Chapter 10

Determination of bifidobacterial carbohydrate utilisation abilities and associated metabolic end-products

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Abstract

Bifidobacteria are able to utilize a diverse range of host-derived and dietary carbohydrates, the latter of which include many plant-derived oligo- and polysaccharides. Different bifidobacterial strains may possess different carbohydrate utilization abilities. These metabolic abilities can be studied using classical bacterial growth assessment methods, such as measurement of changes in optical density or acidity of the culture in the presence of the particular carbohydrate to generate growth and acidification curves, respectively. Scientists may also be interested in the growth rate during the exponential growth phase, and the maximum OD that is reached on a particular sugar, or the length of the lag phase. Furthermore, High-performance liquid chromatography (HPLC) and High-Performance Anion Exchange Chromatography coupled with Pulsed Amperometric Detection (HPAEC-PAD) are extensively used in carbohydrate and metabolic end-product analysis due to their versatility and separation capabilities.

Keywords: carbohydrate, growth curve, acidification, CFU, HPLC, HPAEC-PAD

1. Introduction

Like many intestinal bacteria, bifidobacteria are saccharolytic. They can utilise simple sugars and various complex carbon sources such as mucin, human milk oligosaccharides, xylo-oligosaccharides, (trans)-galactooligosaccharides, soy bean oligosaccharides, malto-oligosaccharides, fructo-oligosaccharides, pectin and other plant derived-oligosaccharides. However, the ability to metabolize particular carbohydrates is species- and strain-dependent. In general, bifidobacteria hydrolyse polymeric carbohydrates to low molecular weight oligosaccharides, which are then further degraded to monosaccharides (1; 2). These monosaccharides are subsequently channelled into the fructose-6-phosphate shunt or 'bifid' shunt, and ultimately converted to short chain fatty acids (SCFAs) and other organic compounds. The most common bifidobacterial metabolic end-products from carbohydrate fermentation include acetate, lactate, formate, ethanol; fucose metabolism was shown to additionally yield 1,2-propanediol (3; 4). As some of these end-products are considered to be beneficial for the host, members of the genus *Bifidobacterium* have a commonly accepted positive effect on human health. Certain bifidobacterial strains are commercially used as probiotics and are included in various food products as functional ingredients. In order to stimulate their establishment, persistence, abundance and growth in the gut, specific dietary carbohydrates, or prebiotics, are commonly used. The influence of these carbohydrates on growth and proliferation of bifidobacteria (as well as other purported beneficial bacteria) in the gut is of increasing interest. Surprisingly, the metabolic utilization pathways of even simple sugars are not completely understood in many gut commensals.

The methods described in this chapter can be used to study carbohydrate utilization and overall cellular metabolism in bifidobacteria, which will be insightful in terms of how this gut commensal manages to be competitive in its specific environmental niche. The scientific information obtained may generate insights that will ultimately allow to develop improved probiotic and prebiotic food supplements with increased efficacy and specificity in terms of their positive effects on human health and targeted microorganism, respectively.

2. Materials

Prepare media according to the manufacturer's instructions. Prepare and handle all the media and saline solutions using sterile technique.

2.1 Growth, CFU and acidification determination

1. Spectrophotometer or a microtiter plate reader suitable for absorbance measurements at wavelengths of 600 nm (OD_{600nm}; NB. some spectrophotometers measure at wave lengths of 595 nm or 620 nm).
2. Sterile 96-well microtiter plates. We recommend polystyrene plates (e.g., Sarstedt, Germany) for growth experiments.
3. pH meter.
4. Anaerobic cabinet or jar.
5. Sterile tubes with cap, sterile petri dishes, cuvettes.
6. Sterile inoculation loop or toothpicks, cell spreader.
7. 0.2- µm or 0.45- µm pore size cellulose acetate or nylon filters.
8. Saline solution: dissolve 0.9 g of NaCl in 100 ml of water, sterilise by autoclaving.
9. When using de Man Rogosa and Sharpe (MRS) growth medium, 0.5 % cysteine should always be added prior to inoculation. A 100 x cysteine stock solution is prepared in water, filter sterilised and stored at +4 °C for up to two weeks.

2.2 HPAEC-PAD

1. Solution A: prepare 200 mM NaOH using 50 % (w/w) Sodium hydroxide solution Fisher Cat. No. SS254-1 (to minimize contamination of the eluent solutions with carbonate, NaOH pellets cannot be used);
2. Solution B: prepare 100 mM NaOH solution with 550 mM sodium acetate (Fluka Cat. No. 71179);
3. Solution C: high quality degassed distilled water

2.3 Growth medium

1. Prepare a chemically-defined or semi-synthetic medium without a carbon source (see Chapter 1 of this book). For example, modified de Man Rogosa and Sharpe (mMRS) medium made from first principles can be used. The selected medium should contain all the ingredients required for bacterial growth except for the carbon source. The latter should be the only growth-limiting compound (*see* **Note 1**).
2. Separately prepare the stock of carbohydrate of interest. Dissolve the powder in distilled water to obtain a 10 % stock (most simple sugars will dissolve readily), filter sterilise or autoclave depending on stability of the carbohydrate. Complex carbohydrates can be added directly to the medium and then sterilised together.
3. Supplement the growth medium with carbohydrate of interest. The final concentration of carbohydrate in growth medium is usually 1 % as this amount is more than sufficient to sustain bacterial growth until the stationary growth phase is reached. Bifidobacterial growth stops when the medium becomes acidified due to accumulation of metabolic end-products such as lactic, acetic or formic acid, which makes the medium unsuitable for growth. Final pH is strain-dependent and usually reaches around pH 4.0-4.5.
4. Pipette the test medium into test tubes or microtiter plate wells. Carbohydrate utilization assays can be performed in different volumes depending on the number of strains and conditions to be tested and availability of the substrate/media.
   Test tubes: Calculate the volume of the test culture taking into account the volume that will be used for OD, acidification and other measurements at different time points. The volume depends on the equipment used and the number of assays performed with the sample. For example, if 1 mL will be removed every hour throughout a 12-hour growth cycle, 30-50 mL of culture will provide sufficient volume for the experiment.
   Microtiter plates: Carefully design the plate layout before starting the experiment (*see* example in **Fig. 1**). Calculate the volume of test medium supplemented with each carbohydrate needed and prepare this medium in a tube. Prepare some spare medium to compensate for the pipetting errors. Then pipette the required amount into each well (e.g., a 96-well plate well can be filled up to 200 µl; this should be the final volume of a well including inoculum). Multichannel pipette can facilitate pipetting.

3 Methods

All procedures should be carried out by applying aseptic techniques. Bifidobacteria are sensitive to oxygen and should not be exposed to aerobic conditions for too long, although most strains will tolerate preparation for the experiment on the lab bench.

3.1 Preparation of the bacterial suspension

A fresh pure culture should be used for the preparation of the inoculum. To refresh the culture originating from a frozen (-80^{o}C) glycerol stock, several passages in fresh medium are typically performed (*see* **Note 2**).

1. Streak the bacterial isolates/strains to be tested onto nutrient-rich agar plates supplemented with a simple favourable carbon source such as lactose or glucose to obtain single colonies.

Most bifidobacteria grow well on solidified de Man Rogosa and Sharpe medium (MRS) or reinforced clostridial agar (RCA). Incubate plates for 18-24 hours 37°C anaerobically.

2. To avoid the selection of an atypical variant clone, bacteria from four to five 'normal-appearing' colonies are used for inoculation. For each isolate, select a few single colonies from the fresh agar plate, touch them using a sterile loop or pipette tip and inoculate a sterile tube containing 10 ml nutrient-rich medium. Incubate the tubes for 16 hours at 37°C anaerobically.
3. Transfer 100 µL of the culture to 10 ml of fresh medium (1:100 dilution). In this step, the growth medium can be changed from commercial rich medium to the medium which will be used for the growth experiment to allow bacteria to adapt to it (*see* **Note 3**). Incubate the tubes for 16 hours at 37°C anaerobically.
4. Centrifuge the cultures. Wash the pellet with saline solution (PBS, 0.9 % NaCl) to remove residual sugar from the overnight culture medium. Resuspend the cells in the initial volume (10 ml) of the test medium without a carbon source. Measure the OD_{600nm} of the inoculum obtained. Because of the loss of linearity at OD_{600nm} values above 1.0, it is necessary to dilute the sample until the OD_{600nm} value is below 1.0.
5. Use this cell suspension to inoculate the tubes/microtiter plate wells.

3.2 Inoculation of the test medium

3.2.2 Microtiter plate assays

The microtiter plate-based assay is a straightforward, high-throughput approach to carbohydrate utilisation screening. However, the volumes used for the assays are usually low (typically, 100 or 200 µl) making subsequent analyses of acidification, metabolites and carbohydrate hydrolysis profile difficult without appropriate equipment. It is important to use a consistent and accurate volume for each experiment as the volume in the well of a microtiter plate determines the length of the light path within which light scattering occurs (5; 6). It contrasts with the measurement in a spectrophotometer, where the sample is put in a cuvette with a fixed light path (usually 1 cm). To monitor growth throughout a continuous bacterial growth cycle, microtiter plate reader must be placed in anaerobic environment. Alternatively, the plate is incubated in an anaerobic cabinet or jar for 16-24 hours, and then taken out to measure. Final growth characteristics (such as final OD_{600nm} and final pH) can be evaluated using this method.

1. Each condition should be tested in triplicate to obtain statistically significant data (*see* **Fig. 1**). Repeating the experiment on three different days ensures reproducibility of growth profile.
2. Inoculate each test well (e.g., 200 µl in a 96-well plate) containing the test medium and growth control wells with 2-5 µl of the bacterial suspension from Step 5 of 3.1 (*see* **Notes 4 and 5**). Make sure that the bacterial suspension is well mixed before inoculating each well. An initial OD_{600nm} between 0.05 and 0.1 is considered a good starting point as it allows a sufficient number of cell divisions before the growth curve reaches a plateau. It allows evaluating the ability of the strain to grow in the test medium. Final inoculum size should contain around 5 x 10^5 colony-forming units (CFU) ml^{-1}.
3. Control wells for bacterial growth containing a favourable carbohydrate (one set for each strain or isolate tested) and a well for sterility (media only) should be included in each experiment.
4. Place the microtiter plate into the microtiter plate reader and program the device as follows:
    a. *Temperature*: does not need to be adjusted if the plate reader is in the anaerobic cabinet.
    b. Select *Kinetic cycle* for a growth curve; *Endpoint measurement* for a single read.
    c. *Shaking*: a short shaking step ensures that the cells are uniformly suspended before each OD_{600nm} reading.
    d. *Wavelength*: 600 nm (or close to this value, some spectrophotometers read at 595 nm or 620 nm).

e. *Reading interval*: 15-30 min depending on the growth rate of the strain in the experimental medium used. A frequent measurement allows obtaining a more accurate growth curve.
f. *Run time*: determines the length of the experiment. Run should be long enough for all cultures to reach their stationary growth phase. A 24-hour growth curve usually provides the information needed. If the strain starts growing after a very long lag-phase (10-12 hours), it may indicate the appearance of mutants or growth of contaminating microorganisms.

5. Save the electronic file with the results. Zero the OD_{600nm} readings using a well that contains the same volume of growth medium as the test wells (blank, sterility control). Plot OD_{600nm} versus time to obtain the growth curve. To calculate growth rate, plot the graph on a logarithmic scale (*see* **Fig 2**). Specific programs can be used to analyse the results and calculate growth rates (e.g., GrowthRates; Hall, 2013).

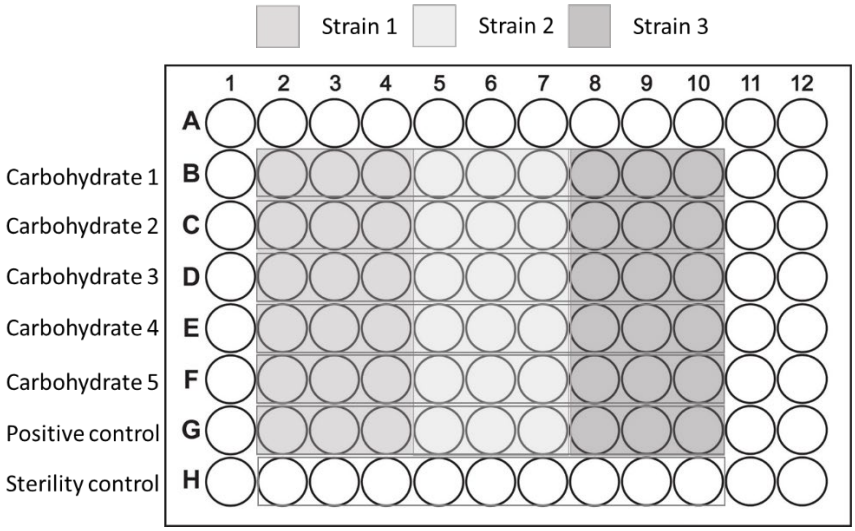

Fig. 1. An example of a layout scheme of a 96-well plate for carbohydrate utilisation screening.

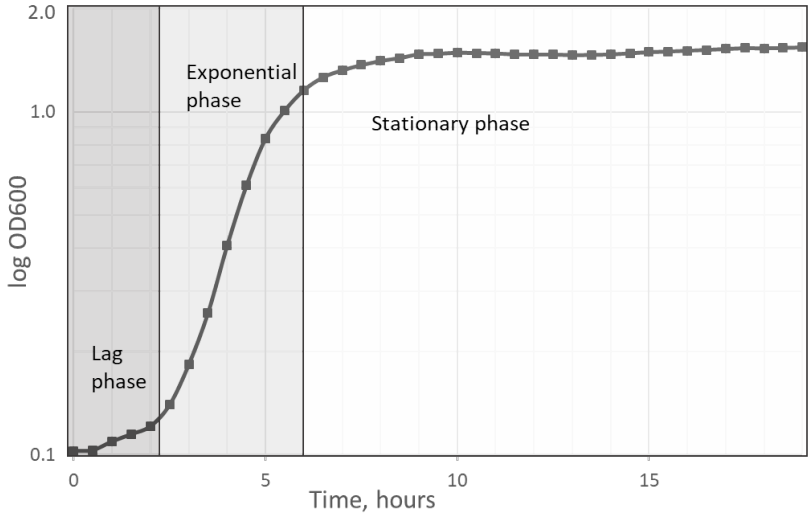

Fig. 2 Growth of *Bifidobacterium pseudocatenulatum* in mMRS supplemented with 1 % glucose, recorded by a microtiter plate reader. A lag phase with no apparent or slow growth is followed by an acceleration phase where the growth rate is increasing until it becomes constant at the exponential phase. When growth conditions become unfavourable due to carbon source exhaustion or accumulation of toxic metabolic end-products, growth rate declines until growth fully stops during stationary phase.

3.2.3 Manual growth and acidification curve generation

Classical methods of growth rate determination are tedious and labour intensive. However, using larger volumes of culture for the assay ensures that enough material for subsequent analyses is collected (*see* **Note 6**).

During their growth, bifidobacteria convert carbohydrates into various organic acids (lactic, acetic, formic) thereby acidifying the growth medium. The pH of the medium is thus indicative of cell metabolism and growth. Acidification curves might be important when the medium is too turbid to observe changes in OD_{600nm} (e.g., in milk). To measure pH without contaminating the culture, an aliquot of the medium is taken out aseptically and is discarded after the measurement. pH-meters that require less than 1 ml of liquid for a measurement are available and can be handy in generation of an acidification curve as they help to save media (*see* **Note 7**).

1. Follow steps 1-3 as described in section **3.2.2** to inoculate the cultures and control tubes (*see* **Note 8**). Measure the starting OD_{600nm} of each culture by a spectrophotometer and adjust if needed. To obtain information about initial acidification, measure the starting pH of each culture. To avoid contamination, aseptically take an aliquot of the medium for a pH measurement.
2. At intervals of 30 min – 1 h aseptically remove an aliquot of the culture, transfer it to a cuvette, measure and record the OD_{600nm}. Make sure that the bacterial suspension is thoroughly mixed before pipetting. Because of the loss of linearity at OD_{600nm} values above 1.0, dilute the sample such that the OD_{600nm} value is below 1.0.
3. Determine the pH of the undiluted aliquot.
4. Repeat steps 2 and 3 until growth stops and the OD_{600nm} and pH values become stable.
5. Plot OD_{600nm} and pH values versus time points to obtain growth and acidification curves.

3.2.4 Colony forming unit (CFU) determination

One of the most fundamental procedures in microbiology is the determination of bacterial cell number by counting Colonies Forming Units (CFU) on plates. Only viable bacteria are counted with this method as it excludes dead or injured bacteria and debris. One major disadvantage of the CFU count is that clumps or chains of bacterial cells form a single colony. For this reason, the results of this method are reported as CFU ml^{-1} rather than bacteria ml^{-1}. Since the CFU determination is relatively tedious and time-consuming, it has limitations for high throughput screening studies.

1. Follow the above-described steps (*see* 3.2.3) to start cultivating the culture on a carbohydrate of interest.
2. Remove an aliquot of the growing culture at the time point of interest. OD and pH measurements can be performed at the same time point to obtain more data about the growth dynamics.
3. Dilute the aliquot of the culture 1:100 using rich medium or sterile saline solution (dilution: 10^{-2})
4. Dilute stepwise the solution obtained five times 1:10, using sterile tubes and nutrition rich broth or sterile saline solution until you reach a dilution of 10^{-7}.
5. Plate 100 µl of the last five of the 1:10 dilutions (10^{-3} to 10^{-7}) onto nutrient-rich agar plates using a sterile cell spreader.
6. Incubate plates at 37°C anaerobically.
7. Count colonies on plates. It is best to determine the CFU based on the dilution that produces between 100 and 400 colonies on the corresponding plate. Higher numbers do not accurately represent the original suspension as errors are created by coincidence of colonies and nutrient limitation. Counting fewer than 100 colonies per plate might result in statistical errors.

8. Calculate the CFU per ml that were in the culture using the following formula:

$$N = \frac{C \times 10}{10^{-D}}$$

where, N = cfu ml^{-1}; C = number of colonies per plate; D = number of the 1:10 dilution.

9. Average the results from three independent tests performed with the microbial isolate.

3.3 Metabolite analyses by high-performance liquid chromatography (HPLC)

HPLC has been widely used to separate simple carbohydrates and organic acids. The separation can be manipulated by varying the make-up and concentration of the mobile phase, and the adsorption properties of the stationary phase. For comprehensive analysis of monosaccharides, sugar alcohols, organic acids, glyphosate, and sugars ion exclusion columns are used (7). The column with the appropriate selectivity must be chosen for each experiment. For instance, REZEX 8 µm 8 % H organic acid column (300 mm × 7.8 mm, Phenomenex, Torrance, CA, USA) can be utilized to separate sugars and organic acids from spent medium. The compounds can be separated and detected at a wavelength of 210 nm, on an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA) or equivalent instrument. Elution is performed for 25 min using a 0.01 M H_2SO_4 solution as a mobile phase at a constant flow rate of 0.6 mL/min at 65°C. The exact elution conditions depend on the HPLC system, the column used and the compounds of interest to be analysed.

1. To assess fermentation end-product profiles by HPLC, collect culture samples at time-points of interest, centrifuge and filter (0.45 µm or 0.2 µm filter) the supernatant to remove cells and other larger particles. These samples can be stored at +4°C or −20 °C.
2. Most common bifidobacterial end-products are lactic acid, acetic acid, formic acid, and in some cases 1,2-propanediol. Prepare the standards of expected metabolites by dissolving them in sterile water or growth medium at a concentration of 10 mM.
3. Sterile test medium and a control sample which contains only cells and medium but no carbon source, serve as indicators of basal metabolite profile before fermentation has occurred.
4. Inject 10-20 µl of sample into the system using the manual injection valve or an auto sampler. Use the separation protocol optimised for the target compound separation (see suggestion above).
5. Each peak of the chromatogram represents a component present in the sample. Retention time is time interval between sample injection and the maximum of the peak. Retention time depends not only on the structure of the specific molecule, but also on factors such as the nature of the mobile and stationary phases, the flow rate of the mobile phase, and dimensions of the chromatographic column. Retention time is usually characteristic for a specific compound in a given separation. For this reason, identity of the component can be established by injecting a standard compound under the same operational conditions. The matching retention time of the standard and the component peak confirms the identity of the unknown sample component.

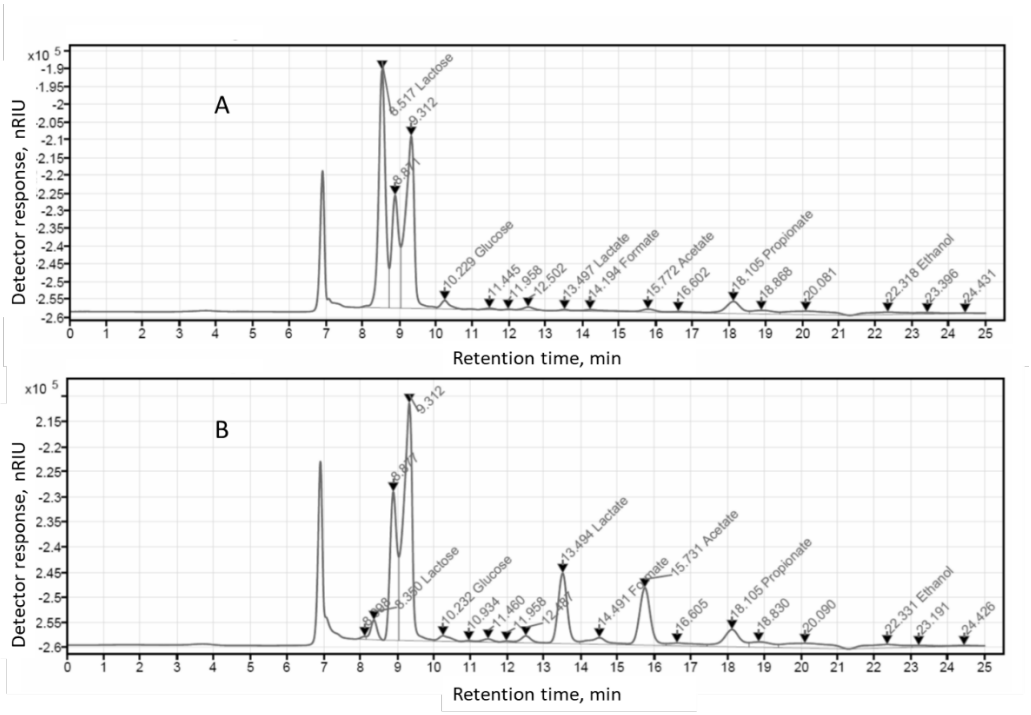

Fig. 3 HPLC analysis of mMRS supplemented with 1% skimmed milk before (A) and after (B) incubation with *Bifidobacterium breve* UCC2003. After 20 h of incubation with the bacterium, a peak representing lactose (around 8 min) decreases in height while those specifying lactate (around 14 min) and acetate (around 16 min) appear.

3.4 Assessing substrate consumption and/or degradation profile using High-Performance Anion Exchange Chromatography – Pulsed Amperometric Detection (HPAEC-PAD)

High-performance anion exchange chromatography (HPAEC) was developed to separate carbohydrates. Coupled with pulsed amperometric detection (PAD), it permits separation, detection and quantification of carbohydrates at low levels with minimal sample preparation and clean-up. HPAEC takes advantage of the weakly acidic nature of carbohydrates to give highly selective separations at high pH using a strong anion-exchange stationary phase. HPAEC-PAD systems such as Dionex ICS-3000 (Sunnyvale, CA, United States) can be used to detect, quantify and/or separate various carbohydrates that appear in the growth medium as a result of microbial sugar degradation. An appropriate column should be selected for a particular analysis. For example, while the Dionex CarboPac PA100 is optimized for oligosaccharide resolution and separation, the Dionex CarboPac PA1 is particularly well-suited to the analysis of monosaccharides and the separation of linear homopolymers.

1. To assess substrate degradation profile, collect culture samples at time-points of interest, centrifuge and filter (using 0.45 nylon filter; cellulose acetate and other filters should be avoided because they may leach carbohydrates) the supernatant to remove cells and other larger particles. These samples can be stored at +4°C or −20 °C.
2. Prepare all media samples for HPAEC-PAD analysis by diluting them with water 1:10. Dilution protects the column from overloading it with rich medium components and improves peak separation. Dilution factor depends on the amounts of metabolites produced and the sensitivity of the method.
3. Prepare a 1 mg/ml stock solution of each of the carbohydrates, as well as their putative breakdown products (where available) to be used as reference standards by dissolving them in sterile water or growth medium.
4. Prepare eluents. To identify monosaccharides, disaccharides, oligosaccharides, linear polysaccharides, and aminoglycosides using the CarboPac PA1 analytical exchange column

(250 mm × 4 mm) with a CarboPAC PA1 guard column (50 mm × 4 mm), these eluents should be used (*see* **Materials**):
A, 200 mM NaOH
B, 100 mM NaOH with 550 mM sodium acetate
C, high quality degassed distilled water

5. Load 25 µl of each sample and standard into the system.
6. Elute at a constant flow-rate of 1.0 ml min^{-1} at 30°C using a linear gradient of sodium acetate with NaOH:
   - from 0 to 50 min, 0 mM;
   - from 50 to 51 min, 16 mM;
   - from 51 to 56 min, 100 mM;
   - from 56 to 61 min, 0 mM.
7. Compare chromatographic profiles of standard carbohydrates to those obtained after sugar incubation with bacterial strains. We use Chromeleon software (Dionex) to analyse the chromatograms obtained.

4 Notes

1. To avoid contamination and ensure selectivity of the medium, mupirocin can be added. This antibiotic inhibits growth of bacilli, lactococci, lactobacilli and streptococci, enterococci, pediococci and propionibacteria while bifidobacteria are naturally resistant. Prepare a stock solution of 10 mg/ml in growth medium, filter sterilize and store at 4 °C. Add this stock solution to the growth medium to obtain final concentration of 50-100 µg/ml.
2. Bifidobacteria strongly acidify their growth medium with their metabolic end-products such as lactic, acetic or formic acid. Final pH of the medium is strain-dependent and usually reaches around pH 4.0-4.5. As most strains are sensitive to low pH, liquid overnight cultures may prove less viable than freshly grown broth cultures. It is thus important to keep the incubation times as short as possible (16 hours for an overnight culture is usually a good incubation time, although it can be shortened if problems with cell viability occur).
3. The carbon source used for the overnight culture should preferably not induce carbon catabolite repression in the strain tested. Repression of catabolic operons might result in a prolonged lag-phase during subsequent growth on the test carbon source.
4. It has been observed in some cases that cultures in the outer wells grow differently than those in the inner wells due to differences in aeration, temperature and evaporation. This property might depend on particular plate reader or volumes used for the experiment. It is important to test the uniformity across the wells in each setup.
5. If the test medium is poor and the carbon source is difficult to metabolise, more inoculum and a higher starting OD_{600nm} (up to OD_{600nm} of 0,1) can shorten the lag phase. Alternatively, removing the washing step can improve strain revival in the test medium.
6. It is not recommended to repeatedly remove the anaerobically growing culture from the anaerobic cabinet for an OD_{600nm} or pH measurement. Ideally, the sample is taken in the anaerobic cabinet. Specialized vials compatible with a spectrophotometer that is placed in the anaerobic cabinet can be used for growth and OD_{600nm} measurements without the need of taking the whole culture out of the cabinet.
7. To monitor bacterial behaviour in test medium without a carbon source, additional control should be added. This tube/well provides information about basal OD_{600nm} and acidification of the medium that can be subtracted from the final values of your experiment.
8. If the aim of the experiment is to test utilisation of multiple sugars by a single strain, the bacterial suspension can be diluted to the final desired OD_{600nm} value, and then added to tubes/wells containing only the carbon source. In this case, the inoculum will be identical, and only sugar type will vary across the tubes/wells.

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