Two techniques for investigation of proteins and short-chain fatty acids of *Lactobacillus plantarum* in presence of galactooligosaccharides

Marusa Bratus
Master thesis in Molecular Biology: Microbiology, 45 ECTS
November 2013 - June 2014
Department of Biology, Lund University
Supervisor: Prof. Bengt Danielsson, Acromed Invest AB,
Magistratsvägen 10, 22643 Lund

Abstract

The long-term aim of this project was to develop synbiotic drinks presenting food supplements with beneficial effects on human health. A synbiotic consists of a combination of probiotic bacteria and one or more prebiotics. For the part of the project that was covered by this Master thesis, a probiotic strain, *Lactobacillus plantarum* F44 and a prebiotic galactooligosaccharide (GOS) were tested *in vitro*. The probiotic *L. plantarum* F44 was selected due to previous systematic studies of the strain characteristics. The selected probiotic strain was cultured with GOS, but also grown without GOS (control), to compare growth efficiency, gene expression, and fermentation products. By monitoring the cell density, it was shown that GOS in a moderate concentration (2 mg/mL) increased the bacterial growth rate. Bacterial proteins play a major role in interaction with the cells in the human gastrointestinal tract. Investigation of these proteins can help understand and predict how probiotics affect the human host. Methods for protein extraction of the *L. plantarum* F44 showed that the strain had a high resistance to osmotic stress but not to lysozyme treatment. Two-dimensional protein maps were obtained of the extracted proteins. However, no distinct differences were noted of the protein patterns of bacteria grown in the presence and absence of GOS. A prospect for the future is to analyse whether there are differences when using other prebiotics, such as pectin and resistant starches, and with other probiotic strains. The concentrations of short-chain fatty acids (SCFAs) in the culture, mainly butyric and propionic acids, produced as a result of bacterial fermentation of the GOS, were determined by high performance liquid chromatography (HPLC). The conditions for the chromatographic separation were carefully evaluated and clear chromatograms of the standard solutions of butyric and propionic acids established. However, a different sample preparation will be required for accurate measurement of the SCFAs produced by the bacteria. The attempted liquid/liquid back-extraction was not sufficient since too many impurities remained in the sample, interfering with the chromatography. Further purification steps are needed.

Key words: HPLC, 2D-PAGE, synbiotics, *Lactobacillus plantarum*, galactooligosaccharides, short-chain fatty acids

Abbreviations

2D-PAGE	two-dimensional polyacrylamide gel electrophoresis
BSA	bovine serum albumin
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
DMSO	dimethylsulfoxide
DTT	dithiotreitol
GIT	gastro-intestinal tract
GOS	galactooligosaccharides
HPLC	high performance liquid chromatography
IEF	isoelectric focusing
MRS	de Man, Rogosa, Sharpe medium
PBS	phosphate buffered saline
SCFAs	short-chain fatty acids
SPE	solid-phase extraction
TCA	trichloroacetic acid

1. Introduction

The long-term aim of this project is to develop synbiotic drinks that would serve as good food supplements with beneficial effects on human health. For the part of the project that was covered by this Master thesis, a probiotic strain; *Lactobacillus plantarum* F44 was cultured with a prebiotic galactooligosaccharide (GOS), to assess whether the bacterial growth increased with the GOS and if the bacteria were able to ferment the prebiotic efficiently into short-chain fatty acids (SCFAs).

The definition of probiotics by WHO (2002) declares that they are "live microorganisms which, when administered in adequate amounts, confer a health benefit to the host" [1, 2]. Prebiotics are defined as "selectively fermented ingredients that result in specific changes in the composition and/or activity of the gastro-intestinal (GI) microbiota thus conferring benefit(s) upon host health" [1, 2]. Synbiotics are artificially created compositions of probiotics and prebiotics, in which the prebiotic selectively supports the growth of the probiotic strain [3].

For the bacterium to be appropriate for use as a probiotic, it needs to meet certain requirements. Probiotic organisms should be GRAS (generally regarded as safe) [1, 2] and be able to survive the conditions of the human gastric tract and colonize the intestine. In order to achieve best results, the probiotic should be able to adhere to the intestinal cells and mucins [1, 4]. The key proteins in the host-probiotic interactions appear to be the LPXTG-anchored proteins and collagen-binding proteins. These proteins are covalently attached to peptidoglycan and their LPXTG motif is recognised by a sortase, which cleaves it and links the protein to the peptidoglycan [5-7]. Suggested beneficial effects of the probiotics are competitively inhibiting the growth of the intestinal pathogens through the production of antimicrobial substances, enhancing the integrity of the intestinal mucosa, lowering the risk of cancer, enhancing the human immune system, and preventing some allergic reactions. In addition, pre- and probiotics are suggested to have an effect in treatment and prevention of obesity, diabetic and cardiovascular diseases [1, 2, 4, 8, 9]. However, effects that the probiotics may have on human health are strain specific and each strain must be tested individually. Most commonly used as probiotics are lactic acid bacteria, mostly from the genera *Lactobacillus* and *Bifidobacterium* [4, 8, 10].

Lactobacillus plantarum can be found in many natural environments, such as dairy, meat and plant material, as well as human intestine. Due to its ability to tolerate highly acidic environment and inability to produce carbon dioxide from hexoses, *L. plantarum* has been widely used as a starter culture. Since it also can survive and persist in the human GI tract it has often been used as a probiotic [11-13]. It was shown that the part of the *L. plantarum* genome that contains the genes of the sugar transport and catabolism is highly variable, which represents its ability to adapt to different environments. The bacterium also exhibits growth phase-dependent differences in expression of metabolic pathways, as shown with the commonly used REB1 and MLBPL1 strains [11]. Koistinen *et al*. [11] found that in the lag phase of growth, the bacteria produced mainly nucleotides and acetyl phosphate, along with the activation of the stress response. In the early log phase, proteins for the simultaneous use

of various carbohydrates were preferentially expressed. Membrane proteins and enzymes involved in the biosynthesis of proteins and cell division were the most expressed proteins during the late log and early stationary phases. In the late stationary phase, most of the stress proteins were produced. Production of proteins involved in simultaneous use of different carbohydrates contradicts the generally accepted view of the metabolism, which states that glucose represses the metabolism of other sugars. These qualities are most likely strain-specific [11, 14].

Galactooligosaccharides are oligosaccharides containing two to five galactose units in the form Glu α 1-4[β Gal 1-6]$_n$, mostly present in cow and human milk or synthesized artificially from lactose syrup [3]. It has been reported that there was an increase of faecal bifidobacteria and lactobacilli in healthy volunteers following ingestion of GOS [3]. In a synbiotic composition, GOS should be fermented selectively by the chosen probiotic strain. Fermentation of the prebiotic results in production of SCFAs, such as acetate, propionate and butyrate commonly in ratio approximately 60:20:20, respectively [2, 3, 15]. These organic molecules can be absorbed by the host mucosal cells, where they play an important role in maintaining the health of the cells. SCFAs stimulate the proliferation of the mucosal cells, inhibit growth of cancer cells, and enhance integrity of the intestinal barrier as well as the host's immune system [2, 9, 15, 16].

This project aimed to detect the production of SCFAs as a result of fermentation of GOS by a selected strain; *L. plantarum* F44 [10]. From the concentrations of the produced SCFAs, the efficiency of the fermentation can be measured. In this study the SCFAs were detected via high performance liquid chromatography (HPLC). A second part of the project was to compare, by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), potential differences in protein expression of the *L. plantrum* F44 strain, grown with or without the presence of GOS. Of most interest are the surface proteins responsible for adhesion of the bacterium to human intestinal cells and mucins and repression of pathogens.

2. Materials and Methods

2.1. Bacteria and chemicals

Bacterial strain used was *Lactobacillus plantarum* F44, donated by Prof. Torkel Wadström at Department of Medical Microbiology, Lund University. Bacteria were grown on MRS agar/broth, prepared by the same laboratory. One litre of MRS medium contained 2 g KH_2PO_4, 20 g glucose, 0.2 g $MgSO_4x7H_2O$, 0.05 g $MnSO_4x4H_2O$, 8 g meat extract, 10 g peptone, 5 g sodium acetate ($C_2H_3NaO_2$), 2 g triammonium citrate ($C_6H_{17}N_3O_7$), and 4 g yeast extract.

The prebiotic galactooligosaccharides was Vivinal GOS Powder Maltodextrin (Domo by Friesland Campina, Amersfoort, The Netherlands). All water was purified by a MilliPore Elix 10 water purification system (Merck Millipore, Billerica, Massachusetts, USA). The chemicals were purchased as follows.

Butyric, hydrochloric, phosphoric, and propionic acid, sucrose (for microbiology), diethyl ether, EDTA, NaCl, DNase I, DTT, $MgCl_2$, NaH_2PO_4, bromophenol blue, Triton X-100 and iodoacetamide: Sigma-Aldrich (St. Louis, Missouri, USA). Acetone, ammonium sulphate, chloroform, DMSO, TCA, polyethylene glycol, Tris base, and glycine: Merck (Darmstadt, Germany). HPLC grade acetonitrile, methanol and toluene: Lab-Scan (Avantor Performance Materials Poland S.A., Gliwice, Poland). Glycerol and KH_2PO_4: BDH Chemicals (VWR, Randor, Pennsylvania, USA). NaOH: Acros Organics (Thermo Fisher Scientific, New Jersey, USA). LiCl, CHAPS and thiourea: MP Biomedicals (Santa Ana, California, USA). BSA fraction V and lysozyme: Boehringer (Petersburg, Virginia, USA). Beta-mercaproethanol: Carl Roth (Karlsruhe, Germany). Urea: Pharmacia Biotech (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

2.2. Bacterial growth

The bacteria were grown in two parallel MRS cultures, one with *L. plantarum* F44 only, and the other supplemented with GOS (2 mg/mL). Initially, a third culture with 10 mg/mL GOS was evaluated, but 2 mg/mL was found to be optimal. The bacteria were grown on MRS agar without GOS for 48 hours at 37 °C then one colony was inoculated into 50 ml MRS broth and grown without agitation for 30 hours at 37 °C. During this time optical density (OD) was measured ($A600_{nm}$), and a growth curve drawn. Prior to each OD measurement the flasks were carefully shaken, to suspend all the cells. Another set of cultures was grown under the same conditions but with agitation (200 rpm). The bacterial cells were harvested by centrifugation for 10 min at maximum speed, 5700 rpm (WiFug, Doctor, 102-06). The supernatant was kept frozen at -20 °C until HPLC SCFA analyses. The cell pellet was frozen at -20 °C for protein analyses.

2.3. SCFA analyses

To evaluate the efficiency of the fermentation of GOS by the *L. plantarum* F44, HPLC was performed. Butyric and propionic acids, products of the GOS fermentation, can be detected in the growth medium and quantified by HPLC [17]. The first step of the experimental set-up was choosing the appropriate column for efficient separation of the analytes. The column Hypersil GOLD aQ 5 μm, 150 × 4.6 mm (Thermo Fisher Scientific, Waltham, Massachusets, USA) was chosen since it has a polar endcapped C18 phase, which provides the additional interaction mechanism for better separation, as well as enables the use of highly aqueous mobile phases. The column was chosen as it was prepared for separation of small polar compounds such as short-chain fatty acids, organic alcohols and sugars. Moreover, it was previously successfully used in the detection and separation of SCFAs produced by bacteria [17]. The HPLC equipment consisted of Gilson 305 pump, 811 dynamic mixer and 806 manometric module (Gilson, Middleton, Wisconsin, USA), Rheodyne 7725i manual sample injector valve (IDEX Health & Science LLC, Rohnert Park, California, USA), and Shimadzu SPD-10A UV-VIS detector (Shimadzu, Kyoto, Japan) connected to a recorder. Separation of the analytes took place in the aforementioned Hypersil Gold aQ column. There was no computer software available for peak analysis, so retention times and peak height were measured manually.

The analyses could not start immediately after preparing all the buffers and sample solutions. The HPLC machine had previously been used for separation of pharmaceutical aromatics and had not been used for several years. Due to that fact, there was no way of knowing neither how clean the system was, nor if there were any technical or mechanical problems. Therefore, a long process of calibrating the system, checking all the parts and optimizing the method took place. A detailed description of the work that had to be done can be read in the Appendix.

The HPLC calibration curve for the propionic and butyric acid was done with prepared standards of 0.5 mM, 1 mM, 2.5 mM, 5 mM, 10 mM, 25 mM and 50 mM solutions of both acids in water, with the ratio of the acids being 1:1. Fifty mM KH_2PO_4 was used as the mobile phase, under a flow rate of 1.25 mL/min at 20 °C. Detection wavelength was 210 nm and recording range was set to 0.2 absorbance units full scale. Prior to use, the mobile phase was filtered through 00H Munktell filter paper (Munktell Filter AB, Falun, Sweden) and degassed by ultrasonication and vacuum pump.

For analyses of the SCFAs produced by the *L. plantarum* F44, supernatants from two cultures, grown with and without GOS as well as pure MRS broth (blank sample) were diluted 5-fold, centrifuged for 10 min at 5700 rpm and filtered through a 0.2 μm syringe filter prior to injection into the HPLC column. This sample preparation resulted in too many interfering peaks, and therefore another method was applied. A liquid/liquid back-extraction (further: liquid extraction), as described by de Baere *et al* [17] was used. Briefly, each millilitre of the bacterial culture supernatant was acidified, and then SCFAs extracted with 5 mL diethyl ether. The organic phase was collected, and the SCFAs were back-extracted into 0.5 mL of 1 M NaOH. The aqueous phase was then collected, acidified again, and analysed with HPLC.

2.4. Protein extraction methods

Attempts to extract proteins from the bacterial cells were made in three different ways. Each method was performed on two parallel bacterial cultures, one grown without addition of GOS (control culture) and the other with addition of 2 mg/mL GOS (experimental culture).

Firstly, surface proteins were extracted using the method described by Sanchez *et al*. [12] with some modifications. Briefly, the bacterial cell pellets were washed with PBS, and then incubated with 5 M LiCl and with a protease inhibitor (Complete protease inhibitor, Roche, Basel, Switzerland) for 1 h at 37 °C. After the incubation, solutions were centrifuged and supernatants filtered through 0.45 μm syringe filters. The extracts were then dialysed (Dialysis tubing, cut off 6 – 8 000 Da; Spectrum Laboratories, Rancho Dominguez, California, USA) against 5 mM EDTA in water. In order to concentrate the solutions, proteins were precipitated with ammonium sulphate.

Secondly, proteins were extracted by sonication. Pelleted cells were re-suspended in 20 mL of PBS and sonicated (Bandelin Sonopuls HD2070 Ultrasonic homogenizer with a 3 mm UW2070 probe), in three cycles of three minutes at 70% power. Cell debris was removed by centrifugation (5700 rpm, WiFug, Doctor, 102-06) and protease inhibitors added to the

protein solution and dialysed (as described above) and concentrated using polyethylene glycol (PEG) 20 000. The PEG flakes were poured in a small box and the dialysis tube, containing the protein extract, was placed into it and kept at 4 °C until the volume of the protein solution decreased about 10-fold.

Thirdly, proteins were extracted using the enzymatic method as described by Koistinen [11], with some modifications. In brief, bacterial cell pellets were washed with a buffer containing 50 mM Tris-HCl, 150 mM NaCl and protease inhibitors. Cells were then treated with 20 mg/mL of lysozyme in 0.5 M sucrose with protease inhibitors for 45 min at 37 °C. Cells were again pelleted and treated with 0.02 mg/mL of DNase I in 50 mM Tris-Cl, 100 mM DTT, 50 mM $MgCl_2$, and protease inhibitors.

Extracts were centrifuged and supernatants treated with 10 volumes of ice-cold acetone with 0.07 % 2-mercaptoethanol and 10 % trichloroacetic acid and the solution kept at -20 °C overnight to precipitate the proteins. The precipitated proteins were centrifuged and pellets washed with ice-cold acetone with 0.07 % 2-mercaptoethanol and then dried. The proteins were resuspended in PBS with 1 % Triton X-100 but the protein pellet was extremely difficult to dissolve and a major part of the proteins did not dissolve at all. The proteins that did dissolve were collected for further analyses. The undissolved proteins were treated with a solution containing 6 M urea, 2 M thiourea, 2 % CHAPS detergent and 2.8 mg/mL DTT. The preparation was stored at -20 °C until further use.

A second enzymatic extraction of proteins was performed with a different final precipitation method. After the DNase treatment, supernatants were collected and proteins precipitated with ammonium sulphate: 1.96 g ammonium sulphate was, under constant stirring, slowly added to 3 mL of the supernatant. Precipitation was done at 4 °C overnight, centrifuged and the precipitate dissolved in PBS.

Protein concentration of all the extracts was estimated using a Bio-Rad Protein Assay for the Bradford method (Bio-Rad Laboratories Inc., Hercules, California, USA) with BSA used to create a standard curve.

2.5. Gel electrophoresis analyses

The proteins extracted from bacterial cultures were analysed and protein patterns compared by 2D-PAGE, to detect potential differences in expression correlated with the presence of GOS. Firstly, a one dimension PAGE was run with both protein extracts, to estimate the number of proteins in the samples and approximate their molecular mass. Electrophoresis was performed in a Criterion cell (Bio-Rad) and the proteins were separated in an 8–16 % Criterion Tris-HCl Gel (Prep+2 well, Criterion precast gels, Bio-Rad). For the one dimension electrophoresis, the preparative well was divided into two parts by a single comb so that both parallel samples could be run in the same gel. This was not sufficient, instead a 12% Tris-HCl Gel with 10 wells (Precast polyacrylamide gel, Bio-Rad) was used for comparison of the protein extraction methods. The electrophoresis buffer contained 25 mM Tris base, 192 mM glycine and 0.1 % SDS (10 % SDS stock solution, Bio-Rad). Before loading the sample into the well, it was mixed with Bio-Rad Laemmli Sample buffer and heated for 5 minutes at 95

°C. One molecular mass ladder (Bio-Rad SDS-PAGE Standards, Low range) was treated in the same way as the sample, the second ladder (Bio-Rad Precision Protein Standards, Prestained, Broad range) was heated for one minute prior to use. Electrophoresis was run for 25 min at 50 V and 150 mA, and then the voltage was increased up to 200 V until the dye front reached the edge of the gel. Proteins were visualized by Coomassie Brilliant Blue R-250 (Imperial Protein Stain, Thermo Scientific).

The 2D-PAGE was performed as follows. Proteins were first separated by their isoelectric point in an 11 cm ReadyStrip, pH 3–10, with an immobilized pH gradient (Bio-Rad) in an integrated instrument (IPGphor isoelectric focusing system, GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The strips were rehydrated for 12 h in a solution containing 8 M urea, 2 % CHAPS, 0.28 % DTT, trace amount of bromophenol blue, together with the protein sample (approximately 10 μg protein per strip). The isoelectric focusing (IEF) was run for a total of 50 kVh. After the IEF, the strips were equilibrated for the second dimension in two steps of 15 min each. The first buffer contained 50 mM Tris-Cl, 6 M urea, 30 % glycerol, 2 % SDS, trace amounts of bromophenol blue, and 1 % DTT. The second buffer had the same composition except instead of DTT, 2.5 % iodoacetamide was added. Strips were then placed on top of the polyacrylamide gels and electrophoresis was performed as described above. The molecular mass markers used were Bio-Rad Silver Stain SDS-PAGE Standards, Low range, and treated prior to loading according to manufacturer's instructions. Proteins were visualised by silver staining.

3. Results and Discussion

3.1. Bacterial growth

L. plantarum F44 was grown in the MRS medium at 37 °C without agitation, with 2 mg/mL of GOS or without the addition of the prebiotic (control). An additional culture with 10 mg/mL GOS was also grown, though later it was decided to use 2 mg/mL of GOS since the bacteria seemed to grow better under this condition (*Figure 1*). Another set of cultures was grown with agitation, giving approximately the same OD results as in Figure 1. From this data it was assumed that the lack of agitation did not result in major cell clumping that could potentially give falsely high cell density.

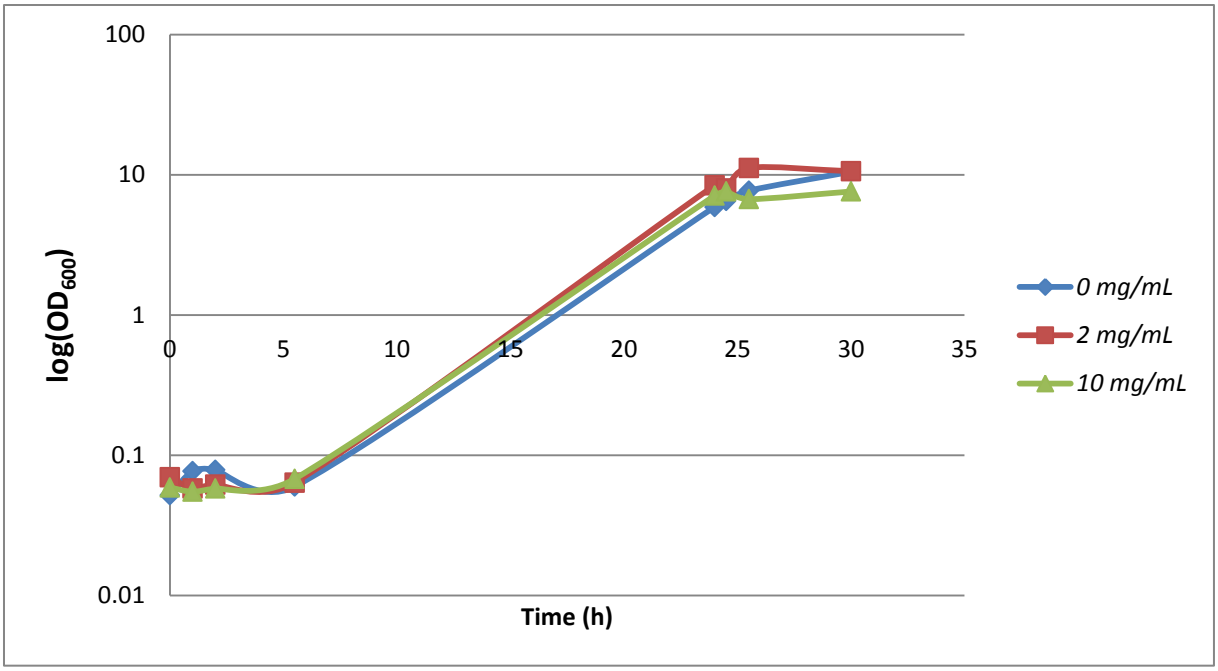

Figure 1: Growth curve of L. plantarum F44 cultured in MRS with 0, 2 and 10 mg/ml GOS (as stated in the chart legend).

3.2. SCFA analyses

In order to determine the SCFAs (of interest were the propionic and butyric acids) produced during bacterial fermentation of GOS, supernatants from the bacterial cultures were collected and analysed by HPLC. After a complicated set-up and method troubleshooting (see the Appendix), the optimal analysis conditions were settled with 50 mM KH_2PO_4 as the mobile phase at 1.25 mL/min flow rate, at 20 °C and detection at 210 nm. The standard curve for the propionic and butyric acid is shown in *Figure 2*.

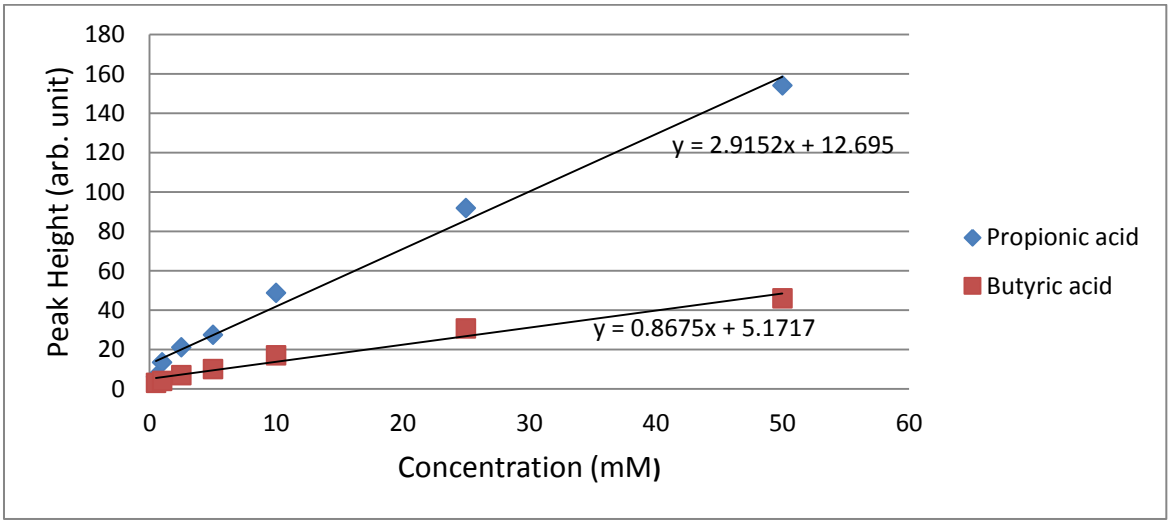

Figure 2: The calibration curves for the propionic and butyric acids are shown as peak height (in arbitrary units) in relation to concentration. Measured concentrations were 0.5, 1, 2.5, 5, 10, 25 and 50 mM and from these points the curve was regressed (linear regression) in Microsoft Office Excel. Chromatographic separation was done with 50 mM KH_2PO_4 as the mobile phase at 1.25 mL/min flow rate, acids were separated on the Hypersil Gold aQ 5 µm, 150 x 4.6 mm (Thermo Scientific) column in the ambient temperature and detected at 210 nm.

Bacterial supernatants were filtered through 0.22 µm syringe filters before being analysed by the HPLC. Many peaks were observed that interfered with the analyses of SCFAs. In order to extract the SCFAs and clean up the samples of interfering substances the supernatants were subjected to a liquid extraction. The same procedure was applied to the standard solutions of the butyric and propionic acids, diluted in MRS. Even after the liquid extraction, there were some additional peaks present, mostly appearing within the first four minutes. These were most likely some remnants from the MRS broth, that were weakly retained in the HPLC column. When the standard solutions were analysed, the peaks for the two acids could be distinguished (*Figure 3A*) and a different pattern was found with the supernatants of *L. plantarum* F44. This was likely due to a metabolic activity of the bacteria that changed the content of the media and the peaks for butyric and propionic acids were not clearly distinguishable from peaks of the substances in the supernatant (*Figure 3B*). It could also be that the acid concentration was much lower than the lowest standard (0.5 mM) and could therefore not be noted.

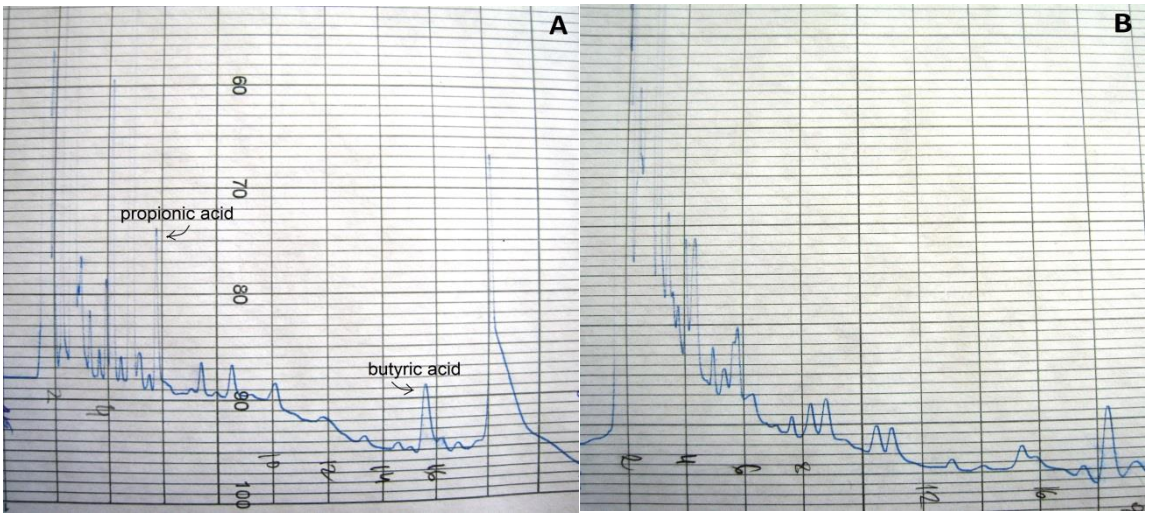

Figure 3: Chromatograms obtained in HPLC analyses of 1 mM standard solution of butyric and propionic acids in MRS (A), and of bacterial supernatant (B) after liquid extraction.

Increasing the recorder resolution did not change the result, as the noise increased too much. If the concentration of the acids was too low, it might depend on the fact that bacteria can utilise the SCFAs after 24 h of growth [16]. The possibility that SCFAs were not produced must also be considered, since MRS is a rich medium and GOS was perhaps not processed by the bacteria.

Another attempt was made to show whether the SCFAs were produced and which peaks represented the propionic and butyric acids. The bacterial supernatant was spiked with a standard solution of the acids, and subjected to the same liquid extraction procedure. Unfortunately, with time and many injections of the samples, different problems were encountered. Some more unexpected and very large peaks appeared and contamination was suspected. The whole HPLC system was thoroughly cleaned; however, the cleaning process did not seem to eliminate the problem. The sample was suspected to be somehow incompatible with the selected HPLC method, especially after it was noticed that at the injection tube small amounts of a green precipitate were forming. Though this seemed like a

strong candidate for the source of the unexpected peaks, it may not have been the sole issue. After the pump was turned off, the signal was still monitored. Instead of the baseline settling at zero, as expected, there was a steadily growing signal present (observed as baseline drift). It may have been an indicator of a detector issue, or very slowly eluting contaminants. To determine whether there were contaminants in the column or the guard column, they were in turn disconnected from the system and signal monitored. Unknown peaks were present when either the column or the guard column was connected to the system; therefore the contamination was believed to be present in these columns. They were thoroughly flushed with 50 % acetonitrile, until the contaminants were removed. This led to the conclusion that either the sample preparation method or components in samples were inappropriate. Components in the MRS broth, or what bacteria produce during culturing, could react with substances within the HPLC system. Therefore, a different approach should be tried for sample preparation prior to the HPLC analyses. Time was limited to attempt other methods in practice, however, suggestions were considered, such as a sample clean-up with solid-phase extraction (SPE). SPE of SCFAs from a bacterial culture has been made with simple C18 columns [18] though there are many options of SPE with bought columns that offer better matrices for a stronger retention of polar analytes, matrices with higher affinity for acidic or alkaline compounds, ion-exchange and others. There is also an option of utilising the volatile nature of the SCFAs and use a liquid-gas-liquid extraction with hollow fibres to purify and concentrate the SCFAs from aqueous samples [19].

3.3. Analyses of extracted proteins

In order to compare the protein patterns and detect possible differences arising due to the presence of the prebiotic, *L. plantarum* F44 was grown with or without the addition of GOS. Bacterial proteins were extracted and a 2D proteome map obtained.

Proteins were extracted by incubation with LiCl. In the first step, cells were incubated with 5 M LiCl for 30 min, and in a subsequent experiment the incubation time was extended to one hour, since very low amount of proteins was measured after 30 min. Longer incubation still resulted in an extremely low protein concentration, if any at all. When assayed with the colorimetric method, no protein was detected. This was confirmed by gel electrophoresis where no visible protein bands were found (*Figure 4*). These results showed that LiCl extraction was not sufficient for *L. plantarum* F44. The chemical and structural characteristics of *Lactobacillus* cell envelopes are not only species, but strain-specific. The macromolecules of the cell envelopes, *i.e.* peptidoglycan, wall teichoic and lipoteichoic acids, exopolysaccharides, and in some cases a paracrystalline layer of proteins outside the cell wall (S-layer), differ in quantity and chemical properties and modifications among *Lactobacillus* strains, explaining why lactobacilli respond to environmental stress differently [5, 6]. During their transit through the GIT, lactobacilli encounter several types of stress, mainly low pH, bile salts and osmotic stress. Lactobacilli have developed intricate responses that allow them to adapt to these stress factors, including a variety of regulators that affect cellular processes such as cell division, cellular transport, and membrane composition. These responses are specific for each strain [5]. Therefore, a cell lysis method, which is efficient for one strain

may need ample modification to be applicable for another strain or may not be applicable at all.

The second method used for protein extraction was sonication. This method yielded a higher protein concentration in the extracts. However, the estimated number of proteins after one-dimensional PAGE was still low (*Figure 5*). 2D-PAGE was performed and gels processed for silver staining. No spots were visible after silver staining (not shown), therefore staining with Coomassie blue was applied. This resulted in visualisation of protein spots (not shown due to broken gels). A new 2D-PAGE was run. The low number of protein spots suggested that *L. plantarum* F44 requires a more vigorous method to break down its cell wall. The reason for low protein yield after sonication could depend on insufficient number of sonication cycles or a too low power of the sonicator. An enzymatic method for protein extraction was decided to perform.

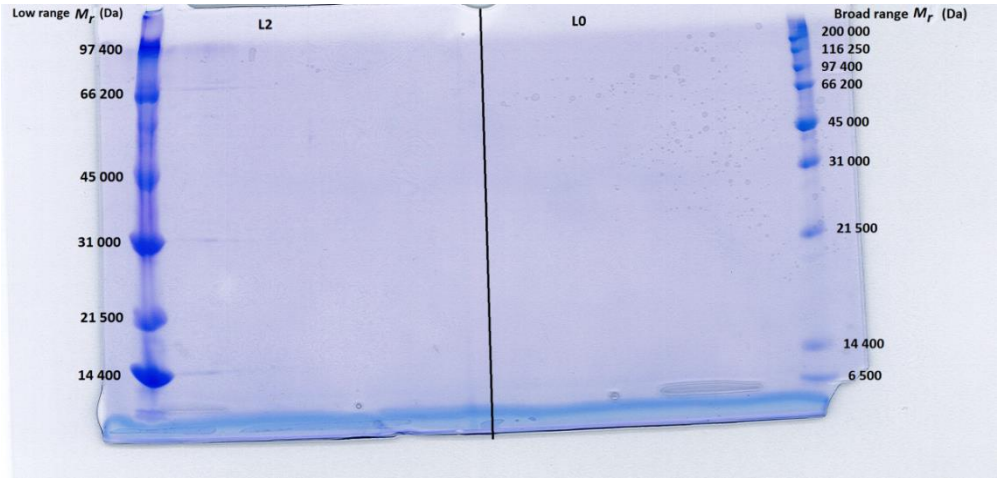

Figure 4: Gel image after electrophoresis of LiCl treated samples. L0 and L2 refer to samples extracted from bacteria that were grown in the absence of GOS and in the presence of 2 mg/mL GOS in the medium, respectively.

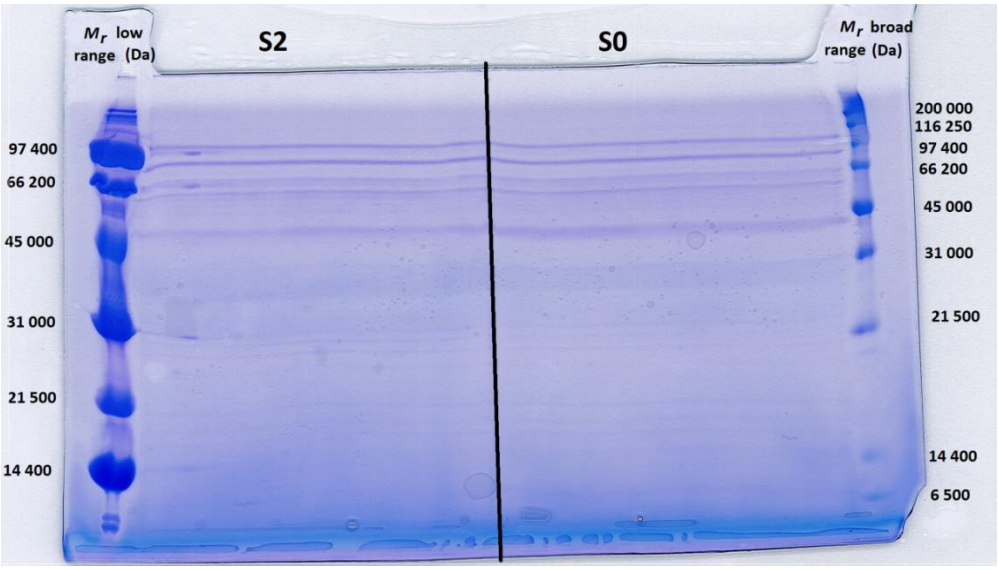

Figure 5: Gel image after electrophoresis of proteins extracted by sonication. S0 and S2 refer to samples extracted from bacteria grown in the absence of GOS and in the presence of 2 mg/mL GOS in the medium, respectively.

Proteins were then extracted using the enzyme method. The proteins were precipitated using acetone/TCA but may get degraded by this treatment due to a low pH. This treatment also caused difficulties to resolve the protein pellet. Different approaches were tried to resolubilise the proteins. PBS alone did not work and only after addition of 1 % Triton X-100 a part of the proteins could be resolved. These proteins were run in a gel and only the lysozyme was stained (*Figure 6*). Next and last attempt was to resolubilise the protein pellet in a urea-based buffer. The pellet dissolved, although not completely, and the proteins run in another one-dimensional electrophoresis. Many protein bands were found, however they were not very clear, probably due to the vigorous treatment of the proteins (*Figure 7*). The combination of PBS and Triton X-100 was not sufficient to dissolve a detectable amount of protein, except for the lysozyme present in a rather high concentration and another very faint band above the lysozyme (*Figure 6*). In the second enzymatic extraction, proteins were precipitated by ammonium sulphate, and the pellet readily dissolved in PBS. This protein extract and all other extracts were loaded on a one-dimensional gel, to compare the protein bands (*Figure 7*). The last enzymatic extraction, with ammonium sulphate precipitation, showed a high number of protein bands in contrast to the other protein extraction methods. In this gel sonicated proteins as well as the molecular mass ladder are very faint due to under-loading and enzymatically extracted proteins were slightly over-loaded. The LiCl protein extraction method, even with additional ammonium sulphate precipitation, showed that the method was not sufficient for protein extraction of *L. plantarum* F44.

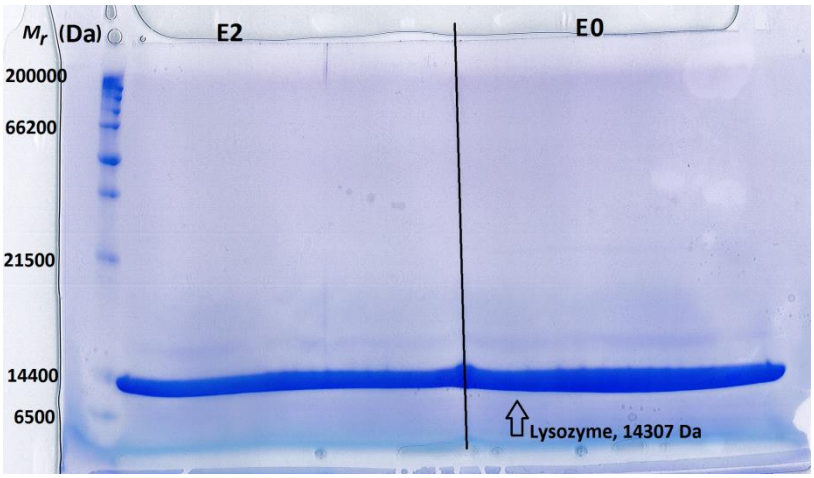

Figure 6: Gel image after electrophoresis of proteins extracted with the enzymatic method, precipitated with acetone/TCA, and resolubilised in PBS with 1 % Triton X-100. E0 and E2 refer to samples extracted from bacteria that were grown in the absence of GOS and in the presence of 2 mg/mL GOS in the medium, respectively. A strong band in the gel corresponds to lysozyme.

The protein samples obtained by sonication (S0 and S2), and proteins from second enzymatic extraction (E0' and E2') were analysed by 2D-PAGE. The 2D-maps are presented in Figures 8 and 9. IEF was run in 11 cm IPG strips with a linear pH gradient 3 – 10. S0 and S2 samples were probably under-focused, hence the horizontal streaking of the spots, which was the reason why no differences in the pattern could be determined. The gel showed that most of the proteins have isoelectric points between pH 3.5 to 6.5. The narrow vertical gap in the pattern in S2 may be the result of a bad contact between the IPG strip and the gel (*Figure 8*). The thin vertical streaks on all the gels are most likely due to impurities in the equilibration solution. A

filtering step of the equilibration solution may be needed. Despite the streaks, the protein pattern can be distinguished in samples E0' and E2' (*Figure 9*). Some differences of the protein spots and intensities were observed between the extracts from bacteria grown with or without GOS. There was no image software available, to analyse the spot positions and intensities, therefore no conclusion can be established about influence of prebiotic presence in the medium. A previous publication of *Lactobacillus acidophilus* showed that the pH range of protein isoelectric points changed in the presence of different prebiotics; proteins were acidic when the bacteria grew in presence of pectin and alkaline in presence of inulin [16]. Such a shift in protein pattern was not noticed when *L. plantarum* was grown with GOS. In future studies, it would be of interest to investigate whether the protein pattern of *L. plantarum* change if grown with other prebiotics.

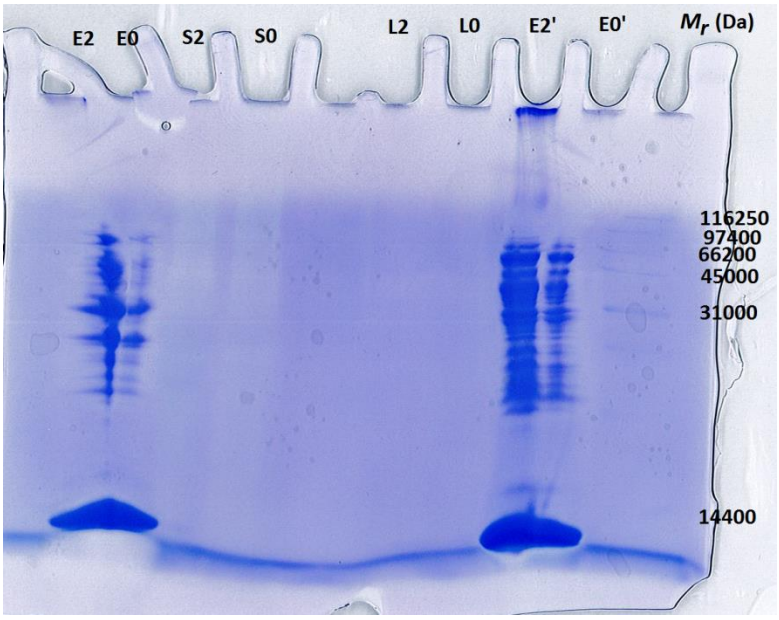

Figure 7: Gel image after electrophoresis of protein extracts obtained with all the described extraction methods. 0 and 2 in all samples refer to the bacterial extracts from L. plantarum F44 grown without GOS or with 2 mg/mL GOS, respectively. From left to right; E refers to proteins extracted by the enzymatic method, precipitated with acetone/TCA and resolubilised in a urea-based buffer. S refers to extracts obtained by sonication, L to extracts obtained by the LiCl treatment and precipitation with ammonium sulphate and E' to samples from the second enzymatic extraction, precipitated with ammonium sulphate.

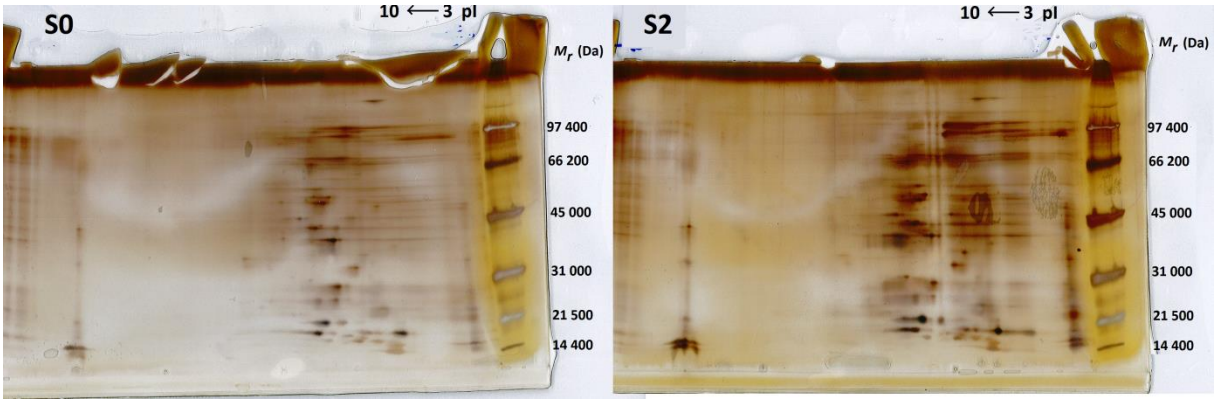

Figure 8: Two dimensional gel electrophoresis of proteins extracted by sonication, from L. plantarum F44 grown without addition of GOS (S0) and with 2 mg/mL GOS (S2). Proteins were focused in 11 cm IPG strips, pH 3 – 10 and separated in an 8 – 16 % gradient polyacrylamide gel and proteins visualised by silver staining.

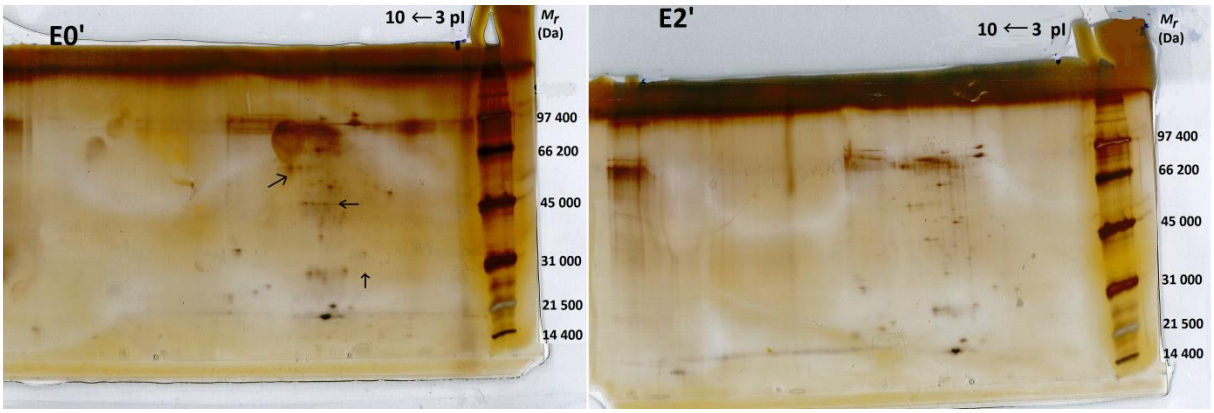

Figure 9: Two-dimensional gel electrophoresis of proteins extracted by lysozyme and precipitated with ammonium sulphate, from L. plantarum F44 grown without addition of GOS (E0') and with 2 mg/mL GOS (E2'). Proteins were focused in 11 cm IPG strips, pH 3 – 10 and separated in an 8 – 16 % gradient polyacrylamide gel and proteins visualised by silver staining. Potential differences in the protein pattern are marked with arrows.

This thesis was a starting point of a bigger project, aimed to develop synbiotic food supplements. The experiments, despite not being conclusive, have provided excellent grounds for further improvements of the methods for investigation of the probiotic bacterium *L. plantarum* in the presence of selected prebiotics. Other methods for isolation of bacterial fermentation products (SCFAs) as well as comparison of the physiology of the bacterium in the presence of other prebiotics should be a future consideration.

Acknowledgements

I would like to thank Prof. Bengt Danielsson for a helpful supervision of the project, Prof. Per-Olof Larsson for help with HPLC, Dr. Ingrid Nilsson for advice and help with 2D-PAGE and Prof. Torkel Wadström for providing bacteria and some of the chemicals and equipment.

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Appendix

HPLC

When the first tests were performed in the HPLC machine, the resulting chromatograms were disordered due to a prior use of the machine with aromatic pharmaceutical compounds. The residual contamination of the column was suspected. After some literature research, the old column was found to be inappropriate for the desired separation of the SCFAs. The column was a XTerra rp18 5.0 μm column, which was replaced with the Hypersil Gold AQ column (see Materials and Methods). The mobile phase was also changed, from 50 % acetonitrile to 20 mM NaH_2PO_4 and acetonitrile in a ratio of 80 to 20 %, respectively, as suggested in literature [17]. The new column was equilibrated with the mobile phase, according to the manufacturer's instructions. A problem with the detector was found; it displayed signals without any sample injection and some attempts to establish the detector were done. The flow cell was removed and washed with toluene. The detector's sensitivity as well as the wavelength, were set to different values. None of these attempts eliminated the problem and the detector eventually had to be completely re-wired. After some deliberation the old guard column was removed. The guard column was of the same type as the XTerra column and therefore deemed inappropriate to be used in pair with the new column. There was also a possibility that the old guard column was contaminated thus releasing unknown compounds, which could be a source of many unexpected and irreproducible peaks in the chromatograms. Baseline noise was successfully diminished by degassing the mobile phase by ultrasonication and vacuum pump.

The problem with the unknown peaks and reproducibility of sample peaks had to be resolved stepwise, because there were a number of possible points in the system that could be interfering. Pumps were controlled for leakage and the connections have been checked. The injector valve could have been dirty as well as the void pockets in the connectors, which could cause loss of the injected sample, whereas the contaminations could reach the column and consequently the detector. Prof. Per-Olof Larsson, from the Department of Pure and Applied Biochemistry, Lund University, kindly helped us with resolving the issues with the HPLC machine. Under his supervision, the detector was again checked, by injecting the sample directly through the flow cell. The absorbance of the mobile phase itself was also checked and determined to not be the cause of disturbance. Some system parts (mainly connectors and a few tubes) were replaced. The injector valve had been cleaned by injecting 2 mL of the following solvents, in order: water, methanol, acetone, toluene, acetone, methanol, water, and the mobile phase, in the "LOAD" and "INJECT" positions. The needle port and the needle were also suspected of contributing to the contamination, therefore it was decided to fill the sample loop from the opposite end, a so-called "filling by suction" (described in the Rheodyne 7725i manual sample injector valve user manual).

The next step was to perform cleaning of the column. Due to the initial use of the old column guard, the new column could have been contaminated, if some residue compound from the guard flushed into it. As described in the Thermo Scientific Phase Overwiev guide, the column was cleaned as follows. Using 50 % of the working flow rate, the column was flushed

with 40 column volumes of each of the solvents, in order: water (during this flush, four aliquots of 200 μL DMSO were injected), methanol, chloroform, and methanol. The next two weeks, after the cleaning step, were dedicated to obtaining reproducible chromatogram peaks for the two acids to be analysed. During these runs, different concentrations were tried to verify if the peak height corresponded to the acids. The detector was set at different sensitivities and recording ranges to determine the optimal settings for the analyses. There was still a problem in obtaining a signal for the analytes.

To verify that the column still was in a working condition, it was tested by injecting theophylline, with a mobile phase acetonitrile and water in the ratio of 60 % to 40 %, respectively. As stated in the example in the column certificate, theophylline eluted after 1.3 min, with a narrow peak, proving that the column was not damaged.

At this point it was decided to change the mobile phase for the analyses of the organic acids. Another example in the column guide by Thermo Scientific used 50 mM KH_2PO_4 as the mobile phase, thus it was decided to use this solution and 50 mM KH_2PO_4 was prepared, filtered, degassed and used to calibrate the column. Then, 10 mM standard solutions of each acid were injected separately, to certify the retention times and verify that each acid eluted in one narrow peak. When the t_r was determined, the 10 mM solution of both acids was separated to finally test the column. Each run was done in triplicate, to assure the reproducibility of the peaks. There were still some occasional, irreproducible peaks in addition to the peaks of the two acids. These peaks might be due to some air bubble that passes the detector as degassing may not be complete. The acids used for preparation of the samples are of >99 % quality and can therefore contain some minute amount of impurities, which get detected as well.