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# DIPLOMARBEIT / DIPLOMA THESIS

Titel der Diplomarbeit / Title of the Diploma Thesis

„Potential new prebiotics in the fields of fructans and galactans - their fermentability and gastro-intestinal stability“

verfasst von / submitted by

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## Kurzfassung

Die positiven Effekte von Pro- und Präbiotika auf die Gesundheit sind Gegenstand aktueller Forschung und von stetig wachsendem Interesse. Im Rahmen dieser Diplomarbeit wurden ausgewählte Kohlenhydrate auf ihr präbiotisches Potential hin untersucht, darunter Vertreter der Fruktane (z.B. aus Agave oder Chicorée) und der alpha-Galactoside aus der Raffinose Familie. Dafür wurde sowohl die Stabilität der Kohlenhydrate im Magen und Dünndarm als Voraussetzung der Eignung als Präbiotika, als auch die Fermentation mit Hilfe diverser probiotischer Bakterienstämme untersucht. Letzteres wurde mit Hilfe einer Trübungsmessung zur Erstellung von Wachstumskurven mit bereits bewährten, auf dem Markt befindlichen Bakterienstämmen durchgeführt.

Die Ergebnisse zeigen, dass der Großteil der getesteten vaginalen Probiotika, aufgrund einer gewissen alpha-Galactosidase Aktivität, in der Lage sind, Raffinose, Stachyose und Verbascose als primäre Kohlenhydratquelle zu verwenden. Neben diesen Raffinose Familie Oligosacchariden wurden auch Guar-Oligosaccharide (aus der Guar Bohne extrahiert) auf ihre Funktion als potenzielles Präbiotikum getestet; zwei Stämme zeigten ein sehr gutes Wachstum, vergleichbar mit Glucose. Hinsichtlich der gastro-intestinalen Stabilität, um so eine Passage des sauren Milieus des Mages zu überstehen, zeigen die Raffinose Familie Oligosaccharide eine hohe Beständigkeit, die ist mitunter das wichtigste Charakteristikum von Präbiotika.

Die in dieser Diplomarbeit präsentierten Ergebnisse zeigen einen Nutzen von unverdaulichen Oligosacchariden für die Lebensmittelproduktion und darüber hinaus auch für pharmazeutische Zwecke. Wegen ihrer Stabilität im Verdauungstrakt und Verwertbarkeit durch die im Dickdarm ansässigen Probiotika, können sowohl Raffinose Familie Oligosaccharide als auch Guar Oligosaccharide als potenzielle Präbiotika angesehen werden, welche einen positiven Effekt auf die allgemeine Gesundheit des Wirtes und dadurch auch auf das Wohlbefinden ausüben.

## Abstract

The positive effects of probiotics and prebiotics on human's health are very important subjects of current research and of steadily growing interest. In this diploma thesis, selected carbohydrates were investigated for their prebiotic potential, including representatives of the fructans (e.g. from agave or chicory) and the raffinose family oligosaccharides. For this purpose, both the stability of the carbohydrates in the stomach and small intestine as a prerequisite for their suitability as prebiotics and the fermentation with the help of various probiotic bacterial strains were investigated. The latter was performed using turbidimetry to generate growth curves with already proven bacterial strains available on the market.

The results show that a majority of the tested vaginal probiotics, due to some alpha-galactosidase activity, are able to use raffinose, stachyose and verbascose as primary carbohydrate source. In addition to these raffinose family oligosaccharides, guar oligosaccharides (extracted from guar bean) were also tested for their function as potential prebiotics; two strains showed particularly good growth, comparable to glucose. Regarding gastrointestinal stability, the raffinose family oligosaccharides showed high stability against the acidic environment of the stomach and the upper intestinal tract, which is among the most important characteristics of prebiotics.

The results presented in this thesis show a usefulness of indigestible oligosaccharides for food production and furthermore for pharmaceutical use. Due to their stability in the digestive tract and usability by the probiotics residing in the colon, raffinose family oligosaccharides as well as guar oligosaccharides can be considered as potential prebiotics exerting a positive effect on the general health of the host and thus also on the well-being.

## List of Abbreviations

| | |
|---|---|
| BV | bacterial vaginosis |
| CA | cellulose acetate |
| cfu | colony forming unit |
| dp | degree of polymerisation |
| $ET_{50}$ | medium effective time |
| FAO | Food and Agriculture Organization of the United Nations |
| FOS | fructooligosaccharides |
| FPS | fructopolysaccharides |
| GOS | galactooligosaccharides |
| *L.* | *Lactobacillus* |
| mg | milligram |
| ml | millilitre |
| MRS | Man – Rogosa - Sharpe |
| OD | optical density |
| $OD_{max}$ | maximum optical density |
| PBS | phosphate buffered saline |
| RFO | raffinose family oligosaccharides |
| rpm | rotations per minute |
| SEC | size exclusion chromatography |
| t | timepoint |
| TLC | thin layer chromatography |
| WHO | world health organisation |

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# 1 Introduction

## 1.1 Role of the intestinal microbiome for the human health

The composition of the intestinal microflora is complex and can be disturbed by several factors such as medication (especially antibiotics), diet or diseases. An intact microflora is not only important for digestion and synthesis of essential vitamins, it is also claimed that some bacterial strains contribute to the host's health in many ways (for example by supporting the immune system). Furthermore, strains like *Lactobacilli* and *Bifidobacteria* prevent the increase of pathogenic microorganisms [1]. Figure 1 shows the population size and diversity of the gut flora which is depending on several factors (for example pH, transit time).

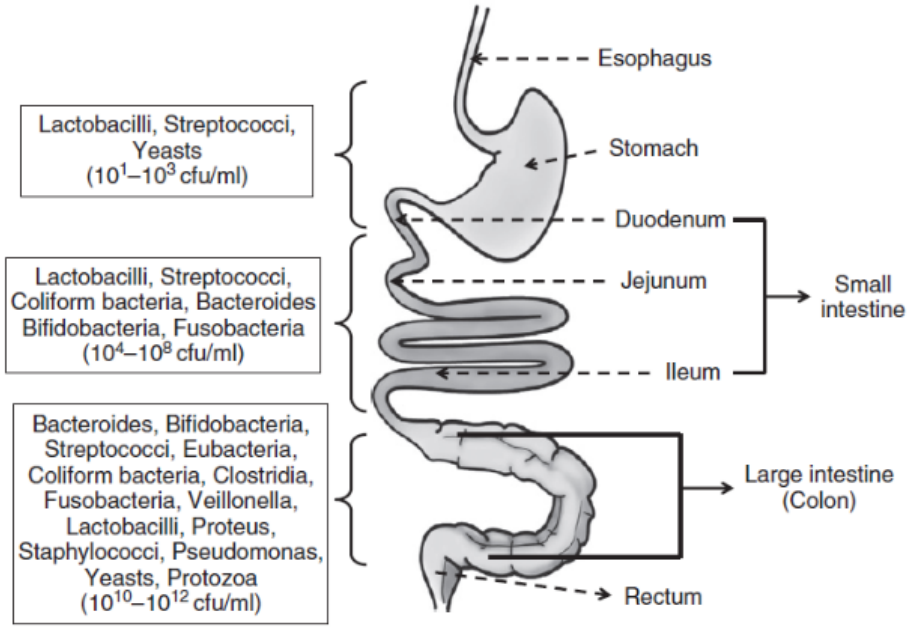


Figure 1: Overview of the distribution of the intestinal human microflora [1].

Due to the better conditions like less acidity and higher transit time, the large intestine contains most of the microbial community. Commensal bacteria are not only found in the intestines, but also on the skin, genitals or in the oral cavity [1].
This work is focussing especially on beneficial bacteria located in the lower female genital tract which is dominated by lactobacilli. Among other beneficial aspects (like $H_2O_2$ production) they lower the pH value in the vagina by producing lactic acid and thus help to prevent the increase of pathogens [2].
A lack of lactobacilli can lead to an overgrowth of pathogenic anaerobic microorganisms which can cause a bacterial vaginosis (BV), the most common vaginal infection in women of reproductive age [3].
High recurrence rates and the danger of resistances in standard antimicrobial treatment (metronidazole and clindamycin) increase the interest in alternative treatments (e.g. probiotics, prebiotics) [4]. A randomized, multicentric, double-blind study with women who suffered from BV investigated the therapy with oral probiotics as a side effect-free alternative to antibiotic therapy. They showed that the vaginal microbiome was intact again in 61.5% of the women in the probiotic treatment group after six weeks, compared to the placebo group with only 26.9%. More importantly, a follow-up of six more weeks also showed a significant benefit for the probiotics group [5].

## 1.2 Probiotics

The correct definition of probiotics is controversial and not uniform although there have been numerous attempts to find a definition of the term.
According to an expert working group of the Food and Agriculture Organization of the United Nations (FAO) and world health organisation (WHO) probiotics can be defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” [6].

Due to their acid and bile resistance, probiotic microorganisms are able to survive the gastric and small intestine passage and then colonize the large intestine. They adhere to the epithelium and thus compete with pathogenic microorganisms [7].
The intake of probiotics can positively influence both the innate non-specific immune defence (e.g. increasing mucin production, activation of macrophages, reduction of intestinal permeability) and the adaptive immune system [8].

## 1.3 Prebiotics

Prebiotics are known as non-digestible food ingredients that contribute to the hosts health and wellbeing by changing the microbial composition of the human gut. Furthermore, they have to be resistant to the acidic and enzymatic conditions in the stomach and the upper intestine [9]. For example, carbohydrates with an α-1,6 linkage (e.g. stachyose, raffinose) cannot be cleaved by the human gut due to a lack of the enzymes (here α-galactosidase). Due to these circumstances they reach the colon where they are broken down by bacteria with α-galactosidase activity [1]. Gibson et al. [9] claims that an update of the criteria defining prebiotics is necessary because many foods or components have been ascribed a prebiotic effect without fulfilling all criteria for it. A prebiotic not only has to be resistant to gastric acid, degradation by enzymes and absorption, but also has to be fermented by the intestinal bacteria and selectively and positively influence their growth [9].
As a result of this degradation process by bacteria, short-chain fatty acids (including butyrate and acetate), lactate and gases are produced [10]. This can lead, especially when administered in higher amounts, to typical symptoms like flatulence [11].
The growth of bacterial strains that are considered safe and beneficial to health (e.g. various kinds of *Lactobacilli* and *Bifidobacteria*) can be promoted by prebiotics. Finding potential prebiotics is thus a target of pharmaceutical and nutritional studies. An increased number of beneficial bacteria in the human gut is associated with many benefits such as improved immune response, controlling of pathogenic bacteria and even a reduced risk of cancer [12].

### 1.3.1 Fructans

Fructans are oligo- or polysaccharides consisting out of fructose units with an optional terminal sucrose unit due to their development process. There are two main types of fructans: inulin-type fructans with a β-2,1 linkage of the fructose units and levan-type fructans with a β-2,6 linkage [13]. In some publications, a third group of fructans is mentioned; the so called graminan-type which contains both kinds of linkages [14]. A regular consumption of prebiotic fructans (e.g. fructooligosaccharides (FOS) or polysaccharides like inulin) contribute to the health in many ways like improving the resistance against pathogens or enhancing the immune response. A positive effect on allergies was also shown [15]. Fructans are one of the most common and best known prebiotics and are found in numerous plants [16].

Their gastrointestinal fermentation never produces fructose but short-chain carboxylic acids, lactate and gases. Due to this fact, they only provide about 40-50% of the energy compared to digestible carbohydrates and are therefore considered to be low-energy ingredients [13].

- Inulin

  Inulin type fructans are herbal reserve carbohydrates that occur in many different species of the Asteraceae family (especially in roots and tubers), such as Jerusalem artichoke [17].

  As prebiotics, they reach the large intestine undigested and are fermented there by bacteria. They have been shown to increase the concentration of bifidobacteria in particular. The exact place of fermentation seems to depend on the degree of polymerization (dp). While inulins with low dp (like FOS) are preferentially broken down in the proximal colon, it appears that those with higher dp survive this transit and are predominantly broken down in the distal colon. Because of this, it can be assumed that those inulins with higher dp

have more positive effects on the distal section of the intestine (see Figure 2) and also have a longer transit time through the intestine [18].

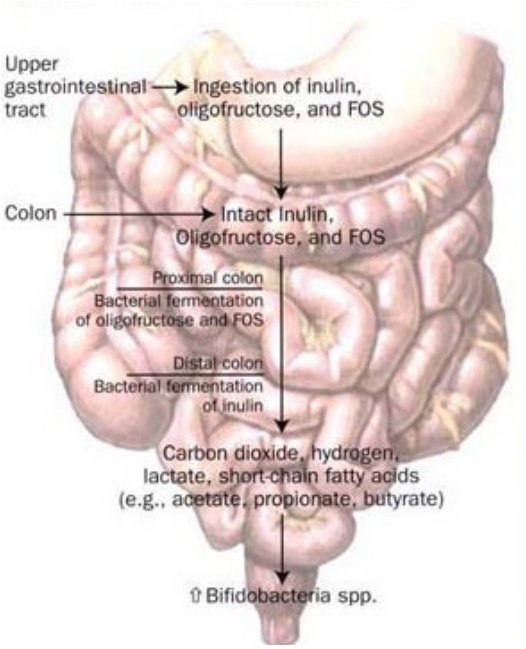


Figure 2: Metabolization of Inulin-type fructans [18].

Since there is no degradation and therefore no resorption in the small intestine, inulin is also suitable for Diabetes mellitus patients as prebiotic food supplement [19]. Figure 3 shows the general structure of an inulin-type fructan.

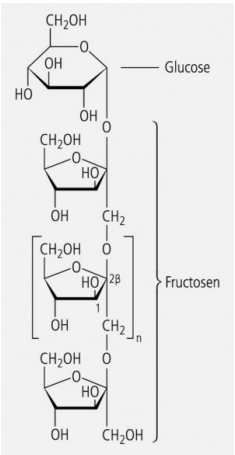


Figure 3: Inulin-type fructan with terminal glucose unit [19].

- Levan
  In contrast to inulin type fructans, which are primarily of plant origin, levans are mainly produced by bacteria (and to a small extent also by fungi or plants) [13]. The pharmaceutical industry uses a number of beneficial characteristics of levans (e.g. hypocholesterolemic and hypoglycemic effects). They are also used as a substitute for blood plasma [20].

- Graminan (mixed type)
  Metlos® (branched, short-chained) and Metlin® (branched, middle-chained) are two examples for the mixed type fructans from agave that contain β-2,6 as well as β-2,1 linkages [21].

### 1.3.2 Galactosides

- Raffinose family oligosaccharides (RFO)
  Oligosaccharides that belong to the raffinose family are non-reducing, soluble sucrose derivates and widely spread in many plants, especially in orders like Lamiales and Cucurbitales. They have important transport and storage functions [22].
  One to three galactose moieties are linked to the glucose unit of sucrose. This α-galactosidic bond is indigestible for humans and fermentation by the microbiome in the colon takes place [23].
  Raffinose (trisaccharide), stachyose (tetrasaccharide) and verbascose (pentasaccharide) are representatives of the raffinose family and are shown in Figure 4 [24].

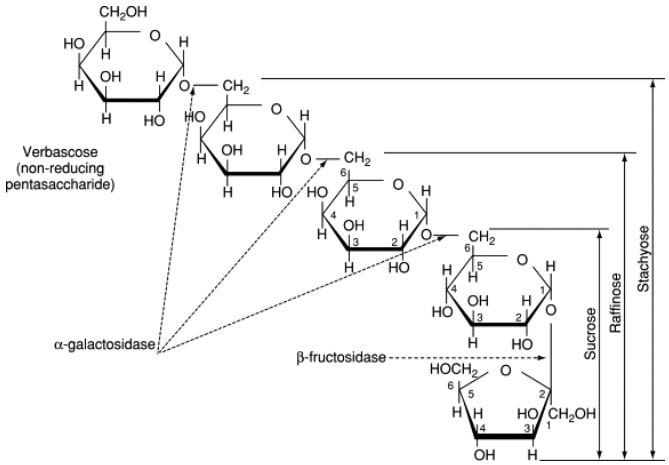


Figure 4: Raffinose family oligosaccharides [24].

RFOs are (along with fructans) the most important types of water-soluble carbohydrates in the plant kingdom [22]. Although they occur in many different plants, seeds of lupin species have the highest content [25].

- Galactomannan

  Galactomannan is a heteropolysaccharide composed of galactose and mannose and is widely distributed in the Leguminosae family. Galactomannans have a basic structure and vary (depending on the source from which they were isolated) in characteristics like molecular weight. For example, Guar beans (*Cyamopsis tetragonolobus* L.) contain guar gum, also known as guar galactomannan. Guar gum consists of unbranched chains of β-D-(1,4)-linked mannose moieties with individual α-D-(1,6)-linked galactose units. Due to its particular characteristics guar gum is widely used in pharmaceutical, food, cosmetic and other industries [26].

  A large number of studies have come to the conclusion that many plant derived polysaccharides, like galactomannan, interact with the immune system and have immune-stimulatory effects [27].

Figure 5 shows the general structure of galactomannan found in guar gum where the ratio of mannose to galactose is 2:1 [28]. In general, this ratio is decisive for the water solubility and bioavailability [29].

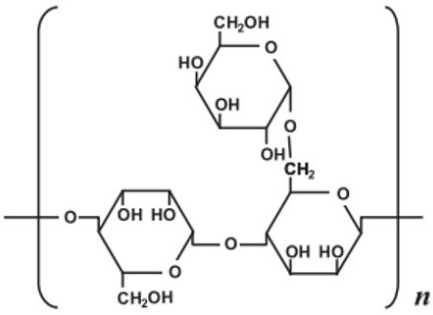


Figure 5: Repetitive basic structure of guar-galactomannan [28].

## 1.4 Synbiotics

The term synbiotic was introduced by Gibson and Roberfroid in 1995 [30]. It defines a combination of probiotics and prebiotics that work synergistically. The aim of research is finding “perfect synergies” that have superior effects compared to administering pro- or prebiotics alone [30, 31].

# 2 Materials and methods

## 2.1 Preparation of media and buffers

### 2.1.1 Media preparation

All media used for the determination of growth curves (BioScreen experiments) were prepared and used under aseptic conditions by the use of the Laminar Air Flow System or autoclaved at 121 °C for two hours to receive accurate and reproducible results. Solutions which were not suitable for autoclaving were filtered sterile (0.22 µm cellulose acetate (CA) filter). After preparation, the solutions were stored at 4 °C until further use.

- MRS media without carbohydrate source ($MRS^-$ and $2xMRS^-$)

Table 1 shows the general composition of Man-Rogosa-Sharpe (MRS) media, all chemicals were of analytical grade form Sigma Aldrich.

Table 1: Composition of MRS media.

| **MRS media** | |
|---|---|
| peptone from casein | 10.0 g |
| meat extract | 8.0 g |
| yeast extract | 4.0 g |
| potassium phosphate dibasic | 2.0 g |
| L-cysteine hydrochloride | 0.5 g |
| Tween 80 | 1.0 g |
| ammonium citrate dibasic | 2.0 g |
| sodium acetate | 5.0 g |
| magnesium sulphate | 0.2 g |
| manganese sulphate | 0.04 g |

For the $MRS^-$ media all components listed above were weighed in and filled to 1000 mL in a volumetric flask with distilled water. The 2x $MRS^-$ media was filled to 500 mL. The solutions were autoclaved at 121 °C and stored at 4 °C [21].

- MRS media with carbohydrate source ($MRS^+$)

The $MRS^+$ broth was prepared with the same quantities (see Table 1), filled to 900 mL with distilled water and autoclaved at 121 °C. After cooling to room temperature, 100 mL of a sterile 20% glucose solution were added under the Laminar Air Flow to avoid a Maillard reaction between the glucose and the amino acids during the autoclaving process.

### 2.1.2 Buffer preparation

- Phosphate buffered saline (PBS) buffer

Table 2 shows the required material for the 10x PBS buffer preparation, all chemicals were of analytical grade from Sigma Aldrich.

Table 2: Composition of 10xPBS.

| **10xPBS** | |
|---|---|
| sodium chloride | 80.0 g |
| potassium chloride | 2.0 g |
| sodium phosphate dibasic heptahydrate | 26.8 g |
| potassium phosphate monobasic | 2.4 g |

The components were weighed in and filled with distilled water to 800 mL. The pH was adjusted to 7.4 with 0.1 M hydrochloric acid, filled up to 1000 mL and autoclaved at 121 °C. Before use, the required amount of 10x PBS was diluted 1:10 with autoclaved distilled water under aseptic conditions.

## 2.2 Used probiotic bacterial strains

Table 3 shows the probiotic bacterial strains and products used.

Table 3: List of used bacterial strains.

| **bacterial strains** | **source/ trade name** | **manufacturer** |
|---|---|---|
| *Lactobacillus casei rhamnosus* Döderlein | Gynophilus® | PROBIONOV |
| *Lactobacillus gasseri (*EB01™*), Lactobacillus rhamnosus (*PB01™*)* | Vagisan® | HÄLSA Pharma GmbH |
| *Lactobacillus plantarum* P17630 | Canesflor® | Bayer |
| *Lactobacillus rhamnosus* R11 | Urocys® forte | Melasan |
| *Lactobacillus crispatus* LBV88*, Lactobacillus rhamnosus* LBV96*, Lactobacillus gasseri* LBV150N*, Lactobacillus jensenii* LBV116 | OMNi-BiOTiC® FLORA plus+ | OMNi-BiOTiC® |

To receive reproducible results, the bacterial strains contained in the preparations listed in Table 3 were cultivated before further use.

## 2.3 Tested fructans and galactans

Table 4 lists all tested carbohydrates including dp, the source and manufacturer.

Table 4: List of carbohydrates – fructans and galactans.

| type | name | source | dp | manufacturer |
|---|---|---|---|---|
| FOS unbranched | Raftilose® | chicory (*Cichorium intybus*, Asteraceae) | 5* | Orafti |
| FPS unbranched (inulin) | Raftiline® | chicory (*Cichorium intybus*, Asteraceae) | 7* | Orafti |
| FOS branched | Metlos® | agave (*Agave tequilana*, Asparagaceae) | 6* | Nekutli |
| FPS branched | Metlin® | agave (*Agave tequilana*, Asparagaceae) | 10* | Nekutli |
| FPS | Levan from *Serratia levanicum* | bacterial | N/A | Carbosynth |
| GOS | raffinose | | 3 | Sigma-Aldrich |
| GOS | stachyose | *Stachys tuberifera,* Lamiaceae | 4 | Sigma-Aldrich |
| GOS | verbascose | bean extract | 5 | Megazyme |
| FOS | 1-kestose | | 3 | University of Vienna |
| GOS | guar-oligosaccharides | purified out of Resource Benefiber®/ guar beans (*Cyamopsis tetragonolobus*, Fabaceae) | | Novartis Nutrition |

* average number dp [21]

### 2.3.1 Preparative size exclusion chromatography

The size exclusion chromatography (SEC) is based on a separation of molecules according to their molecular hydrodynamic volume or size [32].

SEC was applied to fractionate the different types of saccharides in RESOURCE Benefiber® powder. Before that, it was necessary to dissolve the Benefiber® powder

in ultrapure water, precipitate the polysaccharides with methanol (1:4) and centrifuge it at RT for 30 minutes with 10000 rpm. The methanol was evaporated, and the remaining solution was freezed at -20 °C and lyophilized [33].
The freeze-dried sample was then dissolved in ultrapure water and injected into the SEC system. A sample volume of 2 mL was injected, with a pre-run time of 175 min; 42 fractions (10 min/fraction) were collected. The column system consisted out of two columns in series, namely a Biogel P-4 (extra fine, <45 µm, MW range 800 – 4000; column 89 x 2.5 cm) and a Biogel P-2 (fine, 45 – 90 µm, MW range 100 – 1800; column 90 x 2.5 cm) column (gel: Bio Rad Laboratories, Inc.) As mobile phase bi-distilled, degased water was used at a flow rate of 30 – 36 mL/h by peristaltic pump. The system was connected to an RI-detector (Knauer, Berlin, Germany) and a fraction collector and recorder (Healthcare, Co) [21, 33, 34].

To prove the purity, all factions were freeze dried and analysed by thin layer chromatography (TLC). Samples (3 µL of a 1 mg/mL solution in water) were analysed on TLC Silica gel 60 $F_{254}$ (Merck, Darmstadt, Germany) with oligosaccharide eluent system (1-butanol:1-propanol:ethanol:water (2:3:3:2)), twice, and developed and detected with thymol reagent in methanol with 5% sulphuric acid as described previously [21, 33, 35].

## 2.4 Determination of bacterial growth

### 2.4.1 Bacterial cultivation

All working steps were done under the Laminar Air Flow System to exclude contamination with other microorganisms.
The first step was to transfer the bacterial strains in $MRS^+$ media. Therefore, a spatula tip full of bacterial preparation was put into autoclaved test tubes containing 5 mL $MRS^+$ media. Then the obtained bacterial suspensions were incubated at 37 °C under anaerobic conditions for about 24 hours.

Afterwards 1 mL of each culture was transferred into autoclaved test tubes containing 4 mL media without carbohydrate source ($MRS^-$). These cultures were incubated the same way.

### 2.4.2 Test procedure

To determine the growth of the selected bacteria in the presence of the various carbohydrate sources the BioScreen C system, (Oy Growth Curves Ab Ltd, Helsinki, Finland) which is based on a turbidity measurement, was used.

Therefore, the cell density of the overnight $MRS^-$ culture was determined using a photometer (Hitachi U-1100 Spectrophotometer) at 600 nm; as blank $MRS^-$ media was used. To stay in the Beer-Lambert law, the bacterial culture had to be diluted. This step was necessary to calculate the required amount of bacterial suspension to receive a final $OD_{600}$ of 0.1 which was used as the starting point of the BioScreen experiment. The calculated amount was transferred to test tubes, centrifuged (4 minutes, 12500 rpm, 20 °C) and afterwards the supernatant was discarded. The obtained cell pellet was re-suspended in 1 mL 1xPBS. This washing step was repeated twice and the remaining bacteria pellet was re-suspended in 2x$MRS^-$.

As negative control the bacteria cultures were incubated without carbohydrate source. In addition, $MRS^-$ media as well as $MRS^+$ media were incubated to exclude that the derived growth curves were received because of microbiological contamination. The samples were incubated with 1% carbohydrate solutions in special sterile honeycomb plates with a final amount of 200 µL [21].

The plates were incubated for 5 days at 37 °C and the optical density (600 nm) was measured every hour. Before each measurement, the plates were shaken (to distribute the bacteria in the suspension and avoid sedimentation) at low amplitude (to prevent cross contamination) for 15 seconds before every measurement. Every determination was done twice, and the averages and standard deviations were calculated.

Statistical analysis was done with MS Excel and TableCurve 2D Software (Systat Software Inc., USA).

### 2.4.3 Determination of $ET_{50}$ values

TableCurve 2D was used to determine the timepoint after which the medium $OD_{max}$ (medium effective time, $ET_{50}$) is reached. The data points of the graphs obtained in Excel were fitted. Figure 6 shows the growth curve of *L. gasseri*/*L.rhamnosus* (Vagisan®) as an example.

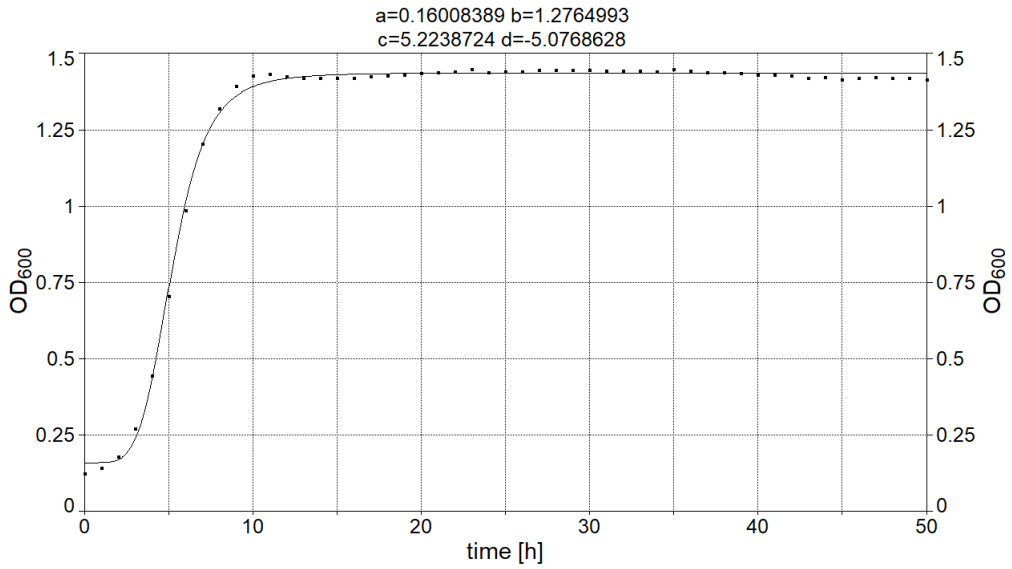


Figure 6: Fitted growth curve of *L. gasseri*/*L. rhamnosus* with glucose.

The continuous curve is surrounded by the original data points. Using the curve three values are determined:

- a corresponds to the starting $OD_{600}$
- b represents the difference between starting $OD_{600}$ and $OD_{max}$
- c shows the turning point of the curve and corresponds to the $ET_{50}$ value

The $ET_{50}$ values were determined for all growth curves (if possible) of all tested bacterial strains and presented as table.

## 2.5 Carbohydrate stability

### 2.5.1 Shelf stability

For the Bioscreen experiments the carbohydrate solutions were prepared once for every double determination and had to stay stable for the duration of the whole experiment. To ensure this, all tested carbohydrates were solved in ultrapure water (1 mg/mL) and stored at 4 °C. Samples were analysed immediately after preparation and after 7 days by thin layer chromatography (TLC). Therefore, following standards were prepared immediately before TLC analysis (1 mg/mL):

- glucose
- fructose
- galactose
- sucrose
- raffinose
- stachyose
- verbascose
- 1-kestose

### 2.5.2 Gastrointestinal stability

It is required that prebiotics must be stable under acid gastric conditions and therefore the stability under gastric conditions was examined. Artificial gastric fluid was prepared freshly (0.08 M HCl containing 0.2% NaCl) and preheated to 37 °C. Selected carbohydrates were solved in artificial gastric fluid (1 mg/mL), incubated at 37 °C and analysed at different timepoints:

- $t_0$ (= immediately after incubation)
- $t_1$ (= after one hour of incubation)
- $t_2$ (= after two hours of incubation)
- $t_3$ (= after three hours of incubation)
- $t_4$ (= after four hours of incubation)
- $t_5$ (= after five hours of incubation)
- $t_6$ (= after six hours of incubation)

As control, all carbohydrates were solved in ultrapure water, incubated for the whole duration (6 hours), and analysed at the end of the experiment. To stop the enzymatic reaction samples were frozen and stored at -18 °C. After that, the stability was analysed by TLC.

### 2.5.3 In-vitro degradation study of galactans and fructans

As an extension to the stability study in gastric fluid digestion experiments were done to ensure that the carbohydrates reach the colon undecomposed. Therefore, three different environments were prepared: mouth, stomach and small intestine. Two different types of amylase solutions were prepared to imitate saliva on the one hand and the conditions in the small intestine on the other hand. Samples were tested at different timepoints using TLC.

Oral conditions – saliva

- Incubation of 1 mg carbohydrate in amylase solution 50 U/L (Sigma Aldrich, α-Amylase isolated from Barley Malt) at 37 °C for 2 minutes under shaking to prevent the formation of concentration zones.
    - $t_1$= after 2 minutes of incubation in amylase solution *

Gastric conditions – gastric juice

- stopping of amylase activity by adding the double amount of synthetic gastric juice (0.08 M HCl + 0.2% NaCl + 0.1% pepsin; Sigma Aldrich, pepsin from porcine gastric mucosa)
- Incubation for 3 hours (sampling every hour) at 37 °C, shaking every 15 minutes for 5 seconds
    - $t_2$= after half an hour of incubation
    - $t_3$= after 1 hour of incubation
    - $t_4$= after 2 hours of incubation*
    - $t_5$= after 3 hours of incubation

Intestinal conditions – intestinal juice

- Addition of amylase solution with a concentration of 80 U/L (double amount of saliva solution; therefore preparing an amylase solution with 120 U/L, 1.5:1 dilution)
- incubation for one hour (sampling every 15 minutes) at 37 °C, shaking every 15 minutes for 5 seconds
- $t_6$= after 15 minutes of incubation
- $t_7$= after 30 minutes of incubation
- $t_8$= after 45 minutes of incubation
- $t_9$= after 60 minutes of incubation*

Negative control: at the beginning the carbohydrates were dissolved in ultrapure water and incubated for the whole duration at 37 °C, analysis took place at the end via thin layer chromatography.

*marks the timepoints where an additional colorimetric determination of glucose was done using test strips (Glucose MQuant™, Sigma Aldrich).

### 2.5.4 Colorimetric determination of glucose

In addition to the TLC analysis, a colorimetric determination of glucose using test strips (Glucose MQuant™, Sigma Aldrich) was performed. Figure 7 shows the different colours of detectible glucose concentrations.

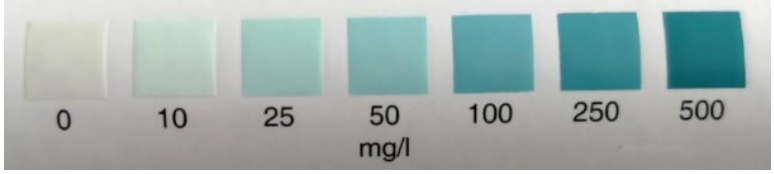


Figure 7: Colour scale of glucose test strips.

## 2.6 Thin layer chromatography

Thin layer chromatography was used for the identification of the guar-oligosaccharides as well as for the different stability studies and for the digestion experiments to see if a degradation of the oligosaccharides has occurred.
The standard solutions were prepared by solving 1 mg in 1 mL ultrapure water. The samples were prepared in the same way and 1 µL was applied to the TLC-plates (TLC silica gel 60 $F_{254}$, Merck, Darmstadt, Germany).
The plates were developed twice with an eluent system suitable for oligosaccharides containing 1-butanol:1-propanol:ethanol:water (2:3:3:2). Afterwards thymol reagent (200 mg thymol in 100 mL methanol containing 5% sulfuric acid) was used to visualize the results (heating to about 105 °C necessary) [35].

# 3 Results

## 3.1 Growth curves of probiotics with selected carbohydrate sources

The results for all carbohydrates listed in Table 4 are shown here. In order to provide a clearer overview, the results are depicted in two separate graphs for fructans and galactans. To preclude growth due to contamination several controls were carried out. MRS$^-$ means Man-Rogosa-Sharpes media without carbohydrate source, MRS$^+$ stands for Man-Rogosa-Sharpes media containing glucose. Additionally, a negative control was done adding just ultra-pure water to the bacterial suspension, so no carbohydrate source was provided. For comparison, each bacterial strain was also tested with glucose and fructose. It is assumed that these references can be used well by all bacterial strains.

### 3.1.1 Growth curves of *L. casei rhamnosus* Döderlein (Gynophilus®)

Figure 8 shows the growth curves of the probiotic contained in Gynophilus® with the tested fructans.

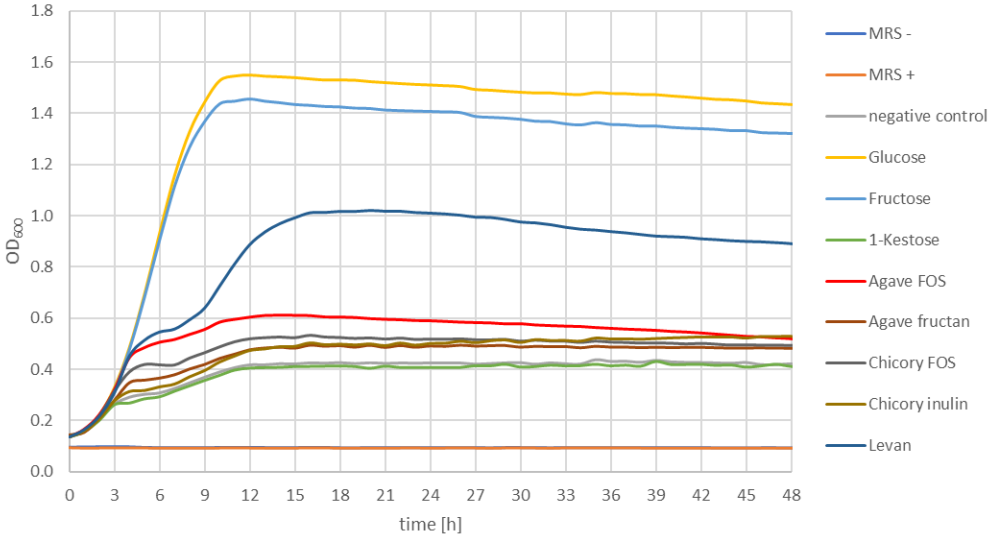


Figure 8: Growth curves of *L. casei rhamnosus* Döderlein with fructans.

It turns out that the highest growth is reached with glucose, closely followed by fructose. The growth with glucose reaches a maximum optical density ($OD_{max}$) of 1.55 whereas fructose reaches 1.46; although the time to reach this plateau is very similar (12 hours). Levan also shows significant, albeit delayed, growth of *L. casei rhamnosus* Döderlein with a maximum $OD_{600}$ of 1.02 after 20 hours.

Figure 9 shows the growth curves of *L. casei rhamnosus* Döderlein with various galactans including guar-oligosaccharides.

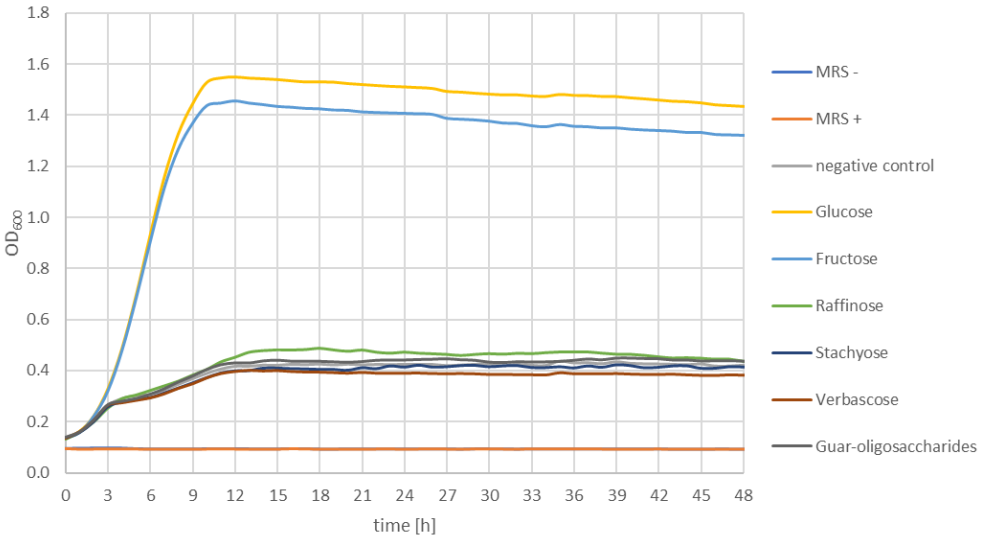


Figure 9: Growth curves of *L. casei rhamnosus* Döderlein with galactans.

All tested galactans could not be used by *Lactobacillus casei rhamnosus* Döderlein significantly; their maximum $OD_{600}$ is about the same level as the negative control.

### 3.1.2 Growth curves of *L. gasseri* (EB01™)/*L. rhamnosus* (PB01™) (Vagisan®)

Figure 10 shows the growth curves of the bacterial strains contained in Vagisan® with the selected fructans.

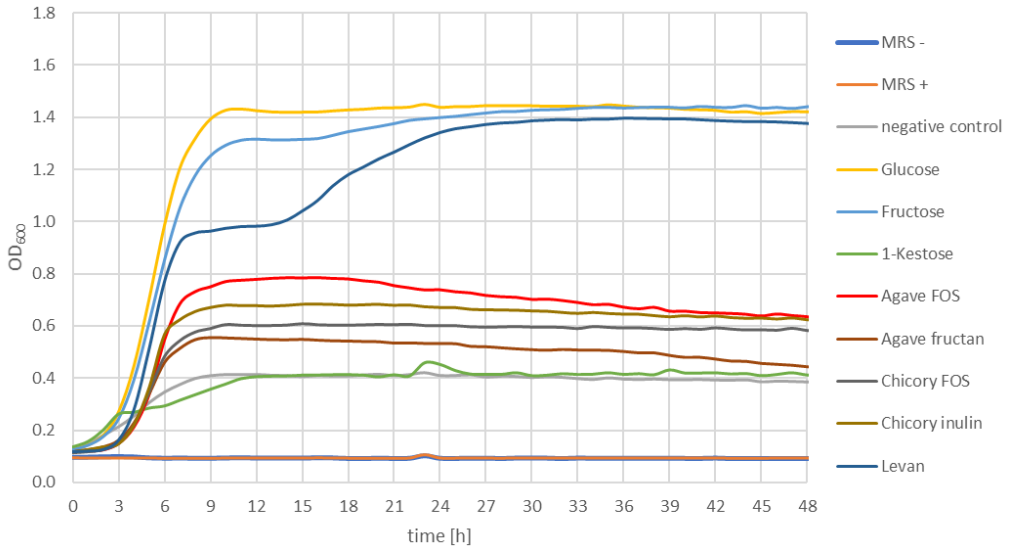


Figure 10: Growth curves of *L. gasseri* (EB01™)/*L.rhamnosus* (PB01™) with fructans.

Glucose and fructose lead to fast and good growth in these combined strains. Both curves reach a maximum $OD_{600}$ of 1.45, but the growth with glucose is faster. With a time leg, the tested levan also reaches a high $OD_{600}$ of 1.4 after 36 hours. Agave FOS (Metlos®), chicory inulin (Raftiline®), chicory FOS (Raftilose®) and agave fructan (Metlin®) ranged in descending order between 0.58 and 0.84 which corresponds to a moderate increase in growth. The probiotics in Vagisan® were not able to cleave 1-kestose.

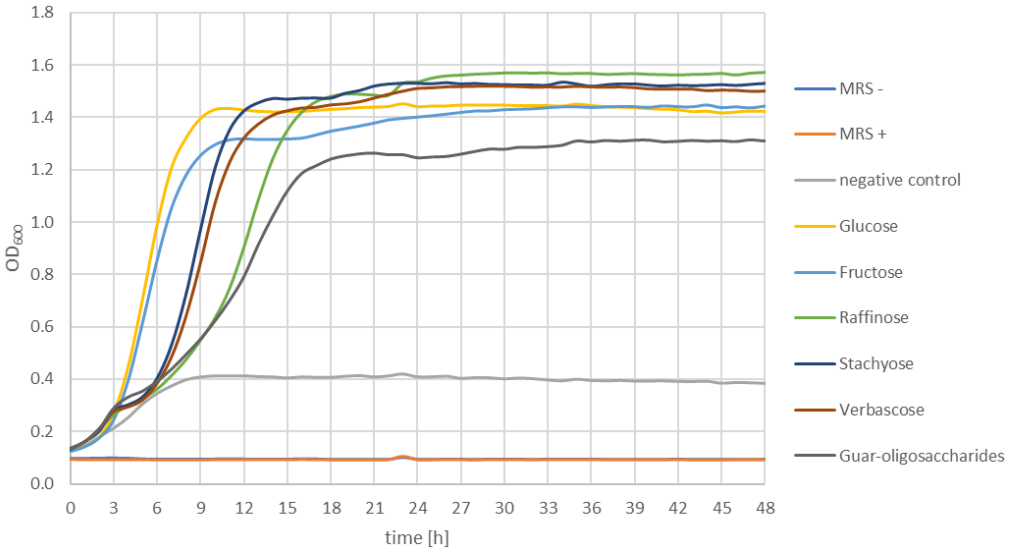


Figure 11: Growth curves of *L. gasseri* (EB01™)/*L.rhamnosus* (PB01™) with galactans.

As shown in Figure 11 the probiotics in Vagisan® grow very well with all tested galactans. Raffinose, stachyose and verbascose show even higher $OD_{600}$ values than fructose and glucose, although the time to reach the maximum took longer compared to fructose and glucose. The growth in presence of the guar-oligosaccharides was slower, but still reached a high maximum of 1.4.

### 3.1.3 Growth curves of *L. plantarum* P17630 (Canesflor®)

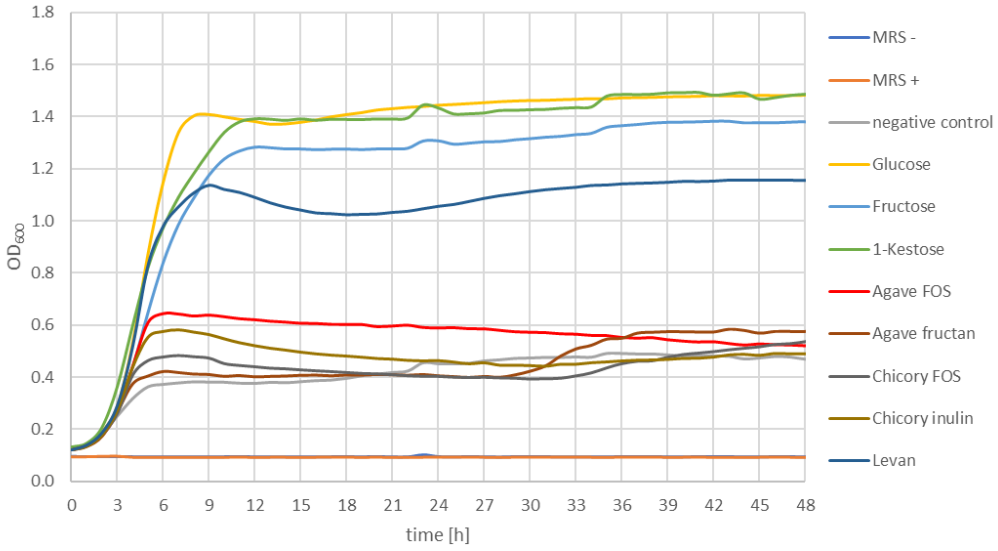


Figure 12: Growth curves of *L. plantarum* P17630 with fructans.

The bacterial strain contained in Canesflor® shows excellent growth in presence of glucose, 1-kestose and fructose (see Figure 12). Levan also causes very rapid growth, although it does not reach that high level. The other tested fructans show fast but only slight growth; none of them could even reach half the maximum optical density of glucose.

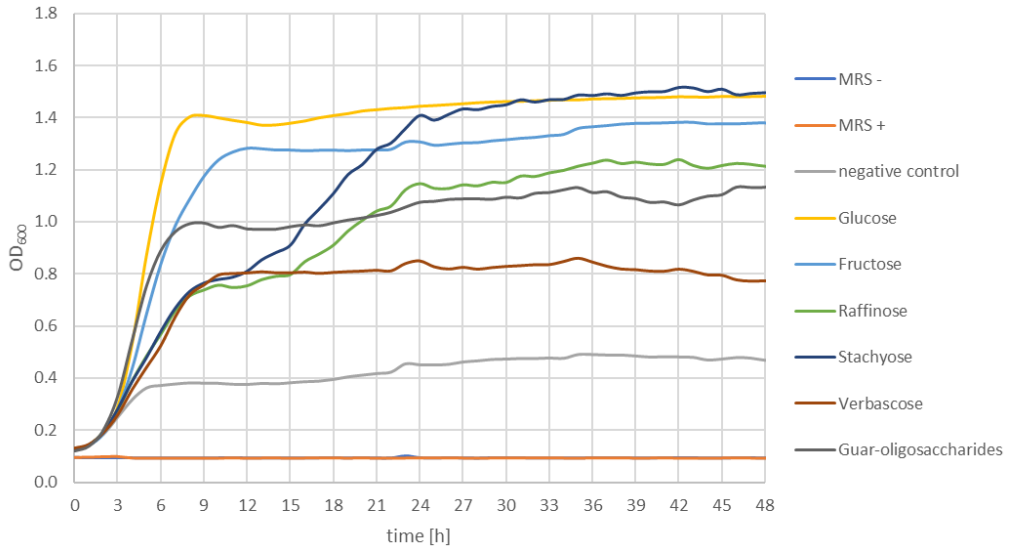


Figure 13: Growth curves of *L. plantarum* P17630 with galactans.

As shown in Figure 13 the guar-oligosaccharides cause a rapid growth (comparable to fructose and glucose), but they do not reach such a high maximum. In comparison, the growth with raffinose and stachyose takes longer, but they reach higher values; stachyose reaches the highest value of all tested galactans. Verbascose also promotes the growth of *L. plantarum* P17630 but it does not get beyond a maximum $OD_{600}$ of 0.89.

### 3.1.4 Growth curves of *L. rhamnosus* R11 (Urocys® forte)

As shown in Figure 14 and Figure 15, neither the tested fructans nor the galactans were used by the bacterial strain contained in Urocys® forte. The only exception is levan, which reaches an $OD_{600}$ of 1.04. The growth of *L. rhamnosus* R11 was significantly promoted by the monosaccharides glucose and fructose with an advantage for glucose.

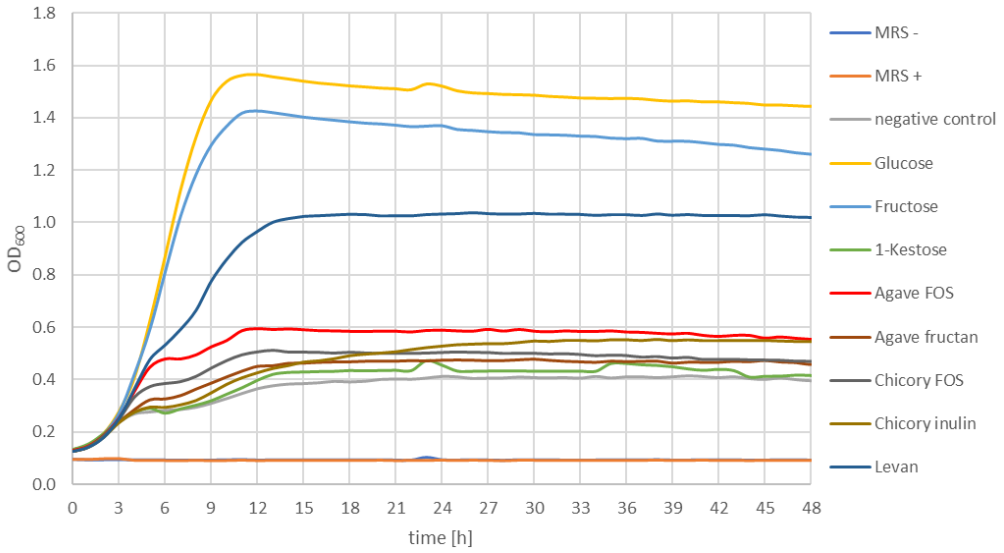


Figure 14: Growth curves of *L. rhamnosus* R11 with fructans.

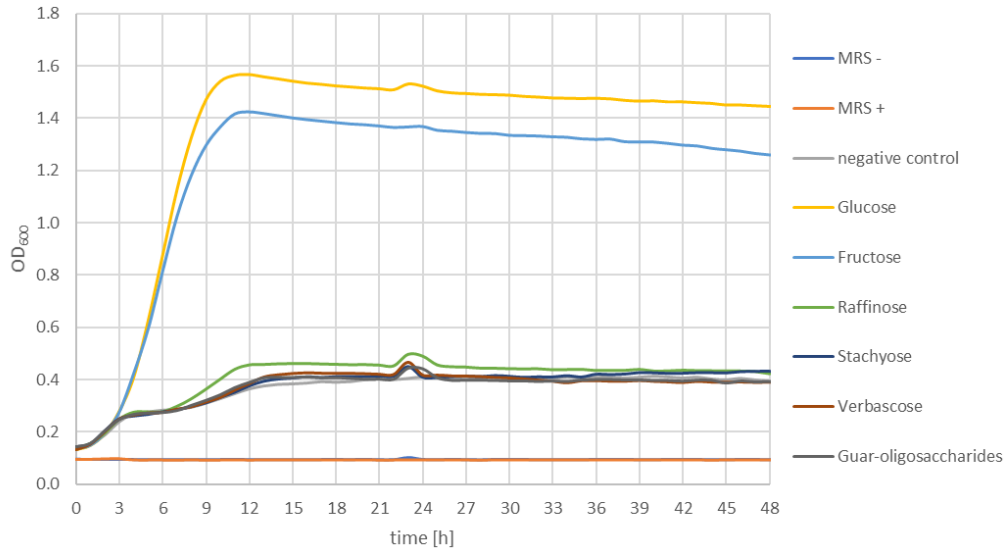


Figure 15: Growth curves of *L. rhamnosus* R11 with galactans.

### 3.1.5 Growth curves of *L. crispatus* LBV88, *L. rhamnosus* LBV96, *L. gasseri* LBV150N, *L. jensenii* LBV116 (OMNi-BiOTiC® FLORA plus+)

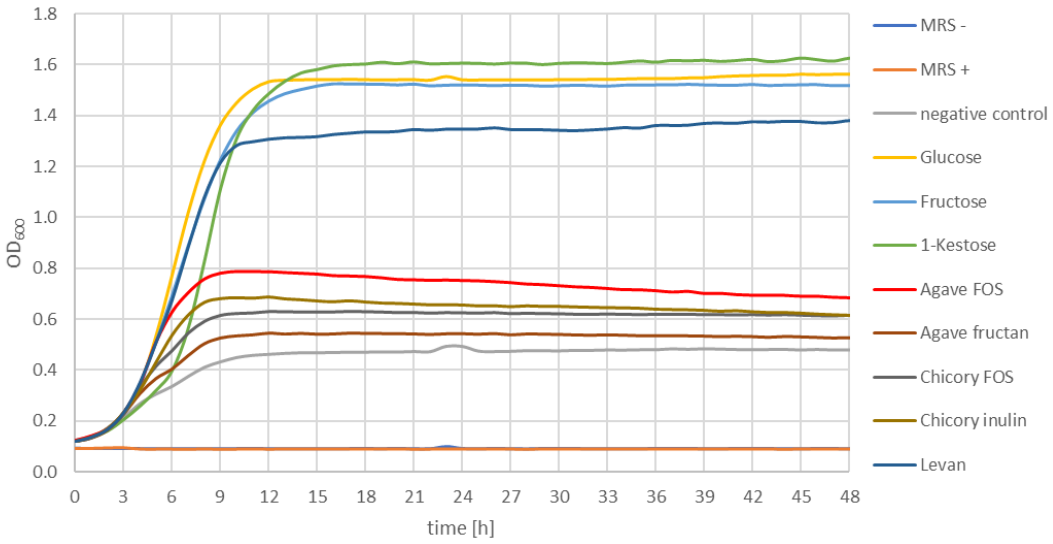


Figure 16: Growth curves of *L. crispatus* LBV88, *L. rhamnosus* LBV96, *L. gasseri* LBV150N, *L. jensenii* LBV116 with fructans.

Figure 16 shows the growth curves of the four combined probiotics contained in OMNi-BiOTiC® FLORA plus+. 1-kestose leads to the highest OD values (1.70), even higher than glucose and fructose which reached nearly the same value (1.57 vs. 1.55) but with an advantage in terms of time compared to 1-kestose. Levan also caused very good growth (maximum OD of 1.44). The other tested fructans show slight growth compared to the negative control, but only agave FOS achieves half the maximum OD of glucose.

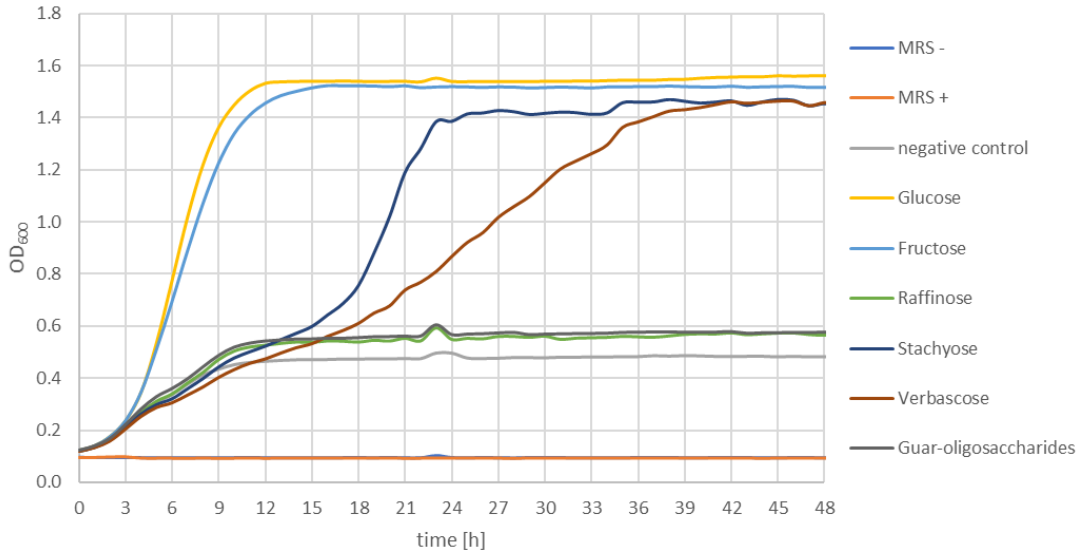


Figure 17: Growth curves of *L. crispatus* LBV88, *L. rhamnosus* LBV96, *L. gasseri* LBV150N, *L. jensenii* LBV116 with galactans.

As shown in Figure 17 glucose and fructose lead to very similar growth curves for OMNi-BiOTiC® FLORA plus+. With some time lag, stachyose and verbascose also caused significantly high growth with an optical density of about 1.5. The delay of growth enhancement is highest for verbascose; it takes over 40 hours to reach its plateau. Interestingly this probiotic supplement hardly used the trisaccharide raffinose, although it showed good utilization of the other alpha-galactosiedes stachyose and verbascose. Growth with guar-oligosaccharides also only achieved slightly higher $OD_{max}$ values than the negative control.

## 3.2 Determination of $ET_{50}$ values [h]

$ET_{50}$ values were determined for all those carbohydrates whose growth leads at least to about half of the growth (half $OD_{max}$) of the reference carbohydrate glucose. The results are shown in Table 5.

Table 5: $ET_{50}$ values of selected probiotic supplements with different carbohydrates.

| | Gynophilus® | Vagisan® | Canesflor® | Urocys® forte | OMNi-BiOTiC® FLORA plus+ |
|---|---|---|---|---|---|
| glucose | 5.5 | 5.2 | 4.7 | 5.7 | 6.2 |
| fructose | 5.3 | 5.6 | 5.4 | 5.6 | 6.6 |
| 1-kestose | - | - | 4.9 | - | 8.2 |
| agave FOS | - | 5.3 | - | - | 4.5 |
| agave fructan | - | - | - | - | - |
| chicory FOS | - | - | - | - | - |
| chicory inulin | - | - | - | - | - |
| levan | 6.6 | 7.1 | 4.3 | 6.5 | 6.2 |
| raffinose | - | 11.6 | 10.9 | - | - |
| stachyose | - | 8.5 | 12.6 | - | 18.0 |
| verbascose | - | 8.9 | 5.3 | - | 26.4 |
| guar-oligosaccharides | - | 11.2 | 4.1 | - | 5.5 |

The maximum optical density at 600 nm ($OD_{max}$) was obtained in Excel by finding the maximum of all values achieved by the Bioscreen measurements. Table 6 lists all $OD_{max}$ values that reached at least about half the maximum $OD_{600}$ of the reference glucose.

Table 6: $OD_{max}$ values of selected probiotic supplements with different carbohydrates.

| | Gynophilus® | Vagisan® | Canesflor® | Urocys® forte | OMNi-BiOTiC® FLORA plus+ |
|---|---|---|---|---|---|
| glucose | 1.5 | 1.5 | 1.5 | 1.6 | 1.6 |
| fructose | 1.5 | 1.4 | 1.4 | 1.4 | 1.6 |
| 1-kestose | - | - | 1.5 | - | 1.7 |
| agave FOS | - | 0.8 | - | - | 0.8 |
| agave fructan | - | - | - | - | - |
| chicory FOS | - | - | - | - | - |
| chicory inulin | - | - | - | - | - |
| levan | 1.0 | 1.4 | 1.2 | 1.0 | 1.4 |
| raffinose | - | 1.6 | 1.2 | - | - |
| stachyose | - | 1.6 | 1.5 | - | 1.5 |
| verbascose | - | 1.6 | 0.9 | - | 1.5 |
| guar-oligosaccharides | - | 1.4 | 1.3 | - | 0.7 |

*L. casei rhamnosus* Döderlein (Gynophilus®) grew fast and good with the references glucose and fructose but was only able to use one of the tested carbohydrates, namely levan.

For the strains in Vagisan® the highest growth ($OD_{max}$) was obtained with raffinose (even higher than the references glucose and fructose), closely followed by the other RFOs stachyose and verbascose; although it took the longest to reach the turning point ($ET_{50}$) with raffinose.

The growth of *L. plantarum* P17630 (Canesflor®) reached high maxima with the references but also with 1-kestose and stachyose. The guar-oligosaccharides lead to a very fast growth (lowest $ET_{50}$ value of all).

*L. rhamnosus* R11 (Urocys® forte) essentially shows the same behavior as the strain contained in Gynophilus®.

The combination of four strains in OMNi-BiOTiC® FLORA plus+ shows, as expected, a fast and high growth with the references. However, the highest $OD_{max}$ is achieved with 1-kestose, but it takes a bit longer to reach the turning point. The growth with the tetra- and pentasaccharides stachyose and verbascose reaches high OD values (comparable to glucose), but the time to reach this plateau is much longer; in case of verbascose even 26.4 hours.

## 3.3 Purification of guar oligosaccharides – SEC analysis

The SEC purification of guar carbohydrates (RESOURCE Benefiber® powder) delivers only small parts of oligo-, di- and monosaccharides, but a higher amounts of high polymer components (results not shown).
The guar polysaccharides obtained from Benefiber® have a weight average molar mass of 26 000 g/mol (with polydispersity index of 18.8). The analysis of low molecular components delivers minor amounts of galactose, saccharose and raffinose as well as certain oligosaccharides (with dp between 3 and 10). The applied oligosaccharides for the detection of growth curves were prepared by preparative SEC. The separation of the guar carbohydrates delivers 15% of the execute sample. Analysis by thin layer chromatography confirmed high purity of galactomannan di- and oligosaccharides (range dp 2-4) [10, 33].
“Purity of isolated fractions was proofed by TLC; out of used 150 mg total guar carbohydrates 23 ± 4 mg guar oligosaccharides (dp of 2 to 4) and around 127 ± 6 mg guar polysaccharides (dp > 5) were yielded with the required purity” [33].

## 3.4 Stability studies of carbohydrate solutions

### 3.4.1 Stability of carbohydrates in water

Table 7 shows the selected carbohydrates and standards for the examination of the shelf stability. Therefore, the samples (dissolved in ultra pure water) were applied directly to TLC Plates and reapplied after storage (7 days, 4 °C). A concentration of

1 mg/mL was chosen. The standards were freshly prepared each time. The assessment of the TLC plates after 7 days shows that all the tested carbohydrate solutions have remained stable over this period (see Figure 18, Figure 19, Figure 20, Figure 21). Therefore, the solutions can be used without hesitation for both determinations using Bioscreen C.

Table 7: Carbohydrates and standards for the shelf stability study.

| **tested carbohydrates** | **standards** |
|---|---|
| levan | glucose |
| stachyose | fructose |
| verbascose | galactose |
| raffinose | sucrose |
| 1-kestose | raffinose |
| Metlos® | stachyose |
| Metlin® | verbascose |
| Raftilose® | 1-kestose |
| Raftiline® | |

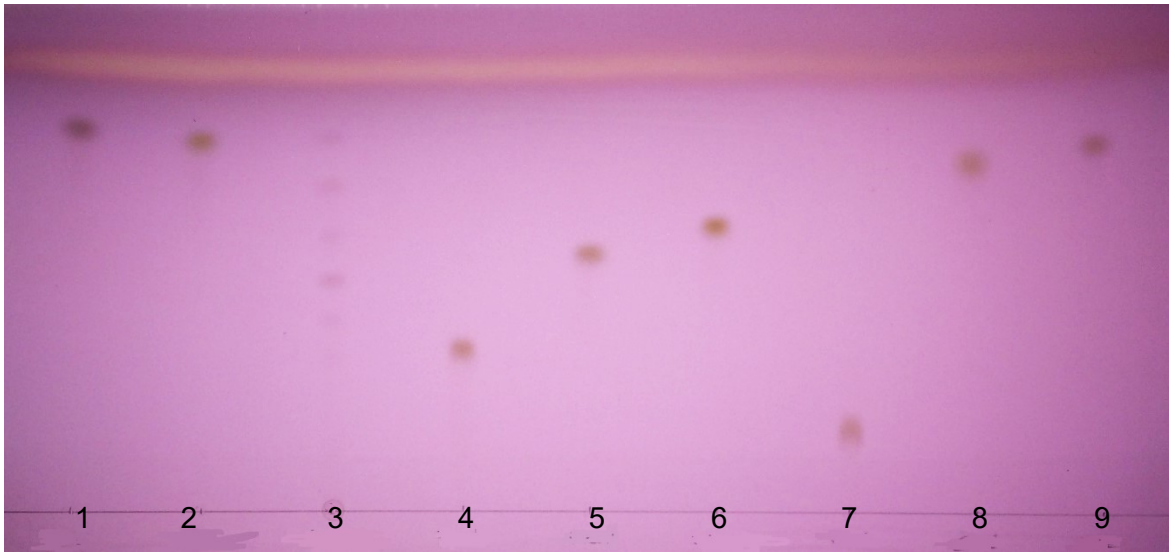


Figure 18: TLC on day 1 in water of the following carbohydrates.
1 = glucose, 2= fructose, 3= levan, 4 = stachyose, 5 = raffinose, 6 = 1-kestose, 7 = verbascose, 8 = galactose, 9 = sucrose.

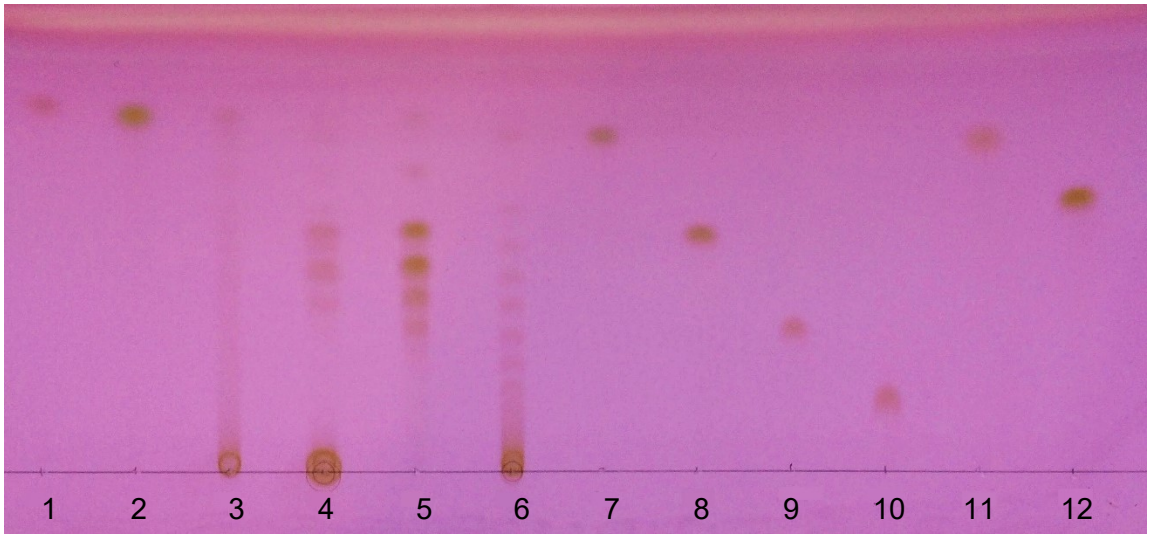


Figure 19: TLC on day 1 in water of the following carbohydrates.
1= glucose, 2= fructose, 3= Metlos®, 4= Metlin®, 5= Raftilose®, 6= Raftiline®, 7= sucrose, 8= raffinose, 9= stachyose, 10= verbascose, 11= galactose, 12= 1-kestose.

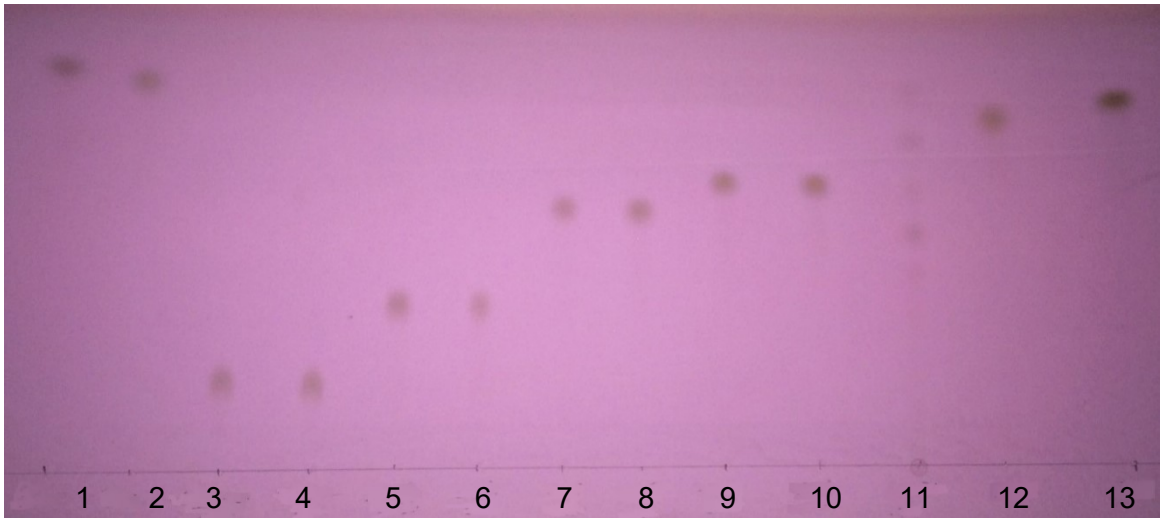


Figure 20: TLC after 7 days in water of the following carbohydrates.
1= glucose, 2= fructose, 3= verbascose 7 days, 4= verbascose, 5= stachyose 7 days, 6= stachyose, 7= raffinose 7 days, 8= raffinose, 9= 1-kestose 7 days, 10= 1-kestose, 11= levan, 12= galactose, 13= sucrose.

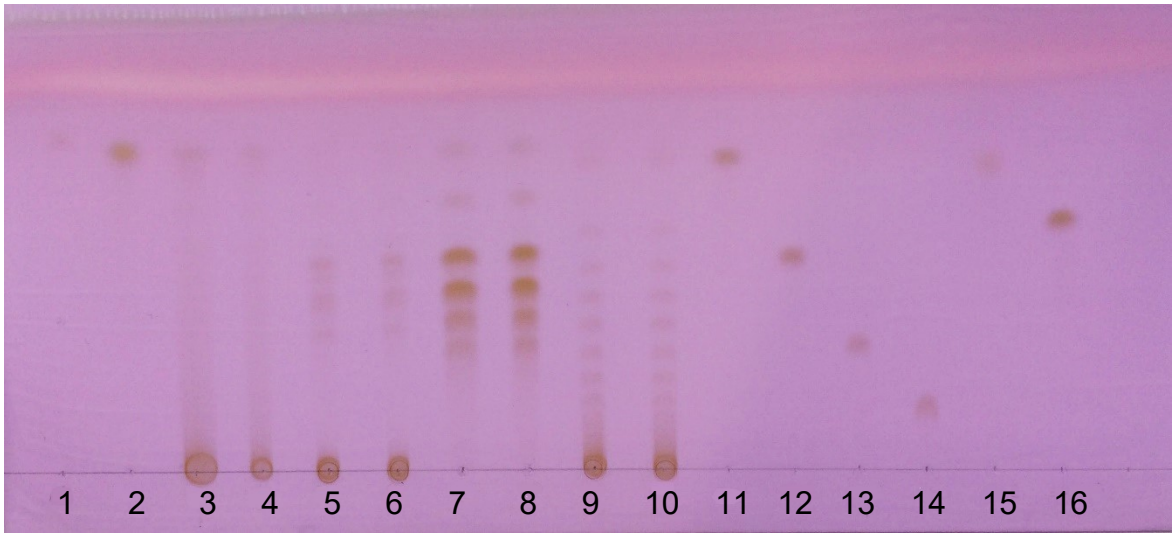


Figure 21: TLC after 7 days in water of the following carbohydrates.
1= glucose, 2= fructose, 3= Metlos® 7 days, 4= Metlos®, 5= Metlin® 7 days, 6= Metlin®, 7= Raftilose® 7 days, 8= Raftilose®, 9= Raftiline® 7 days, 10= Raftiline®, 11= sucrose, 12= raffinose, 13= stachyose, 14= verbascose, 15= galactose, 16= 1-kestose.

### 3.4.2 Stability of carbohydrates in gastric fluid

Table 8 lists the carbohydrates that were selected for the determination of the stability in gastric acid as well as the standards that were prepared for the TLC analysis including the abbreviations used for description on the TLC plates.

Table 8: Tested carbohydrates and standards for stability study in gastric fluid (incl. abbreviations).

| | test substances | | standards |
|---|---|---|---|
| raf | raffinose | glu | glucose |
| stach | stachyose | fru | fructose |
| verb | verbascose | gal | galactose |
| kest | 1-kestose | suc | sucrose |
| | | raf | raffinose |
| | | stach | stachyose |
| | | kest | 1-kestose |

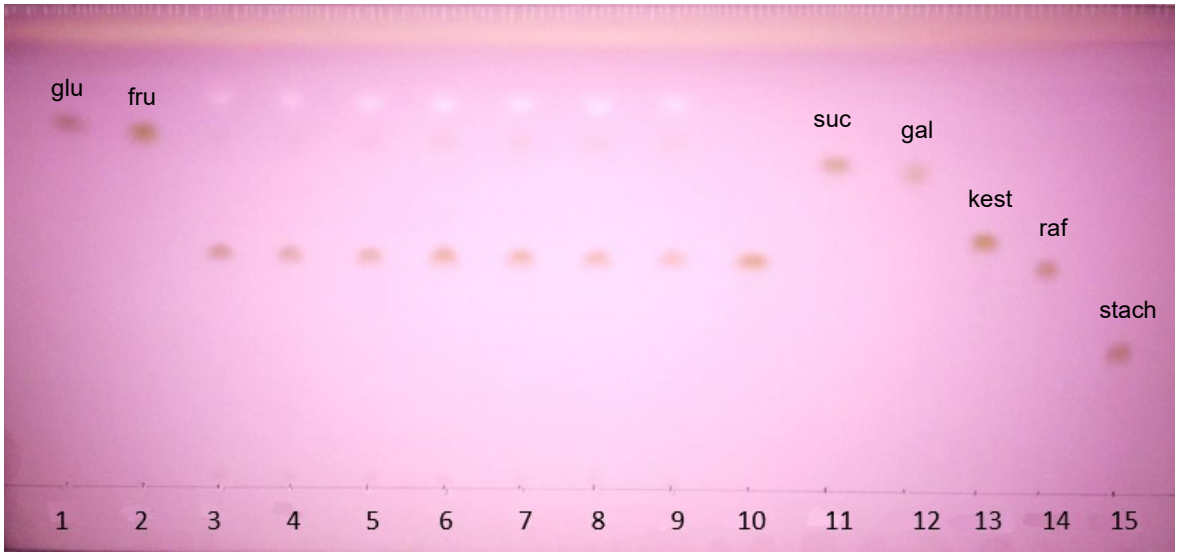


Figure 22: TLC of raffinose in gastric conditions.
1= glucose, 2= fructose, 3= raffinose $t_0$, 4= raffinose $t_1$, 5= raffinose $t_2$, 6= raffinose $t_3$, 7= raffinose $t_4$, 8= raffinose $t_5$, 9= raffinose $t_6$, 10= raffinose control, 11= sucrose, 12= galactose, 13= 1-kestose, 14= raffinose std., 15= stachyose.

As shown in Figure 22, raffinose is stable during the whole duration of 6 hours, but the intensity of the band is slightly decreasing beginning at timepoint $t_1$. It is assumed that minimal degradation to di- and monosaccharides took place but the concentration of these is below the detection limit of TLC; thus, the majority remains stable. The lighter bands at the top are caused by the hydrochloric acid.


Figure 23: TLC of stachyose in gastric conditions.
1= glucose, 2= fructose, 3= stachyose $t_0$, 4= stachyose $t_1$, 5= stachyose $t_2$, 6= stachyose $t_3$, 7= stachyose $t_4$, 8= stachyose $t_5$, 9= stachyose $t_6$, 10= stachyose control, 11= sucrose, 12= galactose, 13= 1-kestose, 14= raffinose std., 15= stachyose.

As Figure 23 shows, there is also a slight degradation in case of stachyose. This seems to be above the detection limit of the TLC because there are barely visible bands at the same hight as the monosaccharide standards. But it can be assumed that the majority remains stable.

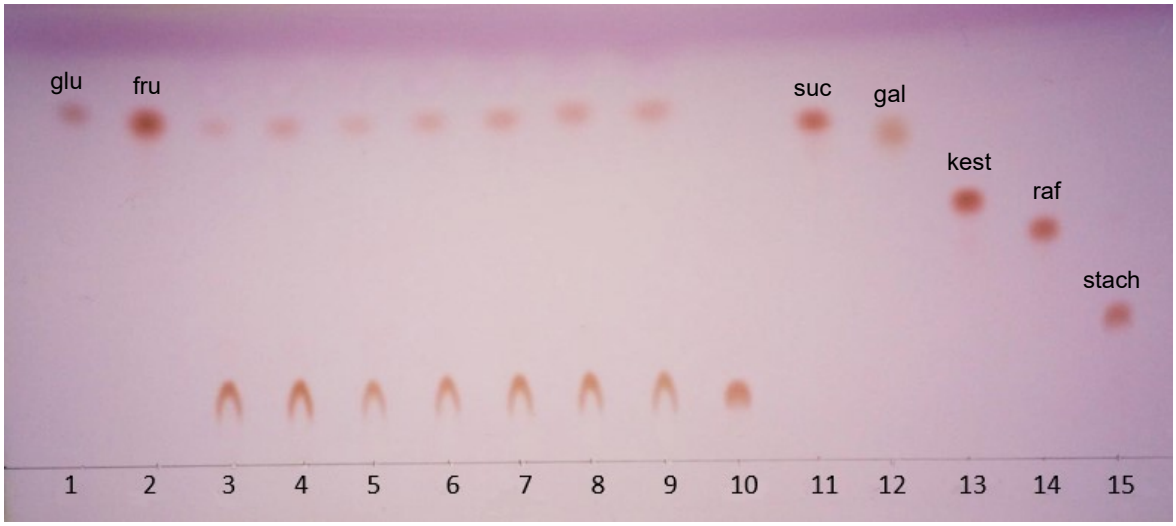


Figure 24: TLC of verbascose in gastric conditions.
1= glucose, 2= fructose, 3= verbascose $t_0$, 4= verbascose $t_1$, 5= verbascose $t_2$, 6= verbascose $t_3$, 7= verbascose $t_4$, 8= verbascose $t_5$, 9= verbascose $t_6$, 10= verbascose control, 11= sucrose, 12= galactose, 13= 1-kestose, 14= raffinose, 15= stachyose.

Figure 24 clearly shows that an increasing breakdown of the verbascose in gastric fluid has taken place. There are visible bands at the height of monosaccharides. Nevertheless, a large part of verbascose seems to remain stable.

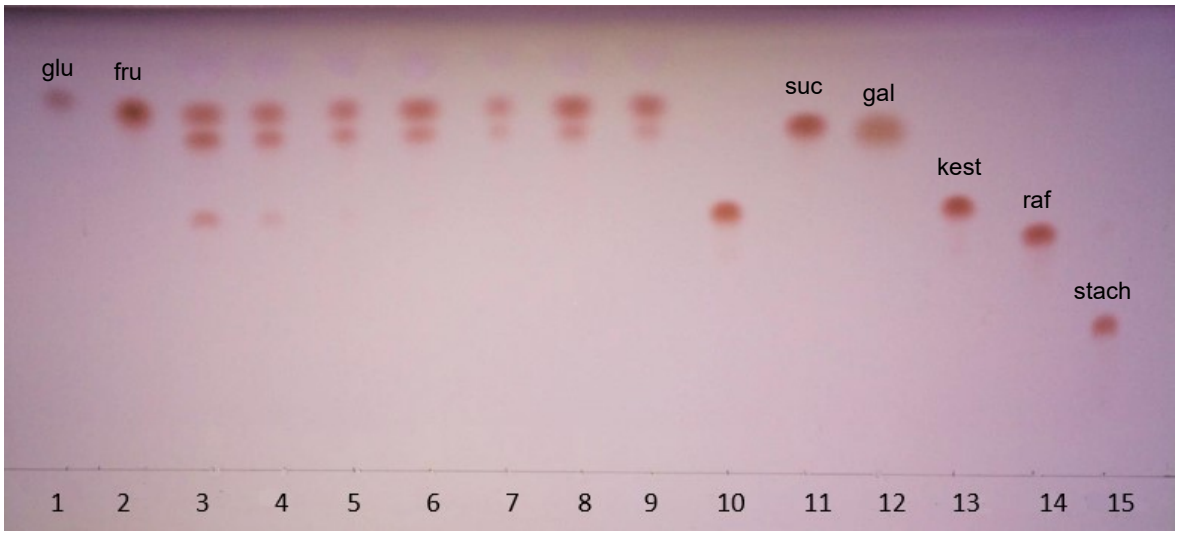


Figure 25: TLC of 1-kestose in gastric conditions

1= glucose, 2= fructose, 3= 1-kestose $t_0$, 4= 1-kestose $t_1$, 5= 1-kestose $t_2$, 6= 1-kestose $t_3$, 7= 1-kestose $t_4$, 8= 1-kestose $t_5$, 9= 1-kestose $t_6$, 10= 1-kestose control, 11= sucrose, 12= galactose, 13= 1-kestose, 14= raffinose, 15= stachyose.

From Figure 25 it can be deducted that already at timepoint $t_0$ there is a clear reduction in 1-kestose, which increases further in the following course. At timepoint $t_3$ (lane 6) 1-kestose is almost completely broken down into fructose and sucrose.

### 3.4.3 Stability of fructans and galactans under simulated gastrointestinal conditions

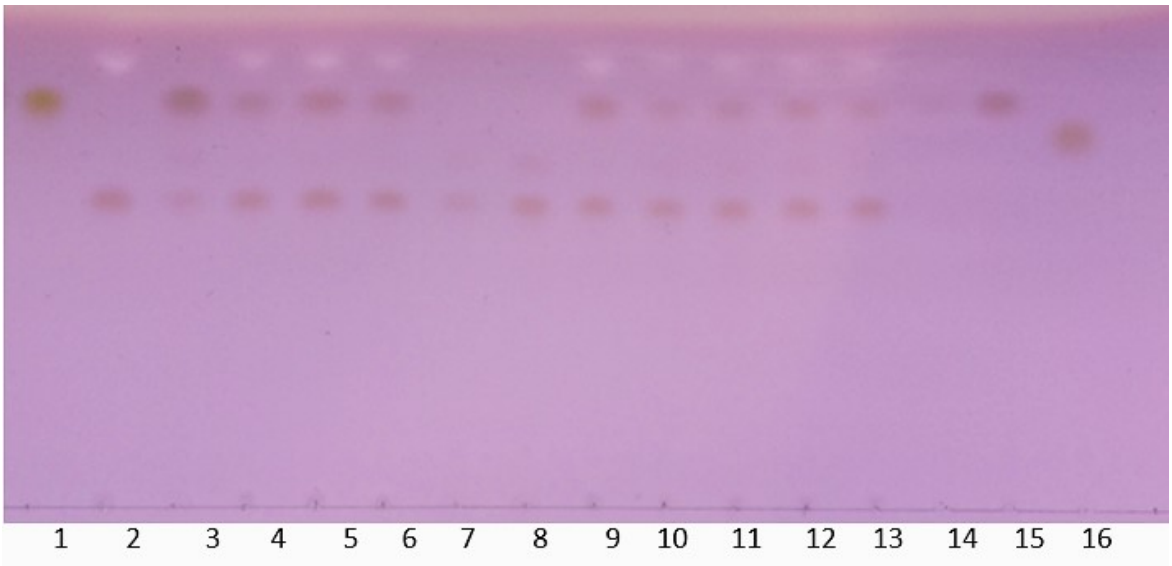


Figure 26: Gastrointestinal stability – TLC of glucose.
1= fructose, 2= gastric fluid, 3= glucose $t_1$, 4= glucose $t_2$, 5= glucose $t_3$, 6= glucose $t_4$, 7= amylase solution 50 U/L, 8= amylase solution 120 U/L, 9= glucose $t_5$, 10= glucose $t_6$, 11= glucose $t_7$, 12= glucose $t_8$, 13= glucose $t_9$, 14= glucose blank, 15= glucose standard, 16= galactose.

As Figure 26 shows glucose stays stable during all conditions of testing. On the top there are bright bands caused by the hydrochloric acid. The gastric fluid also contains pepsin that runs about the same height as a part the amylase solution which yields two bands.

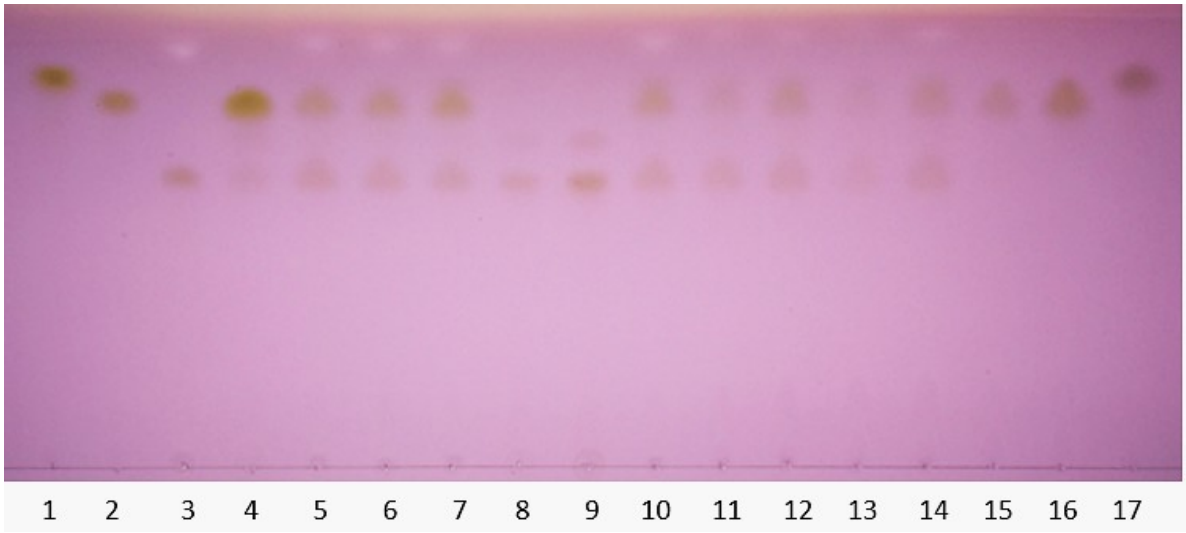


Figure 27: Gastrointestinal stability – TLC of sucrose.
1= fructose, 2= sucrose, 3= gastric fluid, 4= sucrose $t_1$, 5= sucrose $t_2$, 6= sucrose $t_3$, 7= sucrose $t_4$, 8= amylase solution 50 U/L, 9= amylase solution 120 U/L, 10= sucrose $t_5$, 11= sucrose $t_6$, 12= sucrose $t_7$, 13= sucrose $t_8$, 14= sucrose $t_9$, 15= sucrose blank, 16= sucrose standard, 17= glucose.

Figure 27 shows the gastrointestinal stability of sucrose. As mentioned before, bands starting from lane 3 (gastric fluid) are caused by the hydrochloric acid. Particularly noteworthy here is the clear band of sucrose in lane 4; however, that band becomes less distinct in the course of the experiment and tends to tailing. Tailing would normally be suspected if too much substance has been applied. If even smaller amounts do not cause any improvement, the running agent and/or the stationary phase should be changed. This could be the case with the combination of sucrose and the stationary and mobile phase selected here. A clear optimization approach can be seen for further trials.

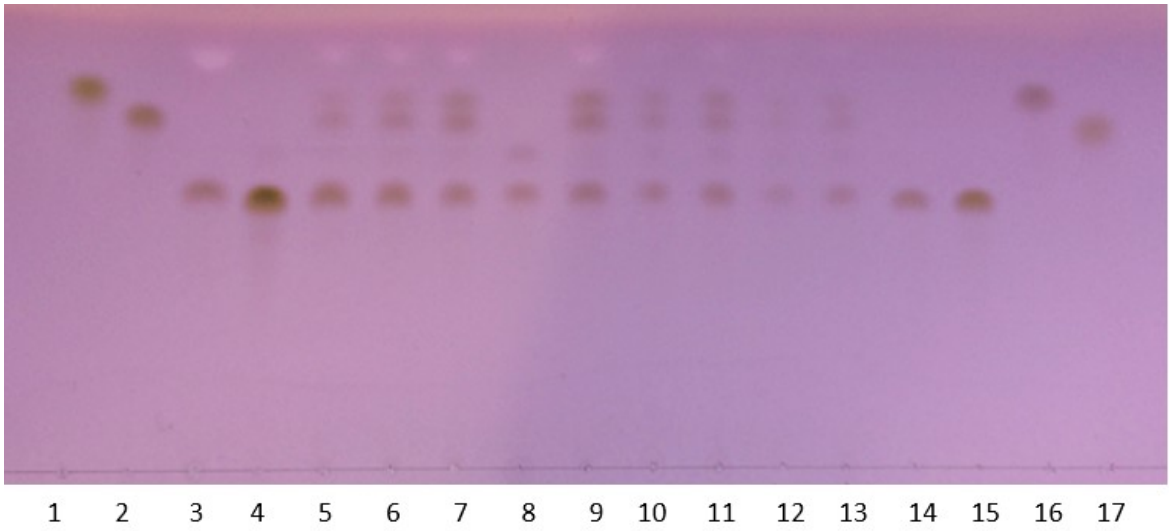


Figure 28: Gastrointestinal stability – TLC of 1-kestose.
1= fructose, 2= sucrose, 3= gastric fluid, 4= 1-kestose $t_1$, 5= 1-kestose $t_2$, 6= 1-kestose $t_3$, 7= 1-kestose $t_4$, 8= amylase solution 120 U/L, 9= 1-kestose $t_5$, 10= 1-kestose $t_6$, 11= 1-kestose $t_7$, 12= 1-kestose $t_8$, 13= 1-kestose $t_9$, 14= 1-kestose blank, 15= 1-kestose standard, 16= glucose, 17= galactose.

After 2 minutes under simulated oral conditions (lane 4) no detectible degradation in case of 1-kestose has taken place (see Figure 28). In contrast, under gastric conditions (lane 5 to 7 and 9) an increasing breakdown is taking place from the beginning. In the mimicked environment of the small intestine (lane 10 to 13) no further degradation is visible.

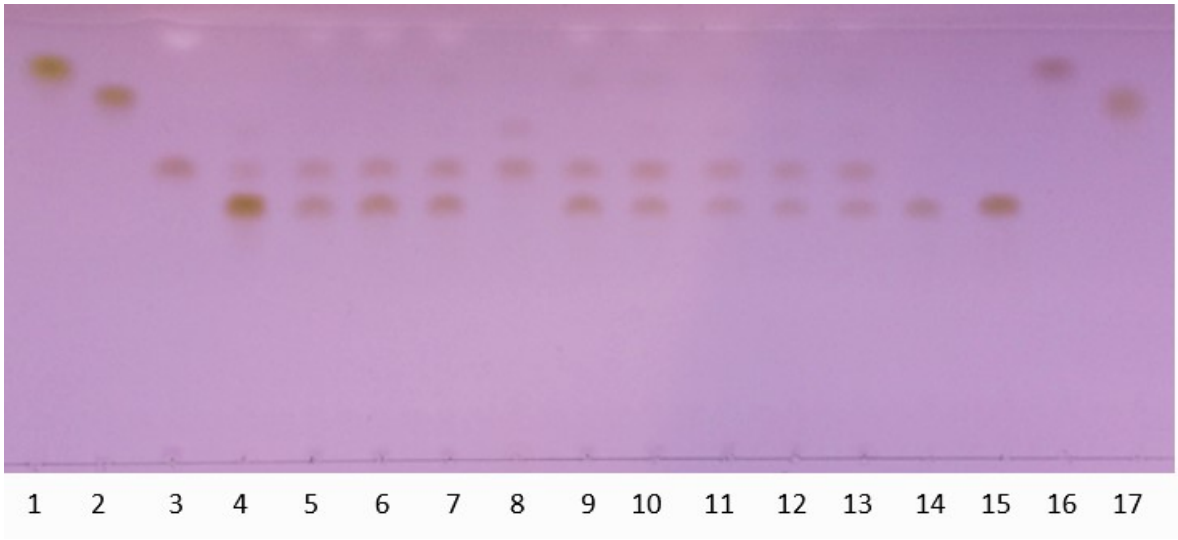


Figure 29: Gastrointestinal stability – TLC of raffinose.
1= fructose, 2= sucrose, 3= gastric fluid, 4= raffinose $t_1$, 5= raffinose $t_2$, 6= raffinose $t_3$, 7= raffinose $t_4$, 8= amylase solution 120 U/L, 9= raffinose $t_5$, 10= raffinose $t_6$, 11= raffinose $t_7$, 12= raffinose $t_8$, 13= raffinose $t_9$, 14= raffinose blank, 15= raffinose standard, 16= glucose, 17= galactose.

As Figure 29 shows the intensity of the raffinose bands is slightly decreasing from timepoint $t_1$, although there are no clearly visible bands at the level of the mono- or disaccharides. Since the intensity of the blank is also significantly paler than the raffinose band at timepoint 1, it can be assumed that raffinose remains largely stable under gastrointestinal conditions and only a minor depletion takes place.

### 3.4.4 Colorimetric determination of glucose

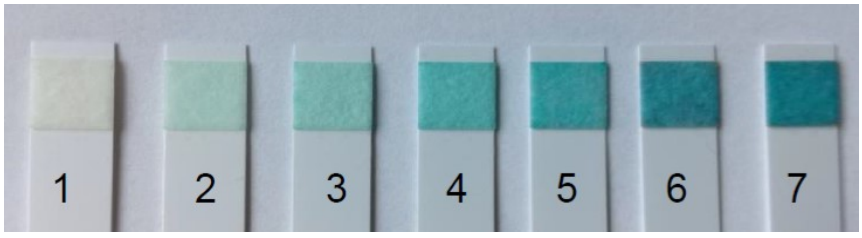


Figure 30: Calibration of glucose test strips using standard series of glucose.
1= 0 mg/L, 2= 10 mg/L, 3= 25 mg/L, 4= 50 mg/L, 5= 100 mg/L, 6= 250 mg/L, 7= 500 mg/L

At the beginning a calibration of the glucose test strips is done. Therefore, different concentrations of glucose solutions are prepared (500, 250, 100, 50, 25 and 10 mg/L). As Figure 30 shows the dilution series provides a good match with the given colour scale on the packaging (see Figure 7).
Table 9 lists the results of the colorimetric determination of glucose.

Table 9: Results of colorimetric determination of glucose using test strips.

| timepoints | glucose | sucrose | 1-kestose | raffinose |
|---|---|---|---|---|
| [h] | [mg/mL] | | | |
| $t_{1,\ mouth}$ | 500 | 25 | 0 | 0 |
| $t_{4,\ stomach}$ | 500 | 250 | 50 | 0 |
| $t_{9,\ intestine}$ | 500 | 250 | 50 | 10 |

High stability under simulated gastrointestinal conditions is shown for raffinose because there is no detectable glucose release when exposed to acidic gastric conditions. A small amount of glucose only shows up in intestinal conditions.
Another result is shown for 1-kestose. It is not cleaved down by α-amylase solution, but it cannot withstand the acidic conditions in the stomach. This result is also confirmed by TLC analysis (see Figure 28).

# 4 Discussion

With one exception, namely levan from *Serratia levanicum*, the tested fructans showed hardly any significant prebiotic effect on the selected bacterial strains. The enzymes necessary to cleave the β-2,1 (inulin-type) or β-2,6 (levan-type) linkages are either not produced in sufficient quantities by these strains or they are produced too slowly and the bacteria thus starve to death. Further research has to be applied in this context.

The reason why all tested probiotic supplements showed growth with levan from *Serratia levanicum* (Carbosynth), regardless of whether they were able to use the rest of the tested fructans, was initially unclear. But during the stability study of selected carbohydrates in water, it turned out that in case of levan from *Serratia levanicum*, degradation had occurred (see Figure 18). Considering the TLC result, it can be concluded that this product contains degradation products (presumably mono- and oligosaccharides) or impurities. The manufacturer has been contacted regarding this and the processing of this problem is ongoing.

The contained mono- and oligosaccharides are a possible explanation why all tested bacterial strains showed good growth with this levan as sole carbohydrate source.

As previously shown by Jacqueline Reiner [36] separated fractions (by SEC) of agave fructan as well as chicory inulin were used by different types of lactobacilli, especially the ones with lower dp. In contrast to this, the lactobacilli strains tested here could not utilize the complete FOS and FPS from agave or chicory. A possible explanation for this discrepancy could be that in this case the linkage type is less important than the molecular mass.

In 1973 Mital et. al reported alpha-galactosidase activity of lactobacilli for the first time. They stated that these enzymes hydrolyze naturally occurring galactosides with α-1,6-linkage (like raffinose or stachyose) rapidly [37]. The probiotic strains in this publication differ from those in this work, but the general conclusion that many lactobacilli show good alpha-galactosidase activity can be confirmed. In this thesis

utilization of RFOs was shown for 60% of the tested probiotic preparations (in detail Vagisan®, Canesflor®, OMNi-BiOTiC® FLORA plus+). In some cases, the tested alpha-galactosides (raffinose, stachyose and verbascose) even lead to higher $OD_{max}$ values than the references glucose and fructose. These are very promising results in terms of pharmaceutical application as pre- and synbiotics.
Significant growth with the guar-oligosaccharides was shown for two probiotic supplements (Vagisan®, Canesflor®). The growth curve for the strains contained in OMNi-BiOTiC® FLORA plus+ also showed minimal usability of the guar-oligosaccharides.
Two preparations that contain only a single probiotic strain (Gynophilus® and Urocys® forte) showed no α-galactosidase activity in the Bioscreen C experiments. Only the standard carbohydrates glucose and fructose were used by all tested bacterial strains.

Gastrointestinal stability is a very important factor for potential prebiotics to be administered orally. Therefore, selected carbohydrates were exposed to simulated gastrointestinal conditions and analyzed by TLC and colorimetric determination of glucose content. The stability under gastrointestinal conditions is given for a majority of the here tested carbohydrates. In case of 1-kestose, which could not withstand the acidic conditions in the stomach, it could make sense to choose an enteric-coated formulation when used as an orally administered prebiotic. 1-kestose showed very good results with *L. plantarum* P17630 (highest $OD_{600}$ value of all); they would therefore be a very promising combination.
In summary, it can be concluded that especially the alpha-galactosides, but also the guar-oligosaccharides (extracted from guar bean), showed a high prebiotic potential. They fulfill the requirements of prebiotics in terms of gastrointestinal stability.

Since there is a steadily growing interest in developing new potentially prebiotic carbohydrates, more research on substrate specificities of selected lactobacilli strains is important to enable a selective effect on desired strains.

# 5 Summary

All here tested vaginal probiotic supplements are able to hydrolyze the alpha-1,6-linkage of RFOs as shown by Jasmin Flandorfer [38]. In this study, we stated about the possible usage of selected fructans (unbranched as well as branched FOS and FPS from agave and chicory) and galactans (raffinose, stachyose, verbascose and galactomannan isolated out of the guar bean) as sole carbohydrate sources for probiotics. Therefore, the growth of selected probiotic strains was recorded using turbidity measurement. Raffinose, stachyose and verbascose were fermented by three of the here tested strains or combinations thereof. Very promising results were also obtained with the oligosaccharides isolated from guar bean. Two out of five probiotic products show growth comparable to glucose and a slight growth was also shown for another preparation. The carbohydrate-stability in simulated gastrointestinal conditions is proved for selected prebiotics, especially alpha-galactosides. Stachyose, raffinose and verbascose show a high stability under gastric conditions; only a minor depletion occurred after prolonged exposure to acidic conditions.

Considering the results of this diploma thesis, it can be stated that new prebiotic compounds in the field of fructans and galactans are identified and the findings of this study are very promising. The results shown here could contribute to the development of new prebiotic products and provide initial indications for the formulation of new synbiotics.

# 6 List of Figures

# 7 List of Tables

# 8 References


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Im Rahmen der hier vorliegenden Diplomarbeit konnte zur Publikation “Fermentation of non-digestible raffinose family oligosaccharides and galactomannans by probiotics.” (Zartl et al. (2018). Food & Function, 9(3):p. 1638-1646.) maßgeblich beigetragen werden.