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„Evaluation of the biological activity and the composition of extracts from two Opuntia species“

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Kurzfassung

Die beiden aus Mexiko stammenden Kakteen *Opuntia ficus-indica* und *Opuntia xoconostle* sind schon lange Teil der mexikanischen Standardküche. Sie gelten als traditionelle Heilpflanzen und finden auch heute noch Anwendung in der Volksmedizin. Präbiotika (auch Ballaststoffe genannt) dienen probiotischen Keimen als Nahrungsquelle. Dadurch fördern sie ihr Wachstum und ihre Aktivität und es resultieren positive Auswirkungen auf den menschlichen Körper, wie zum Beispiel Förderung der Verdauung. Im Rahmen dieser Diplomarbeit wurden Extrakte aus *Opuntia ficus-indica* und *Opuntia xoconostle* auf ihre chemische Zusammensetzung und vor allem präbiotische Wirksamkeit untersucht. Die Extrakte der beiden Pflanzen wurden durch präparative Größenausschlusschromatographie in mehrere Fraktionen aufgetrennt und danach deren chemischen Komponenten durch Dünnschichtchromatographie ermittelt. Um das präbiotische Potenzial zu untersuchen wurden die Proben mit Bakterienstämmen versetzt und die Wachstumskurven mit einer turbidimetrischen Messung bestimmt und verglichen. Die schleimhältigen Drogen der beiden Kakteen zeigten eine ähnliche Kohlenhydratzusammensetzung. Das präbiotische Potential hat sich bei *Opuntia ficus-indica* als größer erwiesen als bei *Opuntia xoconostle*. Fraktion 0-100 und 50-80 von *O. ficus-indica* bewirkten ein deutliches Wachstum der Bakterienstämme *L. delbrueckii ssp. bulgaricus, Bifidobacterium longum ssp. infantis, Bifidobacterium lactis HNO19*™ und *Streptococcus salivarius ssp. thermophiles.* Dieses große Potential kann in der pharmazeutischen Industrie und in Form von Nahrungsergänzungsmittel genutzt werden.

Abstract

The two nopal species *Opuntia ficus-indica* and *Opuntia xoconostle*, native to Mexico, have been used in the Mexican cuisine for a long time. They are traditional medical plants and are still used in folk medicine. Prebiotics serve probiotic strains as a source of food. In such a way they stimulate their growth and activity resulting in positive effects for the human body such as a better digestion. Within this diploma thesis the extracts of *Opuntia ficus-indica* and *Opuntia xoconostle* were evaluated regarding their chemical composition and especially their prebiotic potential. The extracts were separated into fractions using preparative size exclusion chromatography and afterwards their carbohydrate structure was examined using thin layer chromatography. To determine the prebiotic potential the carbohydrates were mixed with probiotic bacterial strains and afterwards the growth curves were measured using a turbidimetric method and they were compared. The mucilaginous samples of the plants showed a similar carbohydrate composition. The prebiotic efficacy turned out to be higher with samples of *Opuntia ficus-indica* than *Opuntia xoconostle*. Fraction 0-100 and 50-80 of *O. ficus-indica* lead to a significant growth of *L. delbrueckii ssp. bulgaricus, Bifidobacterium longum ssp. infantis, Bifidobacterium lactis HNO19*™ and *Streptococcus salivarius ssp. thermophiles.* This high potential can be used in the pharmaceutical industry and for dietary supplements.

List of Abbreviations

rpm	rotations per minutes
RT	room temperature
F	fraction
SEC	size exclusion chromatography
dp	degree of depolymerisation
MW	molecular weight
TLC	thin layer chromatography
TFA	trifluoroacetic acid
PBS	phosphate – buffered saline
MRS+	Man – Rogosa – Sharpes medium with glucose
MRS-	Man – Rogosa – Sharpes medium without glucose
MeOH	methanol
OD_{600}	optical density at 600 nm
OFI	*Opuntia ficus-indica*
XO	*Opuntia xoconostle*
STD	standard

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1. Introduction

1.1. *Opuntia ficus-indica*

Opuntia ficus-indica, also known as prickly pear or nopal cactus, is a dicotyledonous angiosperm plant and is the most common member of the *Cactaceae* family. It is characterized by its remarkable adaption to arid and semi-arid climates. [1] The plant is native to Mexico. Since ancient times it has been used in traditional medicine. *Opuntia ficus-indica* contains a high content in polyphenols exhibiting antioxidant and anti-inflammatory properties. [1] Besides it shows hypoglycemic and hypolipidemic properties. The water extract remarkably improves wound healing and has been used for the treatment of burns and wounds.[1] The cladodes and fruits from the nopal have been used as a food staple for centuries in North and Central America [24] and are being consumed as vegetables.

The sugar composition of the prickly pear was already investigated in previous studies and showing glucose, galactose, xylose, rhamnose and arabinose, galacturonic acid and glucuronic acid as main compounds.[2]

Figure 1: Leaf and fruit of *Opuntia ficus-indica*

Picture taken from: http://www.cepolina.com/photo/nature/plants/cactaceae/opuntia-ficus-indica/5/opuntia-ficus-indica-leaf-fruit.jpg

1.2. *Opuntia xoconostle*

Opuntia xoconostle, or *matudae,* is another member of the *Cactaceae* family and grows in arid and semi-arid climates. Xoconostle fruits are being considered as valuable vegetable food in Latin America [3], especially in Mexiko, where they are native. The fruit is composed of the epicarp (the skin), the mesocarp (pulp), and the endocarp (where the seeds are tightly packed together in a mucilaginous structure).[4] It is characterized by a light red-pink color, a white mesocarp and a deep red colored endocarp.[3]

The mesocarp (pulp) is the edible part of this fruit and is used as a condiment in the Mexican cuisine and in the elaboration of candies, jellies and beverages.[4]

O. xoconostle (acidic cactus pear) has recently received considerable attention regarding the *discovery* of its health-promoting potential, such as hypoglycemic, antihyperlipidemic, hypocholesterolemic, anti-inflammatory, antidiuretic, antiulcerogenic and immunostimulating activity.[5] Studies have shown that *O. xoconostle* contains a number of free sugars, such as fructose, glucose and sucrose.[5]

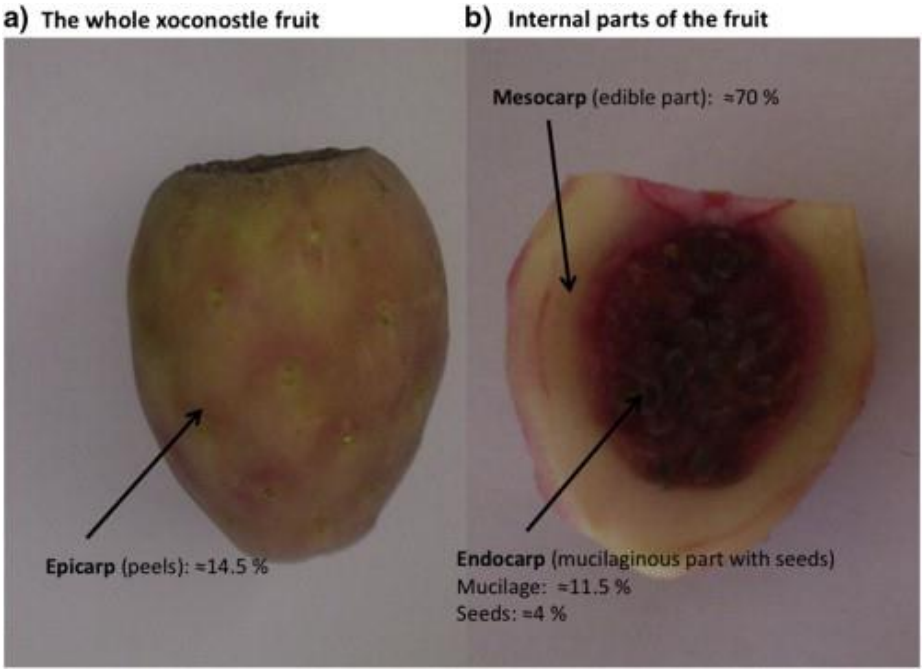

Figure 2: *Opuntia joconostle* fruits. The whole fruit (a) and its parts (b): epicarp, mesocarp (edible part) and endocarp (mucilaginous part with seeds)[3]

1.3. Probiotics

The term “Probiotics” is derived from Greek and means “prolife”.[6] It has been redefined over the years[7] and a lot of definitions developed. Basically probiotics are living microorganisms which when administered in adequate amounts confer a health benefit on the host. [8] Probiotics tend to serve as supplement to the host microflora and provide protection against various enteric pathogens, modulate and regulate the host’s immune response[9] and are also helpful in combating overweight and obesity.[10] Further they cause lower cholesterol levels, an improved lactose tolerance and even a reduced cancer risk.
They are commonly consumed as preparations with active live cultures and contain bacteria, such as *Lactobacilli* and *Bifidobacteria* that has been isolated from natural environments. [11] Sources of probiotics are for example yogurt, cheese, fermented milk and sauerkraut.

Lactobacillus acidophilus LA-5 belongs to the group of gram-positive non-sporulating facultative anaerobic rods and is a natural inhabitant of the gut. It has been used in dietary supplements and fermented milk products worldwide. The bacterium has no adverse effects on the taste or appearance of the product. Furthermore it has many probiotic features, such as ability to survive passage through the stomach and upper small intestine due to its tolerance of stomach and bile acid and resistance to digestive enzymes, [7] therefore it is suitable for oral administration.

Lactobacillus rhamnosus GG is one of the best known probiotic strains [12] and is in use in many various product applications, including several dairy-based products, such as yogurt, semi hard cheese and a few milk-free products (capsules, tablets).[7] The strain has a robust character and shows a good survival in artificial gastric juice.[12]

Lactobacillus delbrueckii ssp. bulgaricus is a Gram-positive bacterium and a member of the acidophilus group of lactobacilli. It is of vital importance to the food industry and is widely used as a starter to produce yogurt and other milk fermented products.[13]

Studies have shown that *Bifidobacterium lactis HNO19,* another probiotic strain, has the necessary prerequisite to survive gastrointestinal transit and that it is resistant to bile, low pH and digestive enzymes.[14] In addition the strain has been found to have excellent stability in supplements, dairy and non-dairy products[15] and provides additional good flavour to fermented products.[7]

Bifidobacterium longum ssp. infantis is another inhabitant of the human intestinal flora and is unique among gut bacteria in its prodigious capacity to digest and consume any human milk oligosaccharide structure. *B. infantis* grows better than other bacterial strains in the presence of human milk oligosaccharides, displays anti-inflammatory activity and is associated with increased vaccine responses. [16]

Streptococcus salivarius spp. thermophilus is a gram-positive, facultative anaerobic and spherical lactic acid bacterium. It is found in fermented milk products such as yogurt and is also used in the production of cheese.[17] As a probiotic strain *Streptococcus salivarius* can soothe the effects of an antibiotic-associated diarrhoea.[18]

1.4. Prebiotics

Prebiotics are non-digestible food ingredients that selectively stimulate the proliferation and activity of desirable bacterial populations *already resident* in the consumer's intestinal tract. Most of the known prebiotics so far are fermentable carbohydrates. [7] Popular prebiotics are fructo-oligosaccharides (FOS), lactulose and gluco-oligosaccharides (GOS). [19] The principle behind prebiotics is therefore to stimulate certain indigenous bacteria residents in the intestinal flora rather than introducing exogenous species as is the case with probiotics. [19] Requirements of prebiotics:

- Stable in food and beverages
- Heat and pH stable
- Resistant to acid, protease and bile during intestinal passage
- Stimulation of fermentative activity of the intestinal flora
- Health benefits [7]

Prebiotics can be obtained naturally from sources like vegetables and fruits consumed in our daily life.[9] They have several health benefits such as reducing the prevalence and duration of diarrhoea, soothing inflammation associated with intestinal bowel disorders and they even exert protective effects to prevent colon cancer.[20] Prebiotics are also associated with a reduced number of risk factors for cardiovascular disease and promoting weight loss. [9] Development in research over the years has led to formation of synbiotics which is a combination of probiotic and prebiotic products.[21] Synbiotics help to enhance the survival of live microbial dietary supplements in the bowel. Due to the awareness of the benefits for gut health and disease prevention, commercial interest in functional food containing synbiotics has consistently increased.[9]

2. Materials and methods

2.1. Sample preparation of *Opuntia ficus-indica* and *Opuntia xoconostle*

For the sample preparation dried cladodes from *O. ficus-indica* and *O. xoconostle* were processed. Both plants grew in the same region (Cuquio, Jalisco, Mexico), were of the same age and after collection, the thorns and glochids were removed.[24]

2.1.1. Extraction

For the extraction process the dried material was ground into a coarse powder using a mortar and a pestle. [24] This powder was extracted overnight in sequential steps with acetone, 95% methanol and deionized water.

2.1.1.1. Extraction with acetone

The nopal powder was mixed with acetone at a ratio of 20:1 and was incubated at room temperature (RT) under continuous stirring overnight. On the next day the liquid was centrifuged at 9000 rpm for 30 minutes, filtrated and evaporated on the rotavapor. The same procedure was performed with an acetone: nopal ratio of 15:1 and 10:1. At last the fractions were pooled and again evaporated. Afterwards the liquid was frozen in a cooling bath, freeze dried and stored for further use.

2.1.1.2. Extraction with 95% methanol

After the extraction with acetone the powder was transferred into a fresh methanol (95%) solution at a ratio of 10:1 – MeOH: nopal and extracted at RT overnight under stirring. The process was repeated three times. The remaining procedure was performed as described in 2.1.1.1.

2.1.1.3. Polysaccharide - extraction with water

For the extraction of polysaccharide, the nopal powder was mixed with deionized water at a ratio of 20:1 and incubated for 4 hours at 40°C under stirring. The procedure was repeated twice at a ratio of 10:1 – water: nopal. After that the method was executed as described in 2.1.1.1.

2.1.2. Mucilage fractionation

The mucilaginous water extract (also marked as Fraction 0-100) was then separated into three fractions: Fraction 0-50 (polysaccharide rich), Fraction 50-80 (oligosaccharide rich) and Fraction 80-100 (oligo – and monosaccharides). The work steps of the mucilage fractionation are shown in the figure below (Figure 3).

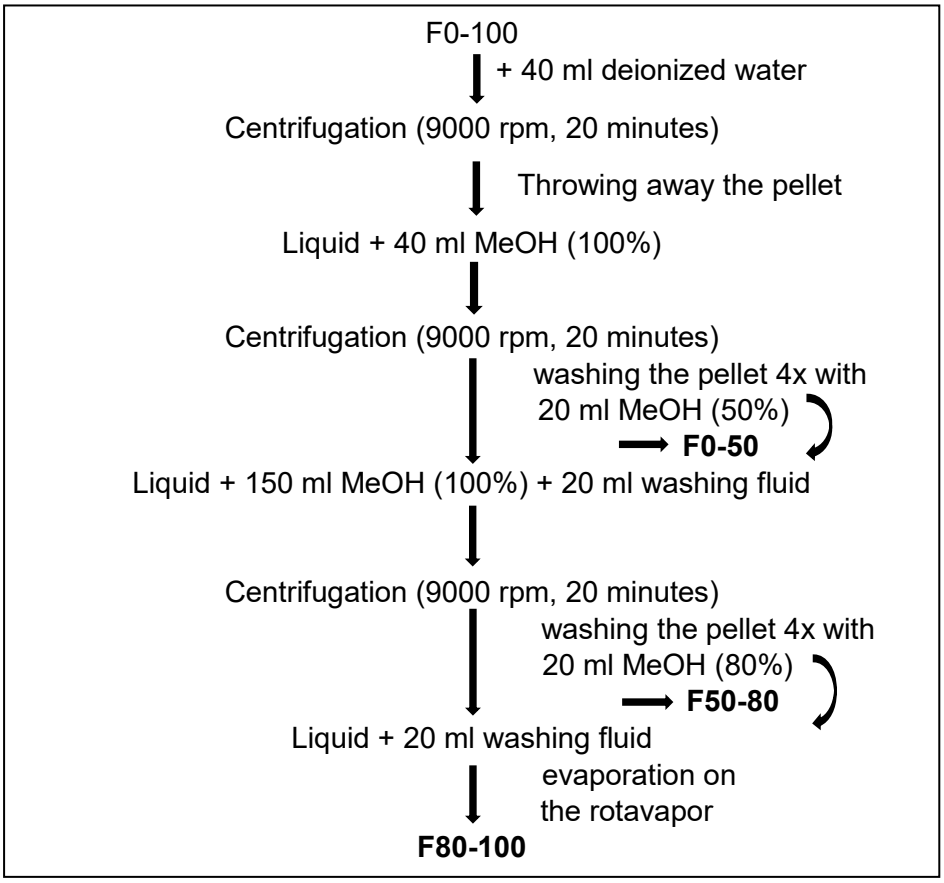

Figure 3: Depiction of the mucilage fractionation process

The obtained pellets from F0-50 and F50-80 were dissolved in deionized water, then frozen on a cooling bath and freeze. The evaporated F80-100 was frozen and lyophilized as well. The dried samples were then stored in the desiccator.

2.1.3. Separation of the extract by size exclusion chromatography

Subsequently fraction 80-100 was separated into many sub-fractions using preparative size exclusion chromatography (SEC).

The basic principle of a preparative size exclusion chromatography is that larger molecules are eluted faster than smaller molecules. Consequently the fragments with a higher degree of polymerization (dp) were eluted first and the fragments with a lower dp at the end.

The SEC was performed by a colleague using the following protocol in brief: 2 columns which were run in series; Column 1 had a diameter of 1.5 cm, a length of 107 cm and was packed with Sephacryl 200 HR; Column 2 had a diameter of 1.5 cm and a length of 124 cm; it was packed with Bio-Gel P2. The flow rate was 0.6 ml/min and the void volume was 210 ml. The process had a duration of 14 hours. Fractions of 6 ml were collected.

The column with the packing material Sephacryl 200 HR was used for the separation of molecules with a size of 1000 – 8000 kDa and with the second column with the packing material Bio-Gel P2 was able to separate molecules with a size of 100 – 1800 kDa.

Calibration of the system was performed using dextran 1 (MW 1080 kDa), dextran 12 (MW 9890 kDa), dextran 25 (MW 23500 kDa) and dextran 410 (MW 276500 kDa). The calibration curve was used for comparison with the collected fractions. On the basis of this comparison the fractions were pooled together by their molecular weight, frozen, lyophilized and stored in the desiccator.

2.2. Characterization of the composition of the extracts of *O. ficus-indica* and *O. xoconostle*

The aim was to determine the composition of carbohydrates in the extracts of dried cladodes of *O. ficus-indica* and *O.xoconostle* and then to test the extracts for prebiotic potential. The former was conducted with thin layer chromatography (TLC) and the later with turbidity measurement using Bioscreen C.

2.2.1. Characterization of the oligosaccharide and monosaccharide composition with TLC

Samples and standards were injected on thin layer chromatography plates (MERCK: HPTLC plates silica gel 60, 20 x 10 cm) using the Linomat (CAMAG) and then developed three times in a row in the mobile phase.
Two different eluents were applied:

- Eluent 1: acetonitrile: 0.3% ammonium hydroxide 17:3 for monosaccharides
- Eluent 2: 1-propanol: distilled water 7:3.5 for oligosaccharides

Detection was achieved by immersing the TLC plate into thymol sulphuric derivatization reagent (2g thymol in 100 ml methanol with 5% sulfuric acid) for 7 seconds. After drying with a hot airstream and to visualize the bands the plates were incubated in a drying oven for approximately 3 minutes at 100°C.

2.2.1.1. Monosaccharide formation through chemical hydrolysis

To determine the monosaccharide composition in the extracts of *O. ficus-indica* and *O. xoconostle* it was necessary to hydrolyse the linkages between the hetero-polysaccharides. That was achieved through chemical hydrolysis. 2 mg of the dried samples were dissolved in 200 µl 2M trifluoroacetic acid (TFA), capped hermetically and incubated at 105°C for 2 hours. After cooling, the samples were centrifuged and the TFA was vaporized. For that methanol (100%) was added and evaporated by bubbling nitrogen gas. After neutral pH of the solution was reached (to ensure that all of the TFA had been removed), the samples were redissolved in 200 µl methanol (60%). The finished samples were stored in the freezer.

2.2.1.2. Oligosaccharide composition

For evaluation of the oligosaccharide composition no hydrolysis was needed.
2 mg of the dried samples were dissolved in 100 µl deionized water and then kept in the freezer. The unhydrolyzed samples were then ran in two different mobile phases, Eluent 1 (acetonitrile: 0.3% ammonium hydroxide 17:3) and Eluent 2 (1-Propanol: water 7:3.5).

2.3. Determination of bacterial growth curves

The bacterial growth curves were determined using Bioscreen C (Labsystems Helsinki Finland). It is a technical device which combines a photometer with an incubator and bases its measurements on turbidimetry.
In the device the samples are incubated at 37°C, shaken and measured every hour at a wavelength of 600 nm. This process was conducted for 120 hours (5 days).

2.3.1. Bacterial strains

Growth curves of six different bacterial strains were determined, including three *Lactobacillus*-species, two *Bifidobacterium*-species and one *Streptococcus*-species.
The used bacterial strains are listed in Table 1.

Bacterial strain	Number
Lactobacillus acidophilus LA-5	DSM 13241
Lactobacillus rhamnosus GG	ATCC 53103
Lactobacillus delbrueckii ssp. bulgaricus	DSM 20081
Bifidobacterium longum ssp. infantis	ATCC 15697
Bifidobacterium lactis HNO19™	
Streptococcus salivarius ssp. thermophiles	ATCC 192588

Table 1: Used bacterial strains

● Cultivation of the bacterial strains

The working steps with the bacterial strains took place under sterile conditions, in a Laminar Air Flow.
On the first day the bacteria (1 ml bacteria suspension from a glycerol stock and 4 ml media *or* a spade point tip of lyophilized and 5 ml media) were incubated overnight in MRS+ media (containing glucose as carbohydrate source) at 37°C under anaerobic conditions. On the next day 1 ml of each culture was diluted with 4 ml of MRS- media (no carbohydrate source) and incubated overnight at 37°C in an anaerobic incubator.

2.3.2. Preparation of media and solutions

2.3.2.1. Preparation of the Man-Rogosa-Sharpes Media

● Man-Rogosa-Sharpes Media without glucose **(1x MRS-)**

casein peptone	10.0 g
meat extract	8.0 g
yeast extract	4.0 g
potassium phosphate dibasic	2.0 g
tween 80	1.0 g
ammonium citrate dibasic	2.0 g
sodium acetate	5.0 g
L-cysteine hydrochloride	0.5 g
magnesium sulfate	0.2 g
manganese sulfate	0.1 g

Table 2: Formulation for 1x MRS- media

The preparation of the 1x MRS- media took place by weighing the substances (Sigma Aldrich) shown in Table 2. Then the substances were dissolved in 1000 ml distilled water and autoclaved at 121°C for 20 minutes. The sterile solutions were stored at 4°C.

● Man-Rogosa-Sharpes Media with glucose **(MRS+)**

The substances (see Table 2) were weighted, dissolved in 900 ml distilled water and autoclaved at 121°C for 20 minutes. Then 100 ml of a 20% glucose solution (also autoclaved at 121°C for 120 minutes and stored at 4°C) was added under sterile conditions (Laminar Air Flow). The finished media was stored at 4°C.

● **2x MRS- media**

For a 2x MRS- media (twice the concentration) the substances as in Table 2 were weighted in, dissolved in 500 ml distilled water and autoclaved at 121°C for 20 minutes. The media was stored at 4°C.

2.3.2.2. Preparation of the carbohydrate solutions

For determination of the growth curves, carbohydrate stocks (2%) were prepared and used as standards. Eight standards were used: glucose, galactose, xylose, rhamnose, arabinose, fucose, glucuronic acid and galacturonic acid.
The carbohydrates were weighed, dissolved in water and sterile filtered. Afterwards the sterile solutions were stored at 4°C.

2.3.2.3. Sample preparation

For evaluation of the prebiotic potential, samples of *O. ficus-indica* and *O. xoconostle* were prepared the day before the test procedure. 2% solutions (dissolved in water) were produced and sterile filtered. Afterwards the stocks were stored at 4°C.

2.3.3. Determination of the effect of different fractions on the growth of probiotics

After incubation of each bacterial culture in MRS- media for 24 hours (as described in 2.3.1.), the optical density at 600 nm was determined by using a photometer (U-1100 Spectrophotometer, HITACHI). The required amount of each bacterial strain was calculated to obtain an OD_{600} of 0.1.

In the sterile workbench the calculated amount of bacteria was then transferred into an autoclaved micro test tube and centrifuged at 12500 rpm for 4 minutes. Thus the bacterial cells were agglomerated as a pellet. The supernatant was discarded and the pellet was washed by suspending it in 1 ml of 1x PBS (phosphate buffered saline) and centrifuging it at 12500 rpm for 4 minutes. This step was conducted three times. After the washing process, the pellet was dissolved in the before calculated amount of 2x MRS- media.

Each well of a sterile microtiter plate (honeycomb) was filled with a volume of 200 µl (100 µl of carbohydrate stock *or* sample stock and 100 µl of bacteria culture). The first four wells of the microtiter plate were always filled with MRS-media and MRS+ media. Negative controls of the bacterial strains were carried out as well. This was achieved by filling the well with autoclaved pure water instead of the carbohydrate or sample stock. The negative control was necessary to determine how much each bacterial culture grows without carbohydrates and to define a baseline.

The microtiter plate containing the samples was incubated at 37°C for 120 hours. The optical density at 600 nm wavelength was measured hourly after shaking the plate for 20 seconds. All samples were tested in duplicates in independent experiments. Averages and standard deviations were calculated and graphs were created.

3. Results and discussion

3.1. Fraction pooling from *O. ficus-indica* and *O. xoconostle* with preparative SEC

After the injection of fraction 80-100 of each plant into the preparative size exclusion chromatography the samples were separated into eight sub-fractions per cactus. Figure 4 and Figure 5 show the sub-fractions obtained by preparative SEC.

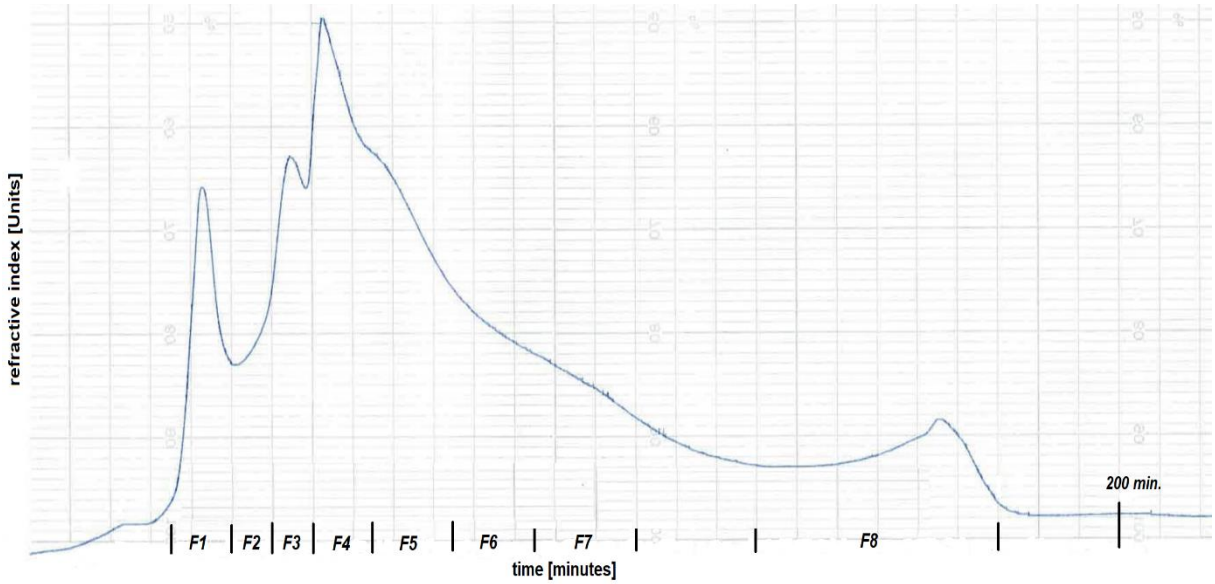

Figure 4: Preparative SEC chromatogram of fraction 80-100 of *O. ficus-indica*

The void volume of the system ends at minute 200. Fraction 8 was the first and fraction 1 was the last to elute. Fraction 4 is the one in highest concentration.

Most of the obtained sub-fractions were further analysed on their mono- and oligosaccharide composition using TLC and on their prebiotic potential with Bioscreen C.

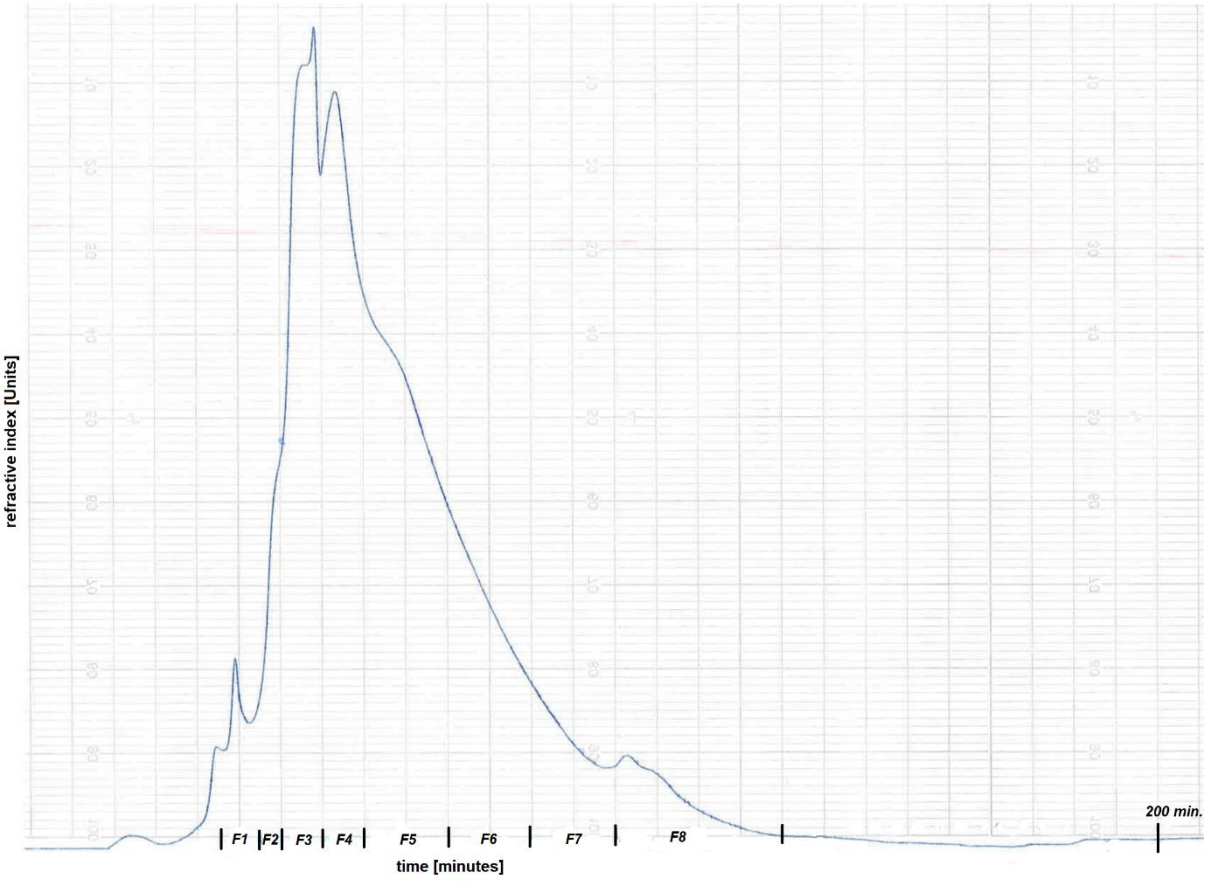

Figure 5: Preparative SEC chromatogram of fraction 80-100 of *O. xoconostle*

Figure 5 shows that fraction 3 is the one with the highest concentration. Most of those sub-fractions were further evaluated on their mono – and oligosaccharide composition and their prebiotic potential.

3.2. Chemical structure of carbohydrates from *O. ficus-indica* and *O. xoconostle*

3.2.1. Monosaccharide composition

Methanol (60%) was added to the hydrolysed samples of *O. ficus-indica* and *O. xoconostle*. Then the samples and standards were injected on the TLC plates with a volume of 2.0 µl, 3.0 µl, 5.0 µl or 7.0 µl. The same standards (STD) were used in all of the TLC plates.

For the preparation of the TLC standards the carbohydrates (as shown in Table 3) were weighted and dissolved in methanol (50%) to get a stock concentration of 10 or 30 mg/ml. The solutions were mixed and the concentration adjusted to 1 mg/ml for the neutral sugars and to 3 mg/ml for the uronic acids.

Carbohydrate	Stock concentration
Rhamnose	10 mg/ml
Xylose	10 mg/ml
Fucose	10 mg/ml
Arabinose	10 mg/ml
Mannose	10 mg/ml
Galactose	10 mg/ml
Glucose	10 mg/ml
Glucuronic acid	30 mg/ml
Galacturonic acid	30 mg/ml

Table 3: Compounds of the TLC standard used in Figure 6, Figure 7 and Figure 8.

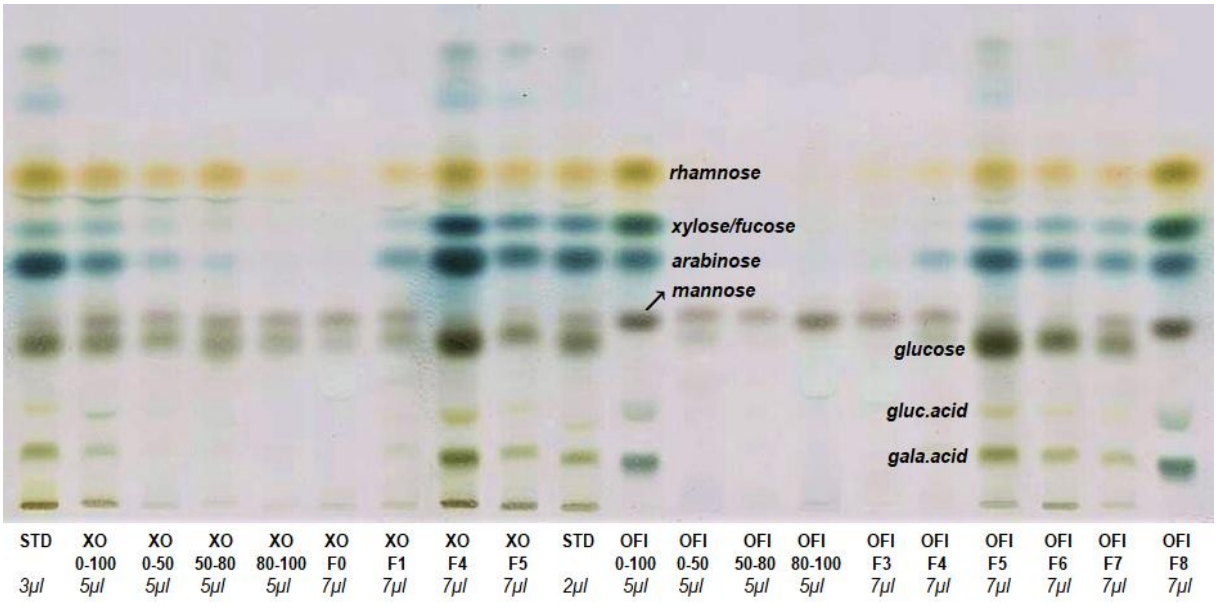

Figure 6: TLC plate of samples of *O. ficus-indica* and *O. xoconostle* after hydrolysis with 2M TFA at 105°C for 120 minutes; mobile phase: acetonitrile: 0.3% ammonium hydroxide 17:3

As seen in Figure 6 the bands of most fractions of *O. ficus-indica* are more intensively coloured than the bands of the fractions of *O. xoconostle*. This indicates that *O. ficus-indica* contains a higher concentration of carbohydrates than *O. xoconostle*.

Most bands of *O. xoconostle* are faded except for fraction 0-100, fraction 4 and 5. The band of fraction 4 of *O. xoconostle* has an especially deep colour, even stronger than the standard. This leads to the conclusion that this fraction contains higher concentrations of the carbohydrates as used in the standard.

Fraction 0-100 is the mucilaginous water extract and the only fraction that wasn't separated. Fraction 80-100 and fraction 0 are the least coloured bands of *O. xoconostle*. They only appear to contain mannose and glucose and eventually rhamnose. In general *O. xoconostle* includes the monosaccharides rhamnose, xylose, fucose, arabinose, mannose, glucose, glucuronic acid and galacturonic acid.

Many samples of *O. ficus-indica* show deep coloured bands except fraction 0-50, 50-80, 80-100 and fraction 4 and 5. Those samples seem to contain only a small concentration of mannose. Fraction 0-100 shows almost all bands of the carbohydrates used in the standard, after all it is the unfractionated mucilaginous water extract. But surprisingly it doesn't seem to contain glucose. Nevertheless the colour of the bands of fraction 0-100 of *O. ficus-indica* is more intense than the one of *O. xoconostle* which suggests that the mucilage of *O. ficus-indica* includes a higher concentration of monosaccharides than the mucilage of *O. xoconostle*.

The SEC fractions 5-8 of *O. ficus-indica* contain a considerable amount of monosaccharides. On the whole *O. ficus-indica* consists of the same carbohydrates as *O. xoconostle* which are rhamnose, xylose, fucose, arabinose, mannose, glucose, glucuronic acid and galacturonic acid.

Table 4 displays the monosaccharides contained in the mucilage (Fraction 0-100) of *O. ficus-indica* and *O. xoconostle.*

Carbohydrate	*Opuntia ficus-indica*	*Opuntia xoconstle*
Glucose		✓
Rhamnose	✓	✓
Xylose	✓	✓
Fucose	✓	✓
Mannose	✓	✓
Arabinose	✓	✓
Galacturonic acid	✓	✓
Glucuronic acid	✓	✓

Table 4: Monosaccharide composition of the mucilage (Fraction 0-100) of *O. ficus-indica* and *O. xoconostle*

3.2.2. Oligosaccharide composition

The dried samples were dissolved in deionized water and plotted on the TLC plate with a volume of 2 µl, 3 µl, 5 µl and 7 µl.

For the determination of the mono– and oligosaccharide composition two different mobile phases were used.

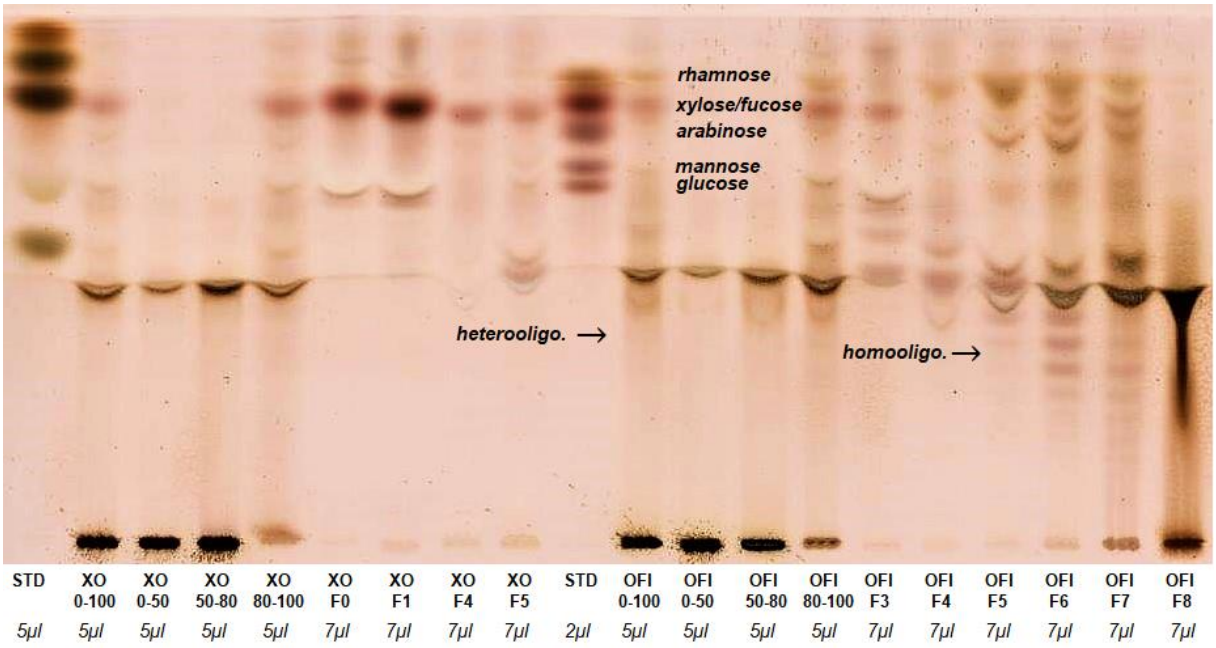

Figure 7: TLC plate of the fractions of *O. ficus-indica* and *O. xoconostle* displaying the contained oligosaccharides; mobile phase: 1-Propanol: distilled water 7:3.5

Figure 7 displays the mono, di- and oligosaccharides included in *O. ficus-indica* and *O. xoconostle*. As an eluent the mixture of 1-propanol: distilled water 7:3.5 was used. In the lower half of the TLC plate homo - and heterooligosaccharides are shown, in the upper half mono - and disaccharides.

Well-defined bands seem to indicate homooligosaccharides as seen for fraction 5, 6 and 7 of *O. ficus-indica*. To determine the monosaccharide components of the homooligosaccharides further analysis would be necessary. Fraction 0-100, 0-50, 50-80 and 80-100 of *O. ficus-indica* show blurred bands leading to the suggestion of the presence of heterooligosaccharides. To determine the composition of the heterooligosaccharides further tests are needed.

O. xoconostle seems to contain heterooligosaccharides as well as seen for fraction 0-100, 0-50, 50-80 and 80-100. Apparently it doesn't contain homooligosaccharides. Some fractions, for example Fraction 0 – Fraction 5 of *O. xoconostle,* appear to have no oligosaccharides at all but only mono – and disaccharides.

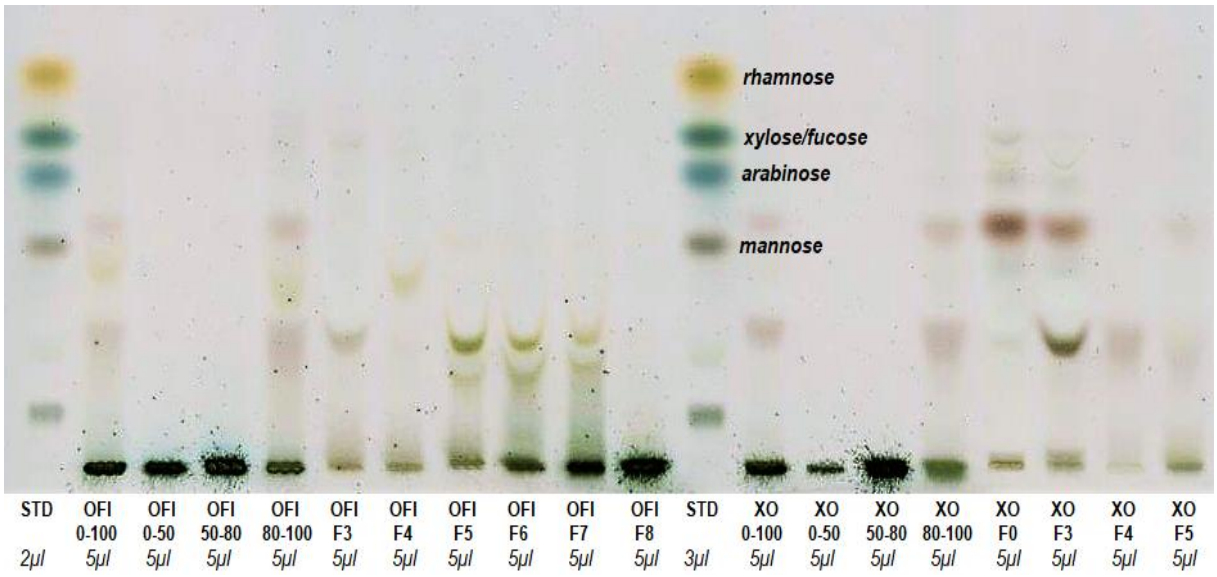

Figure 8: TLC plate of samples of *O. ficus-indica* and *O. xoconostle* without hydrolysis; mobile phase: acetonitrile: 0.3% ammonium hydroxide 17:3

For the TLC plate shown in Figure 8 the same eluent was applied as for the TLC plate used for the determination of monosaccharides (Figure 6): acetonitrile: 0.3% ammonium hydroxide 17:3. As shown in Figure 8 some monosaccharides are visible, for example at fraction 5 – 7 of *O. ficus-indica*.

The carbohydrate composition of the fractions of *O. ficus-indica* and *O. xoconostle* was quite similar but *O. ficus-indica* showed a higher concentration of monosaccharides than *O. xoconostle*. Surprisingly the mucilaginous fraction (fraction 0-100) of *O. ficus-indica* seems to contain no glucose even though glucose and fructose are the most characteristic sugars in *O. ficus-indica*.[22] This could be explained that the glucose got destroyed or was removed during the extraction procedure of this fraction, because other sub-fractions showed the content of glucose. Both plants included oligosaccharides but only *O. ficus-indica* appeared to include homo - and heterooligosaccharides. *O. xoconostle* showed a composition of simply heterooligosaccharides.

3.3. Bacterial growth

For each assay negative controls consisting of Man-Rogosa-Sharpes media without glucose (MRS-) and Man-Rogosa-Sharpes media with glucose (MRS+) diluted with autoclaved pure water have been conducted. Since all negative controls tested negative, those aren't shown in the following diagrams.

3.3.1. Bacterial growth with fractions of *O. ficus-indica*

Every sample - or standard-solution that caused more turbidity increase than the negative control of the bacterial strain (thick red line) has a possible prebiotic potential. The higher the OD_{600} of a sample and the faster the maximum OD_{600} reached, the higher is its prebiotic potential.

● *Lactobacillus acidophilus*

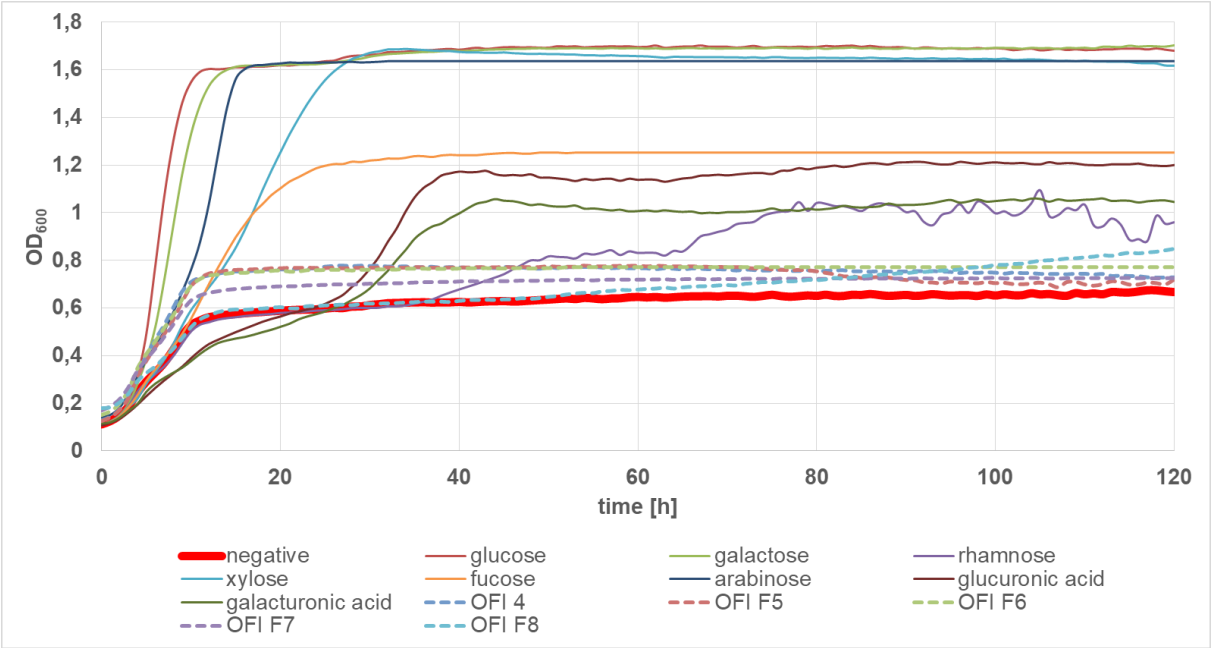

Figure 9: Bacterial growth curves of *L. acidophilus* with fractions from *O. ficus-indica*

Figure 9 shows the growth of *L. acidophilus* after the fractions of *O. ficus-indica* have been added. Glucose, galactose, xylose and arabinose were fermented well by *L. acidophilus*. The growth curves reached a maximum with an OD_{600} of 1.6 and 1.7 and the stationary phase was reached after approximately 15 hours. Fucose, galacturonic acid, glucuronic acid and rhamnose were metabolized by the bacteria as well but the bacterial strain didn't display such a high OD_{600} as in the presence of the other carbohydrates. Most growth curves in the presence of the fractions were characterized by a slight increase of the OD_{600}. The samples of *O. ficus-indica* led to a maximum OD_{600} of 0.8. In general fraction 4, 5 and 6 caused the biggest rise of the bacterial growth curve and the stationary phase was reached after 15 hours.

● *Lactobacillus rhamnosus GG*

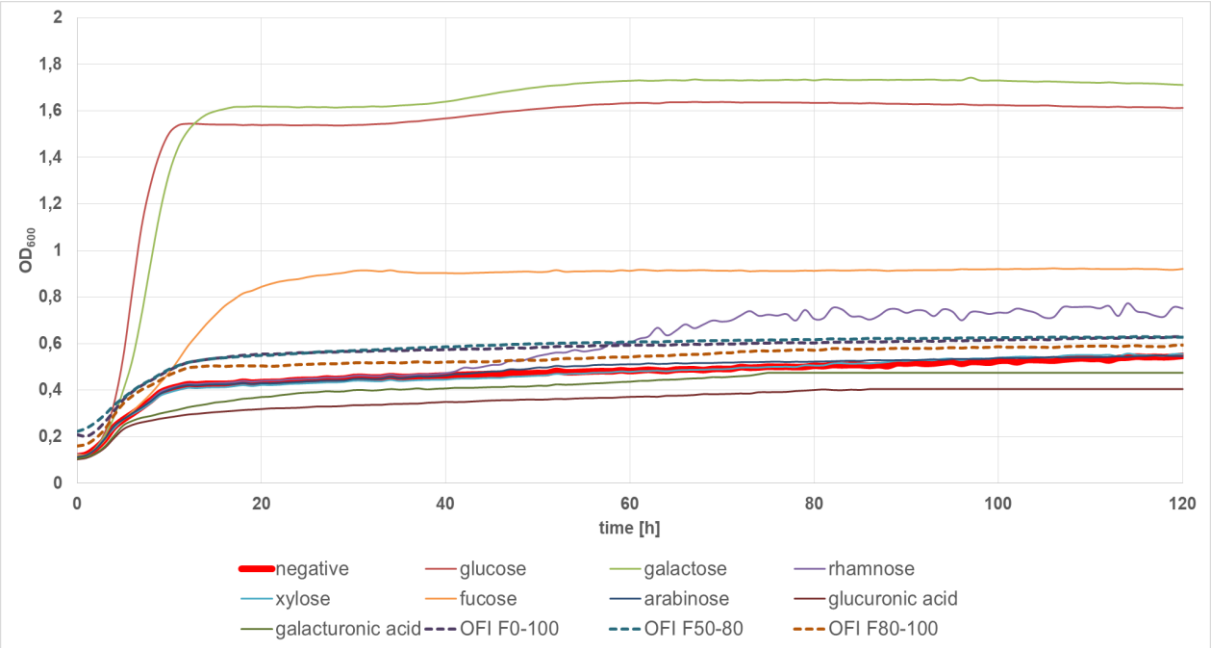

Figure 10: Bacterial growth curves of *L. rhamnosus GG* with fractions from *O. ficus-indica*

Figure 10 presents the growth of *Lactobacillus rhamnosus GG* after the addition of fractions from *O. ficus-indica*.

Glucose and galactose were fermented best leading to a maximum OD_{600} of 1.7. The stationary phase was reached after 15 hours. Fucose was utilized as well, the maximum OD_{600} was at 0.9. The growth curve in the presence of rhamnose displayed once more an inconsistent growth of the bacterial strain. Glucuronic acid and galacturonic acid appear to be unfermentable for *L. rhamnosus GG.*

Fraction 0-100 and 50-80 led to an equal increase of the growth curve. The maximum OD_{600} reached 0.6. Fraction 80-100 didn't cause as a high increase as the other fractions, the growth curve remained slightly below. The maximum OD_{600} was 0.5. The bacterial growth curves of all fractions of *O. ficus-indica* attained its plateau (stationary phase) after 14 hours.

● *Lactobacillus delbrueckii ssp. bulgaricus*

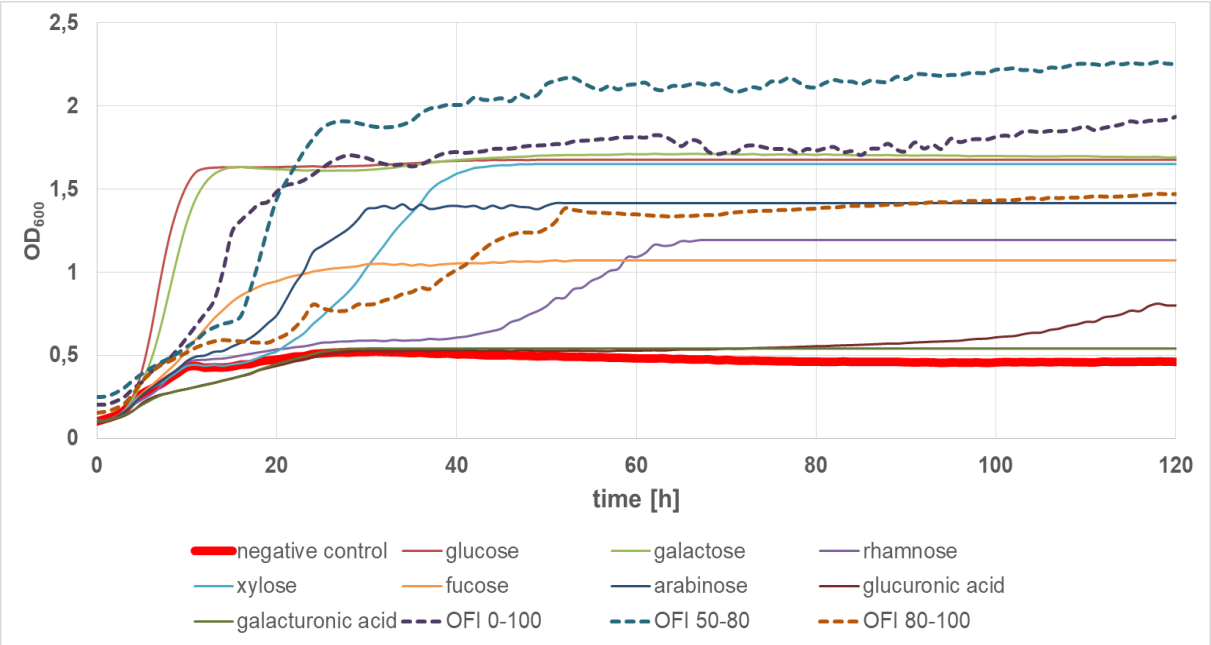

Figure 11: Bacterial growth curves of *L. delbrueckii ssp. bulgaricus* with fractions from *O. ficus-indica*

Figure 11 displays the growth of *L. delbrueckii ssp. buglaricus* after fractions of *O. ficus-indica* have been added.

The bacterial strain showed the highest growth in the presence of fraction 50-80. This fraction was therefore better fermented than the positive control glucose. It caused a maximum OD_{600} of 2.3 while glucose only led to an OD_{600} of 1.7. Fraction 0-100 could achieve a higher bacterial growth than glucose as well. It is unlikely for a nopal fraction to cause a better bacterial growth than the positive control glucose concluding that the fractions have good prebiotic potential but the stock concentrations (2%) were probably too high. Fraction 80-100 led to an uneven growth curve and a delayed maxiumum OD_{600} of 1.5 after 120 hours.

The bacteria showed a similar growth behaviour in the presence of galactose than in the presence of glucose. Xylose was utilized similar to glucose and galactose but the stationary phase was achieved after 40 hours and therefore much later than the stationary phase of glucose and galactose. Arabinose and fucose led to an average growth of *L. delbrueckii ssp. bulgaricus*. Rhamnose caused a more even growth curve than usual but the plateau was reached quite late after 60 hours. Galacturonic acid and glucuronic acid were hardly metabolised by the bacterial strain.

● *Bifidobacterium longum ssp. infantis*

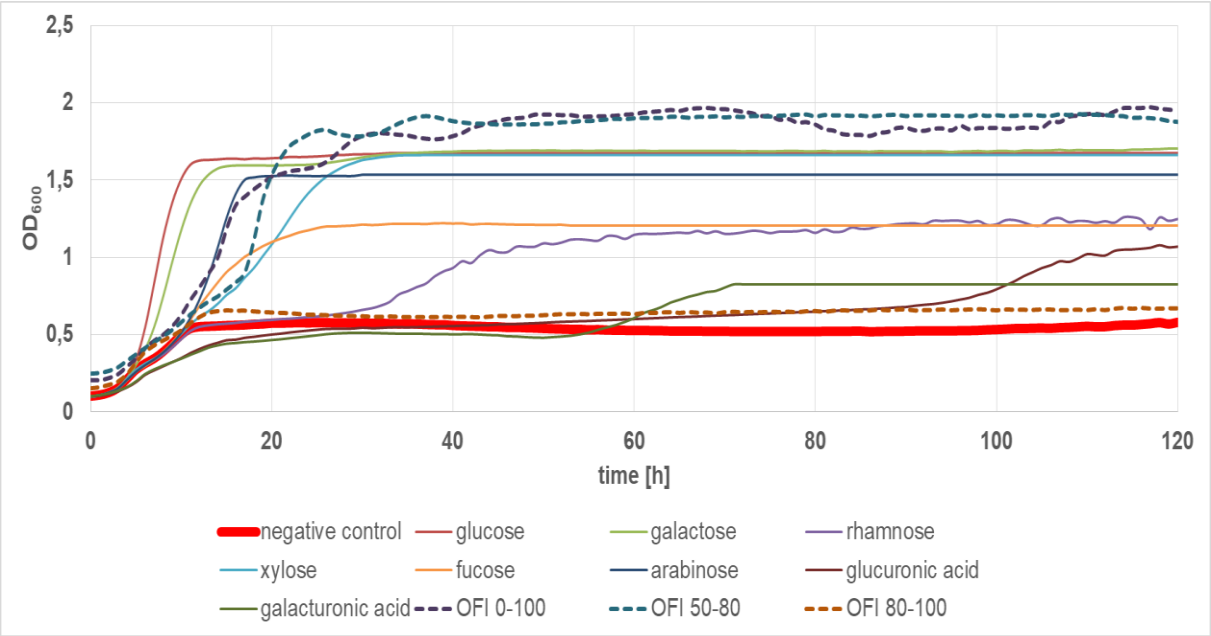

Figure 12: Bacterial growth curves of *Bifidobacterium longum ssp. infantis* with fractions from *O. ficus-indica*

Figure 12 shows the growth curves of *Bifidobacterium longum ssp. infantis* with fractions of *O. ficus-indica*.

Fraction 0-100 and 50-800 led to the best bacterial growth. The growth curves surpassed with a maximum OD_{600} of 2.0 the growth curves of the bacteria in the presence of glucose and galactose which indicates a too concentrated sample solution. Glucose, galactose and xylose showed a similar fermentation by the bacterial strain even though the stationary phase was reached later in the presence of xylose. Arabinose, fucose and rhamnose were utilized mediocrely.

The growth curve with fraction 80-100 run slightly over the growth curve of the negative control. The fraction displayed therefore no noteworthy growth of the bacteria. Glucuronic acid and galacturonic acid led to no significant growth of *Bifidobacterium longum ssp. infanits*.

● *Bifidobacterium lactis HNO19™*

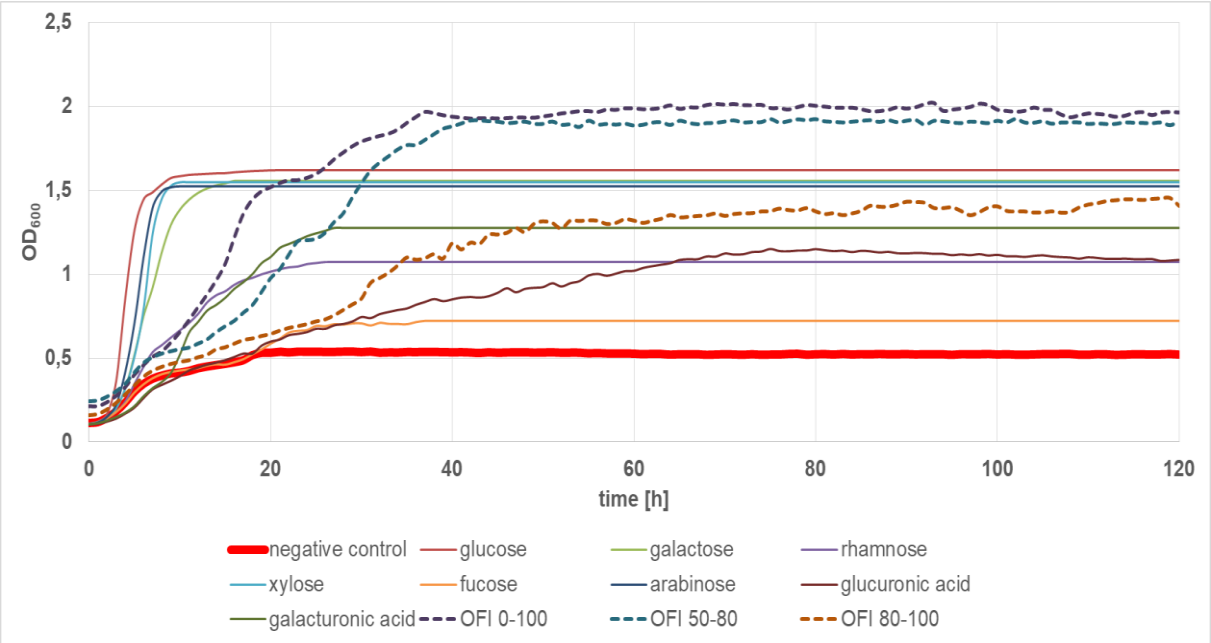

Figure 13: Bacterial growth curves of *Bifidobacterium lactis HNO19™* with fractions from *O. ficus-indica*

Figure 13 presents the growth curves of *Bifidobacterium lactis HNO19™* after fractions of *O. ficus-indica* have been added.

Fraction 0-100 and 50-80 caused once more the best bacterial growth. The growth curves surpassed once more the curves of the bacteria in presence of glucose and galactose which leads to the conclusion that the stock concentration of the sample solution was too high. The maximum OD_{600} was 2.0 after 40 hours.

Glucose, galactose, arabinose and xylose showed a similar fermentation by the bacteria. Glucuronic acid and galacturonic acid were utilized better than usual by the bacterial strain. Rhamnose led to a maximum OD_{600} of 1.0 while fucose caused no noteworthy growth of the bacteria.

Fraction 80-100 was metabolised quite well and even resulted in an OD_{600} of 1.5 but the growth curve increased rather slowly.

● *Streptococcus salivarius ssp. thermophiles*

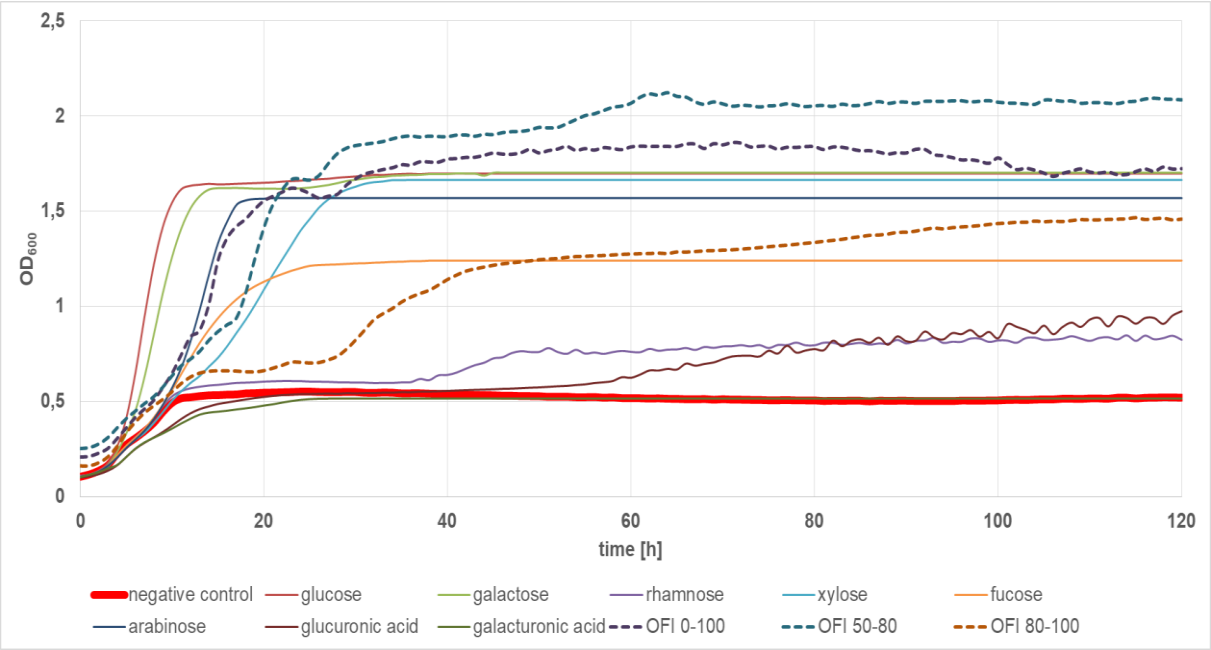

Figure 14: Bacterial growth curves of *Streptococcus salivarius ssp. thermophiles* with fractions from *O. ficus-indica*

Figure 14 graphs the growth curves of *Streptococcus salivarius ssp. thermophiles* after the addition of fractions of *O. ficus-indica*.

Fraction 0-100 and 50-80 caused again the highest bacterial growth curves. The growth curve in the presence of fraction 50-80 even surpassed a maximum OD_{600} of 2.0 while the OD_{600} in the presence of fraction 0-100 stayed beyond 2.0 concluding that the sample - solutions were too concentrated.

Glucose, galactose and xylose showed again similar fermentation by the bacterial strain. Arabinose led to a slightly smaller growth of the bacterial strain than glucose and galactose. Fucose was utilized mediocrely. Rhamnose and glucuronic acid caused the bacterial strain to grow alike unevenly and the curves didn't rise much above the negative control. Galacturonic acid led to no bacterial growth at all.

Fraction 80-100 caused a good growth of *Streptococcus salivarius ssp. thermophiles*. The curve took longer to reach its stationary phase (100 hours) but could attain an OD_{600} of almost 1.5.

3.3.2. Bacterial growth with fractions of *O. xoconostle*

● *Lactobacillus acidophilus*

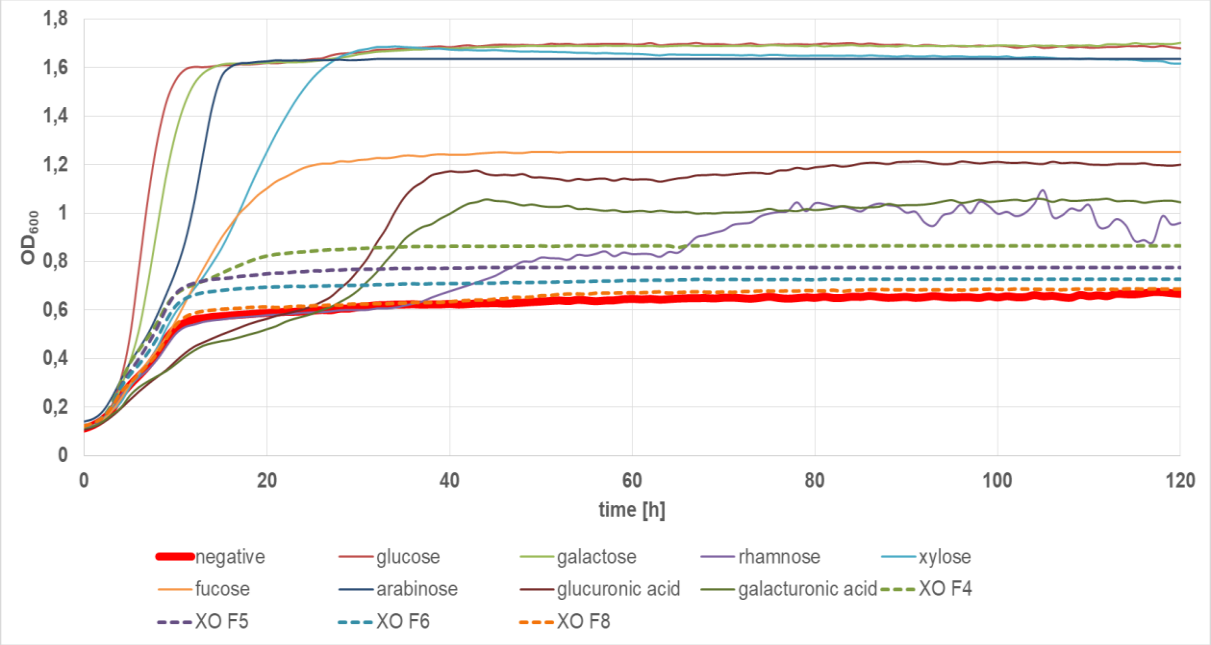

Figure 15: Bacterial growth curves of *L. acidophilus* with fractions from *O. xoconostle*

The diagram pictured in Figure 15 shows the growth of *Lactobacillus acidophilus* with fractions of *O. xoconostle*.

Glucose, galactose, arabinose and xylose were utilized the most by the bacterial strain. They caused a maximum OD_{600} of 1.7. Fucose led to a mediocre increase of the bacteria and the growth curve attained an OD_{600} of 1.2. The rise of the curve in the presence of glucuronic acid and galacturonic acid proceeded slower than in the presence of the other standards, but they caused the bacteria to gain a maximum OD_{600} around 1.1. In the presence of rhamnose the growth curve developed unevenly as usual.

All fractions of *O. xoconostle* except for fraction 8 caused a rise above the negative control and appear to be utilized by *L. acidophilus*. Fraction 4 led to the highest OD_{600} around 0.9 and the stationary phase was reached after 20 hours. The growth curve in the presence of Fraction 8 runs congruent with the negative control, the fraction seems to have no effect on this bacterial strain.

● *Lactobacillus rhamnosus GG*

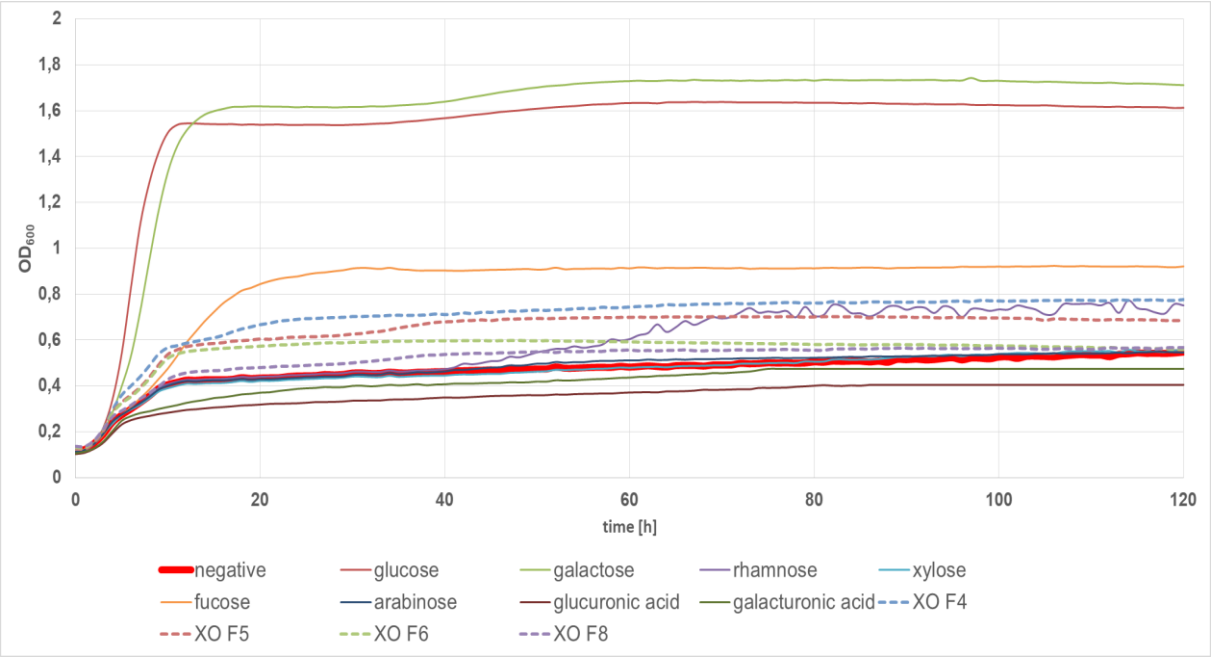

Figure 16: Bacterial growth curves of *L. rhamnosus GG* with fractions from *O. xoconostle*

Figure 16 graphs the growth of *Lactobacillus rhamnosus GG* with fractions of *O. xoconostle*.

Glucose and galactose were utilized the best and led to a peak at an OD_{600} of 1.7.

Fucose caused an OD_{600} of 0.9 while glucuronic acid and galacturonic acid couldn't be fermented by the bacterial strain. Arabinose caused no change as well. In the presence of rhamnose the bacterial growth curve achieved an OD_{600} of 0.8.

Fraction 4 and 5 of *O. xoconostle* led to the best bacterial growth of all the fractions with an OD_{600} of 0.7. The growth curves with addition of fraction 6 and 8 proceed slightly below with a maximum OD_{600} of 0.6.

● *Lactobacillus delbrueckii ssp. bulgaricus*

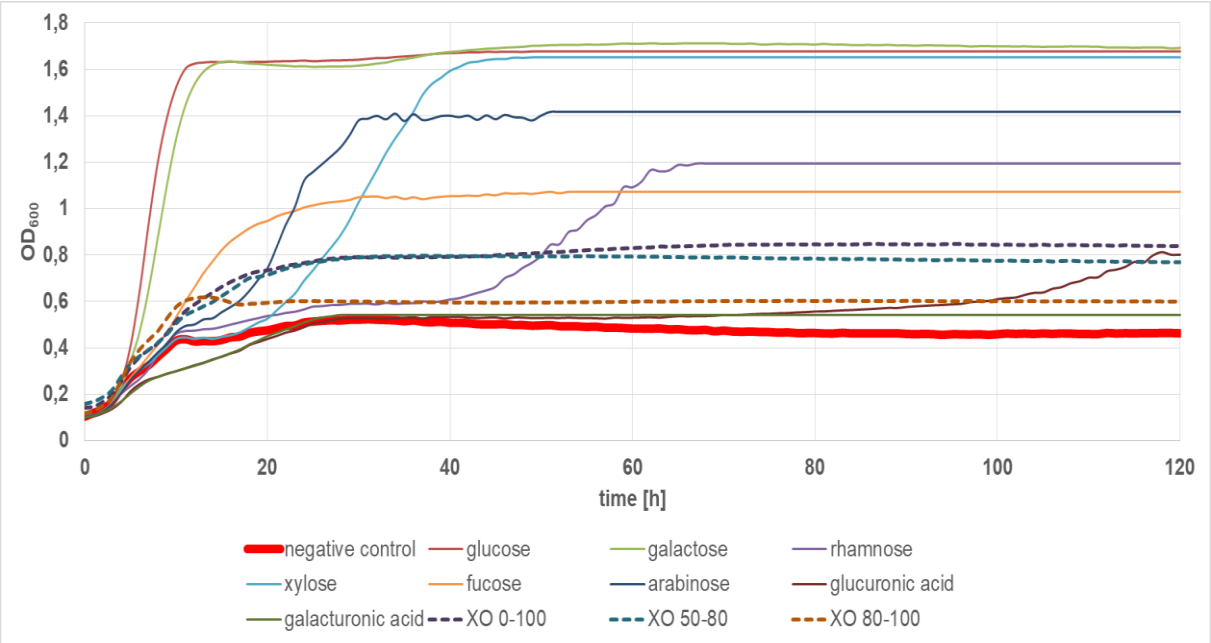

Figure 17: Bacterial growth curves of *L. delbrueckii ssp. bulgaricus* with fractions from *O. xoconostle*

Figure 17 depicts the growth curves of *L. delbrueckii ssp. bulgaricus* after the addition of fractions of *O. xoconostle*.

Glucose and galactose were metabolised as usual by bacterial strains. They caused the highest OD_{600} of 1.7. Xylose led to a high OD_{600} as well but the stationary phase occurred much later. In the presence of arabinose the curve grew inconsistently while fucose caused an even increase of the bacterial growth curve. Rhamnose led to a late rise of the curve, the plateau was attained after 65 hours. Glucuronic acid and galacturonic acid had apparently no or rather hardly any impact on *L. delbrueckii ssp. bulgaricus*.

Fraction 0-100 and 50-80 led to a similar increase of the bacterial growth curve, the maximum OD_{600} was around 0.8. The growth curve in the presence of fraction 80-100 ran slightly over the negative control and caused therefore no considerable growth of the bacterial strain.

● *Bifidobacterium longum ssp. infantis*

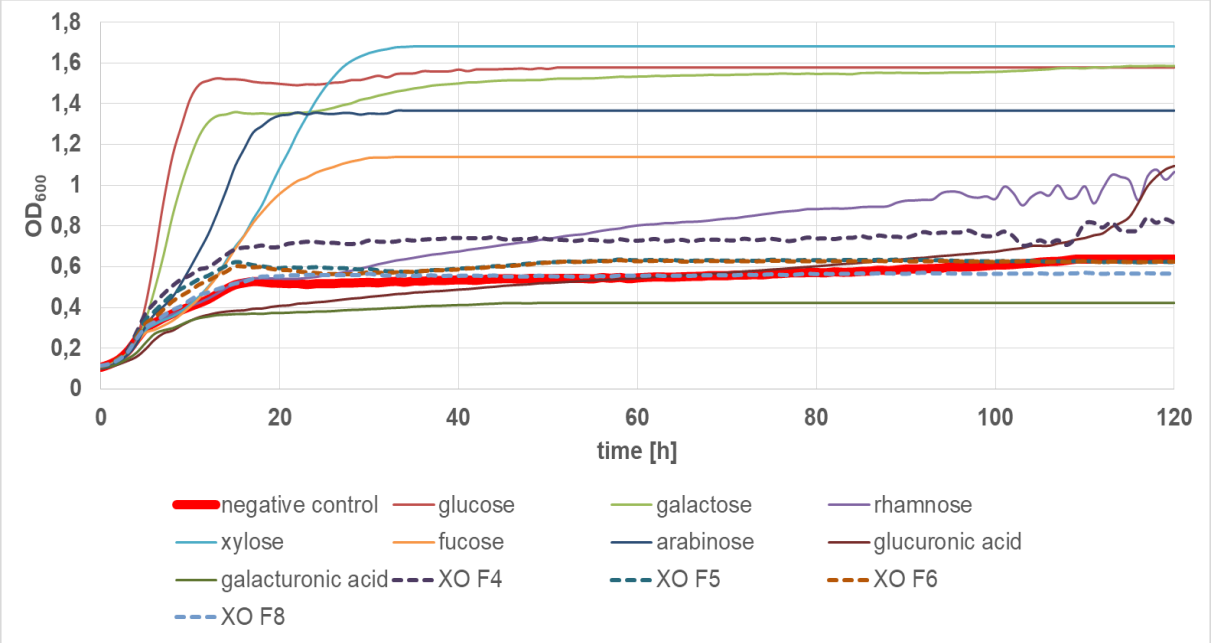

Figure 18: Bacterial growth curves of *Bifidobacterium longum ssp. infantis* with fractions from *O. xoconostle*

Figure 18 illustrates the growth of *Bifidobacterium longum ssp. infantis* with fractions of *O. xoconostle* as source of food.

Of the standard – solutions xylose showed this time the most fermentation while glucose and galactose were metabolised slightly less. Arabinose depicted a good utilization as well and caused a maximum OD_{600} of 1.4. The growth curve in the presence of fucose is located in the middle range. Rhamnose led once more to an irregular growth of the bacteria while fraction 4 of *O. xoconostle* caused the same growth curve just slightly below. Glucuronic acid and galacturonic were hardly or not fermented by the bacteria.

The remaining fractions of O. xoconostle caused no significant growth of *Bifidobacterium longum ssp. infantis*. The growth curves were all located around the negative control.

● *Bifidobacterium lactis HNO19™*

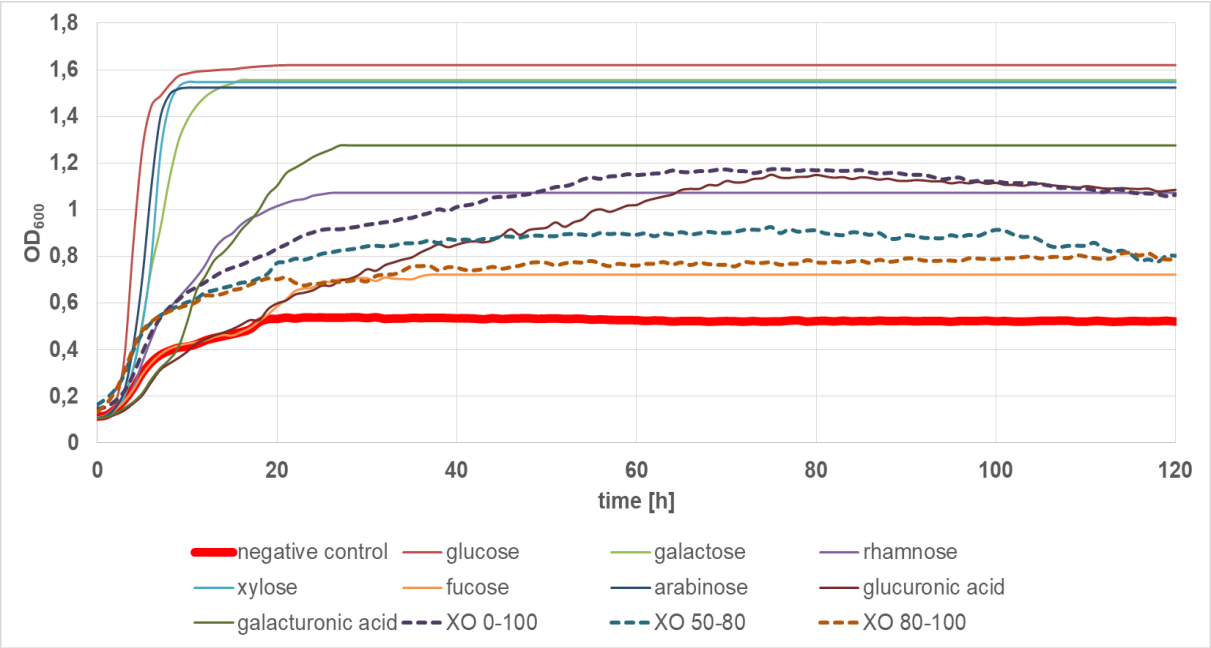

Figure 19: Bacterial growth curves of *Bifidobacterium lactis HNO19™* with fractions from *O. xoconostle*

Figure 19 presents the growth of *Bifidobacterium lactis HNO19™* with fractions of *O. xoconostle* as source of nutrition.

The positive control glucose caused the highest and steadiest growth curve. Galactose, xylose and arabinose were also fermented well. Surprisingly galacturonic acid caused a good bacterial growth with an OD_{600} 1.3. Glucuronic acid was utilized pretty well too but the curve took longer to increase. Rhamnose led to an average OD_{600} around 1.0 while the growth curve in the presence of fucose stayed quite low and parallel to the negative control.

Of all the fractions of O. xoconostle fraction 0-100 led to the best growth of the bacterial strain with an OD_{600} of 1.2. Fraction 50-80 caused an average growth and fraction 80-100 was fermented slightly less. The OD_{600} was around 0.7.

● *Streptococcus salivarius ssp. thermophiles*

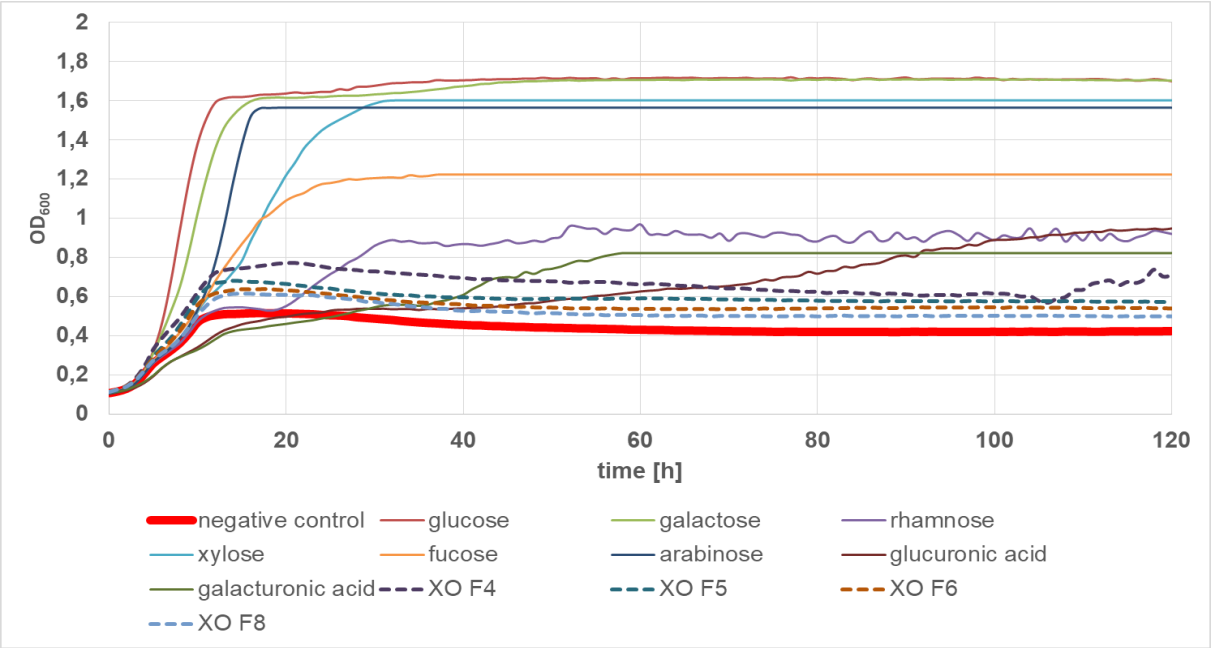

Figure 20: Bacterial growth curves of *Streptococcus salivarius ssp. thermophiles* with fractions from *O. xoconostle*

Figure 20 graphs the growth curves of *Streptococcus salivarius ssp. thermophiles* after the addition of fractions of *O. xoconostle*.

Glucose and galactose displayed almost the same fermentation by the bacteria. Arabinose and xylose were also utilized well while the growth curves in the presence of fucose and rhamnose were located in the middle range. Glucuronic acid and galacturonic acid caused a good increase of the bacterial growth curve.

The fractions of *O. xoconostle* led to a similar growth of the curve but fraction 4 caused once more the highest progression. The highest OD_{600} was 0.8. All samples were utilized by the bacteria and the curves ascended over the negative control. The least fermented fraction was fraction 8 with an OD_{600} of 0.5.

3.3.3. Percentage bacterial growth with fractions of *O. ficus-indica* and *O. xoconostle*

To present the results of the growth curves more clearly the findings were summarised in tables. The percentage of growth of each standard – and sample – solution was calculated by setting the growth of glucose (positive control) to 100%. The other percentages were then determined in relation to glucose. Table 5 shows the percentage of growth of the standards, Table 6 of the samples of *O. ficus-indica* and *O. xoconostle*.

	L.acidophilus LA-5	*L.rhamnosus GG*	*L.delbrue.ssp.bulg.*	*Bifido.long.ssp.inf.*	*Bifido.lact.HNO19™*	*Strept.sal.ssp.therm.*
Negative control	35%	28%	27%	30%	28%	28%
Glucose	100%	100%	100%	100%	100%	100%
Galactose	100%	107%	102%	102%	96%	100%
Rhamnose	62%	43%	69%	74%	64%	46%
Xylose	99%	29%	98%	99%	95%	98%
Fucose	72%	53%	62%	71%	41%	71%
Arabinose	94%	29%	83%	91%	94%	92%
Glucuronic acid	69%	20%	45%	42%	69%	55%
Galacturonic acid	59%	24%	28%	46%	78%	26%

Table 5: Percentage bacterial growth of the standard – solutions in relation to glucose (100%)

	L.acidophilus LA-5	*L.rhamnosus GG*	*L.delbrue.ssp.bulg.*	*Bifido.long.ssp.inf.*	*Bifido.lact.HNO19™*	*Strept.sal.ssp.therm.*
Negative control	35%	28%	27%	30%	28%	28%
OFI F0-100	33%	28%	110%	112%	120%	104%
OFI F50-80	31%	27%	128%	107%	112%	118%
OFI F80-100	34%	29%	84%	33%	86%	82%
OFI F4	41%	33%	39%	38%	41%	36%
OFI F5	41%	32%	38%	36%	46%	33%
OFI F6	39%	33%	36%	34%	46%	33%
OFI F7	35%	28%	34%	33%	43%	31%
OFI F8	42%	28%	31%	38%	33%	34%
XO F0-100	42%	57%	45%	48%	68%	47%
XO F50-80	45%	46%	40%	46%	50%	42%
XO F80-100	38%	39%	32%	45%	45%	78%
XO F4	47%	43%	53%	50%	44%	41%
XO F5	41%	38%	41%	36%	45%	36%
XO F6	38%	31%	35%	35%	39%	33%
XO F8	36%	28%	32%	31%	45%	31%

Table 6: Percentage bacterial growth of the samples of *O. ficus-indica* and *O. xoconostle* in relation to glucose (100%)

As shown in Table 5 *L. rhamnosus GG*, *L. delbrueckii ssp. bulgaricus* and *Bifidobacterium longum ssp. infantis* attained a percentage growth of over 100% only in the presence of galactose.
Xylose also caused a good increase with all bacterial strains except for *L. rhamnosus GG*. Arabinose led to good growth levels of some bacterial strains as well. The remaining standard-solutions caused a mediocre percentage of bacterial growth.

Table 6 displays that fraction 0-100 and 50-80 of *O. ficus-indica* are the best fermented samples. They led to a percentage growth over 100% (relative to glucose) of *L. delbrueckii ssp. bulgaricus, Bifidobacterium longum ssp. infantis, Bifidobacterium lactis HNO19*™ and *Streptococcus salivarius ssp. thermophiles* and were thereby better fermented than glucose. Fraction 80-100 of *O. ficus-indica* was utilized above average by *L. delbrueckii ssp. bulgaricus, Bifidobacterium lactis HNO19*™ and *Streptococcus salivarius ssp. thermophiles*. The sample caused percentage bacterial growth of over 80%.

Fraction 80-100 of *O. xoconostle* led to a percentage growth of 78% of *Streptococcus salivarius ssp. thermophiles* and is therefore the best fermented fraction of *O. xoconostle*. In the presence of the other samples the six bacterial strains showed a mediocre growth.
In general the fractions of *O. ficus-indica* were better utilized than the fractions of *O. xoconostle*.

The examination of the prebiotic efficacy of the two nopal species revealed that some fractions of *O. ficus-indica* have a high prebiotic potential. The growth curves in the presence of some samples increased more than the growth curve in the presence of the positive control glucose concluding in the theory that the samples have a promising prebiotic potential but that the stock concentrations of 2% were probably too high. Those effective fractions were Fraction 0-100 and 50-80 and the bacterial strains were *L. delbrueckii ssp. bulgaricus, Bifidobacterium longum ssp. infantis, Bifidobacterium lactis HNO19*™ and *Streptococcus salivarius ssp. thermophiles.*
This potential can be used in the pharmaceutical or food industry in the future, especially since the consumer's interest in the use of natural ingredients is creating a growing trend leading to more research into the development of natural products.[22]

O. ficus-indica is widely spread and therefore cheap for production. The fruit has adelicious, sweet taste due to its high sugar content and low acidity (pH >4.5.)[23] which makes it even more interesting for the food industry.

O. xoconostle exhibited a smaller prebiotic efficacy. Only fraction 80-100 caused good results with the probiotic strain *Streptococcus salivarius ssp. thermophiles*. It lead to a significant growth of the bacterial strain and could therefore be used as a dietary supplement.

To determine if *O. ficus-indica* and *O. xoconostle* really are suitable for food products or supplements further analysis are necessary. The effective fractions of *O. ficus-indica* have to be retested in different stock concentrations to determine if their prebiotic efficacy really is this promising as shown in the assays. Gastric juice and bile resistance still has to be ensured as well as enzyme stability and the extent of absorption through the organism has to be evaluated. This is the only way it can be assured that the plants have the possibility to unfold their full prebiotic efficacy and that they could be valuable products for pharmaceutical and food industry.

4. Summary

The plants *O. ficus-indica* and *O. xoconostle,* which have been evaluated within the context of this diploma thesis, showed a promising prebiotic potential.
Especially fractions of *O. ficus-indica* lead to a significant growth of the bacterial strains *L. delbrueckii ssp. bulgaricus, Bifidobacterium ssp. bulgaricus, Bifidobacterium lactis HNO19*™ and *Streptococcus salivarius ssp. thermophiles*.
O. xoconostle showed a potential prebiotic efficacy only in the presence of *Streptococcus salivarius ssp. thermophiles*.
The growth curves were measured with a turbidimetric method. Standard – solutions were added as well and caused a strain dependent fermentation. To ensure the effectiveness of those natural products it is necessary to run further tests and analysis concerning their stability to gastric – and bile juice and their bioavailability.
The carbohydrate compositions of the two nopal species showed no major differences. The oligosaccharide compositions of *O. ficus-indica* and *O. xoconostle* is still unknown and should be evaluated further in the future. Moreover it is essential to determine the structure and composition of each fraction to comprehend why some fractions show biological activity and some do not.
Furthermore a greater outcome at the extraction and separation procedures should be in focus as well.

5. References

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6. List of Figures and Tables

6.1. List of Figures

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7. Appendix

Figure 21 shows the growth of *L. acidophilus* with fractions of *O. ficus-indica.*

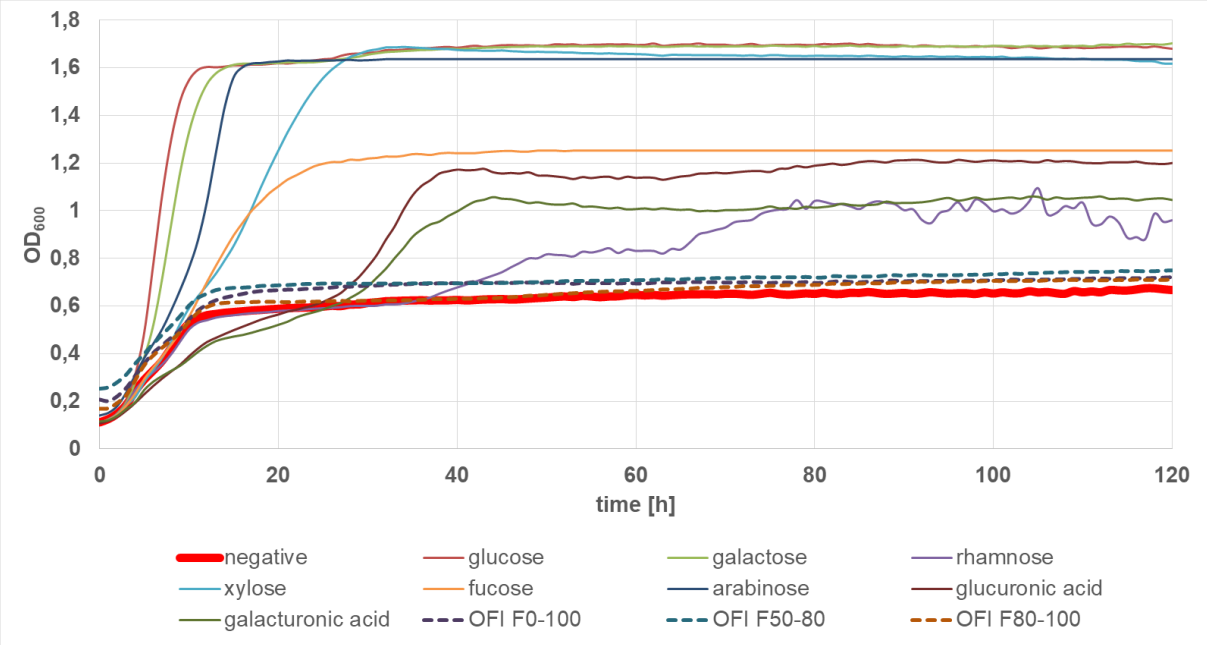

Figure 21: Bacterial growth curves of *L. acidophilus* with fractions from *O. ficus-indica*

Figure 22 depicts the growth of *L. acidophilus* with fractions of *O. xoconostle.*

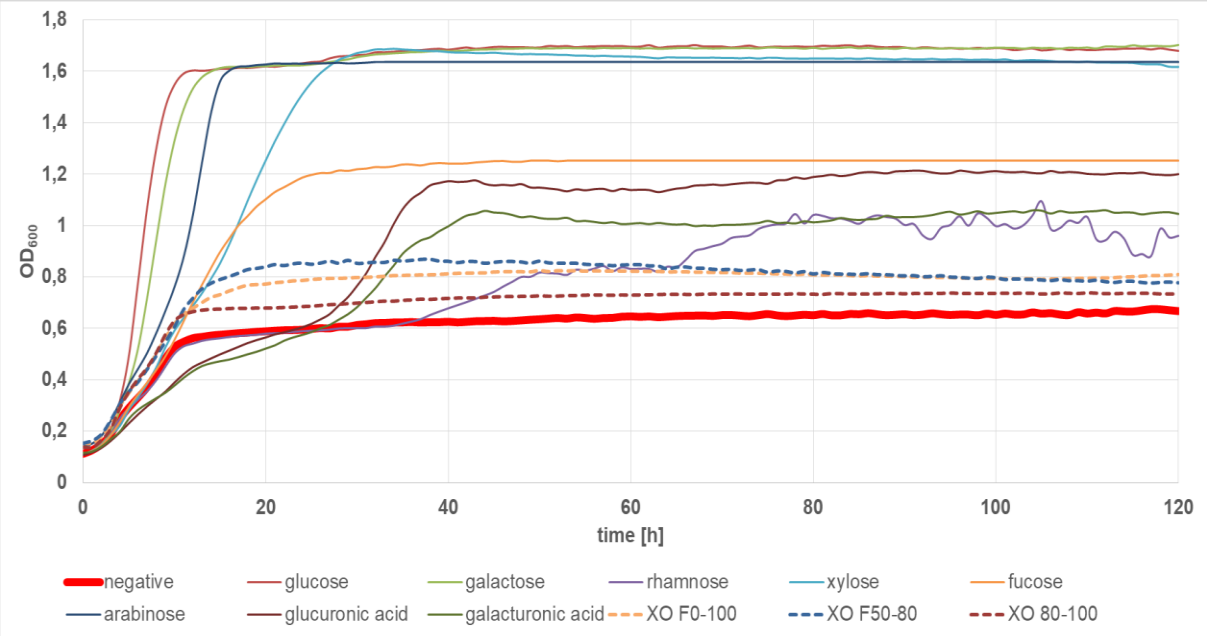

Figure 22: Bacterial growth curves of *L. acidophilus* with fractions from *O. xoconostle*

Figure 23 shows the growth of *L. rhamnosus GG* with fractions of *O. ficus-indica*.

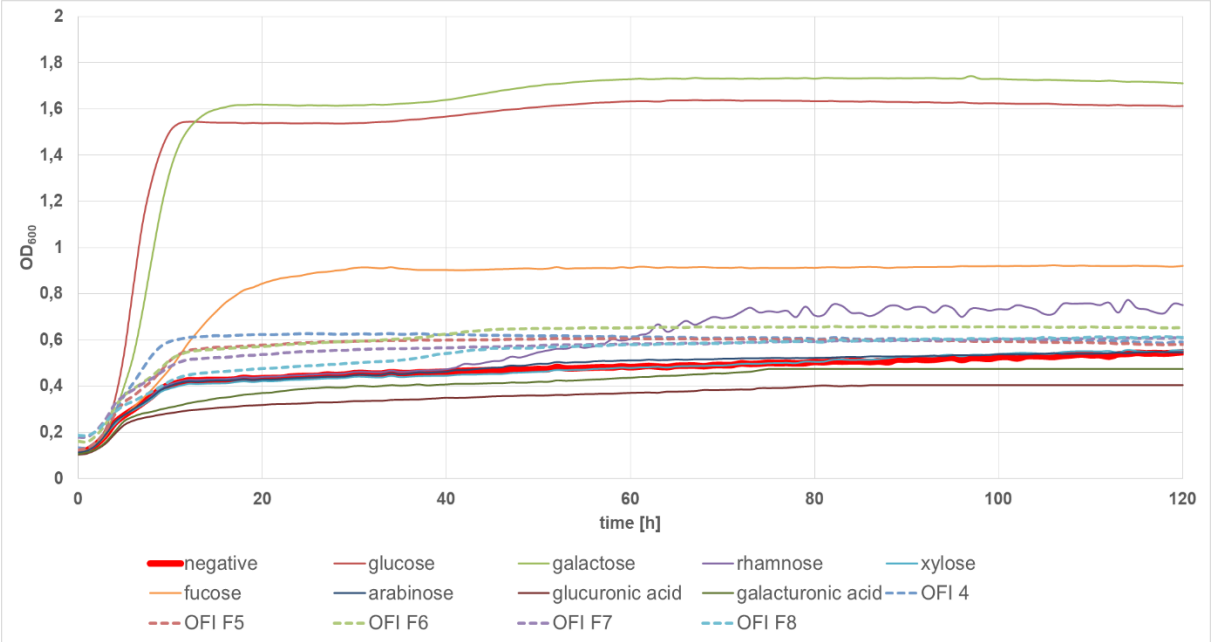

Figure 23: Bacterial growth curves of *L. rhamnosus GG* with fractions from *O. ficus-indica*

Figure 24 graphs the growth of *L. rhamnosus GG* with fractions of *O. xoconostle*.

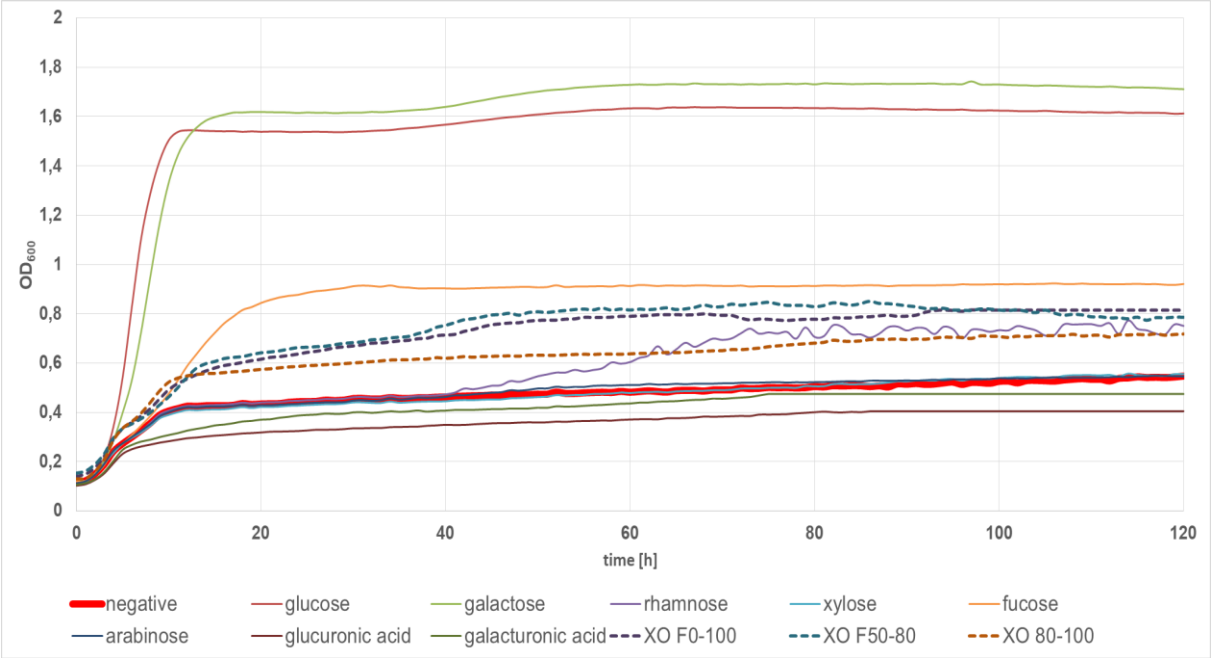

Figure 24: Bacterial growth curves of *L. rhamnosus GG* with fractions from *O. xoconostle*

Figure 25 shows the growth of *L. delbrueckii ssp. bulgaricus* with fractions of *O. ficus-indica*.

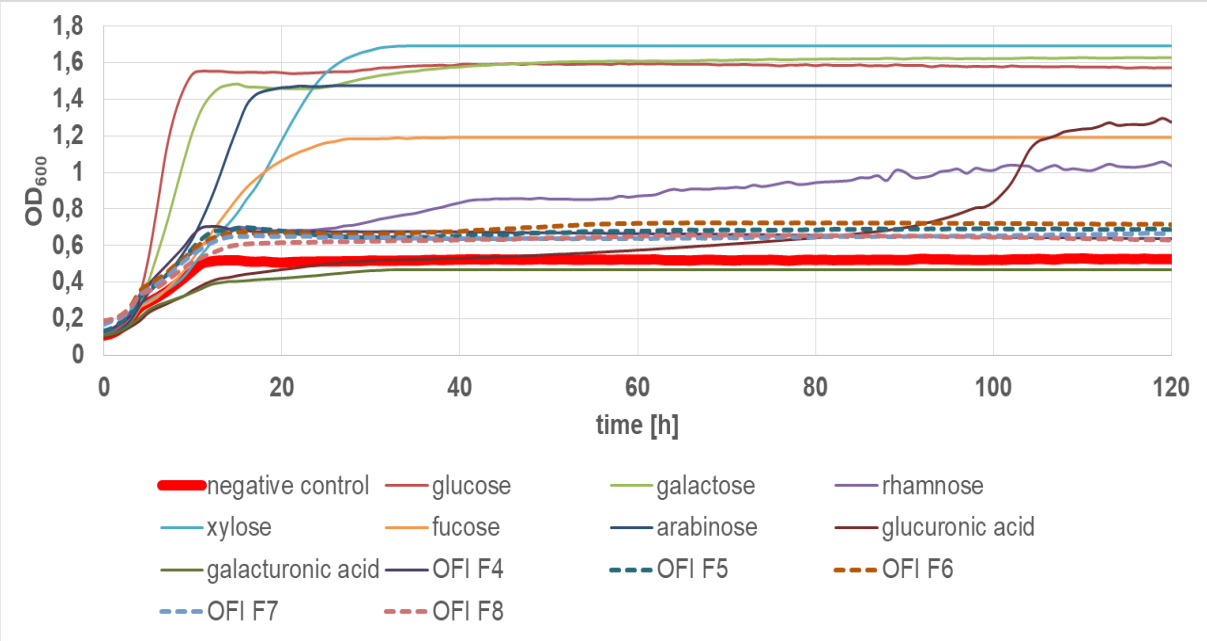

Figure 25: Bacterial growth curves of *L. delbrueckii ssp.bulgaricus* with fractions from *O. ficus-indica*

Figure 26 graphs the growth of *L. delbrueckii ssp. bulgaricus* with fractions of *O. xoconostle*.

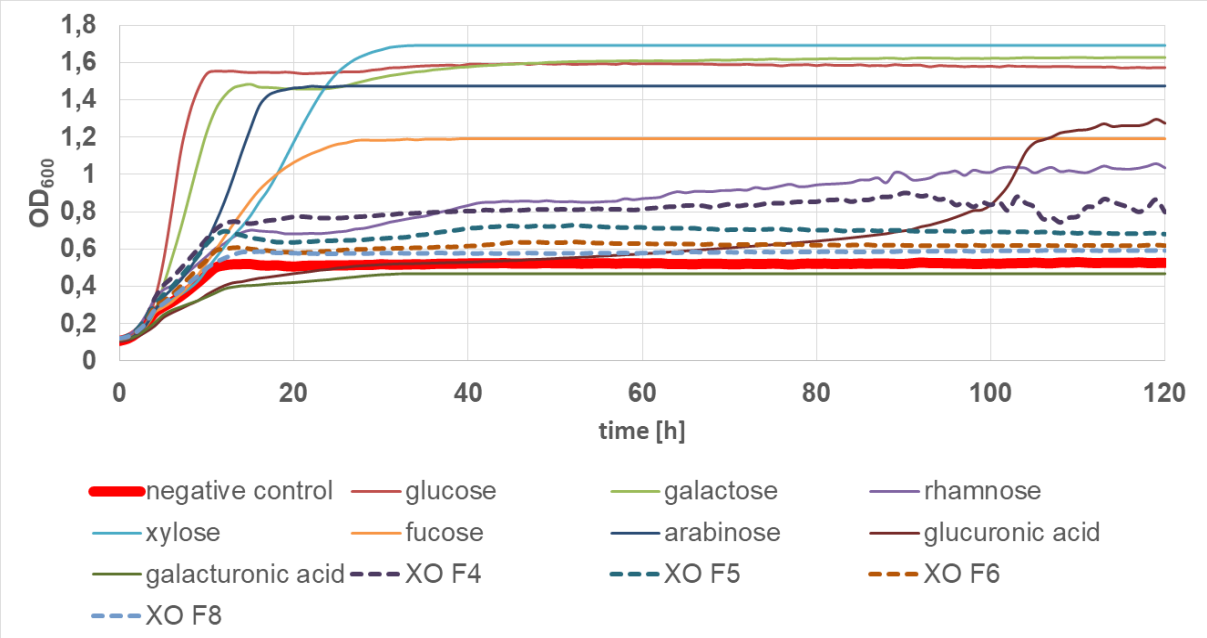

Figure 26: Bacterial growth curves of *L. delbrueckii ssp.bulgaricus* with fractions from *O. xoconostle*

Figure 27 displays the growth of *Bifidobacterium longum ssp. infantis* with fractions of *O. ficus-indica*.

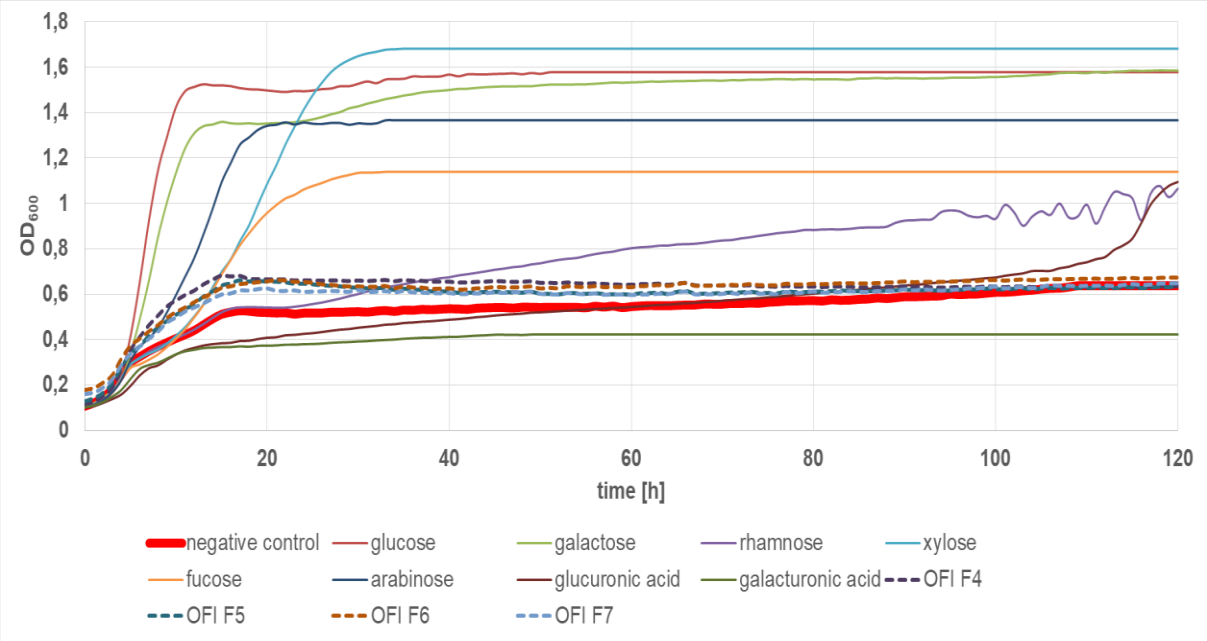

Figure 27: Bacterial growth curves of *Bifidobacterium longum ssp. infantis* with fractions from *O. ficus-indica*

Figure 28 shows the growth curves of *Bifidobacterium longum ssp. infantis* with fractions of *O. xoconostle*.

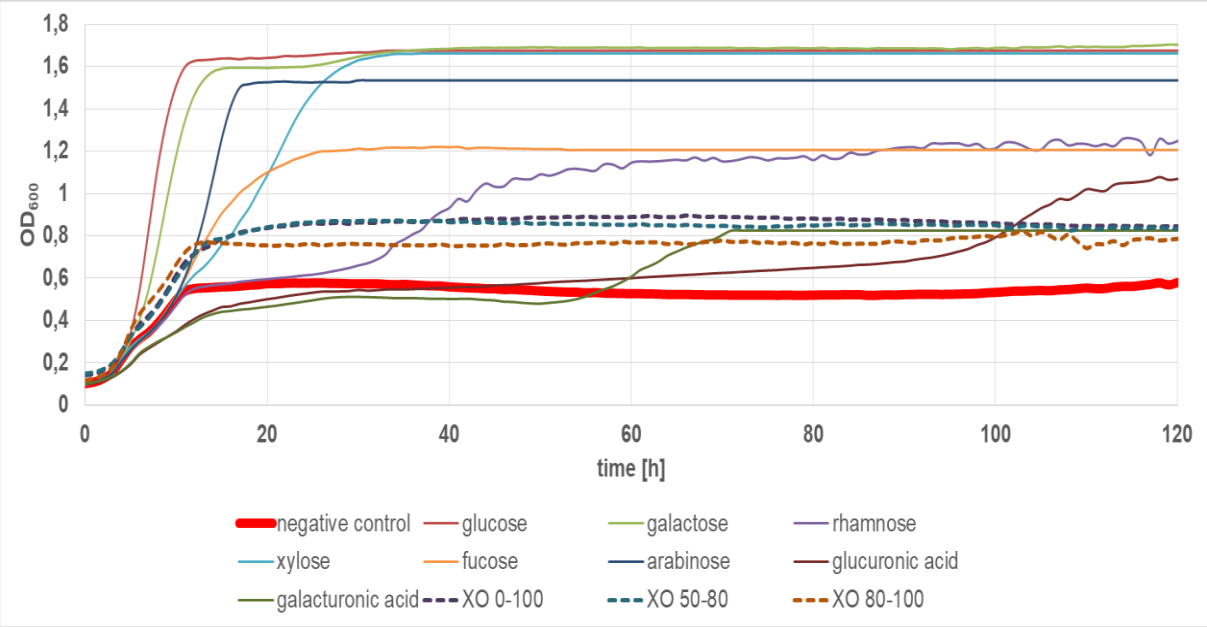

Figure 28: Bacterial growth curves of *Bifidobacterium longum ssp. infantis* with fractions from *O. xoconostle*

Figure 29 depicts the growth of *Bifidobacterium lactis HNO19™* with fractions of *O. ficus-indica*.

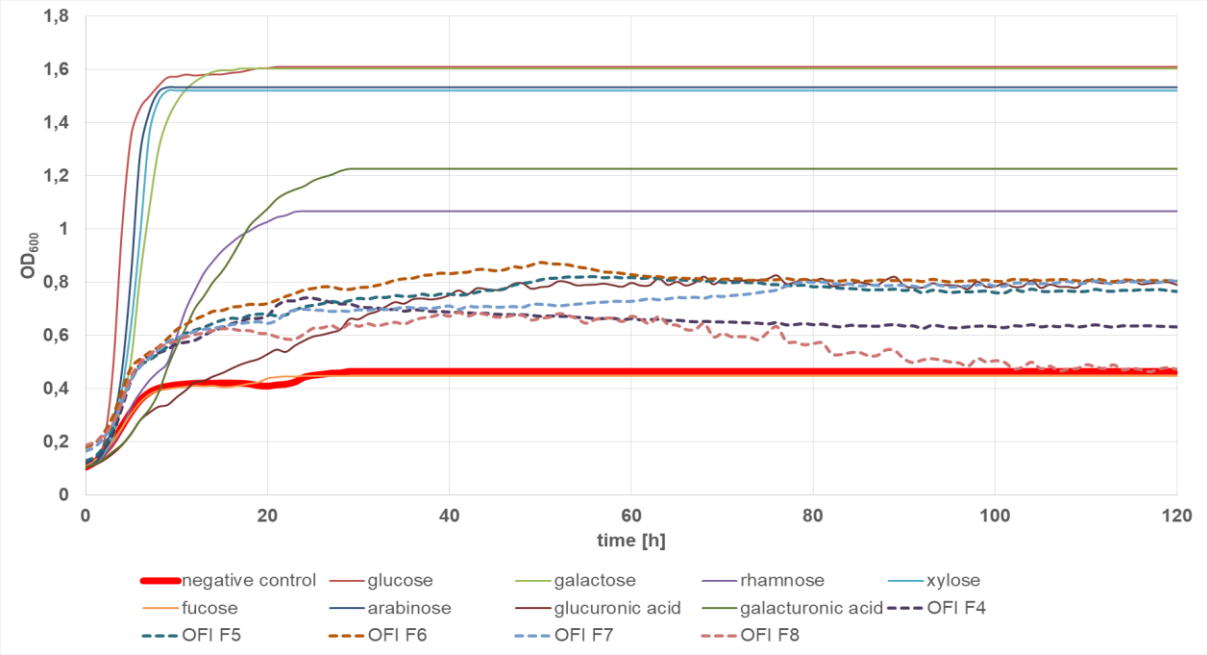

Figure 29: Bacterial growth curves of *Bifidobacterium lactis HNO19™* with fractions from *O. ficus-indica*

Figure 30 shows the growth of *Bifidobacterium lactis HNO19™* with fractions of *O. xoconostle*.

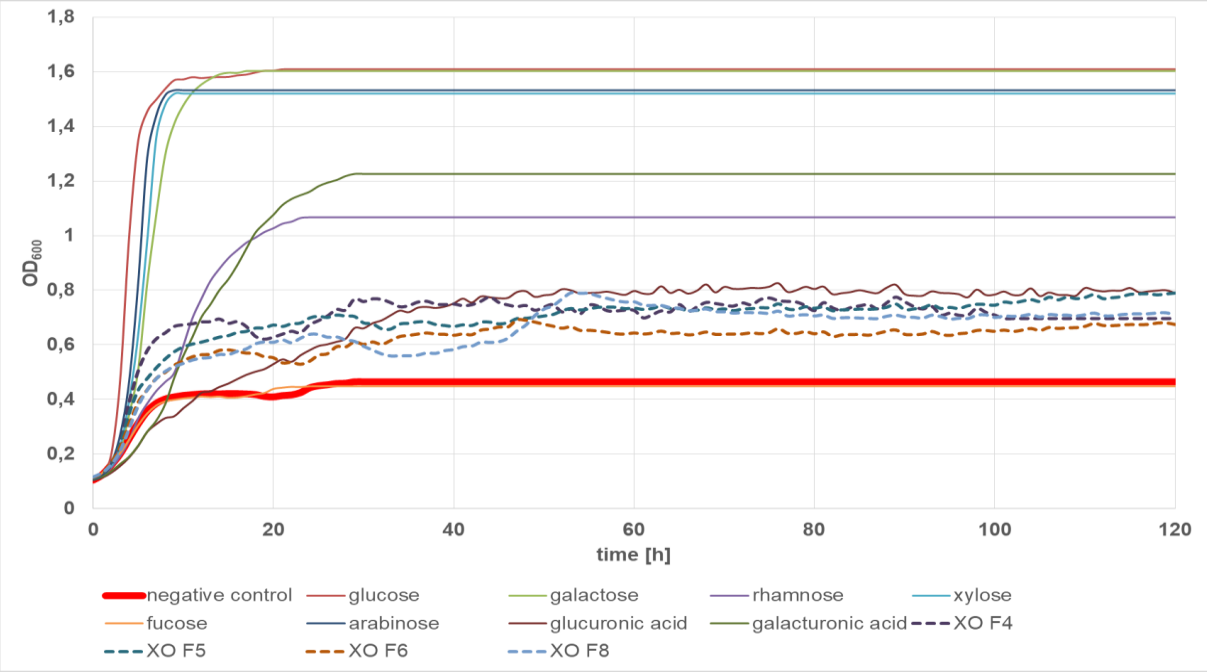

Figure 30: Bacterial growth curves of *Bifidobacterium lactis HNO19™* with from *O. xoconostle*

Figure 31 shows the growth curves of *Streptococcus salivarius ssp. thermophiles* with fractions of *O. ficus-indica*.

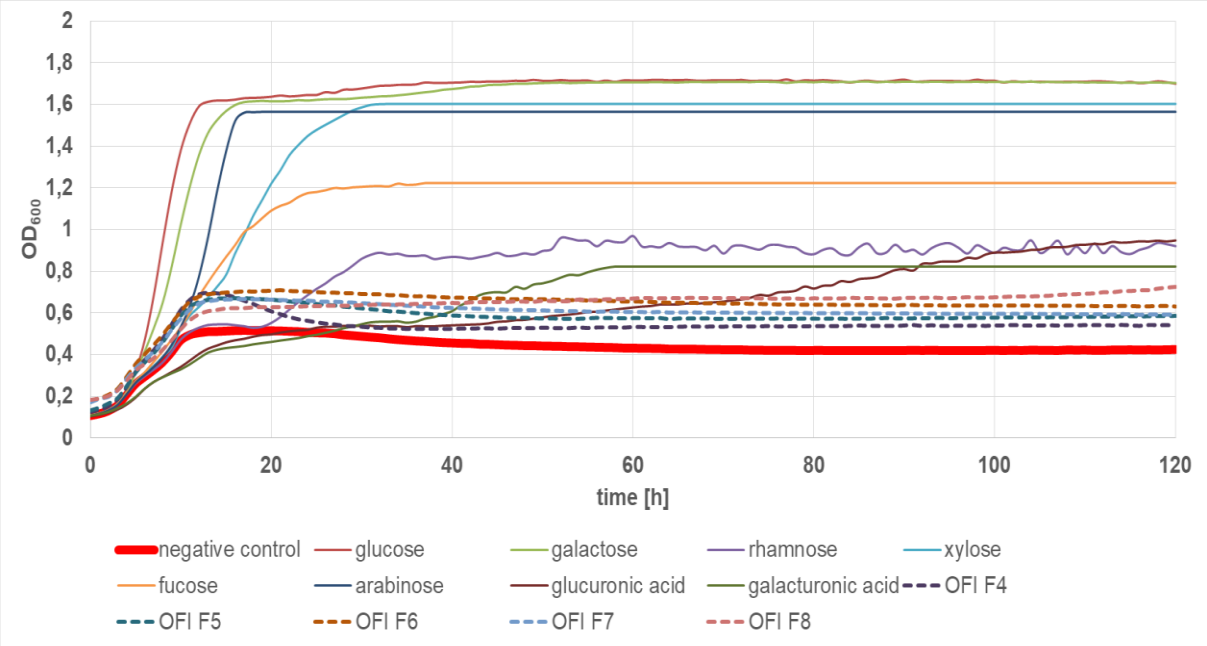

Figure 31: Bacterial growth curves of *Streptococcus salivarius ssp. thermophiles* with fractions from *O. ficus-indica*

Figure 32 graphs the growth of *Streptococcus salivarius ssp. thermophiles* with fractions of *O. xoconostle*.

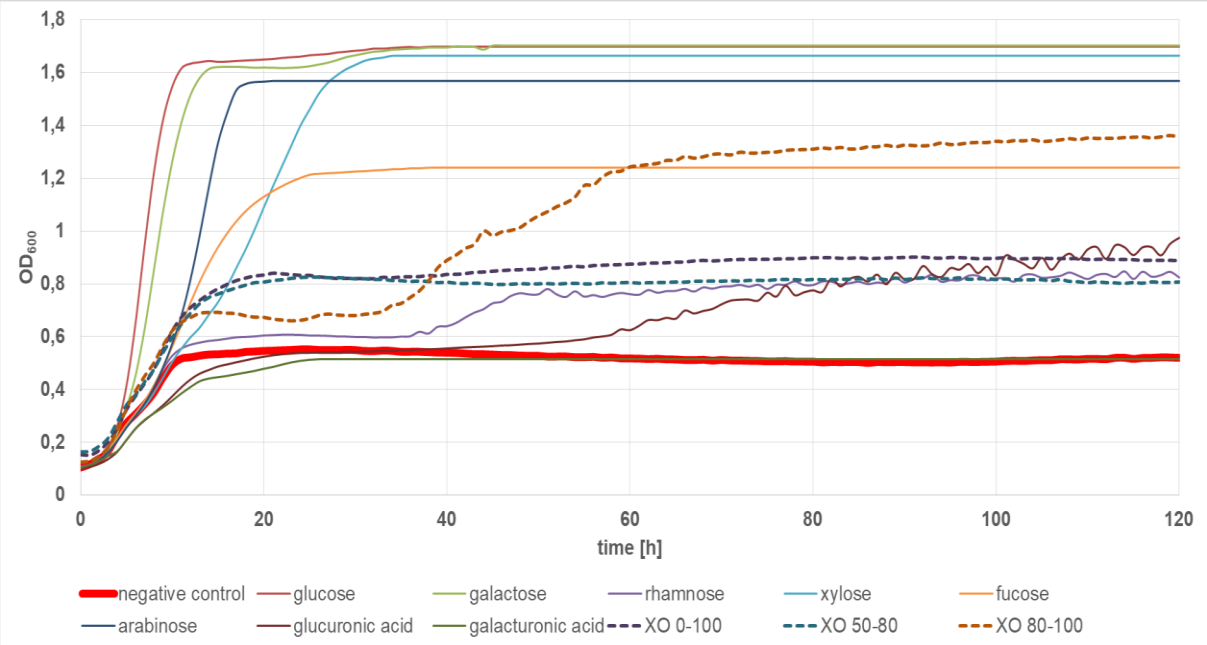

Figure 32: Bacterial growth curves of *Streptococcus salivarius ssp. thermophiles* with fractions from *O. xoconostle*