecal cultures from three donors using COS, and the derivatives COS-Glc1 and COS-Glc2 as a carbon source. Data obtained after 48 h of incubation. Error bars indicate standard deviations. Means within different letters are significantly different ($p < 0.05$). Control does not include carbohydrate source added. Initial Log CFU/mL *Bifidobacterium* counts: donor 1=7.33±0.08, donor 2=7.38±0.06, donor 3=7.43±0.06.

Recently, Rodriguez-Colinas *et al.* (2013) evaluated the *in vitro* fermentation of various purified galactooligosaccharides and observed minor changes in the content of bifidobacteria (increase of 0.15 Log cells/mL) in comparison to those obtained in this study and concluded that these substrates could be considered potentially bifidogenic.

Although it is not yet well known which species of bacteria are involved in maintenance of a healthy human intestinal microbiota, it is generally accepted that bifidobacteria play a relevant role (Tuohy *et al.*, 2005; Connolly *et al*., 2010), and that the increases in their concentration might be beneficial for the host health. The bifidogenic effect of the carbohydrates tested was shown by FISH analyses during incubation of the faecal slurry culture. Since bifidobacteria are acetate producers, beneficial shifts in SCFAs profiles found in faecal cultures point not only toward a unique bifidogenic effect, but also to the modification of other microbial groups that may use acetate and produce propionate or butyrate via a metabolic cross-feeding mechanism (Belenguer *et al*., 2006).

3.5.2.2. Short chain fatty acids production in faecal cultures

Table 3.2 shows the concentration of SCFAs measured by HPLC in the supernatants from the faecal fermentation with the different substrates. The chromatographic analyses of SCFAs were performed at 0, 5, 10, 24 and 48 h of fermentation. As can be observed, the SCFAs concentration profiles were markedly different in either experiment using the same carbon source and inocula from different individuals or in cultures with different carbon sources inoculated with samples from the same donor (see Table 3.2). Because of this, data are presented separately for each donor and a statistical analysis was not carried out.

In general, the increase of total SCFAs after 24 h of incubation was more pronounced in samples with carbohydrates added than in faecal cultures without any external carbon source, thus indicating that all carbohydrates tested were able to act as fermentable substrates for the intestinal microbiota, clearly enhancing the production of SCFAs during incubation.

The different profile of generated metabolic products confirmed that different substrates were metabolized by the various species present in the faecal microbiota. However, this increase was considerably more pronounced in the first 24 h of fermentation. Then, between the 24 and 48 h, the values remained almost constant with only slight variations.

After 48 h of fermentation, the total SCFAs concentrations in cultures from the three donors were similar. At the same fermentation time, the concentrations of total SCFAs achieved in media containing COS were higher in cultures inoculated with inocula from donors 1 and 2 than from donor 3. In experiments containing COS-Glc1 derivative, the concentrations of total SCFAs achieved were higher for inocula from donors 1 and 3 than inocula from donor 2. In the case of experiment containing COS-Glc2 derivative, the total SCFAs concentrations were similar in media inoculated with inocula from donors 1 and 2, while total SCFAs in culture inoculated with faces from donor 3 was considerably higher. The total SCFAs concentrations after 48 h varied in the range 34.35-105.11 mmol/L, confirming that the degree of stimulation varied depending on the carbohydrate type and of intestinal microbiota from the individual, as it was previously established for other carbohydrates by several authors (Salazar *et al*., 2009; Gullón *et al*., 2011b; Cardelle-Cobas *et al*., 2012; Gullón *et al*., 2013). In general, acetic acid was the most abundant SCFA followed by propionic and butyric acids in all experiments (Sarbini *et al*., 2013), with the exception of donor 3 in the presence of COS-Glc2, where a higher concentration of propionic acid was observed in comparison with acetic acid.

It can be noted that the fermentation of all substrates induced the production of acetate. The highest acetate concentration was achieved in media containing COS as the carbon source, followed by the media made with COS-Glc1 and COS-Glc2. Acetic acid has been described as a major product of the bifidobacteria pathway following fermentation by human faecal suspensions, although acetic acid is also formed by many anaerobic bacteria from the human gut (Salazar *et al*., 2009). Thus, it is difficult to relate the production of this acid with one bacterial genus when mixed cultures are studied (Bezkorovainy, 1989). In the present study, the results obtained are different from those reported by Vernazza *et al*. (2005) that evaluated the effects of chitosan and its oligosaccharides on the gut microbiota. These authors found levels of acetic acid lower than those observed in our study.

In the case of propionate, the achieved concentrations were different depending on the carbon source evaluated and the donor. The highest levels of propionic acid and lowest values of the acetic to propionic acid ratio among the three carbon sources tested were obtained for COS-Glc1 and COS-Glc2 and the lowest levels of propionic and highest acetic to propionic acid ratio with COS. Several studies have reported that a reduction in the acetic to propionic acid ratio was linked to inhibition of cholesterol synthesis both in the liver as well as intestinal cholesterol biosynthesis (Delzenne and Kok, 2001). So, Connolly *et al.* (2010) carried out a study using batch cultures inoculated with human faeces to ferment konjac glucomannan hydrolysate and inulin and resulted in a SCFAs molar ratio of 1:0.54:0.21 (acetic:propionic:butyric) and determined this was a high proportion of propionic acid when compared to other SCFAs profiles generated by other carbohydrates (Salazar *et al.*, 2009; Gullón *et al*., 2010; Cardelle-Cobas *et al*., 2012; Sarbini *et al.*, 2013). In the present work, the average molar ratio for the three donors for COS-Glc2 was 1:1.9:0.8, these ratios would be considered to be propionic acid rich SCFAs profiles. Hence, the fermentation with faecal inoculum in the presence of our COS derivatives promoted a shift in the SCFAs profile, causing a decrease of levels of acetic acid and an increase in propionic acid proportions.

The increase in propionate concentration during fermentation of COS derivatives could be related with an increase in *Bacteroides*, which are known to be propionate producers (Macy and Probst, 1979). As known, *Bacteroides* is one of the predominant colonic bacteria genera comprising about 30% of the total culturable microbiota (Woodmansey, 2007).

Moderate increases in butyrate levels were observed in all substrates tested. The butyrate accumulation in experiments with inocula from donor 3 was more pronounced when COS-Glc2 was employed as a carbon source, whereas lower butyrate concentrations were reached in assays using faecal cultures from individuals 1 and 2 with this same substrate. Similar butyrate concentrations were produced in experiments with samples from the three donors for COS. Butyric acid is considered to be a desirable metabolite of gut bacterial function. In fact, it plays an important part in the maintenance of a normal colonocyte population and participates in the energy metabolism and normal development of colonic epithelial cells (Bourquin *et al*., 1992). Also, some *in vitro* studies have shown that it can promote apoptosis of cancer cells (Hague *et al*., 1995). In the present study, the fermentation of various carbon sources tested resulted in butyric acid concentration in the range of 9.47-15.88 mmol/L.

Our results are different from the ones of Vernazza *et al*. (2005), who stated that the *in vitro* use of COS by intestinal bacteria produce low levels of butyrate. However, the high concentration of butyric acid reported in this study is in agreement with literature reports on the use of different oligosaccharides with different structures used as carbon sources for evaluation of the prebiotic potential (Cardelle-Cobas *et al*., 2012; Rivas *et al*., 2012; Gullón *et al*., 2013).

In short, the results presented here allow a preliminary comparison to be made of the bifidogenic properties of different types of synthesized derivatives. The three substrates act as fermentable carbohydrates for intestinal bacteria *in vitro*, enhancing the production of acetic acid and SCFAs and promoting the increase of *Bifidobacterium* numbers in human faecal cultures.

This study, although provides an initial assessment of the prebiotic potential of COS derivatives, should be completed by *in vivo* evaluation of their fermentation properties before being finally used as a functional ingredient for improving the composition of gut microbiota.

Table 3.2. Concentrations (mM) of the three major SCFAs values of faecal cultures from three donors. A= Acetate, P= Propionate, B= Butyrate.

		Donor 1				**Donor 2**				**Donor 3**			
Carbon source	**Time (h)**	**A**	**P**	**B**	**Total SCFA**	**A**	**P**	**B**	**Total SCFA**	**A**	**P**	**B**	**Total SCFA**
Control	0	1.17	3.86	0.00	5.03	0.94	0.00	0.00	0.94	0.00	0.00	0.00	0.00
	5	5.34	4.27	0.80	10.42	4.15	3.47	0.00	7.63	5.98	3.85	1.21	11.03
	10	9.33	3.40	3.07	15.80	11.43	3.69	3.74	18.87	10.42	3.93	3.28	17.63
	24	13.95	6.35	5.00	25.30	14.77	6.45	5.05	26.27	13.23	6.21	4.98	24.41
	48	17.21	7.56	5.37	30.14	17.76	7.39	5.08	30.23	17.60	7.94	5.43	30.97
COS	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	5	6.04	5.09	0.00	11.13	3.91	3.28	0.00	7.19	9.18	3.71	0.00	12.89
	10	13.25	5.16	3.07	21.48	16.29	3.82	3.92	24.03	12.82	3.85	3.66	20.33
	24	41.84	18.07	12.52	72.43	45.97	13.55	11.60	71.12	35.91	8.31	12.30	56.52
	48	51.29	23.33	15.97	90.59	64.81	24.66	15.64	105.11	41.84	12.20	15.99	70.03
COS-Glc1	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	5	11.99	5.24	1.64	18.87	11.65	3.91	1.26	16.83	12.97	4.50	2.14	19.61
	10	31.68	11.44	8.44	51.56	20.46	10.64	5.45	36.55	22.53	14.12	9.92	46.57
	24	27.50	16.54	9.91	53.95	18.42	12.18	5.47	36.07	27.07	26.87	14.93	68.86
	48	24.42	16.82	9.91	51.15	17.60	13.68	5.30	36.58	20.71	25.15	13.20	59.06
COS-Glc2	0	0.00	0.00	0,00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	5	4.28	5.07	1.40	10.75	2.57	3.68	0.89	7.13	4.69	4.46	2.03	11.18
	10	16.02	9.46	5.70	31.18	16.71	14.68	4.86	36.25	10.25	18.65	9.70	38.61
	24	19.05	14.31	7.42	40.78	13.54	16.10	5.09	34.73	10.86	54.35	27.80	93.01
	48	17.02	14.37	7.33	38.73	12.05	17.51	4.79	34.35	18.38	58.86	25.33	102.57

3.6. Evaluation of the cytotoxic effect of COS derivatives by flow cytometry

The biocompatibility of a compound refers to the extent to which its molecule does not have toxic effects or cause injury upon biological systems. Thus, to consider a molecule as biocompatible, it is of great importance to submit it to a number of pre-toxicity tests, *in vitro* or *in vivo* (Keong and Halim, 2009).

In order to evaluate the biocompatibility of the COS derivatives synthesized in the present work, the study of their cytotoxic effect was performed by flow cytometry. For comparative purposes, the cytotoxicity of COS was also evaluated. This assay enables the evaluation of cell viability and also the enumeration of apoptotic and necrotic cells. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin V is a phopholipid-binding protein that has high affinity for PS, and binds to cells with exposed PS. Since externalization of PS occurs in the earlier stages of apoptosis, Annexin V staining can identify apoptosis at an early stage. The conjuction of Annexin V with 7AAD, which penetrates only in dead and damaged cells, enables the differentiation between cells undergoing early apoptosis (Annexin V^+/$7AAD^-$) and cells undergoing late apoptosis and necrosis (Annexin V^+/$7AAD^+$) (Baudouin *et al.*, 2007).

Figure 3.19 shows the results obtained in the evaluation of cell viability. It is possible to notice that for all the compounds and in all the concentrations tested, the percentage of non-viable lymphocytes was clearly lower than the one obtained in the positive control (87%). At 0.05 and 0.005 mg/mL the percentage of non-viable cells was very similar when testing the same compound and also among the different compounds (approximately 20%). As the concentration increased to 0.5 mg/mL, the percentage of non-viable cells also increased to approximately 40% in the case of COS-Glc1, COS-Glc2, and 56% in the case of COS. In fact, this compound showed the highest percentage of non-viable lymphocytes. Also, it is possible to notice that the percentage of non-viable cells obtained at 0.05 and 0.005 mg/mL was approximately the same obtained with the negative control (19%). Only at 0.5 mg/mL the number of non-viable cells was clearly higher than the negative control.

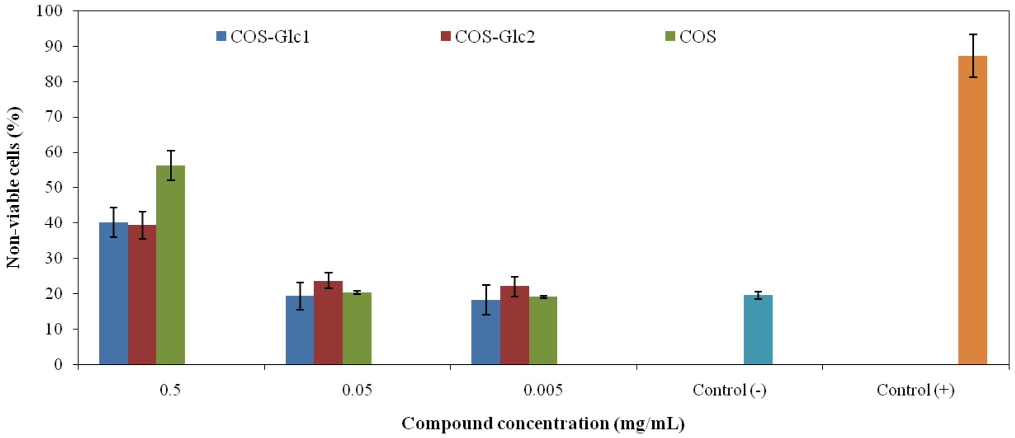

Figure 3.18. Non-viable (Annexin V^+/7AAD$^-$ plus Annexin V^+/7AAD$^+$) lymphocytes following treatment with the COS-Glc1, COS-Glc2 and COS (average ± standard deviation).

There are two parameters which have been suggested to play a crucial role upon induction of cytotoxicity by chitosan and its derivatives: the Mw and the DD or DS (in the case of the derivatives obtained by substitution) (Je *et al.*, 2006; Fernandes *et al.*, 2011). In what concerns the Mw, the analysis carried out by SEC-HPLC showed that COS-Glc1 and COS-Glc2 derivatives present different Mw distribution. In particular, the COS-Glc1 derivative possess fractions with higher Mw. Despite this difference, the cytotoxicity induced by the two derivatives was very similar. On the other hand, due to the cationic nature of chitosan and COS, the surface charge of these molecules has been claimed as an important factor affecting the cytotoxic activity, owing to the electrostatic interaction between the negatively charged groups of the lymphocyte surface and the positively charged amino groups of chitosan and COS (Je *et al.*, 2006). In fact, the cytotoxic effect induced by COS was higher than the one induced by the COS-Glc1 and the COS-Glc2. Since the two derivatives were subjected to substitution of the amino groups it was expected that the cytotoxic effect of these compounds was lower when compared to COS.

Further analyses with flow cytometry were conducted in order to discriminate the percentage of apoptotic and necrotic cells present in the lymphocyte populations treated with the COS-Glc1 and COS-Glc2 derivatives, and COS. The results are illustrated in Figure 3.19.

The results show that the extent of apoptosis in cells treated with staurosporine (positive control) was about 53%. This percentage was clearly higher than that induced by all the compounds at any concentration tested. Also, the extent of necrosis obtained with the COS-Glc1 and the COS-Glc2 at any concentration tested was below the value obtained with the positive control (34%). For COS at 0.5 mg/mL, the percentage of necrotic cells was higher than the positive control. In fact, at this concentration, COS was the compound that induced the highest necrosis rate.

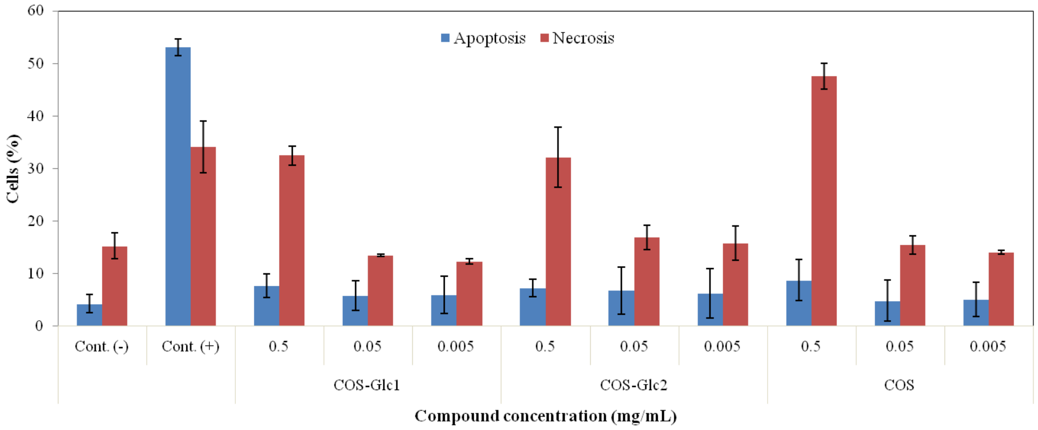

Figure 3.19. Necrotic (Annexin V^+/$7AAD^+$) and apoptotic (Annexin V^+/$7AAD^-$) lymphocytes following treatment with COS-Glc1, COS-Glc2 and COS (average ± standard deviation).

Literature reports on COS cytoxicity are few and inconclusive. The studies are mainly based on the MTT colorimetric assay and reported contradictory conclusions. In fact, Rajapakse *et al.* (2007) reported the absence of toxic effects by COS, at 0.050-1.0 mg/mL upon human and mouse leukocyte cell lines. Xu *et al.* (2008) claimed that at 0.80 mg/mL COS induced apoptosis upon human cells. More recently, Fernandes *et al*. (2011) evaluated the cytotoxicity of COS upon human lymphocyte cultures using flow cytometry. The results suggested that above 0.07 mg/mL COS induced strong cytotoxic effects.

In the present work, at 0.5 mg/mL the synthesized derivatives showed some extent of cytotoxicity. However, none of the derivatives induced cytotoxicity below 0.05 mg/mL, suggesting that at these concentrations these molecules will be harmless to the human cells. Moreover, Chae *et al.* (2005) evaluated the intestinal absorption of water-soluble chitosan and demonstrated that for chitosan of approximately 3 KDa the maximum plasma concentration was of 0.02 mg/mL. According to the results obtained in the present work, at this concentration no significant cytotoxicity was observed which means that it is safe to use the synthesized derivatives as prebiotic ingredients. In fact, this assay shows that the substitution of the amino groups decreased the cytotoxicity of the COS derivatives when compared to unmodified COS and that both derivatives are biocompatible molecules. Nevertheless, further studies are recommended, mainly *in vivo* tests, to eventually confirm these *in vitro* results.

4. Conclusions

In the present work, the detailed study of the different parameters affecting the Maillard reaction (Mw of chitosan, Glc concentration, temperature and reaction time) allowed to establish the optimal reaction conditions for the synthesis of the derivatives of chitosan: 2% (w/v) of LMWC in 1% (v/v) of acetic acid, 2% (w/v) of Glc, 60ºC and 32 h of reaction time.

Two COS derivatives were synthesized in the present work, namely COS-Glc1 and COS-Glc2, which were evaluated in terms of their prebiotic properties. The study of the prebiotic properties of the COS derivatives was carried out in pure cultures and in mixed cultures (human faecal inocula). In general, the results obtained in pure cultures showed some variation according to the strain used. However, it was possible to conclude that the COS-Glc2 was the derivative with more promising results. At a concentration of 0.5% (w/v) this compound did not cause the inhibition of the growth of any of the strains tested. Moreover, most of the lactic acid bacteria tested were able to use COS-Glc2 as a primary source of carbon. The *in vitro* experiments in mixed cultures demonstrated the ability of both COS derivatives to support the growth of bifidobacteria. Indeed, both derivatives promoted an increment in the *Bifidobacterium* population and led to the production of SCFAs upon fermentation.

The evaluation of the cytotoxic effect of the derivatives demonstrated that both derivatives are biocompatible molecules so, it is safe to use them as prebiotic ingredients. Also, the assay showed that the substitution of the amino groups decreased the cytotoxicity of the COS derivatives when compared to unmodified COS. Nevertheless, further studies are recommended, mainly *in vivo* tests, to eventually confirm these *in vitro* results.

Although more studies are necessary, namely *in vivo* studies, the results obtained in the present work demonstrate that the COS derivatives synthesized through the Maillard reaction can constitute an alternative to the current prebiotic ingredients. Additionally, these compounds have proved to be harmless to human cells.

5. Future Work

In the present work, the *in vitro* prebiotic potential of chitosan derivatives was evaluated. However, the final demonstration of a prebiotic effect must be carried out *in vivo* through appropriate nutritional trials and using validated methodologies. Thus, studies using animal models and human trials could be performed with the derivatives produced in the present work in order to demonstrate the *in vivo* prebiotic effect.

Also, a detailed study of the chemical structure of both derivatives is lacking and could be of great importance in order to ascertain which COS derivative (COS-Glc1 or COS-Glc2) has enhanced prebiotic properties.

Another aspect that could be further determined is the antimicrobial activity of both derivatives in order to better understand the results obtained in pure cultures and ascertain if they still maintain certain antimicrobial activity against pathogenic microorganisms.

The evaluation of the antioxidant properties of the synthesized derivatives is another assay that could be performed. In fact, it would be important to compare the antioxidant capacity of the COS derivatives with LMWC and unmodified COS, since the confirmation of this property could expand the future applications of these compounds.

Also, the antiadhesive capacity is a relevant property attributed to some prebiotic oligosaccharides that may confer health benefits. In this view, the study of the ability of the new COS derivatives to inhibit the adhesion of food pathogens to mucin *in vitro* could be conducted in the future.

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