Quinoa Stalks Glucuronoarabinoxylan
Biorefinery, xylooligosaccharides production and potential applications
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Quinoa Stalks Glucuronoarabinoxylan Biorefinery, xylooligosaccharides production and potential applications

DANIEL MARTIN SALAS VEIZAGA | BIOTECHNOLOGY | LUND UNIVERSITY
Quinoa Stalks
Glucuronoarabinoxylan

Bioresintry, xyloooligosaccharides production and potential applications

Daniel Martin Salas-Veizaga

LUND UNIVERSITY

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Quinoa stalks were used as a source of hemicellulose for XO production and further as prebiotic source for probiotic bacteria. Three methods for extraction of hemicellulose were used, of which alkaline extraction (NaOH [0.5 M]) was the optimum method. This methodology allowed the establishment of a sequential extraction platform, including saponins obtention via PHWE, hemicellulose via alkaline extraction and cellulose via acid purification. Maximum yields of 15.4, 120 and 296 mg/g raw material of saponins, hemicellulose and cellulose yield were obtained, respectively. Saponins and hemicellulose extractions were dependent on PHWE conditions used, while cellulose extraction was not dependent on conditions in previous steps and resulted in purest fraction. The purified hemicellulose fraction consisted of glucuronoarabinoxylan (GAX) (HPAE-C-PAD, FT-IR) with an estimated Mw of 1758 ± 31 kDa (SEC). The xylopyranosyl backbone was linked via β-(1,4) bonds, and substituted with 4-O-Methyl glucuronosyl and arabinofuranosyl residues. The later was linked as oligomers via α-(1-5) or as monomers α-(1-3) or α-(1-2) to the xylose skeleton (NMR). The monosaccharides composition of the GAX included xylose, glucuronic acid, arabinoise and galactose in a molar ratio of 114:23:5:1, respectively. The GAX purified from quinoa stalks was treated by two methodologies to produce XOs: an enzymatic method (using xylanases) and a dilute acid method using H2SO4 [0.25 M]. In the enzymatic method, in house produced thermostable endoxylanases from GH10 (from R. marinus and B. halodurans) and the commercial GH11 PENTOPAN®, were used. The maximum yield of linear XOs obtained from the hydrolysis of GAX was 1.217 g XOs/100 g of GAX. Dilute acid treatment resulted in a maximum yield of 0.584 g XOs/100 g GAX. However, the DP in dilute acid treatment was more widely distributed, ranging from xyllose to xylohexaose compared to the enzymatic method. B. adolescentis ATCC15703 and Weissella sp. strain 92 were cultivated using XOs from quinoa stalks GAX as carbon source. After 48 hours of cultivation, B. adolescentis grew to a maximum OD600 of 0.326, producing the followig amounts of SCFA and lactate (g/L): Acetate (1.243); lactate (1.013); propionate (0.812); formate (0.179) and; butyrate (0.124). Weissella sp. 92 grew to an OD600 of 0.626, producing Acetate (0.379 g/L) and lactate (0.259 g/L). The consumption of XOs by Weissella sp. 92 was mainly consuming monosaccharides (xylose and arabinose), and XOs: xylose, xyliotriose and xylohexaose, while B. adolescentis was able to consume all linear XOs at different ratios, and was, moreover, able to consume substituted XOs (according to HPAEC-PAD). A draft genome sequencing of Clostridium boliviense strain E-1 was made, and a number of genes encoding hemicellulose-active enzymes were identified. Two enzymes were fully characterized; one two domain GH43-like endo-β-xylanase (Cbe1Xyn43, Mw 52.9 kDa) and one bifunctional acetyl esterase/endo-β-xylanase (Cbe1Est1XynX, Mw 44.2 kDa), also consisting of two domains (one carbohydrate esterase and one potential glycoside hydrolase). Both enzymes were thermostable and most active at neutral pH. Both enzymes also were active on birchwood glucuronoxylan, wheat bran arabinoxylan and quinoa stalks GAX. No xylosidase activity was determined. Cbe1Xyn43 kinetic parameters was determined to Km (1.587 min⁻¹) and Kmax (0.126 mM) using p-nitrophenol xylobioside (pNPX₂) as substrate. For Cbe1Est1XynX the Km (6.645 min⁻¹) and Kmax (0.233 mM) was determined using pNPX and, using p-nitrophenyl acetate (pNPAc) Km was 243.363 min⁻¹ and Kmax was 2.25 mM. Despite the sequence similarity of Cbe1Xyn43 to enzymes in GH43, the conserved catalytic residues of GH43 could not be identified, making classification of the enzyme difficult. In conclusion, the potential of quinoa stalks as a raw material for valorization has been demonstrated (separating saponins, GAX and cellulose) for further biorefinery applications. GAX in particular, resulted in successfully XOs production by enzymatic and acid methodologies for prebiotic usage. Also additional xylanases were explored, and were demonstrated as potential tools to diversify and increase linear and substituted XOs production from different material.

Key words: Quinoa stalks, Glucuronoarabinoxylan, Xylooligosaccharides, Xylanases, Probiotics.
Quinoa Stalks
Glucuronoarabinoxylan

Biorefinery, xylooligosaccharides production and potential applications

Daniel Martin Salas-Veizaga
Coverphoto: Quinoa (*Chenopodium quinoa* Willd.).

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Abstract

Quinoa stalks were used as a source of hemicellulose for Xylooligosaccharides (XOs) production and further as a prebiotic source for probiotic bacteria. Three methods for extraction of hemicellulose were used, of which alkaline extraction using NaOH [0.5 M] resulted in the highest yields. This methodology allowed the establishment of a sequential extraction platform, to obtain saponins using Pressurized Hot Water Extraction (PHWE), hemicellulose via alkaline extraction and cellulose via acid purification [80 % acetic acid, 69 % nitric acid]. Maximum yields of 15.4, 120 and 296 mg/g raw material of saponins, hemicellulose and cellulose yield were obtained, respectively. Saponin and hemicellulose extraction yields were dependent on the temperature in the PHWE used, while cellulose, was less dependent of previous extractions, and resulted in the purest fraction extracted from the agricultural residues. The purified hemicellulose fraction consisted of glucuronoxarabinoxylan (identified by HPAEC-PAD, FT-IR) with an estimated molecular weight (M_w) of 1758 ± 31 kDa (estimated by SEC). The xylopyranosyl backbone was linked via β-(1,4) bonds, and substituted with 4-O-Methyl glucuronosyl and arabinofuranosyl residues. The latter were linked as oligomers by α-(1-5) or as monomers α-(1-3) or α-(1-2) to the xylose backbone (according to NMR identification). The monosaccharides composition of the extracted glucuronoxarabinoxylan included xylose, glucuronic acid, arabinose and galactose in a molar ratio of 114:23:5:1, respectively.

The glucuronoxarabinoxylan purified from quinoa stalks was treated by two methodologies to produce XOs: an enzymatic method (using xylanases) and a dilute acid method using H₂SO₄ [0.25 M]. The enzymatic method was developed using in house produced thermostable endoxylanases from glycoside hydrolases (GH10) originating from Rhodothermus marinus and Bacillus halodurans and the commercial GH11 PENTOPAN®. The maximum yield of linear XOs obtained from the hydrolysis of glucuronoxarabinoxylan was 1.217 g XOs/100 g of purified xylan. Dilute acid treatment resulted in a maximum yield of 0.564 g XOs/100 g of purified xylan (substituted XOs could not be quantified). The degree of polymerization after dilute acid treatment was more widely distributed than after enzyme treatment, ranging from xylobiose to xylohexaose.

Bifidobacterium adolescentis ATCC15703 and Weissella sp. strain 92 were cultivated using XOs from quinoa stalks glucuronoxarabinoxylan as carbon source
[0.5 % w/v]. After 48 hours of cultivation, *B. adolescentis* grew to a maximum OD$_{600}$ of 0.326, producing the following amounts of Short Chain Fatty Acids (SCFA) and lactate (g/L): Acetate (1.243); lactate (1.013); propionate (0.812); formate (0.179) and; butyrate (0.124). *Weissella* sp. strain 92 grew to an OD$_{600}$ of 0.626, producing Acetate (0.379 g/L) and lactate (0.259 g/L). The carbohydrate source consumption by *Weissella* sp. strain 92 was mainly monosaccharides (xylose and arabinose), and XOs of DP 2-4: xylobiose, xylotriose and xylotetraose, while *B. adolescentis* was able to consume all linear XOs at different ratios, and, moreover, also consumed substituted XOs (according to HPAEC-PAD).

*Clostridium bolivienne* strain E-1 was investigated as a source of hemicellulose-active enzymes, due to presence of a number of genes in its genome that encoded putative hemicellulose active enzymes. From a total of 8 putative xylanases, two enzymes were fully characterized; one putative GH43-like endo-β-xylanase (*CbE1Xyn43*, $M_w$ 52.9 kDa) and one bifunctional two domain acetyl esterase/endo-β-xylanase (*CbE1Est1XynX*, $M_w$ 44.2 kDa), consisting of two separate domains (one encoding the carbohydrate esterase and the other potentially encoding the glycoside hydrolase). Both enzymes were thermostable with their highest specific activity at neutral pH. Both enzymes also were active on birchwood glucuronoxylan, wheat bran arabinoxylan and quinoa stalks glucuronoroarabinoxylan. No xylosidase activity was determined. *CbE1Xyn43* kinetic parameters was determined to $K_{cat}$ (1.587 min$^{-1}$) and $K_m$ (0.126 mM) using $p$-nitrophenyl xylobioside ($p$NPX$_2$) as substrate. For *CbE1Est1XynX* the same parameters were determined using $p$NPX$_2$ ($K_{cat}$ = 6.645 min$^{-1}$ and $K_m$ = 0.233mM) and $p$-nitrophenyl acetate ($p$NPAc) ($K_{cat}$ = 243.363 min$^{-1}$ and $K_m$ = 2.25 mM).

Despite the sequence similarity of *CbE1Xyn43* to enzymes in GH43, the conserved catalytic triad of the family could not be identified, making definite classification of the enzyme difficult.

Quinoa stalks has demonstrated its potential as a valorizable lignocellulosic material allowing separation of saponins, glucuronarabinobioxyan and cellulose sources and further biorefinery applications may be developed from here. In the particular case of glucuronarabinobioxyan, enzymatic and acid methodologies resulted in successful XOs production for prebiotic usage. Also new types of xylanases have been isolated that may complement candidates from more established GH families to diversify and enhance production of linear and substituted xyooligosaccharides.
Quinoa, more particularly its grain, has been a base-diet food in Bolivia, mainly due to its content of proteins and its high nutritional value. Global interest for quinoa has started 40 years ago, an interest that promoted not only the agricultural expansion in Bolivia in terms of cultivation area, but also turned Bolivia into one of the biggest producers of quinoa worldwide. Together with the increasing cultivation areas and grain productivity, the amount of agricultural waste residues has also been triplicated. These residues (mainly stalks) have not had any other subusage by farmers and they are just piled next to the grown fields, not even being consumed by local cattle. These quinoa stalks opened the interest of the present work, on how to use the material as a potential carbohydrate source. The possibility to use quinoa stalks for this purpose, will be, not only an extra-benefit in terms of economical use of this byproduct, but also will contribute to a stronger establishment of the green chemistry methodologies, and of biorefineries as biobased raw materials used to substitute fossil-based products.

The carbohydrates from quinoa stalks are found as cellulose, a polymer that has been used for biofuels production, and also hemicellulose (in this case xylan), that can be used as source of prebiotics. Prebiotics are non-digestable food aditives that have indirect beneficial effect on the hosts that consume them. The beneficial effect is indirect, as these prebiotics are a carbohydrate source for probiotic bacteria, that are microorganisms in the gut, proven to be beneficial for the host, improving its health. Among prebiotic compounds, xylooligosaccharides (XOs) that are short sugar chains formed by units of xylose. To obtain XOs from quinoa stalks, it is first necessary to extract the xylan. To achieve that objective, quinoa stalks were first subjected to Pressurized Hot Water Extraction (PHWE) to remove and obtain saponins (soaplike molecules composed of a water-loving sugar part and a lipid-loving part), followed by separation of hemicellulose and cellulose. When each fraction was separated, their content was analysed to determine their chemical composition. These analyses showed that the xylan extracted from quinoa stalks is a complex polymer (glucuronoarabinoxylan), that however is suitable for XOs production (Papers 1 and 2).

XOs production was performed by enzymes (biological catalysts) that are capable to break the mentioned glucuronoarabinoxylan into shorter sugar chains (XOs). The enzymes that were used to produce XOs, are functional at high temperatures
up to 60 °C, which is very useful for industrial production. These enzymes were found in two bacteria: *Rhodothermus marinus* and *Bacillus halodurans*, and in one fungal strain, an enzyme sold under the name PENTOPAN®. Six variants of the enzymes were used to produce XOs from the glucuronoarabinoxylan purified from quinoa stalks. The enzymes successfully produce XOs, although the production was not as high as was expected. Additional strategies were then tried, in order to improve XOs yields (Paper 1 and 4). Once the XOs were produced, these were used to feed probiotic bacteria (being sources of carbohydrates). Two probiotic bacteria were tested, *Bifidobacterium adolescentis* and *Weissella* sp. strain 92. *Bifidobacterium adolescentis* is a well known probiotic bacteria, several times proven to be beneficial for human health, and *Weissella* sp. strain 92, is a bacterial strain with high potential to be considered as a probiotic. Both of them consumed XOs from quinoa stalks as their only carbon source, demonstrating the potential of the XOs as prebiotics (Paper 4).

A secondary objective was to discover novel enzymes acting on the glucuronoarabinoxylan isolated from the stalks. For this purpose, the bacterium *Clostridium boliviense* strain E-1 was selected, as there was a large number of genes in its genome that could encode enzymes with the capacity to produce XOs form xylan. Two enzymes were successfully cloned and produced in *Escherichia coli* (a bacterium that is used as a host to produce enzymes in an abundant manner). The two enzymes, include one candidate that shows similarity to enzymes that are classified together as a family, called glycoside hydrolase family 43 (GH43) and the other enzyme is a bifunctional esterase/xylanase. When both enzymes were tested using quinoa stalks glucuronoarabinoxylan, both were able to produce XOs. Furthermore, as the two enzymes are also active at 65 °C, their use in industry is promising (Paper 3).
Resumen de Divulgación Científica

La Quinua ha sido una fuente de alimento base en la dieta boliviana por su rico contenido en proteínas y alto valor nutritivo. El interés global por el grano de quinua se ha iniciado 40 años atrás, interés que impulsó la expansión agrícola de este grano en Bolivia, convirtiéndolo en uno de los mayores productores a nivel mundial. De la misma manera que el crecimiento en cantidad anual de grano producido y de hectáreas de cultivo de quinua ha incrementado, así también lo hicieron sus residuos agrícolas. Estos residuos son principalmente tallos, no apetecidos por el ganado, no tienen ningún otro uso para los agricultores y terminan acumulándose en las periferias de las áreas de cultivo. Por este motivo, los tallos de quinua han despertado el interés del presente estudio por su potencial transformación en productos de alto valor añadido, tales como saponinas, celulosa, hemcelulosa y sus derivados, incluyendo prebióticos. Los prebióticos son sustancias que favorecen el crecimiento de la flora intestinal bacteriana beneficuosa para la salud. La hemcelulosa extraída de los tallos de quinua es transformada en prebióticos tipo xilooligosacáridos (XOs) (pequeños polímeros de azúcares tipo xilosa). Los procesos de transformación se basan en tecnología enzimática, que, entre otros beneficios, es amigable con el medio ambiente.

La presente tesis se basó en dos partes principales. La primera parte consistió en extraer las diferentes fracciones de los tallos de quinua. Primero, se realizó una extracción con agua caliente a alta presión (PHWE en inglés) para la recuperación de saponinas, compuestos con propiedades emulsificantes, detergentes y de actividad biológica. Posteriormente, se extrajeron hemcelulosa y celulosa. La pureza y composición de las fracciones de hemcelulosa y celulosa fueron analizados por distintos métodos, en el caso de las fracciones de hemcelulosa y celulosa fueron analizadas por distintos métodos que determinaron, en el caso de la hemcelulosa, la presencia de xilosa, ácido glucurónico y arabinosa (monosacáridos), deduciendo así que el principal componente es un glucuronoxarabinoxilano, atractivo para la producción de XOs con potencial prebiótico (Publicaciones 1 y 2).

La producción de XOs fue realizada a través de enzimas (catalizadores biológicos) capaces de romper las cadenas largas de azúcares constituyentes del glucuronoxarabinoxilano, para tal efecto se utilizaron enzimas termotolerantes (capaces de ser activas y resistentes a 65 °C) de bacterias tales como
Rhodothermus marinus y Bacillus halodurans y de hongo PENTOPAN®. Las seis enzimas probadas fueron capaces de producir exitosamente los XOs, sin embargo, la cantidad producida no fue tan alta como fue esperada, teniendo entonces que buscar fuentes alternativas de mejoramiento de los rendimientos de producción de XOs (Publicación 1). Estos XOs entonces fueron utilizados como única fuente de energía (carbohidratos) para el crecimiento in vitro de bacterias probióticas (Bifidobacteria sp. y Weissella sp.) observando que los XOs producidos a partir de tallos de quinua resultaron beneficiosos para su crecimiento (Publicación 4).

La segunda parte de la tesis consistió en buscar nuevas enzimas con capacidad de producir XOs, para ello se aisló una bacteria anaerobia con capacidad de crecer en xilanos: Clostridium boliviense cepa E-1. En el genoma de esta bacteria, se detectaron un total de 8 genes putativos codificantes de enzimas con capacidad productora de XOs, genes que fueron clonados en Escherichia coli para la producción de las enzimas con tecnología recombinante. Así, dos enzimas producidas demostraron poseer actividad sobre el glucuronoaarabinoxilano extraído de los tallos de quinua, y por tanto con un potencial importante para la producción de XOs (Publicación 3).
Populärvetenskaplig sammanfattning

Quinoa-frön har länge varit en basföda i Bolivia, främst på grund av deras innehåll av proteiner och allmänt höga näringsvärde. Det globala intresset för quinoa började för 40 år sedan, ett intresse som främjat jordbruksutvecklingen i Bolivia och gjort Bolivia till en av världens största producenter av quinoa. Ökad odlingsareal och ökad produktivitet har också medfört att mängden avfall från quinoa-odlingen tredubblats. Detta avfall (huvudsakligen stjällkar) används inte ens som foder till boskap, utan deponeras bredvid de odlade fälten. Detta initierade ett intresse att utnyttja quinoa-stjällkar som kolhydratkälla. Kolhydraterna i quinoa-stjällkar är främst cellulosa och hemicellulosa, och förekommer tillsammans med lignin och saponiner. Dessa substanser kan separeras med hjälp av olika tekniker och var och en av dem kan användas för olika ändamål, exempelvis som biobränsle (utgående från cellulosa) eller prebiotika (från hemicellulosan). Xylooligosackarider (XO) från hemicellulosan är små sockerkedjor som människor inte direkt kan smälta, men de kan användas av nyttiga bakterier (kallas probiotika) i våra tarmar.

Denna avhandling består av två huvuddelar. Den första delen handlar om extraktion av saponiner (surfaktanter sammansatta av en kolhydratdel och en fettdel), hemicellulosa och cellulosa-fraktioner från quinoa-stjällkar. Saponiner extraherades genom hettvattenextraktion, följt av extraktion av hemicellulosa och slutligen renades den kvarvarande cellulosen upp. Varje fraktion isolerades och deras kemiska sammansättning bestämdes. Dessa analyser visade att hemicellulosa-fraktionen från quinoa-stjällkar är en komplex polymer (glukuronarabinoxylan), som är lämplig för XO-produktion (Artikel 1 och 2).

XO-produktion utfördes med hjälp av enzymer (biologiska katalysatorer) som kan sönder dela nämda glukuronarabinoxylan till kortare sockerkedjor (xylooligosackarider, XO). De enzymer som användes kan fungera vid temperaturer upp till 60 °C (de är alltså termotabila) och hade isolerats från två bakterier: Rhodothermus marinus och Bacillus halodurans. Dessutom användes ett kommersiellt enzym (PENTOPAN®). Sex enzymer utvärderades för att producera XO från glukuronarabinoxylan uppnaden från quinoa-stjällkar. Enzymerna producerade XO, men produktiviteten var inte så hög som förväntat. Olika strategier testades då för att effektivisera XO-produktionen (Papper 1 och 4). Producerade XO användes som näring för probiotiska bakterier. Två probiotiska
bakterier testades, Bifidobacterium adolescentis och Weissella sp. stam 92 och båda förbrukade XO (olika mängder). Detta visar potentialen hos de producerade XO att gynna tillväxten av probiotiska bakterier (Artikel 4).

List of Papers

This doctoral thesis is based on four papers (two published and two in manuscripts format) included in the end of the thesis summary. They are referred to as follows:


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1 First Authorship shared.
My contribution to the papers

**Paper 1:** I planned and performed all the experiments including extraction and xylan characterization. I wrote the draft manuscript, and took active part in the revision. Rodrigo Villagomez made the characterization of xylan through NMR and wrote that section of the paper.

**Paper 2:** Javier Linares-Pastén and I planned and designed the paper outline. I made all experimentations and HPAEC-PAD characterization of hemicellulose and cellulose fractions. I wrote the sections of the manuscript in which I was involved in the experimentation and took part in the revision. Alicia Gil-Ramírez performed math model design, FTIR analysis and experimentation for saponin extraction. Carl Grey did the MS characterization of saponins.

**Paper 3:** I performed and planned all the experimentations and wrote the draft manuscript. Elisabet Eik Gudmundsdottir made the DNA genome sequencing of *Clostridium boliviense* strain E-I. Javier Linares-Pastén made the computational 3D modeling design and the annotation of the genes codifying enzymes from *Clostridium boliviense* strain E-I genomic DNA.

**Paper 4:** I planned, performed and wrote the manuscript. I made all the experimentations regarding optimization of XOs production and cultivation of *Weissella* sp. strain 92. I also performed analysis of the XOs consumption and metabolic products of *Weissella* sp. strain 92 and *Bifidobacterium adolescentis* through HPAEC-PAD and HPLC. Abhishek Bhattacharya made the microbial cultivation of *Bifidobacterium adolescentis*.
1. Introduction

Quinoa (*Chenopodium quinoa* Willd.) is the second biggest crop produced in Bolivia. The global interest for this plant, in specifically for its seeds, started in early 80s’ when its nutritional value and traditional usage was expanded to the world (Carimentrand & Ballet, 2010). The quinoa plant accepts a wide range of growth conditions in terms of altitude, temperature, pH, soil salinity and droughtness (González et al., 2009). All these characteristics makes quinoa a complex plant in terms of structure and composition; in the same way, these characteristics provides quinoa all the properties it posseses. Moreover, these mentioned characteristics have not only offered alimentary and nutritional interest but have also opened an opportunity of exploitation of byproducts from a biorefinery point of view.

Biorefining of quinoa agricultural waste, particularly stalks, emerged as an interesting raw material to be investigated. Quinoa stalks have not had other sub usages in agriculture, and then this byproduct is just piled next to the fields. The option of utilizing quinoa stalks, to obtain new utilities from this particular material and all its derivatives components, has developed the interest in this investigation. Further application in different areas, will strengthen the status of quinoa, as a valuable plant for alimentary and also biorefinery approaches. One potential use of quinoa stalks extracted from this material is to obtain further valorization by enzymatic conversion of the polymer into xylooligosaccharides (*Paper 1*).

Xylooligosaccharides (XOs) are sugar oligomers that in food related fields are used as prebiotics (Aachary & Prapulla, 2011). XOs are derived from the hemicellulose (i.e. xylan) fraction of various raw materials, making the step of xylan extraction a key point for its further use. Moreover, development of an integrated extraction process that include other fractions from the quinoa stalks (e.g. cellulose) is also of high interest. Enzymatic production of XOs is done mainly by glycoside hydrolases (GH) of family 10 and 11, hemicellulose-acting enzymes that mainly include endo-xylanase members (Motta et al., 2013). XOs production can also be promoted by enzymes with complementary activities (e.g. glucuronidases and arabinofuranosidases) (Saha, 2003). When complex xylan structures are extracted, such as glucuronoxarabinoxylans, these enzymatic activities are required and used to enhance the production yields of linear XOs.
Enzymes that are members of GH family 30, 43 and bifunctional enzymes, such as the esterase/xylanase investigated here (Paper 3) are of interest and, search for these types of GHs from novel and never studied microbes increase the opportunities to diversify the XOs (including linear non-substituted as well as substituted oligosaccharides) and improve the XOs yield.

XOs have already been produced and characterized, from other sources (Broekaert et al., 2011), and have been proven to have prebiotic effect (i.e. have stimulated growth of probiotic bacteria and secretion of Short Chain Fatty Acids (SCFA) beneficial for health). However, enzymatically produced-XOs with substituents in their structure and their consumption by probiotics are less commonly reported (Nordberg Karlsson et al., 2018). Investigations of substituted structures, and their interaction with the microorganisms provide new hints on how substituents could represent a new factor for selection of probiotic bacteria that may lead to further host health improvement.

The present work is the first attempt to investigate the quinoa stalks raw material as a potential source of mainly xylan, without discarding other lignocellulosic components. Moreover, enzymatic production of XOs from the extracted xylan, and their consumption by probiotic bacteria, proves its use as prebiotic. In addition, the characterization, activity measurement and product profile of two novel enzymes, a GH43-like xylanase and a bifunctional esterase/xylanase, can be potential candidates with complementing activities for use to produce XOs from quinoa stalks xylan.

1.1. Scope of the thesis

The thesis has been divided into four articles, two published (with one additional data publication), and two in the form of manuscripts.

Paper 1 describes the extraction and characterization of the quinoa stalks glucuronoarabinoxylan and a first approach to XOs production by thermostable enzymes from families GH10 and GH11. Paper 2 describes the design of an integrated extraction method for saponins, hemicellulose and cellulose fractions from quinoa stalks, and includes the publication of Data in Brief. Paper 3 is focused on the identification, cloning, production, characterization and product profile determination of novel xylanases. One novel GH43-like enzyme and one bifunctional esterase/xylanase enzymes from Clostridium boliviense strain E-1. Finally, Paper 4 studies the production of XOs from quinoa stalks through different techniques and includes their consumption by potential probiotic strains from genus Weissella sp. strain 92 and Bifidobacterium adolescentis.
2. Quinoa (*Chenopodium quinoa* Willd.)

2.1. Origin and usage

Quinoa (*Chenopodium quinoa* Willd.) is a plant member of Amaranthaceae family (Adolf *et al.*, 2013; Ruiz *et al.*, 2014), Chenopodiaceae subfamily (Iglesias-Puig *et al.*, 2015), which origins and uses are described since 1560 (Tapia, 2015) (Figure 1). First classified by Willdenow in 1797, quinoa crops extended alongside the west coast of South America, from Colombia to Argentina (Jellen *et al.*, 2015). Comsume by Aymaras and Quechus (native american cultures) quinoa grains are believed to be cultivated and domesticated since 5000 – 7000 years ago (Vega-Gálvez *et al.*, 2010; Small, 2013). Based to their agroecological distribution, quinoa variants can be classified as: (1) Inter-Andean valleys quinas (Colombia - Ecuador - Peru); (2) Altiplano quinas (Peru - Bolivia); (3) Salare quinas (Bolivia - Chile - Argentina); (4) Coastal quinas (Chile) and, (5) Yunga quinas (Bolivia) (Bazile and Baudron, 2015). Each agroecological type possesses their own climatic characteristics and specific quinoa phenotypes (Rojas *et al.*, 2015).

Quinoa is not a cereal, although it shares most of the characteristics of real cereals. It is an annual crop, of variable size reported from 0.5 to 3 m, with roots that penetrate 1.5 m into the soil, stalks that could be branched or unbranched and variable amounts of leaves according to the ecotype (Small, 2013). Quinoa is considered as an “exceptional” crop and nutrional food for its content of protein, aminoacids, minerals and gluten-free grain properties (Bazile *et al.*, 2015). Furthermore, United Nations considered quinoa as an important alternative to contribute to global food security and, established 2013 as the International Year of quinoa (Chavarria-Lazo *et al.*, 2015; Gil-Ramírez *et al.*, 2018). In the last 20 years, the production of quinoa has expanded from just the Andean territory in South America to the other four continents. South American countries (mainly Peru and Bolivia) are, however, still the major producers of quinoa around the world. Countries such as United States, Canada, Australia, France, Morocco and
India also became in intermediate producers by 2013 (Iglesias-Puig et al., 2015; Bazile and Baudron, 2015).

Due to the possession of different genotypes and phenotypes, quinoa plants are able to growth at different climate conditions, not only related with the temperature, but also altitude and droughtness (Bosque Sanchez et al., 2003). This variability and, robustness in cultivation, made quinoa and its grain a food of interest specially because the feasibility to grow the plant without a specific ecotype (Jacobsen, 2003).

2.2. Quinoa crops in Bolivia

Bolivian geography is divided into three major zones taking into account the vegetation and fauna: Highlands (in spanish Altiplano), valleys and lowlands/tropics (Corporación OSSO, 2007). Quinoa crops, in Bolivia, are typical for highlands (Kerssen, 2015), in areas from 2500 to 4500 meters above the sea level (López et al., 2011), temperatures that varies from – 11 °C at night to 20 °C at day light, variable droughtness according to the season, and moderate to high soil salinity (Jacobsen, 2011; Apaza et al., 2013).

Quinoa grain is one of the most important sources of food for the Bolivian population, particularly in the Andean region (Jacobsen, 2011). Its production for the international market started in 1983 and since then, Bolivia has maintained the position as the major producer of this product (Carimentrand et al., 2015). Quinoa crops has been increasing, mainly due to the global interest for the grain. In terms
of production it has increased from 5 000 up to 60 000 tons, and the production area has increased from 10 000 to 120 000 ha, in the time frame from the 1980’s until 2013 (Jacobsen, 2011; Gandarillas et al., 2015a).

2.3. Biorefinery of quinoa

2.3.1. Quinoa “bran” and leaves

The increasing quinoa grain production brought together some side effects not only related with the production of the grain itself, but also with an excessive generation of byproducts from the quinoa plant, such as seed coats “bran” (mojuelo), leaves and stalks (Carrasco et al., 2015; Salas-Veizaga et al., 2017).

Seed coats of quinoa are rich in saponin content, in fact, some grain varieties and ecotypes are designated based on the content of saponins as “bitter” grains (1 – 3 % dry weight), “semi-sweet” grains (0.1 – 1 % dry weight) and, “sweet” grains (0 – 0.1 % dry weight) (López et al., 2011; Quiroga et al., 2015). Many methods have been developed to remove this component from the seeds to aid further utilization (Troisi et al., 2015). Saponins from quinoa have been particularly investigated and have been proven as fungal growth inhibitor, antiviral compounds, and biopesticides (Lozano et al., 2012).

In terms of amount of “mojuelo” (seed coats), Lozano et al. (2012) estimated a total average of 891 tons of seed coats generated per year from the total sieved grains, which represents approximately the 5 % of the total raw material. Over twenty saponins have been identified for quinoa seeds and seed coats (“bran”) (Quiroga et al., 2015), all of these saponins are triterpene glycosides, having potential in brewing, detergents and cosmetics (Paper 2) (Gil-Ramírez et al., 2018), interestingly quinoa saponins has no oral toxicity in humans (Zhu et al., 2002).

Leaves, which are byproducts after harvesting, represent 55 % of the residues. Their potential use as forage as animal feed has been suggested and also evaluated in Andean cattleing (Blanco, 2015). From a biorefinery point of view, leaves of quinoa have not been yet investigated. However, a potential usage of leaves from quinoa as feed and also in other areas are limited, especially due to quinoa -plant pests, such as fungal growth, nematodes and moths (Gandarillas et al., 2015b; Gómez-Pando et al., 2015). Although all these diseases invade the whole plant causing economical and productivity losses, leaves are the most affected part (Gandarillas et al., 2015b). In addition, this problem represents not only extra
steps in a possible application of this byproduct, but also in terms of productivity of the derived product that would be wanted to be produced.

2.3.2. QUINOA STALKS

Quinoa stalks residues represent the 45 % of the generated byproducts produced after harvest (Blanco, 2015). Quinoa stalks are porous and cylindrical, and because of the presence of saponins (Paper 1 and 2) stalks are not suited as cattle feed (Carrasco et al., 2015). After harvesting, farmers leave the stalks next to the fields (Figure 2). Contrary to seed coats, stalks have no other use or sub-use. However, the lignocellulosic compounds in the stalks have potential in biorefining, and separation of key lignocellulosic components have been studied in this thesis.

Quispe (2013) and Carrasco et al. (2015) also established a first approximation of quinoa stalks usage, in those studies, chemically hydrolyzed quinoa stalks were tested in order to extract monosaccharides that worked as a platform for bioethanol production. These data, together with this study, are the first characterization of the stalks that has been made (Table 1).

![Figure 2. Quinoa (Chenopodium quinoa Willd.) cultivation field. Challapata, Oruro – Bolivia. Quinoa stalks are left next to the cultivation area without any other sub usage (© 2014 Salas-Veizaga).](image-url)

The presence of hemicellulose (here xylans) and cellulose in the stalks has been verified, and the formed foam also suggested the presence of saponins (Paper 1). Saponins are interesting products to be investigated and can be used for different
purposes. Both saponins and cellulose are possible platforms for different types of applications (Paper 2). The hemicellulose (never extracted and studied from quinoa stalks) is a putative xylan source that can be further developed into xylooligosaccarides (XOs) (Paper 1 and 3). XOs are prebiotics oligosaccharides (stimulating probiotic bacteria) with application in food and feed sector (Paper 4).

Table 1. Quinoa stalks composition [g per 100 g Dry Matter] established in previous studies.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemicellulose</td>
<td>20.31 ± 0.00</td>
<td>25.7 ± 0.65</td>
<td>23.01</td>
</tr>
<tr>
<td>Xylan</td>
<td>ND</td>
<td>16.65 ± 0.25</td>
<td>NC</td>
</tr>
<tr>
<td>Arabinan</td>
<td>ND</td>
<td>3.35 ± 0.15</td>
<td>NC</td>
</tr>
<tr>
<td>Galactan</td>
<td>ND</td>
<td>3.40 ± 0.25</td>
<td>NC</td>
</tr>
<tr>
<td>Mannan</td>
<td>ND</td>
<td>2.30 ± 0.00</td>
<td>NC</td>
</tr>
<tr>
<td>Cellulose</td>
<td>42.19 ± 1.15</td>
<td>36.80 ± 0.30</td>
<td>39.50</td>
</tr>
<tr>
<td>Lignin</td>
<td>12.94 ± 0.12</td>
<td>22.45 ± 1.25</td>
<td>17.70</td>
</tr>
<tr>
<td>Klason</td>
<td>ND</td>
<td>19.45 ± 1.10</td>
<td>NC</td>
</tr>
<tr>
<td>Acid-Soluble</td>
<td>ND</td>
<td>3.00 ± 0.15</td>
<td>NC</td>
</tr>
<tr>
<td>Ash</td>
<td>1.60 ± 0.10</td>
<td>4.80 ± 0.35</td>
<td>3.20</td>
</tr>
</tbody>
</table>

ND: Non Determined.  
NC: Non Calculated.
3. Complex hemicellulose and lignocellulose from quinoa stalks

3.1. Hemicellulose

Hemicellulose is the second most abundant polysaccharide in the world (Saha, 2003; Chakdar et al., 2016). Even though it is characterized as heteropolymers, many hemicelluloses are formed by mainly pentoses such as xylose and arabinose (Castanares et al., 1995; Hu & Ragauskas, 2012). Moreover, and in constrast to cellulose, hemicelluloses can possess substituents composed of oligosaccharides from a single monosaccharide type, single monosaccharide substitutions or acid sugars in form of uronic acids (Paper 1). Hemicelluloses are grouped into D-xylans and D-xyloglucans, D-mannans and D-(galactomannans, β-D-glucans and L-arabino-D-galactans (Ebringerová et al., 2005; Mamman et al., 2008; Sedlmeyer, 2011). Xylose-based hemicelluloses (xylans) have a backbone of β-(1-4) linked D-xylopyranosyl units and; according to its substituent abundance and chemical structure, xylans are divided in three major groups: glucuronoxylans (e.g. Birchwood xylan), arabinoxylans (e.g. Wheat xylan) and glucuronoarabinoxylans (e.g. Corn or quinoa stalks xylan) (Rogowski et al., 2015; Chakdar et al., 2016) (Paper 1 and 3).

Based on the structure and how and which substituents are disposed alongside the xylopyranosyl backbone, the grade of complexity of different hemicellulose increases, especially if enzymatically hydrolysis is of interest (Vardakou et al., 2004; Tenkanen et al., 2012).

In line with this point of view, homoxylans (polymer based on only xylanopyranosyl β-(1-4) linked units), tend to be more effectively hydrolyzed by major active-xylanases enzyme groups. On the contrary, homoxylans are rarely present in terrestrial plants (Ebringerová, 2005).

Heteroxylans, on the other hand, which are the most common xylan forms found in terrestrial plants, varies in complexity depending on the amount and position of the decoration(s) alongside the xylose backbone (Ebringerová, 2005; Girio et al., 2010). Arabinoxylans are xylans commonly found in cereals (Sarvesh et al., 2018). Characterized arabinoxylans, are found to include single, double or both
types of arabinofuranoside substituents, α-(1,3), α-(1,2) linked to the xylopyranosyl residues in the backbone. The degree of substitution will also determine the xylose/arabinose ratio (Ebringerová, 2005).

Glucuronoxylans, more common in hardwood plants, are characterized for having 4-(methyl)-O-glucuronic acid substituents α-(1,2) linked to the xylose backbone, although literature mentioned other neutral monosaccharides in minor amount as substituents (Gírio et al., 2010; Hilpmann et al., 2016).

Complex hemicellulose-xylans are common in softwoods and agricultural wastes, characterized for having varying amounts of substituents, differing in type and molar ratio (Ebringerová, 2005; Van Dyk & Pletschke, 2012). Examples of the most common substituents include arabinofuranose monomers α-(1,3), α-(1,2) linked to the backbone (although α-(1,5) linked arabinofuranoside oligomers has been also reported), and, 4-(methyl)-O-glucuronic acid α-(1,2) linked to the xylopyranosyl β-(1,4) backbone (Labavitch & Ray, 1978; Darvill et al., 1980; Luonteri et al., 1995). Units of ferulic acid, galacturonic acid, and neutral monosaccharides such as L-galactopyranosyl α-(1,2) linked to the xylose backbone are also constituents of these xylans (Linares Pastén et al., 2014).

Quinoa stalks hemicellulose is characterized for being a complex glucuronoarabinoxylan, due to its structure that possesses high amounts of 4-O-methylglucuronic acid, higher than any other substituent, such as arabinose or galactose (Paper 1). These substituents and the xylose main backbone have been identified and characterized by High-Performance Anion Exchange Chromatography with Pulse Amperimetric Detection (HPAEC-PAD), Nuclear Magnetic Resonance (NMR), Fourier Transformation Infra-Red Spectroscopy (FT-IR) and Size Exclusion Chromatography (SEC). The estimated mass was $1758 \pm 31$ kDa$^2$ and the polymer was composed mainly of xylose, glucuronic acid, arabinose and galactose in molar ratio of 114:23:5:1. In comparison with other hemicelluloses, quinoa stalks glucuronoroarabinoxylan possesses a high amount of uronic acids, in higher amounts than the substituents such as arabinose and galactose. Literature determined ranges of uronic acids:xylose 1:7.5-10.5 for agricultural waste-based hemicelluloses. The corresponding ratio for the xylan isolated from quinoa stalks was 1:5 (uronic acids:xylose) (Paper 1) (Timmel & Syracuse, 1967; Gírio et al., 2010). The major substituent in xylans from agricultural waste is often arabinose, which is also present in the xylan from quinoa stalks, although in lower amounts. In that sense, based on the high amount of glucuronic acids, quinoa stalks glucuronoroarabinoxylan posses a monosaccharide composition more similar to softwoods and hardwoods than to cereals or other

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$^2$ The value of Molar Mass has been calculated as extrapolation of pullulan standards, which maximum value was 642 kDa. However, the value here presented is also based on six experimental sample repetitions.
agricultural residues (Gírio et al., 2010). The major advantage of a structure with high uronic acid content is the increasing polarity of the hemicellulose structure of quinoa stalks, this characteristic improved its water solubility and make this substrate more accesible to further enzymatic treatments to different applications. For example, to ensure the precision/repeatability of the analytical assays, the water solubility of this polymer for allowed solubilisation to approximately 2 g/L, which for other commercial hemicelluloses (particularly xylans) has been difficult to accomplish.

The structural analysis showed that the backbone of the glucuronoarabinoxylan is formed of β-xylopyranosyl units bond each other by (1-4) glycosidic bond (Heteronuclear Multiple Bond Correlation (HMBC) spectrum) (Paper 1). On the other hand, arabinofuranose units were determined to be bond through α-(1-5) linkage each other, suggesting their presence as substituents alongside the xylopyranosyl backbone in oligomeric and also monomeric form. The specific linkage(s) of the arabinofuranosyl residues to a particular xylopyranosyl unit, could not be determined by NMR due to the high molecular weight of the polymer, but are suggested to be present in both α-(1-2) and α-(1-3) forms (Figure 3) (Paper 1).

![Diagram](attachment:image.png)

**Figure 3. Schematic design of quinoa stalks glucuronoarabinoxylan.**

Xylopyranosyl units are β-(1-4) linked internaly (black boxes), 4-O-glucuronic acid is methylated in position C-4 (red hexagones) and, arabinofuranoses are link each other through α-(1-5) bonds (green pentagons) (Paper 1).
Determination of the identity and quantity of monosaccharides was estimated and determined using HPAEC-PAD (Paper 1, 2 and 4). However, the hydrolysis methods to obtain the total monosaccharide from the polymeric material had variations dependent on the type to be quantified. To obtain both neutral sugars and uronic acids, Saeman hydrolysis was used, and followed by, the detection and quantification by TriFluoroacetic Acid (TFA) or hydrochloric acid (De Ruiter et al., 1992) (Figure 4).

![Figure 4. Effect of the sulphuric acid treatment over uronic acids quantification.](image)

TFA and HCl (2 M) were used to quantify Uronic Acids instead sulphuric acid (Saeman hydrolysis), increasing uronic acid quantification.

The major difference between the two methodologies referred to above, is the strength of the acid that is used. Sulphuric acid can be harmful for the xylan itself and also for the acid sugars that could be the substituents present on it (Modenbach & Nokes, 2012). Nevertheless, the amount of quantifiable, detected monosaccharides content is higher than using other chemicals (Figure 4). In contrast, TFA or HCl acids hydrolysis resulted in a milder option of hydrolysis, maintaining acid sugars into the quantifiable monosaccharides, but reducing the total amount of neutral monosaccharides that could be detected in the xylan (Data not published).
3.2. Lignocellulosic material from quinoa stalks

The hemicellulose described above, is a main component of the lignocellulosic material in quinoa stalks. Lignocellulosic material is basically divided in three major components: Cellulose, hemicellulose and lignin (Rojas et al., 2005; Mamman et al., 2008; Nigam et al., 2009). Each of these fractions in the lignocellulose is characterized for their own qualities in terms of composition and nature. These fractions can be separated from one another through different methods (Paper 1). From these methods, chemical-extracted pretreatments are the most common and also most used methods to separate each fraction effectively (Paper 1 and 2). However, the choice of the chemical compound, the concentration and the exposure time of the selected chemical method will determine the efficiency and success of the extraction process.

Moreover, these methods tend to extract one fraction only, and eliminate or reduce drastically the recovery yield of the others (Gil-Ramírez et al., 2018) (Paper 2) (i.e. alkaline pretreatment extractions successfully separate the cellulose and hemicellulose fractions from the remaining lignin fraction, but then lignin is dissolved and lost) (Brodeur et al., 2011). Other methods, such as autohydrolysis or enzymatic methods represent “environmentally friendly” options for extraction and recovery of the different lignocellulosic components (Garrote et al., 1999). Nevertheless, they show other drawbacks, such as reduced yields, as well as other effects, related to the nature of the lignocellulosic source itself (Paper 1).

3.2.1. Quinoa stalks lignocellulosic sequential extraction material

The process of extracting quinoa stalks glucuronoarabinoxylan was demonstrated to recover approximately the 66 % of the total hemicellulose in the plant material, when hemicellulose was the only focused fraction to be extracted (Paper 1). Nevertheless, during the extraction process, the presence of foam was noticed when the stalks were washed. It was also noticed that when the hemicellulose extraction finished, the remaining material did not look damaged, suggesting the presence of both, saponins and an intact remaining cellulose fraction, respectively.

In order to take advantage of saponin, hemicellulose and cellulose fractions, a sequential methodology was designed and proposed to extract as much of each fraction as possible, without affecting the others (Figure 5). Pressurised Hot Water Extraction (PHWE) was used as the first methodology, followed by alkaline glucuronoarabinoxylan extraction and acid purification of cellulose (Paper 2). Different time and temperatures of PHWE resulted in significant variations in the sequential extraction and in the recovery yields that were obtained for the respective fraction. It has been established that higher temperatures and short time
conditions of PHWE treatment is necessary for extracting the major amount of saponins, however these conditions affected glucuronoarabinoxylan extraction drastically (Table 2) (Paper 2). On the other hand, it was demonstrated that hemicellulose and cellulose extractions and recovery yields are less affected by the downstream methods that were used specifically for their separation (alkaline and acid purification, respectively). Furthermore, PHWE conditions of 110 °C and 70 minutes improved the glucuronoarabinoxylan recovery yield from the 66 % reported in Paper 1 to 74 % reported in Paper 2, without loosing the structural properties of the hemicellulose and cellulose polymers.

Figure 5. Sequential extraction of fractions of interest from quinoa stalks. Saponins extraction by PHWE, xylan alkaline extraction and acid cellulose purifications are described. Figure published in Gil-Ramirez et al. © Elsevier (2018).

3.2.2. Saponins

Saponins are glycoside metabolites containing a steroidal or triterpenal aglycone group linked to monomeric or oligomeric monosaccharide chain (Güclü-Üstündag & Mazza, 2007). Saponins have a dual polar non-polar structural component that make them water-soluble (Vincken et al., 2007), but are forming colloidal structures, which in the presence of agitation generates foam (Sparg et al., 2004). Saponins appear in nature as a natural plant defense to possible plant pathogens including bacteria, fungi or insects (Cheok et al., 2014).
In the previous chapter, saponins have been described as a component of quinoa “bran” (seed coats), and its usage from a biorefinery point of view has started to be exploited decades ago in the production areas in South America, and specifically in Bolivia (Lozano et al., 2012; Troisi et al., 2015). The worldwide expansion of the grain and the access to it, has also contributed to the extraction and identification of saponins in quinoa seeds (Woldemichael & Wink, 2001; Zhu et al., 2002; Verza et al., 2012).

Table 2. Hemicellulose (xylan) and Cellulose fraction yield and compositions extracted after different PHWE temperature and times. Table published in Gil-Ramirez et al. © Elsevier (2018).

Extraction Conditions made reference to the temperature and time of PHWE used for saponin first-step extraction. Monosaccharides composition, xylose (green), arabinose (blue), galactose (red) and, glucose (orange).

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Extraction Conditions</th>
<th>Hemicellulose Fraction</th>
<th>Cellulose Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Temperature [°C]</td>
<td>Time [min]</td>
<td>Yield [g xylan/100 g Raw Material (Recovery [%])</td>
</tr>
<tr>
<td>1</td>
<td>170</td>
<td>60</td>
<td>1.9 (11.71)</td>
</tr>
<tr>
<td>2</td>
<td>110</td>
<td>1</td>
<td>8.9 (54.84)</td>
</tr>
<tr>
<td>3</td>
<td>195</td>
<td>35</td>
<td>2.9 (17.87)</td>
</tr>
<tr>
<td>4</td>
<td>110</td>
<td>70</td>
<td>11.1 (68.39)</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
<td>60</td>
<td>9.0 (55.45)</td>
</tr>
<tr>
<td>6</td>
<td>110</td>
<td>35</td>
<td>11.0 (67.78)</td>
</tr>
<tr>
<td>7</td>
<td>50</td>
<td>11</td>
<td>12.0 (73.94)</td>
</tr>
<tr>
<td>8</td>
<td>25</td>
<td>35</td>
<td>8.4 (51.76)</td>
</tr>
<tr>
<td>9</td>
<td>110</td>
<td>35</td>
<td>8.8 (54.22)</td>
</tr>
<tr>
<td>10</td>
<td>170</td>
<td>11</td>
<td>6.5 (40.05)</td>
</tr>
</tbody>
</table>

Quinoa stalks has, to our knowledge, never been studied as probable and potential source of saponins. The foaming presented in the raw material, when hot water washing method was done, however, suggested the presence of saponins in the stalks (Paper 1). In the present study, conventional and previously used successful saponin extraction methods for quinoa “mojuelo” (seed coats) were tested. Nonetheless when such methodology was tested in stalks, saponins could not be detected (Paper 2). When Pressurised Hot Water Extraction (PHWE) was used as an alternative for saponin extraction, MS analysis of the extracted product and a diosgenin equivalents method for quantification of saponins showed the presence of saponins in quinoa stalks, reaching values of 15.82 ± 1.55 mg of saponins per gram of raw material (representing 16 g of saponins per kilogram of agricultural
waste). This methodology has not only, for the first time, been developed for saponin extraction in quinoa stalks, but was also used as starting point for a successful sequential extraction of saponins, hemicellulose and cellulose fractions from this material (Paper 2).

3.2.3. Cellulose

Celluloses, also known as glucans, are homopolymers composed exclusively of β-(1-4) linked β-D-anhydroglucopyranose chains (Kumar et al., 2009; Brodeur et al., 2011). These homopolymers are the most abundant carbohydrate reservoir in nature (Rojas et al., 2005; Jørgensen et al., 2007), present even in bacteria, fungi and algae. The cellulose degree of polymerization varies from 10 000 to 15 000 in hardwoods and softwoods, respectively (Agbor et al., 2011). Cellulose has poor solubility in water and in inorganic solvents, and is considered a recalcitrant component (Mood et al., 2013; Linares-Pastén et al., 2014). In contrast, the possibilities and usages that cellulose has are very well known (Gil-Ramírez et al., 2018) (Paper 2). In plants, cellulose can be found in crystalline and amorphous forms. Crystalline forms of cellulose are the most abundant form in nature, although amorphous cellulose is easier to degrade by enzymes (Béguin & Aubert, 1994).

Quinoa stalks has been studied by Quispe (2013) and Carrasco et al. (2015) as source of monomeric sugars to produce bioethanol, and mainly focused on the cellulose fraction. Hence, the interest in this lignocellulosic fraction as a whole set of polymers has never been studied until the current work. Based on the obtained results, it was evident that cellulose from quinoa stalks was not only proven to be very resistant to previous extractions and pretreatment processes but could also be extracted in a very pure form, and in fact resulted in the purest lignocellulosic fraction extracted (Table 2) (Paper 2).

The monosaccharide content determination of the cellulose fraction, made by HPAEC-PAD, showed glucose to be the only component, and after acid hydrolysis most of the polymer was totally hydrolyzed and quantified (even to 100 %), which represent a good opportunity for further biorefinery applications. Moreover, in contrast to other processes or/and purification attempts, the cellulose obtained was, (which was likewise observed for glucuronoarabinoxylan fraction), a “cotton like” product (Figure 6 - Right).
3.2.4. Alkaline-based glucuronoxarabinoxylan extraction method

Methodologies previously proposed and tested to extract the hemicellulose (xylan) fraction from the lignocellulosic materials include chemical, physical and enzymatic methods (Vázquez et al., 2000; Gáspár et al., 2007; Chapla et al., 2012).

The alkaline method is the most used and effective for extraction of xylan from different materials (Table 3), mainly in hardwoods, herbaceous crops and agricultural residues (Chen et al., 2013). This method, in short, mixes the raw material with different concentrations of alkali at different temperatures and times of exposure (Hespell, 1998; Modenbach, 2013). The alkali component reacts and cleaves the ester and ether linkage between lignin and hemicellulose (Galbe and Zacchi, 2007; Reyes et al., 2013; Kim et al., 2016). At the same time, due to the high pH (>12) of the reaction medium, the hemicellulose is solubilized, and hence separated from the cellulose fraction (Kim et al., 2016). After the alkaline treatment, the reaction medium is neutralized, and the solubility of the hemicellulose fraction reduced to finally, after the addition of ethanol, be
precipitated forming two (solid-liquid) phases (Kilpeläinen et al., 2012) (Figure 6 - Left).

In the specific case of quinoa stalks glucuronoaarabinoxylan, alkaline hydrolysis using sodium hydroxide (0.5 M = 2 % w/v) was suitable to extract the maximum amount of hemicellulose without interfering with or affecting the monosaccharide composition (Paper 1, 2 and 4). Utilization of lower or higher NaOH concentrations resulted in a marked variation in composition and recovery yields, turning those parameters inversely proportional each other. This means that a high NaOH concentration increased the recovery yield but reduced the proportion of arabinose and galactose in the total monosaccharide content (Figure 7). Furthermore, an increasing amount of glucose directly proportional to the increase of NaOH, suggest a starting degradation of cellulose (Modenbach, 2013) (Paper 4).

In the present study, consecutive extractive processes have been established, meaning repeated use of the remaining material as source in a second and third alkaline extraction, using the same extraction conditions as the first process (Paper 1). Recovery yields improved significantly when the consecutive extractions were used, as compared to a single extractive processes either static or with reflux (Table 4) (Paper 1 and 2). The alkaline extracting process did not affect the content or recovery of the remaining cellulose fraction, irrespective of the number of repetition of the alkaline extraction step. This was indeed also the case when PHWE had been used, prior to the hemicellulose extraction (Table 2) (Paper 2). Then, the alkaline extraction here suggested represents a good methodology to extract both glucuronoaarabinoxylan and the cellulose.

![Graphs](image)

**Figure 7. Effect of the NaOH concentration in quinoa stalks Glucuronoaarabinoxylan extraction.**

(Left) Extraction yield and; (Right) Monosaccharides composition of the extracted glucuronoaarabinoxylan at different NaOH concentrations.

Other suggested methodologies that shown high recovery yields for other agricultural residues, like autohydrolysis (Vázquez et al., 2005; Immerzeel et al., 2014) or enzymatic treatment (Rico et al., 2014) did not result in good yields for
quinoa stalks xylan, despite the fact that some precipitated solid material was detected by these methods (Paper 1).

Table 3. Different alkaline treatment used in different material to hemicellulose extraction. Adapted from Paper 1 and Paper 2.

<table>
<thead>
<tr>
<th>Raw material</th>
<th>Alkali (pre) treatment</th>
<th>Alkali concentration</th>
<th>Time of exposition</th>
<th>Temperature</th>
<th>Recovery yield (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quinoa Stalks</td>
<td>0.5 M (2 %v/v) NaOH</td>
<td>90 min</td>
<td>80 °C</td>
<td>66 %</td>
<td>Salas-Veizaga et al. (2017).</td>
<td></td>
</tr>
<tr>
<td>Tobacco stalk</td>
<td>24 % KOH</td>
<td>3 h</td>
<td>35 °C</td>
<td>ND</td>
<td>Akpinar et al. (2009).</td>
<td></td>
</tr>
<tr>
<td>Cotton stalk</td>
<td>24 % KOH</td>
<td>3 h</td>
<td>35 °C</td>
<td>ND</td>
<td>Akpinar et al. (2009).</td>
<td></td>
</tr>
<tr>
<td>Sunflower stalk</td>
<td>24 % KOH</td>
<td>3 h</td>
<td>35 °C</td>
<td>ND</td>
<td>Akpinar et al. (2009).</td>
<td></td>
</tr>
<tr>
<td>Wheat straw</td>
<td>2 % NaOH</td>
<td>1.5 h</td>
<td>80 °C</td>
<td>41 %</td>
<td>Faryar et al. (2015)</td>
<td></td>
</tr>
<tr>
<td>Corn fiber</td>
<td>2 % KOH – 2 % Ca(OH)₂</td>
<td>5 h</td>
<td>70 °C</td>
<td>40 %</td>
<td>Hespell (1998)</td>
<td></td>
</tr>
<tr>
<td>Corn stover</td>
<td>15 % NH₃</td>
<td>1.5 h</td>
<td>121 °C</td>
<td>73.6 %</td>
<td>Zhu et al. (2006)</td>
<td></td>
</tr>
<tr>
<td>Maize bran</td>
<td>0.8 M KOH</td>
<td>2 h</td>
<td>95 °C</td>
<td>74 %</td>
<td>Chanliaud et al. (1995)</td>
<td></td>
</tr>
<tr>
<td>Hardwood kraft pulp</td>
<td>1 M NaOH</td>
<td>2 h</td>
<td>Room Temperature 30, 60 and 75 °C</td>
<td>61 %</td>
<td>Hakala et al. (2013)</td>
<td></td>
</tr>
<tr>
<td>Barley hull</td>
<td>15 - 30 % Ammonia</td>
<td>12 h – 11 weeks</td>
<td>81.5 %</td>
<td>Kim et al. (2008)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forage sorghum</td>
<td>1 Kg Ammonia (AFEX)</td>
<td>5 min</td>
<td>90 °C</td>
<td>ND</td>
<td>Li et al. (2010b)</td>
<td></td>
</tr>
<tr>
<td>Rice straw</td>
<td>6 % NaOH</td>
<td>3 weeks</td>
<td>20 °C</td>
<td>13 %</td>
<td>Hu &amp; Ragauskas (2012)</td>
<td></td>
</tr>
</tbody>
</table>

ND: Non Determined.
* The referred value is the sum of alkaline and PHWE extracted hemicellulose fraction.

3.2.5. Purification of glucuronoarabinoxylan and recovery yields

One of the key points in the hemicellulose extraction for further characterization or modification for application (in the present study represented by production of xylooligosaccharides (XOs)), is to extract the purest possible glucuronoarabinoxylan fraction. Particularly, after the alkaline treatment method using NaOH, neutralization to pH 5.0 to 6.0 (by glacial acetic acid), results in a considerable amount of sodium acetate. This side product is kept into the freeze-dried glucuronoarabinoxylan product and was detected by H-NMR analysis.
Table 4. Comparison between reflux and static methods for glucuronoarabinoxylan extraction from quinoa stalks. Adapted from Paper 1 and 2.

<table>
<thead>
<tr>
<th>Vessel</th>
<th>Reflux method(^a) g/100 g Dry Matter</th>
<th>Static method(^b) g/100 g Dry Matter</th>
<th>Three consecutive extractions g/100g Dry Matter</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.940</td>
<td>5.790</td>
<td>10.410</td>
</tr>
<tr>
<td>2</td>
<td>5.340</td>
<td>6.050</td>
<td>11.150</td>
</tr>
<tr>
<td>3</td>
<td>4.990</td>
<td>6.400</td>
<td>11.000</td>
</tr>
<tr>
<td>Average</td>
<td>4.40 ± 1.30</td>
<td>6.08 ± 0.31</td>
<td>10.85 ± 0.39</td>
</tr>
</tbody>
</table>

\(^a\) Treatment: 100 °C, 60 min.  
\(^b\) Treatment: 80 °C, 90 min.

Washing the hemicellulose product with mixtures of water and a low concentration of ethanol (Paper 1), (sequential centrifugation - water washing) had been suggested in previous studies (Faryar et al., 2015). These methodologies were however noticed to be not totally effective to reduce the formed acetates. Dialysis using commercial membranes was shown to be a suitable option for glucuronoarabinoxylan purification, at least at lab-scale operations. The main advantages of dialysis purification were not only the elimination of any excess of unwanted side-product obtained after xylan precipitation, but also to reduction or at least a balancing of the cost that is spent on ethanol consumption (suggested in previous procedures). Furthermore, and also tested by H-NMR, dialysis will ensure obtaining a chemically pure product (Figure 6 - Right) (Paper 1).
4. Xylooligosaccharides (XOs) and use of novel xylanases

4.1. Xylooligosaccharides

Xylooligosaccharides (XOs) are sugar oligomers with a degree of polymerization (DP) from 2 to 10 xylopyranoside units (Zhou et al., 2008; Yang et al., 2011). XOs can be classified as non-substituted and substituted XOs (Yoo et al., 2012). XOs can be used in different industries and for different purposes (Vázquez et al., 2000; Aachary and Prapulla, 2011). In food related applications XOs are used as prebiotics (Paper 1, 3 and 4), however, according to the previously mentioned DP production and type, not all XOs are taken up by probiotic bacteria (Paper 4). This consumption, in fact, is even more reduced when substituted XOs are tested (Kabel et al., 2002; Aachary and Prapulla, 2011). The limitation in substituted XOs consumption open new opportunities to investigate not only the specificity of the known prebiotics, but also the effect of the substituted XOs on candidate probiotic bacteria selection (capability of uptake and consumption) (Chapter 5) (Paper 4).

Strategies to produce XOs include: (1) chemical production directly from the lignocellulosic material (raw material); (2) chemical production indirectly made from previously-xylan-extracted product and (Paper 4); (3) chemical-enzymatic mixed treatments, that consists in a previously extracted xylan process through a chemical method and further XOs production by xylanases or other enzymatic mixtures (Akpinar et al., 2010; Aachary and Prapulla, 2011; Chapla et al., 2012; Wei et al., 2016) (Paper 1 and 3). Enzymatically produced XOs has gained more interest during the last years not only for the more specific DP range (Akpinar et al., 2010; Hakala et al., 2013), but also for offering a “green” processing alternative (Yoon et al., 2006; Salas-Veizaga et al., 2017) (Paper 1).

Non-substituted or linear XOs are more common to be produced by enzymatic methods, since most of the known xylanases are displaying a xylan backbone cleaving activity (e.g. glycoside hydrolase (GH) family10 and 11) (Carvalho et al., 2013). Nevertheless, the main drawback in the production of these linear XOs is precisely the substituents present in xylan structures, which reduces (sometimes
drastically) XOs yields (Paper 1). Nowadays, new sources of microorganisms and additional enzyme characterizations reveal new insights into xylan enzymatic degradation, adding new families (and groups) of enzymes with glycoside hydrolase activities and also offering complementary hydrolysis options that could improve or promote a better XOs production (Paper 1 and 3).

4.2. Quinoa stalks XOs: Production by GH10 and GH11 families

Glycoside hydrolases (GH) (EC 3.2.1.) are the enzymes that catalyse reaction that involves the cleavage of O-glycosidic bond. These enzymes are responsible in nature of the metabolism of carbohydrates, resulting in enzymes that are present in most of groups of organisms on Earth (Naumoff, 2011). Among all the enzymes that belong to this group, hemicellulose-active enzymes hydrolyze hemicellulosic polymers to small sugars either oligomers or monomers. Xylans, composed of a β-(1,4)-xylopyranosyl-unit backbones with different substituents are the most common type of polymers presented in hemicellulosic fractions (Dodd & Cann, 2009). Glycoside hydrolases with xylanolytic activity are grouped in: (1) endoxylanases; (2) β-xylosidases; (3) α-glucuronidases; (4) α-arabinofuranosidases and; (5) acetylxylan esterases (Carvalho et al., 2013; Juturu et al., 2014). To produce XOs enzymatically, endoxylanases are the enzymes that have been more studied (Motta et al., 2013). Arabinofuranosidases and glucuronidases have also been studied in production of XOs as complementary enzymes to improve XOs yields (Pollet et al., 2010).

GH10 and GH11 were the first two major groups of xylanas described (Liu et al., 2003; Brienzo et al., 2010; Verma and Satyanarayana, 2012; Pokhrel et al., 2013). GH10 are considered xylanas with high molecular mass and low pl values, while GH11 are characterized as low molecular mass and high pl enzymes (Biely et al., 1997). GH10 are (β/α)8-barrel fold structures and GH10 enzymes has more versatile action patterns (Paës et al., 2012). GH11 enzymes, on the other hand, have β-jelly-roll and have a more limited activity profile. This specificity is related to the small size of their structures (few GH11 xylanas are able to be active on substituted xylans) and how the enzymes are interacting with the substrates (Biely et al., 1997). Nevertheless, this mentioned specificity also gives GH11 enzymes the quality of being considered the “true xylanas” (Motta et al., 2013).

In the present study, two xylanas from GH10 family and one from GH11 have been tested in their ability to hydrolyse glucuronooarabinoyxlan from quinoa stalks to produce XOs. The family GH10 xylanas belonged to Rhodotermus marinus
Among the possible explanations of why the yields of produced XOs were so low, is that the complex structure of glucuronoxarabinoylan of quinoa stalks was hindering access to the active sites of the enzymes used. This was suggested to be the most probable reason for such features (Paper 1), and more specifically the considerable amount of uronic acids in the polymer. However, even though the XOs production seemed relatively low, the product profile of the mentioned enzymes showed the presence of unidentified peaks in the chromatograms (unidentified in terms of comparison with commercially available standards) (Figure 8). These unidentified peaks varied according to the GH10 or GH11 enzyme chosen, showing that in fact the enzymes to a certain extent could access the polymer and hydrolyse the backbone with resulting substituted oligosaccharides. Interestingly, GH10 produced more of these unidentified peaks than GH11, which are most likely substituted XOs (Paper 1).
4.3. Bifunctional and additional types of xylanases

Additional types of xylanases, such as members of the families GH3, 8, 30, 39, 43, 51, 52, 54, 62, 116, and GH120 have been described as glycoside hydrolases with xylanase, arabinofuranosidase or dual activities (Lagaert et al., 2014; Yang et al., 2014). The majority of GH43 enzymes have a dual xylanase/arabinofuranosidase activity, and also GH30 possess a catalytic activity on xylans substituted with glucuronic acids, with the substituent as a necessary recognition site to mediate the hydrolysis of β-1,4 xylosyl linkages in its active form (Nishitani & Nevins, 1991). Besides the mentioned families, another common group of xylanases that are involved in the hydrolysis of hemicelluloses are the bifunctional esterase/xylanases (Shallom & Shohan, 2003). This particular group of bifunctional enzymes are characterized to not only participate in the hydrolysis of the xylans into small components, either monomeric or oligomeric, but also in the de-esterification of the xylopyranosyl backbone and the arabinofuranosyl substituents from acetyl and feruloyl groups, respectively (Blum et al., 1999; Wong, 2006). The mentioned characteristics among these groups, makes them suitable to investigate concerning use on the quinoa stalks glucuronoarabinoxylan.
4.3.1. GH43

GH43 has more than 5000 genes listed in 2016 (Linares-Pastén et al., 2017), which is now expanded to >10000 (www.cazy.org), although still less than 160 enzymes have been characterized. A crystal structure from a GH43 enzyme was firstly described by Nurizzo et al. (2002), and this structure revealed a five-bladed β-propeller fold. Its activity was described as α-1,5-linked-L-arabinofuranosidase able to act on arabinans. However, the activities discovered for this family now include: β-D-xylosidase, endo-β-D-xylanase, endo-α-L-arabinanase and, 1,3-β-galactosidase, which means that the catalytic properties and substrate binding vary according to the particular GH43 enzyme studied (Mewis et al., 2016).

The catalytic mechanism involved in GH43 arabinofuranosidase/endo-β-xylanase requires an inversion and one-step single displacement reaction (Falck et al., 2016). Independent of the substrate specificity, three conserved residues formed the active site of the enzymes, two aspartates and one glutamate (Lagaert et al., 2010). One aspartate and the glutamate are the acid and base, and the second aspartate may be responsible to modulate pKa and the orientation of the catalytic acid (Nurizzo et al., 2002).

The catalytic activities reported also included removal of substituents, mainly arabinofuranosyl residues, and showed that GH43 arabinofuranosidases, selectively were able to act in double substituted xylose cleaving only (1→3)-α-L-arabinofuranosyl, but not in (1→2) bond (Sorensen et al., 2006). Other GH43 arabinofuranosidases are instead specifically hydrolyzing 1,5-α-L-arabinobiose and release the monomeric form of arabinose (Linares-Pastén et al., 2017).

4.3.2. Bifunctional Esterase/Xylanases

Bifunctional Esterase/Xylanases occur in nature mainly by combination of two genes encoding close related activities, resulting in separate domains with the respective activity (Khandeparker & Numan, 2008; Escuder-Rodriguez et al., 2017). Referring to the enzyme itself, bifunctional esterase/xylanases usually catalyzes two consecutive reactions grouped by literature as follows: primary reaction: (1) feruloyl esterase/xylanases, that are responsible of breaking bonds between ferulate and arabinofuranosyl substituents present in xylans, separating lignin from hemicellulose fractions (Christov & Prior, 1993; Wong, 2006) or; (2) acetyl esterase/xylanases responsible to break ester linkages present between xylopyranose backbone and acetyl substituent (Margolles-Clark et al., 1996). The secondary reaction in both cases is either endo-xylanase or β-xylosidase that hydrolyse the xylopyranosyl backbone into small xyooligomeric structures or releasing monomeric xylose (Prates et al., 2001). Furthermore, the catalytic
domain of the esterase posses a α/β hydrolyse fold and catalytic triad Serine – Histine and Aspartate (Bornscheuer, 2002; Ulaganathan et al., 2015).

Although these bifunctional enzymes have been described in nature (Christov & Prior, 1993; Shallem & Shohan, 2003; Xie et al., 2007), only few of them has already been fully characterized. Furthermore, it has been yet difficult to determine the mechanisms involved in the bifunctionality of these enzymes (substrate-based mechanisms) (Biely et al., 1985). In the present study, the acetyl esterase/xylanase bifunctionality of one enzyme cloned from Clostridium boliviense strain E-1 has been proved and its kinetic parameters measuring acetyl esterase activity and endo-xylanase activity has been determined (Paper 3).

4.2.3. GH30

GH30 display (β/α)_S-barrel fold (same as GH10 family) and are classified under the same clan of enzymes (Clan A, www.cazy.org). GH30 xylanases are characterized as methylglucuronic acid specific (Pães et al., 2012) (Paper 4). A GH30 xylanase was first described by Nishitani and Nevins (1991), and this enzyme was extracted from Bacillus subtilis. In this type of enzyme, recognition of the negative charged carboxyl group from 4-O-methyl-D-glucuronic acid is by the positive charged guanidinium group of arginine from the enzyme (Biely et al., 2015). Enzymes from family GH30 have lately been divided in different subfamilies; and from these subfamilies, subfamily 8 requires strictly the gluconic acid substituent for activity, demonstrating the key role of gluconic acid possess in the activity of this enzyme (Urbaniková et al., 2011; St John et al., 2014; Rogowski et al., 2015). Literature also suggested a 300-fold reduction in activity in absence of the carboxyl group or the whole gluconic acid (Biely et al., 2015).

The present study characterized a hemicellulose structure extracted from quinoa stalks that include a considerable number of substituents of mainly 4-O-methyl-gluconic acid in its structure and also arabinofuranosyl residues which NMR analysis suggested to be present as single, double or in short oligomeric forms alongside the xylose backbone (Figure 3) (Paper 1 and 2). Enzymes belonging to the GH family 43, bifunctional esterase/xylanase (Paper 3) and GH30 (Paper 4) were interesting sources of complementary acting enzymes to be searched for and produce, not only to obtain substituted XOs (Paper 4), but also enzymes to improve the yields of GH10 and GH11 produced XOs (Paper 3 and 4).
4.4. New microbial sources of xylanases: *Clostridium boliviense* strain E-1

*Clostridium boliviense* E-1 (=CCUG 50824\(^\text{T}\), =DMS 17227\(^\text{T}\)) (AY943862) had been isolated in Bolivia, more specifically on an river bank with algae presented at Lakajahuira River in Oruro, Bolivia. This particular region in Bolivia has temperatures that vary in ranges from – 15 °C during nights to 20 °C at midday along the year, moreover, the region is also located above 4000 m from the sea level. Strain E-1, as all *Clostridia* strains, is a gram-positive bacterium, anaerobic, which optimum temperature of growth is in the range of 30 – 37 °C, pH 6.8 to 7.4 and it is spore-former (Álvarez Aliaga, 2005).

Due to its ability to grow on xylans as only carbon source and to produce detectable amounts of XOs, this microorganism was chosen as potential source of hemicellulose active enzymes (**Paper 3**). The draft genome of the microbe was sequenced, putative hemicellulose active enzymes were annotated, and after searching available web databases, eight genes were selected for cloning (Table 6). Six clones were successfully transformed in production vectors (pET21a), but only two enzymes were produced in active form. Both were predicted as potential endo-β-xylanase; one being most similar to enzymes in GH43 (*CbEI\textit{Xyn}43*) and the other later assigned as a bifunctional esterase/xylanase (*CbEI\textit{Est}1\textit{Xyn}X*) (**Paper 3**).

<table>
<thead>
<tr>
<th>Code</th>
<th>Predicted Activity</th>
<th>MW (KDa)</th>
<th>GH</th>
<th>Trans. pET21a</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1X1</td>
<td>Arabinan endo-1,5-α-L-arabinosidase</td>
<td>35.218</td>
<td>43</td>
<td>✓</td>
<td>x</td>
</tr>
<tr>
<td>E1X2</td>
<td>Bifunctional xylanase/acetylase</td>
<td>30.725</td>
<td>ND</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>E1X3</td>
<td>Xylosidase/arabinocydase</td>
<td>38.753</td>
<td>43</td>
<td>✓</td>
<td>x</td>
</tr>
<tr>
<td><strong>E1Xyn43</strong></td>
<td><strong>Endo-1,4-β-xylanase</strong></td>
<td>54.534</td>
<td>43</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>E1X5</td>
<td>Arabinoxylan arabinoarabohydrolase</td>
<td>64.145</td>
<td>43</td>
<td>✓</td>
<td>x</td>
</tr>
<tr>
<td><strong>E1Est1XynX</strong></td>
<td><strong>Endo-1,4-β-xylanase</strong></td>
<td>47.117</td>
<td>ND</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>E1X7</td>
<td>Endo-1,4-β-xylanase</td>
<td>91.476</td>
<td>ND</td>
<td>✓</td>
<td>x</td>
</tr>
<tr>
<td>E1X8</td>
<td>Endo-1,4-β-xylanase</td>
<td>75.417</td>
<td>ND</td>
<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>

**ND:** Non Determined.

4.5. Recombinant production of *CbEI\textit{Xyn}43* and *CbEI\textit{Est}1\textit{Xyn}X*

*CbEI\textit{Xyn}43* and *CbEI\textit{Est}1\textit{Xyn}X* from *C. boliviense* strain E-1, are enzymes that were difficult to produce in active form using *E. coli* production system. The genes of both enzymes had a considerable number of rare codons to *E. coli*. This
characteristic made their expression difficult into the standard recombinant *E. coli* system BL21(DE3) using isopropyl β-D-1-thiogalactopyranoside (IPTG) as inducer (Novagen®, Merck KGaA, Darmstadt, Germany). The host for expression of both recombinant plasmids (based on pET21a) was changed to Rosetta(DE3) (Novagen®), a host that possess an extra plasmid carrying tRNA with rare codons for *E. coli* and in this system expression of the enzymes was successfully achieved, with the amendments of increasing the expression time (after IPTG addition) to an overnight culture, and reducing the temperature from 37 °C to 30 °C (Rosano & Ceccarelli, 2014). Despite the successful expression of the genes from pET21a::*CbE1Xyn43* and pET21a::*CbE1Est1XynX* no activity could be observed.

Only when Rosetta-Gami(DE3) (Novagen®) was used as *E. coli* host, both enzymes got active configuration (Paper 3). The Rosetta-Gami™ 2 host possess a mix of features of the other *E. coli* hosts, specifically the presence of the plasmid that supplies rare codons to *E. coli* (from Rosetta(DE3)) and enhancing of disulphide bond formation (from Origami™, Novagen® Competent Cells User Protocol, Merck KGaA, Darmstadt, Germany). *CbE1Xyn43* and *CbE1Est1XynX* have a molecular weight of ~53 and ~44 kDa, respectively (Figure 9), and in the cloning design a C-terminal histidine tail had been added to enable purification by Immobilized Metal Ion Affinity Chromatography (IMAC).

![Figure 9. CbE1Xyn43 and CbE1Est1XynX identification in SDS-PAGE.](image)

Molecular Weight marker (Rows 1 and 3); Molecular Weight of *CbE1Xyn43* (Row 2): 52.902 kDa and; Molecular Weight of *CbE1Est1XynX* (Row 4): 44.291 kDa.
4.5. Enzymatic characterization of CbE1Xyn43 and CbE1Est1XynX

Both enzymes showed high temperature optima (65 °C) and relatively broad pH optima (Figure 10). The residual activity was measured and both enzymes remained active at 65 °C for prolonged periods. Even after 7 day of incubation both enzymes kept over 70 % of activity (Paper 3). Thermostability is one of the most important properties for industrial and commercial applications, especially when enzymes are the target product. The thermostability currently observed in both enzymes allows relatively long reactions, and still a possibility to inactivate the enzyme by a further temperature increase, which can be important in controlling the length of the reaction in application trials. (Linares-Pastén et al., 2014).

![Figure 10. CbE1Xyn43 and CbE1Est1XynX optimum temperature and optimum pH determination.](image)

(L) Optimum temperature and; (R) Optimum pH for CbE1Xyn43 and CbE1Est1XynX was determine using pNPXα (Paper 3).

CbE1Xyn43 was expected to carry motifs typical of subfamilies in GH43, as analysis by BLAST (blast.ncbi.nlm.nih.gov), showed significant sequence similarity to this GH family. However, trials to model the structure did not result in good quality models. One of the two potential domains encoded by CbE1Xyn43 could be homology modelled (data not shown), and corroborated the presence of more than one domain, with a good fit to the Carbohydrate Binding Module family 6 (CBM6) (Figure 11). BLAST searches of the remaining coding sequence (when the sequence encoding the CBM was omitted) still resulted in significant homology only to GH43 enzyme candidates (Paper 3). CbE1Xyn43 had better specific activity on debranched wheat arabinoxylan than to birchwood xylan and quinoa stalks glucuroarabinoxylan. CbE1Xyn43 did not show activity on para-nitrophenyl-arabinofuranoside (pNPA) or p-nitrophenyl-xylopyranoside (pNPX). The HPAEC-PAD product profile analysis, showed very little xylose or arabinose in monosaccharide form, corroborating the lack of xylosidase activity by this CbE1Xyn43 (Table 7) (Paper 3). On the other hand, endoxylanase activity was
confirmed using three methods: 3,5-dinitrosalicylic acid (DNS), HPAEC-PAD and 
p-nitrophenyl-xylobioside (pNPX₂) (Paper 3).

Figure 11. Carbohydrate Binding Module Family 6 (CBM6) from Clostridium boliviense strain E-1 Xyn43. 
The amino acid sequence alignment of CbE1Xyn43 was compared with data bases (blast.ncbi.nlm.nih.gov) resulting 
in a high match with structure determined CBM6 (> 90 % identity) allowing computational modelling with high 
accuracy.

Although CbE1Xyn43 had been predicted to have only endo-β-xylanase activity, 
additional substrates were tried to investigate if the enzyme could hydrolyze either 
α-(1-2), α-(1-3), or double α-(1-2) (1-3) arabinofuranosyl residues linked to the β-
(1-4) xylanopyranosyl backbone in arabinoxylooligosaccharides (AXOs) 
(Megazyme, Ireland) (Paper 3). The enzyme was only shown to hydrolyse the 
residues from the xylose backbone, but interestingly, it seemed to be selective in 
hydrolyzing only AXOs with arabinofuranosyl residues that were α-(1-3) linked 
(with no activity on oligosaccharides containing either arabinofuranoses that were 
α-(1-2) or double substituted α-(1-2) (1-3) xylose residues). The absence of 
arabinofuranosidase activity and the lack of affinity to arabinofuranosyl 
substituents suggested that this enzyme rather acts on non-substituted 
xylopyranosyl backbone xylans than decorated ones. This hypothesis was 
corroborated when evaluation of hydrolysis products showed birchwood xylan 
(the least substituted substrate) to be the best substrate for CbE1Xyn43 in terms of 
XOs yield (Paper 3).
Table 7. Neutral XOs produced by Clostridium boliviense strain E-1: Bifunctional esterase/xylanase CbE1Est1XynX and putative GH43: CbE1Xyn43.

<table>
<thead>
<tr>
<th>Degree of Polymerization</th>
<th>Relative Production (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BX</td>
</tr>
<tr>
<td></td>
<td>E1Est1XynX</td>
</tr>
<tr>
<td>(X) Xylose</td>
<td>0.27</td>
</tr>
<tr>
<td>(Xc) Xylobiose</td>
<td>23.34</td>
</tr>
<tr>
<td>(X3) Xylotriose</td>
<td>36.33</td>
</tr>
<tr>
<td>(X4) Xyloolotraose</td>
<td>22.85</td>
</tr>
<tr>
<td>(X5) Xylopentaose</td>
<td>10.57</td>
</tr>
<tr>
<td>(X6) Xylohexaose</td>
<td>6.64</td>
</tr>
<tr>
<td>Total XOs (DP 2-6)</td>
<td><strong>99.73</strong></td>
</tr>
</tbody>
</table>

CbE1Est1XynX, here for the first time reported, exhibit not only the production of linear XOs, but also the production of high molecular weight unidentified peaks that could represent the presence of branched XOs (Paper 3). The activities here reported together with the molecular modelling showed an enzyme with separate domains homologous to Carbohydrate Esterase (CE) family 1 from Clostridium thermocellum and the CBM6 domain (with a folding similar to a GH11) from Clostridium cellulolyticum, for the esterase domain and xylanase domain, respectively (Figure 12). Authors have established that the presence of bifunctional esterase/xylanase enzymes allowed not only the deacetylation or defferoulation of the xylopyranosyl backbone or arabinofuranosyl substituents, respectively, but also enhanced the accessibility of other enzymes to the internal lignocellulosic fractions continuing degradation of the residual lignocellulosic fractions (Wong et al., 2006). The separation of hemicellulose from the other lignocellulosic fractions (lignin and cellulose) by esterase/xylanases also increases solubility of hemicellulose in water (Zhang et al., 2011; Huy et al., 2013).

The CbE1Est1XynX bifunctionality was tested using synthetic substrates: p-nitrophenyl acetate (pNPAc) to measure esterase activity and pNPX2 to measure endoxylanase activity. CbE1Est1XynX seems to have esterase activity as the primary activity over xylanase activity, showing higher turnover and catalytic efficiency for this substrate (Table 8). The enzyme showed higher specific activity when debranched wheat arabininoxylan was used than when using birchwood xylan and quinoa stalks glucuronoroarabinoxylan (Paper 3). In contrast with these results, when CbE1Est1XynX product profile was measured, a major amount of XOs were produced from quinoa stalks glucuronoroarabinoxylan and birchwood xylan, and minor amounts in debranched wheat arabininoxylan (Table 7) (Paper 3). Exo-xylanase activity was not significant for CbE1Est1XynX, since neither HPAEC-PAD analysis after use of the polymers nor p-nitrophenyl arabinofuranoside/xylopyranoside showed presence of xylose of p-nitrophenol release, respectively.
CbE1Est1XynX on the other hand, had higher catalytic efficiency on the substrates used than CbE1Xyn43 (Table 8).

![Bifunctional esterase/xylanase Est1XynX from Clostridium boliviense strain E-1.](image)

Figure 12. Bifunctional esterase/xylanase Est1XynX from *Clostridium boliviense* strain E-1.
Computational modelling of Carbohydrate Esterase family 1 domain (left) close related to CE1 from *C. thermocellum* and Carbohydrate Binding Module 6 (CBM6) domain (right) structurally close related to GH11 from *C. cellulolyticum* (Mr = 44,293 kDa). Esterase domain was proved functional using pNP-Acetate. Glycoside hydrolase domain proved to be functional using pNP-Xylobioside and on three different xylan types, Birchwood glucuronoxylan, wheat bran debranched arabinoxylan and, quinoa stalks glucuronoxarabinoxylan.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>V&lt;sub&gt;max&lt;/sub&gt; (µmol × min&lt;sup&gt;-1&lt;/sup&gt; × mg&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>K&lt;sub&gt;m&lt;/sub&gt; (mM)</th>
<th>K&lt;sub&gt;cat&lt;/sub&gt; (min&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>K&lt;sub&gt;cat&lt;/sub&gt;/K&lt;sub&gt;m&lt;/sub&gt; (min&lt;sup&gt;-1&lt;/sup&gt; × mM&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CbE1Xyn43</td>
<td>pNPX&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.030</td>
<td>0.126</td>
<td>1.587</td>
<td>12.598</td>
</tr>
<tr>
<td>CbE1Est1XynX</td>
<td>pNPX&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.150</td>
<td>0.233</td>
<td>6.645</td>
<td>28.517</td>
</tr>
<tr>
<td>CbE1Est1XynX</td>
<td>pNPAc</td>
<td>5.525</td>
<td>2.250</td>
<td>243.363</td>
<td>108.161</td>
</tr>
</tbody>
</table>

**Table 8. Michaelis-Menten kinetic parameters stimulation of CbE1Xyn43 and CbE1Est1XynX.**

4.6. Xylooligosaccharides produced by CbE1Xyn43 and CbE1Est1XynX

Common XOs produced by GH10 and GH11 are xylobiose and xylotriose (Xu *et al.*, 2012; Linares-Pastén *et al.*, 2018) (*Paper 1*). These XOs are not substituted oligosaccharides and the amount of each XOs is dependent on the specific enzyme used for their production (Akpinar *et al.*, 2009; Carvalho *et al.*, 2013). Highly
substituted structures in xylans has been determined to be a limiting factor in enzymatic production of XO (Paper 1 and 4), and despite that GH10 enzymes are able to act on substituted xylans, the activity decreases. Moreover, GH11 enzymes seem to have a considerable reduction in activity when xylans possess large amounts or substituents in their structure (Carvalho et al., 2013).

Novel types of xylanases show, not only capacity to produce XO from more complex xylan structures, but also indeed require of these substitutions on the xylan structure to reach their maximum activity profile (St John et al., 2011). GH43 and GH30 families are reported to be able to hydrolyze substituted xylans along with an ability to produce both linear-unsubstituted and XO including such substituents (Wei et al., 2016). This opens new possibilities to produce and test oligosaccharides with different types of substrate decorations in different application areas. In this study in particular, their influence on probiotic growth was investigated (Chapter 5) (Paper 4).

Literature refers to three common xylan-types in vascular plants: (1) glucuronoxylans, commonly present in hardwoods; (2) arabinoxylans, mainly present in cereals and softwoods and; (3) glucuronoorabinoxylans, also present in several cereals and grasses (Rogowski et al., 2015). CbE1E1Xyn43 and CbE1Xyn43 were hence tested on Birchwood xylan, debranched wheat arabinoxylan (30 % Arabinose) and quinoa stalks glucuronoorabinoxylan (Paper 3). The major amount of neutral XO that were produced by CbE1E1Xyn43 that were possible to identify and quantify by HPAEC-PAD were xylotriose and xylotetraose. Xylose was almost not produced by CbE1E1Xyn43 and was much lower than the amount produced by the GH10 and GH11 enzymes used in Paper 1 (Table 7). Quinoa stalks glucuronoorabinoxylan resulted in better yields in terms of XO production as compared to birchwood xylan or debranched wheat arabinoxylan (Paper 3).

The ability of different GH43 enzymes to produce XO varies with the enzyme specificity, determining a broad spectrum of activities and catalytic affinities in the family (Michlmeyer et al., 2013; Jordan et al., 2013). Not all GH43 enzymes required substituents to be active (Huy et al., 2013), but some can be very selective and accept specific types of substituents. Sørensen et al. (2006) e.g. demonstrate that certain xylanases from GH43 were able to hydrolase (1→3) substituents, that were situated on either mono or double substituted xylose residues in the xylan backbone. Vandermarliere et al. (2009) and Lagaert et al. (2010) reported other GH43 arabinofuranosidase that indeed were able to hydrolyze arabinofuranosyl bonds that were either (1→2) or (1→3) linked, but only in monosubstituted residues. Furthermore, some enzymes in GH43 were shown to display both exo and endo activities at the same time (Brüx et al., 2006).
In the present study, *Cbe1Xyn43* showed activity on birchwood xylan, debranched wheat arabinoxylan and quinoa stalks glucuronarabinobioxylan ([Paper 3](#)). *Cbe1Xyn43*, as was expected, exhibited better activity on debranched wheat arabinoxylan than on the uronic acids substituted xylans. The neutral (linear) XOs product profile showed that this enzyme was able to produce xylotriose, xylobiose and xylotetraose (Table 7). *Cbe1Xyn43* appears to be specifically cleaving only the xylan backbone and did not display any arabinofuranosidase activity ([Paper 3](#)). The ability of *Cbe1Xyn43* to produce XOs of large DP and some monosaccharides, demonstrated endo-xylanase activity on the measured substrates ([Paper 3](#)).

### 4.7. Enzymatic GH30 XOs production from quinoa stalks glucuronarabinobioxylan

Previous investigations established a cleavage mechanism for xylanases in GH30 in which the second glycosidic bond of the xylose backbone after the glucuronic acid substituent is targeted for hydrolysis (Nishitani & Nevins, 1991; Urbániková et al., 2011). It has also been demonstrated that the product profile in terms of substituted XOs exceeded the DP 10, which means that one glucuronic acid substituent is present each 10 – 12 xyloses units (Wei et al., 2016). Authors also suggested mandatory presence of the glucuronic acid substitution in the xylan backbone to get a functional form of GH30 enzymes (Padilha et al., 2014; Sainz-Polo et al., 2014; Verma & Goyal, 2014). In the present work, a commercial GH30 glucuronoxylanase from *Bacteroides ovatus* (NZYTech Ltd., Portugal) was tested over the mentioned xylan substrates. As was expected, no activity was detected either using DNS or HPAEC-PAD when debranched wheat arabinoxylan was used as substrate. On the other hand, glucuronosyl xylans (from birchwood and quinoa stalks), resulted in oligosaccharide production directly related with the amount of glucuronic acids present in each structure. Quinoa stalks glucuronarabinobioxylan resulted in higher production of oligosaccharides in comparison with birchwood xylan ([Paper 4](#)). The HPAEC-PAD product profile showed no-linear quantifiable XOs (Figure 13); however, later retention times showed a marked production of oligosaccharides, especially using quinoa stalks glucuronarabinobioxylan as substrate, suggesting that these peaks represent different DP of glucurononxyooligosaccharides ([Paper 4](#)). Moreover, the pattern of peak distribution is similar between the data obtained with birchwood xylan and quinoa stalks glucuronarabinobioxylan, but different to the previously discussed GH10 and GH11, indicating selective hydrolysis at defined positions after the glucuronosylation for GH30, which differs from the substituent acceptance in the active site in the GH10 and GH11 enzyme candidates (Figure 8).
Figure 13. Glucuronoxyloligosaccharides (GXOs) produced from quinoa stalks glucuronoarabinonoxylan by *Bacteroides ovatus* GH30 glucuronoxylanase.

(Black line) Commercial standards for linear XOs: Xylose (X); Xylobiose (X2); Xylotriose (X3); Xylotetraose (X4); Xylopentaose (X5) and Xylohexasose (X6). (Brown line) Product profile of GH30 *B. ovatus* over quinoa stalks glucuronoarabinonoxylan.
5. Quinoa stalks XOs application: The case of prebiotics

Previous chapters had focused on using quinoa stalks glucuronoarabinoxylan as a substrate for XOs production, especially enzymatically produced XOs (Papers 1, 2 and 3). Independent on the capacity of the tested enzymes to produce XOs (as defined by standards), one of the common features showed is the presence of unidentified peaks that suggest substituted XOs (Figure 8). Linear XOs have been reported by literature as promoters of growth of target probiotic bacteria (e.g. Lactobacilli and Bifidobacteria) (Kontulla et al., 1998; Gullón et al., 2008; Madhukumar & Muralikrishna, 2012; Immerzeel et al., 2014). However, few studies have focused on using the substituted XOs as potential prebiotics, and those that have, have focused on arabinofuranoside-branched AXOs (Pastell et al., 2009; Falck et al., 2013; Rivièrè et al., 2014). In the particular case of quinoa stalks glucuronoarabinoxylan, the major substituent present in its structure is glucuronic acid (Paper 1), and glucuronoxylooligosaccharides (XOs substituted with glucuronic acid), a term first used by Rogowski et al. (2013) has rarely reported as a prebiotic source to be used as food additive.

5.1. Prebiotics

The term prebiotic has for the first time been described by Gibson and Roberfroid in 1995 when they established that a prebiotic is a nondigestible 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 improves host health. On that condition, prebiotic food or candidate prebiotic component has to fulfill a series of requisits in order to be considered as a truly “prebiotic”, in such condition a prebiotic ought to (1) have resistance to gastric acidity; (2) not being hydrolyzed by gastrointestinal tract enzymes; (3) not being absorbed in the upper gastrointestinal tract; (4) be consumed by intestinal microorganisms and; (5) selective stimulate the growth of those microorganisms associated with the well-being and health of the host (probiotics) (Kopp-Hoolihan, 2001; Gibson et al., 2004; Roberfroid, 2007; Aachary & Prapulla, 2011). The concept of prebiotics has
also been suggested to be limited to the gastrointestinal tract, which means that the
term prebiotic only applies to food or additives that modulates beneficial bacteria
in that specific body region and not in other sites where these beneficial bacteria
are also present (e.g. skin) (Schrezenmier & de Vrese, 2001). This adds
requirements for any food that would like to be considered as prebiotic, in that
sense, prebiotic also has to ensure (6) being from a natural source, mainly plants or
synthesized by enzymes or microorganisms; (7) not generate any side-effect or
residual problem and; (8) ideally be used as food additive (Samanta et al., 2015).

Despite some proteins and lipids that are also considered as prebiotics,
carbohydrates have gained more interest and attention (Ziemer & Gibson, 1998).
Fructooligosaccharides and galactooligosaccharides have been the oligomeric
carbohydrate most studied for their intestinal inabilty of being consumed; and
mainly because they are found in nature without any extra processing (particularly
fructooligosaccharides) (Gibson & Roberfroid, 1995).

XOs belongs to a group of “recently” established prebiotics. Interestingly,
although they were reported to be used as food ingredient in Japan in 1990
(Mäkeläinen et al., 2009; Crittenden & Playne, 1996), by 2007 they were still
considered as potential prebiotic due to lack of necessary information to fulfill the
prebiotic criteria (Gibson et al., 2004; Roberfroid, 2007). Only in 2011, literature
reported XOs as a prebiotic oligosaccharide, for their capacity to selectively
stimulate probiotic bacteria, resist gastric acids, an incapability of being absorbed
by the intestine and, to improve the host health (Broekaert et al., 2011). Likewise,
arabinixylo-oligosaccharides (XOs with arabinofuranoside(s) groups) are also
now grouped into prebiotics (Aachary & Prapulla, 2011; Broekaert et al., 2011;
Hoseinifar et al., 2016). XOs have been demonstrated to be fermented selectively
by Bifidobacteria. Nevertheless, the fermentation of these oligosaccharides is
limited when Lactobacilli were tested; only few strains were able to grow in
presence of XOs (Moura et al., 2007; Mäkeläinen et al., 2010). Literature has also
demonstrated that Bifidobacteria are able to consume AXOs (Falck et al., 2013).

5.2. Acid produced XOs from quinoa stalks
glucuronoarabinoxylan

The up-scaling of enzymatic processing to produce XOs remains as a task still to
be solved in prebiotic production (Galbe & Zacchi, 2007; Carvalho et al., 2013).
In order to scale up the production of XOs with good efficiency, many approaches
have been proposed by literature, including chemical hydrolysis, or a combined
chemo-enzymatic hydrolysis (Akpinar et al., 2009). Chemically produced XOs
possess two major advantages; (1) up-scaling is more established, simplifying
work with large amount of material and; (2) accelerating the time needed, which means both fast development and acceleration of processing time, taking into account the amount of the chemical compounds needed (acid or alkali), temperature and, sometimes, pressure used for the hydrolysis (Galbe & Zacchi, 2007). However, every single variable described must be carefully measured and evaluated in order to get as much oligosaccharides as possible, not only for the time of hydrolysis itself, but also to avoid; (1) production of excessive amount of monosaccharides; (2) formation of inhibitory or toxic byproducts (e.g. furfural) and; (3) too random degree of polymerization production (Carvalho et al., 2013).

Here, dilute sulphuric acid [0.25 M] was chosen to produce XOs from the previously extracted glucuronoarabinoxylan (Paper 4). In order to get as much XOs as possible, time of hydrolysis were measured at 15, 30 and 60 minutes at the mentioned acid concentration and fix temperature 90 °C (Paper 4). Literature suggested that these parameters ensure the production of XOs (Akpinar et al., 2010; Carvalho et al., 2013), however time was not taking into account and was the parameter that was measured (Paper 4). Quinoa stalks glucuronoarabinoxylan turned into a maximum amount of linear and quantified XOs production when it was hydrolyzed for 30 minutes. Shorter times of exposition promoted low rates of XOs production, and longer times led to reduction of the higher DP XOs, but also in monomeric xylose (Figure 14). The conditions selected in the present study for XOs production resulted reproducible in terms of DP pattern, which means that scaling up should be possible.

5.3. Probiotics

The definition of “probiotic” has been modified since the first time that the word was used in 1965 by Lilley and Stillwell (Fuller, 1989). FAO/WHO in 2001 formulate the term probiotic as live microorganisms that, when administrated in adequate amounts, confer a health benefit on the host (Hill et al., 2014). Similar to prebiotics, probiotics also have a certain number of requirements to be filled in order to consider any microorganisms as a (potential) probiotic. However probiotics embrace not only microorganisms present in gastrointestinal tract, but also in all mucosal areas of the body (Reid et al., 2003). Among the properties suggested for probiotics, they include: (1) Biosafety, meaning that the microorganisms must not be pathogenic, allergenic, mutagenic or carcinogenic; (2) Probiotics shall be from human origin or from animal target; (3) Possess resistance for in vitro and in vivo conditions (e.g. bile and acidic stability); (4) Adherence and colonization of mucosal surfaces; (5) Must be microorganisms with genetically stable properties; (6) Must have antagonist effects towards pathogenic and carcinogenic microbes; (7) Must be reproducible, resistant and
remain viable after processing and storage and; (8) Probiotics must validate, clinically, health improvement and benefits for the host (Ouwehand et al., 1999; Kosin & Rakshit, 2006).

![Figure 14. Dilute sulphuric acid [0.25 M] production of XOs from quinoa stalks glucuronoarabinoxylan. Treatment made at 90 °C for (Blue) 15 min; (Red) 30 min and (Green) 60 min. Quantified mono and oligosaccharides: (Ara) Arabinose; (Gal) Galactose; (Xyl) Xylose; Xylobiose (X2); Xylotriose (X3); Xylotetraose (X4); Xylopentaose (X5) and Xylohexaose (X6).]

Regarding this last point, authors also suggest that the mentioned benefits shall be proved by (1) restauration of the intestinal microflora; (2) improving colonisation resistance and diarrhoea; (3) diminution of serum cholesterol, faecal enzymes, potential mutagens; (4) enhancement of lactose metabolism and immune system response and; (5) improving the calcium absorption and synthesis of vitamins (Ziener & Gibson, 1998; Ouwehand et al., 1999). In such way, probiotics are more difficult to get accepted as such, and the microorganisms that follows the criteria and that are accepted as probiotics are reduced to only few genera and species.

Lactobacilli and Bifidobacteria are the most studied probiotic genera (Ouwehand et al., 2002; Soccol et al., 2010). However, not all the members of these groups are accepted as probiotics, mainly due to that the probiotic quality does not apply to specific species but rather to strains (Borriello et al., 2003; Azaïs-Braesco et al., 2010). Moreover, only Bifidobacteria is described as non-risk safe probiotic as
present no contraindications in their consumption are known (Kligler & Cohrssen, 2008). 

*Lactobacilli* have on the other hand, been recommended not to be consumed by persons with hypersensitivity to milk (Williams, 2010). Although more of the studies and new probiotic bacteria development is focused in the mentioned two groups, the search for novel microorganisms with probiotic effect in host health has turned into one important goal nowadays, trying to expand the range of bacteria or yeast with beneficial effects to more taxonomic and phylogenetic groups, such as *Weissella* spp. (Lee et al., 2012) or even *E. coli* Nissle (Behnse et al., 2013), to help to understand better the gastrointestinal microbiome, and expand the range of not only probiotic microorganisms, but also prebiotic sources to feed them.

### 5.3.1. *Bifidobacterium adolescentis*

*Bifidobacterium adolescentis* is member of probiotic bacteria able to utilize not only linear, but also branched XOs as carbon source for its growth (Lagaert et al., 2011; Falck et al., 2013). Moreover, according to Gullón et al. (2008), *B. adolescentis* has also the quality of having the ability to use XOs for its metabolism. Like the rest of Bifidobacteria, it is a gram-positive, non-spore former with low oxygen tolerance (anaerobe) (Soccol et al., 2010). *B. adolescentis* possess a number of enzymes that allow the hydrolysis of XOs (degree of polymerization (DP) 2-4) (Gullón et al., 2008), first transforming the oligosaccharide into xylose. Xylose is converted in xylulose, xylulose-5-phosphate and then incorporated into its metabolism to produce acetate, ethanol, formate and lactic acid as fermentation product (Pokusaeva et al., 2011; Egan & Van Sinderen, 2018).

### 5.3.2. *Weissella* spp.

*Weissella* spp. are gram-positive, non-spore forming, facultative anaerobic bacteria (Collins et al., 1993). They possess a glucose-heterofermentative metabolism (De Bruyne et al., 2010) and they are able to metabolize XOs with DP 2 and 3 (Falck et al., 2016; López-Hernández et al., 2018). Although this group is still considered as a candidate probiotic group, literature is starting to test different *Weissella* spp., specifically *W. confusa* and *W. cibaria* as potential probiotic bacteria. Lee et al. (2012) demonstrated that both species fullfill the statements that are necessary to qualify them as probiotics. *W. confusa* and *W. cibaria* have also been demonstrated to possess active arabinofuranosidases (Linares-Pastén et al., 2017) and β-xylanase (Falck et al., 2016) from family GH43, suggesting the possibility to also be active over branched XOs.
5.4. Probiotic bacteria and quinoa stalks XOs

5.4.1. Consumption of quinoa stalks XOs by *B. adolescentis* ATCC15703 and *Weissella* sp. strain 92

*Bifidobacteria* spp. is one of the most studied probiotic species (Behnsen *et al.*, 2013), when consumption of XOs is searched (Rastall & Gibson, 2015; Reddy & Krishnan, 2016). In the present study the strain *Bifidobacterium adolescentis* ATCC15703 has been tested for its capacity to grow using XOs, produced by dilute acid treatment of quinoa stalks glucuronoarabinoxylan, as carbon source in the culture medium. Moreover, the metabolic products (Short Chain Fatty Acids) produced from the XOs consumption were determined (Paper 4). *B. adolescentis* ATCC15703 could grow in medium containing the XOs as carbon source, but there was a marked difference in cell density (OD$_{600nm}$) compared to growth in glucose (preferred substrate) (Figure 15 – Left). XOs of all DPs analysed were consumed, but the consumption rates varied with the DP, with xylobiose and xylotriose being the most preferred XOs, a pattern also previously observed in literature (Madhukumar & Muralikrishna, 2012; Falck *et al.*, 2013).

*Weissella* spp. is a bacterial genus that has been included in the list of potential probiotics (Lee *et al.*, 2012). In the present study, *Weissella* sp. strain 92 (a strain previously studied at the Division of Biotechnology) was used to explore in more detail its suggested probiotic quality. In comparison with *B. adolescentis*, *Weissella* sp. strain 92 was capable to consume XOs until DP 4 (xylotetraose), but it was incapable to consume higher DP XOs (Paper 4). (Figure 15 - Right).

![Graph](image1.png)

**Figure 15.** Consumption of lineal XOs and monosaccharides from dilute-acid-hydrolyzed quinoa stalks glucuronoarabinoxylan by probiotic bacteria. (Left) Consumption of XOs by *Bifidobacterium adolescentis* ATCC15703. (Right) Consumption of XOs by *Weissella* sp. strain 92.
The available literature on both tested probiotic strains corroborates the same results presented here (Egan & Van Sinderen, 2018). One characteristic that was also shown here was the ability of the *B. adolescentis* strain to consume the substituted oligosaccharides (Figure 8, Figure 15), suggesting that *B. adolescentis* consumes linear XOs, AXOs and potentially also GXOs (Figure 16). On the other hand, *Weissella* sp. strain 92 was able to only consume monomeric and linear XOs of low DP, but not other substituted products present in the complex XOs mixture reported here. These results suggested that XOs produced by dilute-acid of the quinoa stalks include components that selectively promote growth of the *Bifidobacterium* sp.

![Figure 16. Consumption of unspecific peaks suggested to be AXOs or GXOs from dilute H₂SO₄ [0.25 M] treated quinoa stalks glucurononarabinoxylan by Bifidobacterium adolescentis ATCC15703.](image)

Quantified lineal XOs, DP 2-6, appears at early retention time (0-8 min). Here, unspecific peaks are shown at retention times later than 8 min. (Black line) Oligosaccharides produced after dilute acid hydrolysis of quinoa stalks glucurononarabinoxylan. Remaining oligosaccharides present in culture media after *B. adolescentis* cultivation: (Pink line) 8 hr; (Green line) 12 hr and; (Blue line) 24 hr.

### 5.4.2. Metabolic SCFA produced by *B. adolescentis* ATCC15703 and *Weissella* sp. strain 92

Short Chain Fatty Acids (SCFA) are metabolic fermentation products of colonic bacteria that constitute an energy source for human colonic epithelium (Candela *et al.*, 2010). Probiotic bacteria produce SCFA that aids in the maintaining of pH in the colon (Reddy & Krishnan, 2016), allowing mineral absorption, which is one of the selection criteria for probiotic bacteria (Ziemer & Gibson, 1998; Ouwehand *et al.*, 1999). A major part of the human gut microbiota, and specifically probiotic bacteria; produce acetate, propionate and butyrate (Baruah *et al.*, 2017). Acetate and propionate are involved in the metabolism of lipids and cholesterol (Patel & Prajapati, 2015), while butyrate is important for colonocytes function (Rastall &
Gibson, 2015). Although propionate, acetate and butyrate are the most common SCFA fermentation products of probiotic microbiota in the gut, other (sometimes strain specific) metabolic products are also found, such as lactate, formate and succinate (Shen et al., 2010; Silva et al., 2017).

The XOs produced from quinoa stalks and consumed by *B. adolescentis* ATCC15703 resulted in production of acetate, lactate and propionate after 48 hr of incubation. (Paper 4). Moreover, after 24 hr of cultivation, minor amounts of formate was also detected (Figure 17 - Left). The SCFA produced in this study using *B. adolescentis* ATCC15703 were in accordance with fermentation products reported previously for the same species (Table 9). The ability of this particular strain to growth on quinoa stalks XOs confirms the usage of quinoa stalks XOs as a potential prebiotic material; promoting not only the total consumption of the prebiotic itself, but also the production of useful SCFA for gut health improvement.

Despite to the fact that the *Weissella* sp. strain 92 used in this study has not been characterized on species level yet; it has been studied as potential probiotic (Patel et al., 2013; Immerzeel et al., 2014). In comparison with those studies, quinoa stalks XOs extracted by dilute acid treatment clearly promoted growth and production of lactate and acetate (Table 11), showing that quinoa stalks XOs also, to a certain extent, promotes this strain.
Table 9. Short Chain Fatty Acids produced by different *Bifidobacteria* spp. Strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Subs.</th>
<th>SCFA [% Rel] (mM/mL)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Acetate</td>
<td>Formate</td>
</tr>
<tr>
<td><em>B. adolescentis</em></td>
<td>ATCC15703</td>
<td>(1.00)</td>
<td>(0.14)</td>
</tr>
<tr>
<td></td>
<td>QSXOs</td>
<td>[36.90]</td>
<td>[5.30]</td>
</tr>
<tr>
<td><em>B. bifidum</em></td>
<td>XOs</td>
<td>(1.00)</td>
<td>(0.22)</td>
</tr>
<tr>
<td><em>B. angulatum</em></td>
<td>XOs</td>
<td>(1.00)</td>
<td>(0.27)</td>
</tr>
<tr>
<td><em>B. gallicum</em></td>
<td>XOs</td>
<td>(1.00)</td>
<td>(0.35)</td>
</tr>
<tr>
<td><em>B. breve</em></td>
<td>XOs</td>
<td>(1.00)</td>
<td>(0.52)</td>
</tr>
<tr>
<td><em>B. infantis</em></td>
<td>XOs</td>
<td>(1.00)</td>
<td>(0.25)</td>
</tr>
<tr>
<td><em>B. longum</em></td>
<td>XOs</td>
<td>(1.00)</td>
<td>(0.25)</td>
</tr>
<tr>
<td><em>B. pseudolongum</em></td>
<td>XOs</td>
<td>(1.00)</td>
<td>(0.00)</td>
</tr>
<tr>
<td><em>B. catenulatum</em></td>
<td>XOs</td>
<td>(1.00)</td>
<td>(0.00)</td>
</tr>
<tr>
<td><em>B. adolescentis</em></td>
<td>DSMZ 18350</td>
<td>(1.00)</td>
<td>(0.00)</td>
</tr>
<tr>
<td></td>
<td>XOs</td>
<td>(1.00)</td>
<td>ND</td>
</tr>
<tr>
<td><em>B. adolescentis</em></td>
<td>NDRI 236</td>
<td>[99.2]</td>
<td>ND</td>
</tr>
<tr>
<td><em>B. bifidum</em></td>
<td>ATCC29521</td>
<td>[100]</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>WBO</td>
<td>[0.03]</td>
<td>ND</td>
</tr>
<tr>
<td><em>B. bifidum</em></td>
<td>ATCC29521</td>
<td>[100]</td>
<td>ND</td>
</tr>
<tr>
<td><em>B. bifidum</em></td>
<td>NCD02715</td>
<td>[100]</td>
<td>ND</td>
</tr>
<tr>
<td><em>B. bifidum</em></td>
<td>NCD02715</td>
<td>[100]</td>
<td>ND</td>
</tr>
</tbody>
</table>

**ND**: Non Determined.

*: Amounts expressed as ratios of produced acetate. Values calculated according to Palframan et al. (2003).

Table 10. Short Chain Fatty Acids produced by probiotics using quinoa stalks xylooligosaccharides as carbon source.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Time [hr]</th>
<th>OD600</th>
<th>SCFA [g/L] Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lactate</td>
</tr>
<tr>
<td><em>B. adolescentis</em></td>
<td>ATCC15703</td>
<td>0</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>0.127</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>0.322</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>0.326</td>
</tr>
<tr>
<td>Weissella sp. strain 92</td>
<td>0</td>
<td>0.122</td>
<td>0.02 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>0.474</td>
<td>0.17 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.503</td>
<td>0.19 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.626</td>
<td>0.26 ± 0.05</td>
</tr>
</tbody>
</table>

**ND**: Non detected.
Table 11. Short Chain Fatty Acids produced by *Weissella* spp. Strains.

QSXOs: Quinoa Stalks Xylooligosaccharides; WBXOs: Wheat Bran Xylooligosaccharides; BXOs: Birchwood Xylooligosaccharides.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Substrate</th>
<th>OD&lt;sub&gt;600&lt;/sub&gt;</th>
<th>SCFA (g/L) [mM]</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Weissella</em> sp. Strain 92</td>
<td>QSXOs</td>
<td>0.626</td>
<td>(0.38) [6.33]</td>
<td>Present Work</td>
</tr>
<tr>
<td><em>Weissella</em> sp. (92)</td>
<td>WBXOs</td>
<td>&gt; 1.2</td>
<td>(0.31) [0.30]</td>
<td>Immerzeel et al. (2014)</td>
</tr>
<tr>
<td><em>Weissella</em> sp. (AV1)</td>
<td>WBXOs</td>
<td>&gt; 1.2</td>
<td>(0.33) [0.34]</td>
<td>Immerzeel et al. (2014)</td>
</tr>
<tr>
<td><em>Weissella</em> sp. (92)</td>
<td>BXOs</td>
<td>0.4</td>
<td>[11.1] [9.1]</td>
<td>Patel et al. (2013)</td>
</tr>
<tr>
<td><em>Weissella</em> sp. (AV1)</td>
<td>BXOs</td>
<td>0.5</td>
<td>[11.2] [9.0]</td>
<td>Patel et al. (2013)</td>
</tr>
<tr>
<td><em>Weissella</em> sp. (85)</td>
<td>BXOs</td>
<td>0.4</td>
<td>[11.1] [8.3]</td>
<td>Patel et al. (2013)</td>
</tr>
</tbody>
</table>
6. Concluding remarks

Quinoa stalks and the lignocellulose in this raw material was demonstrated as a source of cellulose, hemicellulose and saponins, and these components could be separated in an integrated process. The hemicellulose fraction was also used as a material for further refining, and production of xylooligosaccharides from the material was evaluated using enzymatic and chemical processing. The obtained oligosaccharides were used as a potential prebiotic to feed probiotic bacteria.

XOs from quinoa stalks are possible to produce, combining an alkaline method to extract the hemicellulose fraction from the raw material and in second step using enzymatic hydrolysis (Paper 1). The hemicellulose fraction from quinoa stalks consisted of glucuronoarabinoxylan, that was highly uronic acid substituted. These acidic sugars increased solubility of the extracted xylan up to 2% (w/v) which should (in theory) increase accessibility of the substrate to the water-soluble enzymes used for the hydrolysis to XOs.

The foaming observed during the optimization of the hemicellulose extraction and the intact remaining solid fraction after alkaline extraction of xylan, led to the interest in designing an integrated extraction process focusing on separation of the three components saponin, xylan and cellulose from quinoa stalks (Paper 2). Hemicellulose (xylan) and cellulose were feasible to extract and purify without affecting their structure and nature. If the saponin fraction is desired, Pressurized Hot Water Extraction (PHWE) is suggested as a method which provide this component from the stalks. However, as high temperatures were required to obtain the saponins, this affects the hemicellulose fraction. Irrespective of the parameters used in the previous steps, the cellulose obtained in the final step was a very pure fraction.

A new microbial source (Clostridium boliviense strain E-1) of hemicellulose active enzymes has also been explored (Paper 3). Two novel types of xylan-active enzymes were recombinantly produced and biochemically characterized: CbE1Xyn43 and CbE1Est1XynX. Both enzymes are active on different types of xylan including quinoa stalks glucuronoarabinoxylan. Both enzymes were shown to act on the xylose backbone and could accept some substituents in the active site. On the other hand, the GH30 enzyme from Bacteroides ovatus, that specifically requires uronic acid substituents for function, resulted in better activity using
quinoa stalks glucuronoarabinoxylan, and according to HPAEC-PAD product profile, only substituted oligosaccharides were produced, which are most likely GlucuronoXylooligosaccharides (GXOs).

Growth of (potential) probiotic strains *Bifidobacterium adolescentis* and *Weissella* sp. strain 92, using XOs from quinoa stalks glucuronoarabinoxylan as carbon source has also been demonstrated (Paper 4). The capacity of *B. adolescentis* to consume oligosaccharides in a relatively broad DP-range including substituted XOs reinforced the applicability of oligosaccharides from quinoa stalks glucuronoarabinoxylan as a functional prebiotic.

Quinoa stalks are a low-value and high-volume agricultural byproduct which in the present work has being proved as lignocellulosic source for XOs production and also with additional biorefining potential and additional possible uses (saponisins and cellulose). XOs, here produced and tested to growth *B. adolescentis* and *Weissella* sp. strain 92, demonstrate its potential (a strain selective) use as prebiotic. The continuous search for novel hemicellulose active enzymes is also of enormous importance not only for specific XOs production, but also for opening different strategies for valorization of agricultural residues.

### 6.1. Future perspectives

The present work has demonstrated the possibility to use a previous agricultural waste, i.e. Quinoa stalks as lignocellulosic source. Paper 2 has demonstrated the possibility of extraction of saponins, hemicellulose and cellulose from quinoa stalks. At lab-scale, the results showed interesting yield recoveries and highly pure products. Moreover, the extracted products preserved their natural composition. Scaling up to pilot plant scale and verify the same behaviour, extraction efficiency and purity of the products is of interest to further analyse the feasibility of using this raw material.

Paper 4 demostrate the usefulness of XOs produced from quinoa stalks glucuronoarabinoxylan as potential prebiotic. Quinoa stalks XOs showed a marked selectivity between *B. adolescentis* and *Weissella* sp. strain 92, although both strains were able to consume low DP linear XOs. *B. adolescentis* also grew on various substituted oligosaccharides, while *Weissella* sp. strain 92 did not. Expanding this apparent selectivity to non-probiotic and to pathogenic gut bacteria will complement the knowledge on the selective capacity of the quinoa XOs.

Two novel hemicellulose active enzymes from *Clostridium boliviense* strain E-1 were also studied (Paper 3). However, at least six other potential enzymes were also encoded in the genome and four of them were cloned into a production vector
(pET21a). Exploring and fully characterizing these enzymes might provide new catalytic improvements for many biorefinery application, including XOs production.
Acknowledgement

For some type of weird premonitions, it was on my “destiny” to come to Sweden. Sweden is an interesting country where I discovered many positive things and met amazing people. If I had to mention in this section to all persons who were involved in certain way in this thesis, the contents of this part would be larger than the thesis itself (and maybe more interesting).

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There are lot of things and places that I saw here, and there are even many more to see. Maybe in another time, perhaps in another life…
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“My passionate sense of social justice and social responsibility has always contrasted oddly with my pronounced lack of need for direct contact with other human beings and human communities. I am truly a ‘lone traveler’ and have never belonged to my country, my home, my friends or even my immediate family, with my wholeheart; in the face of all these ties, I have never lost a sense of distance and a need for solitude”.

- Albert Einstein -