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ORIGINAL PAPER

# Xylose utilization and short-chain fatty acid production by selected components of the intestinal microflora of a rodent pollinator (*Aethomys namaquensis*)

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**Abstract** Namaqua rock mice (*Aethomys namaquensis*) consume nectar xylose when visiting *Protea* flowers. Whole-animal metabolism studies suggest that the gastrointestinal microflora plays an important role in xylose metabolism in *A. namaquensis*. We collected caecal contents under anaerobic conditions, cultured caecal microflora both aerobically and anaerobically, and assessed caecal microbial xylose utilization using a $^{14}$C-xylose incubation assay. All four mice sampled hosted culturable caecal micro-organisms that tested positive for xylose utilization. These were classified by 16S rRNA based taxonomy as: *Bacillus subtilis*, *Bacillus pumilus*, *Bacillus licheniformis*, *Shigella boydii, Arthrobacter* sp. and members of the fungal genera *Aspergillus* and *Penicillium*. Cultures of these isolates were then analyzed by gas chromatography to determine the types and quantities of short-chain fatty acids produced by xylose fermentation. These results are discussed in the context of other studies of gut microflora in vertebrates.

**Keywords** Gut symbionts · Fermentation · $^{14}$C-labeled xylose · Proteaceae · Cape Floristic Kingdom

**Abbreviations**
CFU Colony forming units
SCFA Short-chain fatty acids

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## Introduction

In two South African genera of the Proteaceae, *Protea* and *Faurea*, xylose constitutes as much as 39% by mass of total nectar sugars, the remainder of which are the more common nectar sugars sucrose, glucose and fructose (Nicolson and van Wyk 1998). Before nectar sugar analyses among the Proteaceae (van Wyk and Nicolson 1995; Nicolson and van Wyk 1998), the pentose sugar xylose had not been reported as a floral nectar sugar and was only known to occur in the extrafloral nectar of grasses (Bowden 1970). Its presence in the nectar of Proteaceae prompted studies of sugar preferences and sugar absorption efficiencies among

insect, bird and rodent pollinators of the Proteaceae, reviewed by Jackson and Nicolson (2002). These studies suggested that xylose is of some importance in the diet of the Namaqua rock mouse *Aethomys namaquensis*, which pollinates certain geoflorous *Protea* species but is unlike other pollinators of the Proteaceae in that it consumes pure xylose solutions and gains energetic benefit from this sugar (Johnson et al. 1999). A high total proportion (78%) of xylose doses given to rock mice was processed in some way, with only 2% of ingested xylose simply excreted as feces (Johnson et al. 1999, 2006). Of the processed fraction, some 42% is likely metabolized in the gut lumen, and a further 34% absorbed from the intestine into the blood, then filtered and excreted in the urine (Johnson et al. 2006). The fate of the remaining 22% is unknown, but it is more likely to be retained in the blood and slowly excreted rather than efficiently metabolized by the mouse tissues, which probably lack the necessary enzymes for this process (xylose isomerase and xylulokinase). Certain bacteria, yeasts and fungi possess these enzymes and are well known to be efficient xylose utilizers (Demetrakopolous and Amos 1978; Chandrakant and Bisaria 1998; Ho et al. 1999; Jeffries and Shi 1999; Aristidou and Penttilä 2000).

Gastrointestinal microflora are essential components of the digestive system in vertebrates that consume plant material (Stevens and Hume 1995). Plant polysaccharides, such as cellulose and hemicellulose, can only be utilized by vertebrates if the necessary gastrointestinal microbes first degrade the polysaccharides into components that are metabolically useful to the host. The hemicellulose component of plant cell walls, xylan, is a polysaccharide made up of xylose monomers. The degradation of xylan and the microbial utilization of xylose have been well studied in ruminants (e.g. Turner and Roberton 1979; Hespell et al. 1987; Matte et al. 1992; Marounek and Kopečný 1994) and less thoroughly investigated in non-ruminants where, particularly in small herbivores, the major part of microbial plant digestion occurs in the caecum (Stevens and Hume 1995). Microbial degradation and fermentation of plant material in the vertebrate gut produces, predominantly, short-chain fatty acids (SCFA), which the host animal is able to use in oxidative metabolism. The general nutritional contributions of gut microbes to vertebrates, and SCFA production and contributions to the energy requirements of vertebrates have been comprehensively reviewed by Stevens and Hume (1995, 1998).

Xylose utilization in situ by intestinal bacteria of *A. namaquensis* has been previously confirmed using captive mice fed $^{14}$C-labeled xylose first with their natural gut flora intact and then again after antibiotic treatment to reduce gut flora populations (Johnson et al. 2004, 2006). Reduction of gut microbe populations reduced xylose metabolism to 57% of pre-treatment values, highlighting the importance of gut symbionts in complete utilization of this sugar by the rodent. Here, we further investigate this relationship by isolation and identification of some important xylose-utilizing microbes from the cecum of this mouse species. Diet plays an important role in the structure and metabolic activity of intestinal microbial communities; we investigated bacterial xylose utilization in the gut contents of mice in their natural habitat, eating their natural diet. We cultured obligate and facultatively anaerobic microbes isolated from the mouse caecum under strict anaerobic conditions, to simulate conditions in the mouses' caeca as closely as possible. We also sampled caecal contents anaerobically and assessed bacterial xylose utilization using a $^{14}$C-xylose incubation assay. We identified positive xylose utilizers from these cultures to species level by DNA sequencing. This approach highlights a novel role for rodent endosymbionts in the context of plant–pollinator relationships.

## Materials and methods

### Sample collection and isolation of microflora

*Aethomys namaquensis* individuals were caught under permit (numbers 180/2003 and 001-201-0002, Western Cape Nature Conservation) in Sherman live traps at Wolfieskop (33°55′S, 19°24′E) and Jonaskop (33°56′S, 19°31′E), near Villiersdorp in the Riviersonderend Mountains, South Africa. These sites were chosen because of the presence of *Protea humiflora*, a ground-flowering species known to be pollinated by *A. namaquensis* (Fleming and Nicolson 2002), and in which xylose comprises 5–9% of total nectar sugars (Nicolson and van Wyk 1998). Animals were captured during the flowering season (July–September) when xylose, in the form of *P. humiflora* nectar, was present in their diet. Ethical approval for animal capture and the protocol below was obtained from Sub-Committee B of the Research Committee of the University of Stellenbosch.

Four animals (Mouse 1–Mouse 4) caught during the night were taken back to the laboratory and housed individually in standard rodent cages (44 cm L × 28 cm B × 23 cm H) at a constant temperature of 22°C with ad libitum access to water and food pellets (Prestige Products, Crispy Rat Muesli). Within 24 h of capture, animals were killed by $CO_2$ inhalation and placed in an anaerobic chamber (atmosphere: 85% $N_2$, 10% $CO_2$

and 5% $H_2$) (Forma Scientific, OH, USA) where the caecum of each was excised. One 0.5 g sample of caecal contents for each animal was weighed into a sterile tube containing glass beads and 4.5 ml sterile pre-reduced rumen fluid-based liquid medium (Campbell et al. 1997). The sample was vortexed to homogenize the caecal contents and resuspend bacteria from caecal material. Isolation of caecal samples was carried out under strictly anaerobic conditions within the anaerobic chamber.

### Cultivation of microflora

Enumeration and growth of culturable caecal microbes was done both aerobically and anaerobically to obtain as many different microbial species in culture as possible. Serial 10-fold dilutions ($10^{-2}$–$10^{-7}$) of the single sample from each mouse suspended in liquid medium were plated out in duplicate on a rumen fluid-based medium, [a medium used to specifically culture rat caecal and fecal microflora (Campbell et al. 1997)]. Plates were incubated at 37°C for 48 h. Anaerobic incubation was carried out in anaerobic jars. The number of colony forming units (CFU) per gram of caecal content was calculated for each plate.

### Xylose utilization

For each of the four mice, single representatives of all culturable colonies were selected from the plates at the highest dilution that yielded sufficient numbers of colonies ($30 < n < 300$). We selected representatives on the basis of colony size, color, and shape, and re-streaked these onto rumen fluid-based medium to produce pure cultures (Mouse 1: 10 isolates, 4 aerobic and 6 anaerobic; Mouse 2: 13 isolates, 10 aerobic and 3 anaerobic; Mouse 3: 16 isolates, 7 aerobic and 9 anaerobic; Mouse 4: 6 isolates, 2 aerobic and 4 anaerobic). All these isolated cell lines ($n = 45$; 23 aerobic and 22 anaerobic isolates) were used for assessment of xylose utilization using a $^{14}$C-xylose radio-isotopic incubation assay that we modified from procedures described by Krishnan et al. (1980). The $^{14}$C-assay was performed by placing a 2 ml microtube (Quality Scientific Plastics, Porex Bio Products Group, Fairburn, GA, USA) into a 20 ml glass vial. The vials and microtubes were autoclaved and 1.25 ml aliquots of a 1 M NaOH solution were placed into the microtube to trap $CO_2$ released by growing cultures during incubation. NaOH traps $CO_2$ as sodium hydrogen carbonate ($NaHCO_3$) which remains in solution. The assay medium, dispensed in 1 ml aliquots into each vial, contained: 0.05 g xylose, 0.05 g casitone, 40 ml clarified rumen fluid (filtered through cheesecloth, flushed with $CO_2$, autoclaved, stored at 4°C and centrifuged at 10,000×$g$ for 20 min before use in medium), 7.5 ml solution A (g/l: 6.0 $K_2PO_4$ and 2.0 $Na_3C_6H_5O_7{\cdot}2H_2O$) and 7.5 ml solution B (g/l: 12.0 NaCl, 12.0 $(NH_4)_2SO_4$, 6.0 $KH_2PO_4$, 1.2 $CaCl_2$, 2.46 $MgSO_4{\cdot}7H_2O$ and 20.0 $Na_3C_6H_5O_7{\cdot}2H_2O$) made up to 100 ml with distilled water. Prior to inoculation, 0.1 $\mu$Ci uniformly labeled $^{14}$C-xylose (specific activity: 542 $\mu$Ci/mg, Amersham Life Science, IL, USA) was added to the assay medium in each vial. Isolated cultures were inoculated into the assay medium in three separate vials, which were sealed with a sterile rubber stopper and metal crimp seal. Vials inoculated with anaerobic cultures were flushed with $CO_2$ before being crimp sealed. Vials were incubated at 37°C oscillating at 70 rpm for 48 h.

After incubation, vial contents were prepared for liquid scintillation counting as follows: all NaOH was removed from the microtube by micro-pipette. The low-retention plastic of the micro-tubes ensured that no liquid was lost during this process. The NaOH solution was dispensed into a glass scintillation vial, to which 14 ml Hionic Fluor scintillation cocktail (Perkin Elmer, MA, USA) was added; 0.25 ml aliquots of each sample's assay medium were dispensed into four scintillation vials and each mixed with 16 ml Hionic Fluor; 1 ml distilled water was used to rinse the assay vial and was dispensed into a scintillation vial and mixed with 15 ml Ultima Gold scintillation cocktail (Packard, Connecticut, USA). Radioactivity of samples was determined on a Beckman liquid scintillation counter (LS 5000 TD, Beckman Instruments, CA, USA). The protocol counted radioactivity in the full $^{14}$C window (0 to 154 keV) to ensure the highest counting efficiency. Counting efficiency was calculated from the $C^{14}$ quench curve generated for the scintillation counter using manufacturer's standards. Xylose utilization was assessed by determining the percentage of the original 0.1 $\mu$Ci $^{14}$C-xylose dose converted to $^{14}CO_2$ and trapped in the NaOH solution.

### Microbe identification

Positive bacterial xylose utilizers, identified by the $^{14}$C-xylose assay, were processed for identification. DNA was extracted using the Dneasy Tissue Kit (QIAGEN catalogue no. 69504). DNA encoding the 16S rRNA gene was amplified by PCR with two 16S rRNA primers, one forward (F1:S16,1) and one reverse (R5:S16,12) primer. Amplifications were carried out on an Applied Biosystems (CA, USA) 27000 thermal cycler. PCR products were purified using the Wizard SV Gel and PCR Clean-up System (Promega Corporation,

WI, USA). Strands were cycle sequenced using BigDye dye termination chemistry (Perkin Elmer, CT, USA) and all unincorporated dye label was removed by Sephadex columns (strip of eight columns, Princeton Separations, NJ, USA) according to the manufacturers' protocol, before the samples were run on an ABI Prism 3100 sequencer (Applied Biosystems). Nucleotide sequences obtained were analyzed using Sequence Navigator software (ABI, version 1.01). Nucleotide homology searches were performed in the GenBank database with BLAST.

Fungal colonies that proved to be xylose utilizers were identified to genus level based on the morphology of the colonies and microscopic analyses of conidiophore morphology (Domsch et al. 1980; Raper and Fennel 1965).

### Short-chain fatty acid production

After taxonomic identification, the caecal microflora species isolated as positive xylose utilizers were further investigated by gas chromatography in order to identify the types of SCFA they each produce during xylose metabolism. Colonies from stock tubes were streaked onto rumen fluid-based medium to reproduce cultures. After incubation at 37°C for 24 h, colonies from the plates were used to inoculate pre-culture test tubes containing 5 ml rumen fluid medium. Pre-culture tubes were incubated at 37°C for another 24 h and optical density was measured at 600 nm, in order to determine the volume of pre-culture, with an optical density of 0.1, required to inoculate experimental tubes. For each caecal microflora species, duplicate experimental tubes were prepared for two xylose concentrations that were tested. The medium used to cultivate cultures for gas chromatographic analysis was the same as the assay medium used in the xylose utilization assay described above, except that the concentration of xylose was either 1% or 2% (w:v). Duplicate experimental tubes of each concentration were inoculated with culture from the pre-culture tubes. Control tubes were not inoculated with culture. All tubes were incubated at 37°C for 12 h. After incubation the pH of each tube was determined using a pH meter, for comparison with the initial pH of culture medium (7.2) before incubation. Each tube was then centrifuged at 10,000×*g* for 15 min at 4°C. The supernatant was filter-sterilized with 0.45 μm filters and stored at –20°C until analysis by gas chromatography. Samples of the rumen fluid used to make up the culture medium were also analyzed by gas chromatography to determine the concentrations of SCFA contributed by rumen fluid to the freshly made medium.

### Gas chromatographic analysis

A standard solution of SCFA (acetic, propionic, butyric, iso-butyric, valeric and iso-valeric) was prepared by dissolving 1 ml of each fatty acid and 0.5 ml of *n*-hexanol in 1 l of formic acid solution (one part 35% formic acid and three parts distilled water). Samples were prepared for analysis by diluting 1 ml of 35% formic acid with 3 ml of aqueous, filtered sample and adding 2 μl of *n*-hexanol as internal standard. The injection volume was 1 μl. Samples were analyzed using a Varian 3700 gas chromatograph equipped with a flame ionization detector and a 30 m bonded phase Nukol (Supelco, Inc., Belafonte, PA) fused silica capillary column (0.53 mm diameter and 0.50 μm film thickness). The column temperature was initially held at 105°C for 2 min, increased at 10°C $min^{-1}$ to 190°C and held for 10 min. The injector temperature was set at 150°C, while the detector was set at 300°C. Nitrogen was used as carrier gas at a flow rate of 6.1 ml $min^{-1}$. SCFA were quantified by means of Borwin Version 1.2 integration software (JMBS Developments, Le Fontanil, France), operating in the internal standard mode. Results were converted to original sample concentration.

### Statistics

One-sample *t* tests were used to compare the total amounts of acid produced in the 1 and 2% xylose culture media after incubation to the total amount of acid in control media before incubation, in order to detect significant increases in acid production. One-sample *t* tests were also used to compare the final pH values in the 1 and 2% xylose media to the pH of the culture media before incubation to detect significant decreases in the pH as a result of acid production. Finally, we used stepwise multiple regression with pH as the dependent variable and concentrations of each different SCFA by each species as independent variables, to ascertain which SCFA accounted for the greatest proportion of the pH variation between species. These analyses were performed using the Statistica 7 software package (Statsoft Inc., Tulsa, OK, USA).

## Results

### Isolation and cultivation of microflora

The total number of CFU cultured from caecal samples on rumen fluid-based medium ranged from $5.7 \times 10^8$ to

$5.2 \times 10^9$ per g caecal content (coefficient of variation = 82%) for aerobic cultures, and from $2.6 \times 10^8$ to $9.1 \times 10^{10}$ per g caecal content (coefficient of variation = 89%) for anaerobic cultures.

## Xylose utilization

For all 45 isolates assayed in triplicate, total recovery of the label, expressed as a percentage of the original dose, ranged from 60.8 to 99.9% (mean = 87.2% ± 11.7 SE). Of the 45 isolates assayed, 24 showed evidence of xylose utilization as label trapped in the NaOH solution. Of those 24 positive xylose utilizers, 4 were isolated from Mouse 1, 7 from Mouse 2, 10 from Mouse 3 and 4 from Mouse 4 (Table 1). The mean percentage of the label trapped in NaOH for the 24 isolates, each assayed in triplicate, ranged from 6.4 to 60.1% of the original dose. The rest of the isolates all had less than 0.8% of the label trapped in NaOH. Counts obtained from these samples were close to background counts obtained for blank NaOH solutions.

## Microbe identification

Of the 24 isolates identified as positive xylose utilizers, 21 were bacterial and three were fungal isolates. The 21 bacterial isolates were made up of 16 isolates belonging to the genus *Bacillus* (which comprised three different species: *Bacillus subtilis*, *Bacillus pumilus* and *Bacillus licheniformis*), four *Shigella boydii* isolates and one isolate belonging to the genus *Arthrobacter* (Table 1). Morphological analyses of the three fungal isolates identified one as an *Aspergillus* species and two as members of the genus *Penicillium*. Thus four bacterial isolates were identified to species level and one bacterial and two fungal isolates identified to genus level. These seven isolates are hereafter referred to as the xylose-utilizing isolates. Results are presented and discussed for each xylose-utilizing isolate in turn, rather than as communities within each mouse. A group of possibly pathogenic microbes, which included *S. boydii*, the *Arthrobacter* species and *Penicillium* fungi were all isolated from one mouse, and another pathogenic fungus, *Aspergillus*, from another mouse. The

**Table 1** Isotope recovery from each of the 21 bacterial and 3 fungal xylose-utilizing isolates, grouped first by species, growth conditions, and individual mouse, and the mean percentage ± SD of $^{14}C$ dose recovered from each isolate (assayed in triplicate) in the NaOH trap, culture medium, vial wash and in total

| Taxon (GenBank accession codes for strains with > 99% sequence match) | Growth conditions of initial isolates | Mouse number | NaOH trap (CE = 93.82%) | Culture medium (CE = 92.71%) | Vial wash (CE = 96.18%) | Total |
|---|---|---|---|---|---|---|
| *B. licheniformis* (AY842874) | Aerobic | 1 | 40.5 ± 1.4 | 42.9 ± 2.8 | 1.9 ± 0.7 | 85.2 ± 12.6 |
| *B. licheniformis* (AY842874) | Anaerobic | 1 | 34.4 ± 9.2 | 60.2 ± 8.9 | 4.2 ± 0.9 | 98.8 ± 17.8 |
| *B. pumilus* (AY260861) | Aerobic | 1 | 6.7 ± 1.1 | 64.5 ± 6.4 | 7.7 ± 1.1 | 78.8 ± 7.5 |
| *B. subtilis* (AY030331) | Aerobic | 1 | 34.4 ± 4.2 | 31.4 ± 3.8 | 3.1 ± 1.1 | 98.9 ± 8.2 |
| *B. licheniformis* (AY842874) | Aerobic | 2 | 24.3 ± 1.8 | 36 ± 3.9 | 7.5 ± 1.2 | 67.8 ± 5.9 |
| *B. licheniformis* (CP000002) | Aerobic | 2 | 43.6 ± 10.4 | 54.2 ± 14.1 | 2.1 ± 0.5 | 99.9 ± 24.9 |
| *B. pumilus* (AB098578) | Aerobic | 2 | 6.4 ± 1.2 | 81.4 ± 1.4 | 5 ± 1.1 | 92.8 ± 3.3 |
| *B. subtilis* (AY030331) | Aerobic | 2 | 17.1 ± 2.9 | 75.2 ± 6.9 | 4.1 ± 1.5 | 96.4 ± 7.7 |
| *B. subtilis* (AY304996) | Aerobic | 2 | 17 ± 2.1 | 55.6 ± 8.1 | 4.8 ± 1.4 | 77.4 ± 9.4 |
| *B. subtilis* (AY162127) | Anaerobic | 2 | 36.1 ± 9.0 | 60.6 ± 24.7 | 2 ± 1.3 | 98.8 ± 35.1 |
| *Aspergillus* sp. | Aerobic | 2 | 24 ± 0.8 | 36.6 ± 13.3 | 2.8 ± 1.4 | 63.4 ± 14.5 |
| *B. licheniformis* (AY842874) | Aerobic | 3 | 19.1 ± 12.8 | 41.3 ± 14.5 | 7.7 ± 3.8 | 68.2 ± 5.0 |
| *B. licheniformis* (AY842874) | Anaerobic | 3 | 48.1 ± 8.2 | 40.6 ± 4.8 | 2.2 ± 0.5 | 90.8 ± 5.7 |
| *B. subtilis* (AY162127 ) | Aerobic | 3 | 19.3 ± 4.8 | 55.8 ± 10.6 | 4.8 ± 3.1 | 79.9 ± 11.6 |
| *Penicillium* sp. | Aerobic | 3 | 60.1 ± 0.1 | 37.7 ± 2.8 | 2.1 ± 1.5 | 99.8 ± 4.9 |
| *Penicillium* sp. | Aerobic | 3 | 34.4 ± 7.7 | 40.9 ± 20.1 | 3.6 ± 2.9 | 79 ± 16.9 |
| *S. boydii* (AY696668) | Anaerobic | 3 | 14.7 ± 8.9 | 67.6 ± 5.3 | 0.8 ± 0.2 | 83 ± 14.5 |
| *S. boydii* (AY696668) | Anaerobic | 3 | 35.6 ± 2.3 | 62.2 ± 4.2 | 1.1 ± 0.2 | 98.9 ± 6.8 |
| *S. boydii* (AY696681) | Anaerobic | 3 | 37.7 ± 15.1 | 43.3 ± 19.5 | 4.8 ± 1.5 | 85.8 ± 37.7 |
| *S. boydii* (AY696668) | Anaerobic | 3 | 27.9 ± 19.2 | 39 ± 24.9 | 1.2 ± 0.7 | 68.2 ± 44.7 |
| *Arthrobacter* sp. (AY864629) | Anaerobic | 3 | 6.8 ± 2.8 | 70.9 ± 3.9 | 0.7 ± 0.1 | 78.4 ± 11.7 |
| *B. licheniformis* (AJ880762) | Anaerobic | 4 | 6.5 ± 0.8 | 78.8 ± 1.4 | 0.7 ± 0.1 | 86 ± 2.2 |
| *B. subtilis* (AY304996) | Aerobic | 4 | 15.4 ± 1.4 | 78.2 ± 10.9 | 4.8 ± 2.7 | 98.4 ± 13.9 |
| *B. subtilis* (AY162128) | Aerobic | 4 | 8.5 ± 1.5 | 79.1 ± 4.8 | 4.3 ± 0.9 | 92 ± 7.6 |

GenBank accession codes in parentheses after species names. *B.*: *Bacillus*, *S.*: *Shigella*. CE = mean counting efficiency for each type of sample. All $^{14}C$ incubation assays were performed under anaerobic conditions. Fungal isolates do not have GenBank accession codes as they were not identified by DNA analysis (see Materials and methods)

pathogenicity of these microbes to wild mice is not known.

## Short-chain fatty acid production

The concentrations of SCFA in the autoclaved rumen fluid were comparable to the concentrations of SCFA present in freshly made media and in the control tubes after the incubation period. Thus, the initial concentrations of SCFA in the culture medium are due to the use of rumen fluid in the medium, and higher concentrations of SCFA in experimental tubes after incubation indicated SCFA production. Each of the seven xylose-utilizing isolates produced the SCFA that are typically end-products of microbial fermentation, namely acetic, propionic and butyric acids. Low, but measurable concentrations of iso-butyric, valeric and iso-valeric acids were also detected. After incubation all acids were present in the culture medium at concentrations greater than those present in the controls. Consequently, for each acid we subtracted the quantities of SCFA measured in the controls from those measured in experimental tubes to estimate the amount of acid produced by each species during incubation. The production of fatty acids by the five bacterial species was generally greater than that by the two fungal species (Table 2).

The amounts of acid produced depended on substrate concentration but not consistently in the expected direction of more substrate yielding more acid. The three *Bacillus* species produced between two and six times as much acetic acid when grown in the 2% xylose medium compared to 1% xylose. The *Arthrobacter* species, as well as the *Penicillium* isolate, also produced more acetic acid in the 2% medium. *Bacillus pumilus*, *S. boydii* and the *Penicillium* isolate produced as much as twice the amount of propionic acid in the 2% medium. *S. boydii also* produced more butyric acid in the 2% medium. Species that produced higher concentrations of acid in the 1% xylose medium were *S. boydii* and the *Aspergillus* species, which produced more acetic acid, and *B. subtilus*, *Arthrobacter* and *Aspergillus* species which produced more propionic acid. The *Arthrobacter* species also produced more butyric acid in the 1% medium relative to the 2% xylose medium. The remaining acids, iso-butyric, valeric and iso-valeric, were produced in much lower quantities than the other three acids, except for *B. pumilus* and *B. licheniformis*, which produced 4.41 and 19.61 μM/12 h, respectively, in the 2% medium. The production of iso-valeric acid by *B. licheniformis* at 2% exceeded production of this acid by all other isolates by an order of magnitude, but we are unable to explain this result. Across all the acids only *B. licheniformis* was consistent in producing more acid in response to more xylose in the medium. *B. pumilus* did so for five out of the six acids, *S. boydii*, for four out of the six, the *Penicillium* isolate for three, *B. subtilus* for two and the *Arthrobacter* species for one out of the six. In both media, the total amount of acid produced was significantly greater than the initial amount of acid in the medium ($P < 0.05$), a difference largely due to the production of acetic acid. Mean molar ratios of acetate:propionate:butyrate across all isolates in the 1% medium were 52:35:13, with acetate ranging from 26 to 71% of total acid, proprionate from 22 to 58%, and butyrate from 7 to 23%. In the 2% medium, mean molar ratios of these acids, respectively, were 62:29:9, with acetate ranging from 38 to 79% of total acid, propionate from 16 to 62%, and butyrate from 0 to 19%.

The production of acid during incubation was also reflected in the decrease in pH from the initial medium

**Table 2** Amounts of short-chain fatty acids (μM/12 h) produced by each species during incubation at 37°C in 1 and 2% xylose media, total SCFA production (μM/12 h) and final pH after incubation

| | Control | *Bacillus subtilis* | | *Bacillus pumilus* | | *Bacillus licheniformis* | | *Shigella boydii* | | *Arthrobacter* species | | *Aspergillus* species | | *Penicillium* species | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1% | 2% | 1% | 2% | 1% | 2% | 1% | 2% | 1% | 2% | 1% | 2% | 1% | 2% |
| Acetic acid | 10.83 | **9.17** | **55.00** | **18.33** | **34.17** | **29.17** | **62.5** | 30.00 | 23.33 | **14.17** | **17.50** | 4.17 | 1.67 | **10.83** | **25.00** |
| Propionic acid | 1.42 | 20.27 | 12.16 | **11.49** | **22.30** | **10.14** | **12.84** | **8.78** | **14.19** | 15.54 | 6.76 | 4.05 | 2.70 | **3.38** | **5.41** |
| Butyric acid | 2.27 | **5.11** | **5.68** | **5.11** | **6.82** | **5.68** | **7.95** | **4.55** | **8.52** | 9.09 | 2.84 | 0.57 | 0 | 1.14 | 1.14 |
| Iso-butyric acid | 0.28 | 4.55 | 1.70 | **2.27** | **2.84** | **2.27** | **6.82** | 2.84 | 1.70 | 1.70 | 1.14 | 0.57 | 0 | **0.57** | **1.14** |
| Valeric acid | 0.25 | 2.45 | 0.98 | **0.49** | **4.41** | **0.98** | **2.45** | **0.98** | **1.47** | 1.47 | 0.49 | 0.49 | 0 | 0.49 | 0 |
| Iso-valeric acid | 0.29 | 2.94 | 2.45 | 2.45 | 2.45 | **2.94** | **19.61** | **1.47** | **2.45** | 1.96 | 0.98 | 0.49 | 0.49 | 0.98 | 0.49 |
| Total acid | 15.34 | 44.49 | 77.97 | 40.14 | 89.82 | 51.18 | 112.17 | 48.62 | 51.66 | 43.93 | 29.71 | 10.34 | 4.86 | 17.39 | 33.18 |
| pH | 7.2 | 7.12 | 5.89 | 7.04 | 5.94 | 7.03 | 5.55 | 5.57 | 5.47 | 6.39 | 5.86 | 6.24 | 6.06 | 6.2 | 5.88 |

Instances where isolates produced higher concentrations of acid in the 2% xylose medium are indicated in bold. Values are means of two independent determinations across all acids. There was no difference in the concentration of acids between the 1 and 2% control tubes; thus, only one value is given in the control column. The control value was subtracted from the amount of acid measured in experimental tubes to determine the amount of acid produced by each species from xylose

pH of 7.2. Final pH values in the 1% medium ranged from 5.57 to 7.12, and in the 2% medium from 5.47 to 6.06 (Table 2). In both media the final pH differed significantly from the initial pH of 7.2 ($P < 0.05$). The stronger production of acids in the 2% medium was also reflected in the pH readings, with a highly significant decrease in the 2% medium ($P < 0.00001$). Stepwise multiple regressions revealed that across all seven isolate species, the molar concentrations of four SCFA emerged as significant predictors of pH. For 1% xylose medium, acetic and iso-valeric acids accounted for more than 97% of all pH variance ($R = 0.973$, adjusted $R^2 = 0.8934$, $F_{(3,3)} = 17.766$, $P < 0.021$; for acetic acid, $P < 0.037576$, and for iso-valeric acid, $P < 0.006532$). For 2% xylose medium, propionic and butyric acid concentrations together accounted for 95.8% of pH variance ($R = 0.9583$, adjusted $R^2 = 0.8366$, $F_{(3,3)} = 11.239$, $P < 0.03866$; for propionic, $P < 0.036$, and for butyric acid, $P < 0.014$).

## Discussion

We have shown here how gut symbionts enable Namaqua rock mice to obtain energy from xylose, an unusual nectar sugar whose presence in Proteacea nectar is still enigmatic (Jackson and Nicolson 2002). In addition to nectar, rock mice eat plant material (floral structures and seeds), and like other rodents, have a caecal fermentation chamber where cellulose in this material is broken down by gut bacteria. Gut morphology and fermentative capacity may thus be a pre-adaptation sensu Shelley (1999), allowing mice that consume xylose-rich nectar to access the energy in this sugar.

In birds, the other vertebrate pollinators in the Cape Floristic Kingdom (''fynbos''), large populations of intestinal bacteria and specialized caecal bacteria enhance the efficiency of cellulose digestion (Mead 1989; Lan et al. 2005). Caeca therefore tend to be large in herbivorous and omnivorous birds, and small, vestigal or absent in carnivorous, insectivorous, piscivorous, granivorous and nectarivorous species (Clench and Mathias 1995; Preest et al. 2003). Since the presence of xylose-fermenting microbes in host animal's digestive systems appears to be linked to a more widespread fermentative capacity for long-chain carbohydrates, it is not unexpected that nectarivorous bird pollinators of the Proteaceae lack xylose-fermenting gut microflora. The third group of pollinators of the fynbos, insects such as the green protea beetle (*Trichostetha fasicularis*), may well possess xylose-utilizing endosymbionts (Johnson and Nicolson 2001): xylose-fermenting gut yeasts of the *Pichia stipitis* clade are associated with wood-ingesting beetles (Nardon and Grenier 1989; Suh et al. 2003).

### Xylose utilization by rodent gut microflora

Removal of the caecum under anaerobic conditions and exposure of caecal contents in an anaerobic environment optimized survival and culture of obligate anaerobic microflora. Moreover, our use of a rumen-fluid based medium, previously used to culture rat caecal and fecal microflora (Campbell et al. 1997) created growth conditions favorable for positively identified xylose utilizers: *Bacillus subtilis*, *B. pumilus*, *B. licheniformis*, *S. boydii*, an *Arthrobacter* species and fungal isolates belonging to the genera *Aspergillus* and *Penicillium*. We acknowledge the potential bias introduced when experimental strains are selected using a synthetic medium and grown in isolation. In our study, this approach may have favored *Bacillus* species, common to caecal samples from all four mice. However, to enable replication of experiments and because of the potential for shifts of microbial community composition over time, we chose to work with dominant community members in pure culture as a first step to characterizing this particular microbe community.

The bacteria identified here probably contribute modestly to the nutrition of *A. namaquensis*, particularly during the austral winter and spring: the community of xylose utilizers in the digestive tract of Namaqua rock mice is enriched when xylose is included in the diet (Johnson et al. 2006). This community is likely not unique among mammals: domestic pigs are known to possess gut bacteria that ferment xylose (e.g. Kiriyama et al. 1992). It *is* unique in terms of its occurrence in a herbivore rather than an omnivore, and in that the context for this occurrence is the remarkable Cape Floristic Kingdom (''fynbos''), the biodiversity of which is mirrored by a diversity of plant–pollinator relations that are still being explored.

The *Bacillus* species isolated from Namaqua rock mice in this study have also been isolated from the gastrointestinal tract of broiler chickens (Barbosa et al. 2005). Mouse gut *B. subtilis* and *B. licheniformis* grew under both aerobic and anaerobic conditions in the laboratory. *B. pumilus* isolates grew only aerobically, but are most likely capable of anaerobic growth as well, since they were isolated from the anaerobic environment of the gut, although the isolation and assaying process revealed two of the aerobic cultures as positive xylose utilizers. More than 90% of *B. subtilis*, *B. pumilus* and *B. licheniformis* strains are capable of producing acid from xylose (Sneath et al. 1986). Xylose

utilization, expressed as the percentage of the dose recovered in NaOH during the radio-isotopic incubation assay, ranged from 8.5 to 36.1% for the seven *B. subtilis* isolates and from 6.5 to 48.1% for the seven *B. licheniformis* isolates. The two *B. pumilus* isolates showed very low xylose utilization (6.4 and 6.7%).

The pathogenic bacterium *S. boydii* was isolated under anaerobic conditions from one of the four mice used in this study. The genus *Shigella* comprises Gram-negative rods that are facultatively anaerobic (Sneath et al. 1986). Within the genus 10% or less of the strains are positive for acid production from xylose, and xylose fermentation among different *S. boydii* strains is variable (Sneath et al. 1986). The four *S. boydii* isolates cultured here all grew only anaerobically and showed xylose utilization ranging from 14.7 to 37.7% of the original dose.

A single isolate identified as *Arthrobacter* sp. was not identified to species level since it matched most significantly with an unidentified *Arthrobacter* species in the GenBank database. Species within the genus *Arthrobacter* are Gram-negative rod shaped bacteria in young cultures and Gram-positive cocci in older cultures (Sneath et al. 1986; Eschbach et al. 2003). Most species of *Arthrobacter* are obligate aerobes and make up an important fraction of the indigenous bacterial flora of soils; however, some species (*Arthrobacter globiformis* and *Arthrobacter nicotianae*) have shown an adaptive capacity to grow anaerobically (Eschbach et al. 2003). Ninety percent or more strains of *Arthrobacter nicotianae* utilize xylose (Sneath et al. 1986). Some *Arthrobacter* species are regarded as opportunistic pathogens (Funke et al. 1996). Recently a number of new *Arthrobacter* species, isolated from human and other animal sources, have been described: *A. luteolus* and *A. albus* from human clinical specimens (Wauters et al. 2000); *A. rhombi*, from organs of Greenland halibut (*Reinhardtius hippoglossoides*) (Osorio et al. 1999); *A. nasiphocae* from the nasal cavities of the common seal (*Phoca vitulina*) (Collins et al. 2002), and *A. gandavensis* from mastitic milk and the uterus of dairy cows (Storms et al. 2003). Of all of these new *Arthrobacter* species, only *A. luteolus* utilized xylose (Wauters et al. 2000). The single *Arthrobacter* species that we isolated was not a strong xylose utilizer (only 6.8% of the $^{14}$C-xylose dose was recovered).

Gastrointestinal fungi also play an important role degrading plant fiber during digestion in herbivores. However, the two fungal isolates that we cultured from caecal samples are opportunistic pathogens rather than belonging to the known group of beneficial fungi found in the gut. *Aspergillus* species and *Penicillium* species are commonly found in the environment worldwide. They grow on plant material and may contaminate cereal grains and animal feeds by secreting mycotoxins (Yiannikouris and Jouany 2002). *Aspergillus* species in the gastrointestinal tracts of ruminants are associated with hemorrhagic bowel syndrome, in which hemorrhagic lesions develop along the intestinal tract (Jensen and Shoenheyder 1989; Jensen et al. 1989, 1991). *Penicillium* species also cause infections and intestinal mycoses in humans (Fantry 2001). Although the fungal genera isolated in our study are not known to be beneficial intestinal microbes, they utilize xylose. Both *Aspergillus* and *Penicillium* species have xylan-degrading enzyme systems (Filho et al. 1991; Kormelink et al. 1993; McCrae et al. 1994), the control of which has been studied in *Aspergillus* to improve fermentation yields (Prathumpai et al. 2003). Xylose utilization, as the percentage $^{14}$C-labeled xylose recovered during the incubation assay, was 24.0% for the *Aspergillus* isolate and 34.4 and 60.1% for the two *Penicillium* isolates.

## Short-chain fatty acid production

Mammals do not possess the enzyme xylanase which is required to hydrolyze xylan, but bacteria, yeasts and fungi do. Xylans represent a major energy source for microbial fermentation within ruminants and non-ruminants (Hespell et al. 1987). Xylose released during xylan degradation in the gut lumen is utilized inside the microbial cell and the predominant end-products, SCFA, are absorbed by the host, providing energy for body functions (Stevens and Hume 1995, Johnson et al. 2006). Other carbon sinks resulting from colonic microbial fermentation are the microbes themselves, plus $CO_2$ and $CH_4$. The radio-isotopic assay used here assesses the utilization of a labeled substrate, $^{14}$C-xylose, by recovery of the label in $CO_2$ released during fermentation. Label not recovered as $CO_2$ remained in the assay culture as SCFA, other by-products of fermentation, and unutilized $^{14}$C-xylose and was recovered and quantified as label in the culture. Of this fraction, it is the SCFA that are the major contributors to the energy requirements of the host animal, analogous to but not as significant as the contribution made by the fermentation of long-chain carbohydrates to the nutrition of herbivorous mammal species consuming cellulose (Bergman 1990). In ruminants, SCFA contribute 70% to the total energy requirements (Stevens and Hume 1998). In non-ruminants, such as the rock hyrax (*Procavia habessinica*) and naked mole-rat (*Hetercephalus glaber*), contributions to total energy requirements are 44 and 22%, respectively (Rübsamen et al. 1982; Buffenstein and Yahav 1991).

The main types of SCFA produced by microbial fermentation of carbohydrates are acetic acid, propionic acid and butyric acid, and each is utilized by different tissues in the host's body (Bergman 1990). Pathways of SCFA metabolism differ slightly between species. The types and proportions of SCFA produced from long-chain carbohydrate (largely cellulose) fermentation are dependent on the site of fermentation (fore-gut or hind-gut), the bacterial species inhabiting the gastrointestinal tract and on the host's diet, and in mammals vary from 75:15:10 to 40:40:20 when expressed as molar proportions of acetate:propionate:butyrate (Bergman 1990). Our results for SCFA production from xylose by rock mouse gut symbionts fall within this range, with acetic acid dominating as it does across all mammal species studied to date (see Bergman, 1990 for a review of mammals). Acetic acid constitutes as much as 98% of all SCFA in the rock hyrax stomach (Rübsamen et al. 1982), and 70 and 80% respectively of fecal fatty acid concentrations of free-living marine and land iguanas, hind-gut fermenters (Mackie et al. 2004). Tropical marine fish fed on microalgae reflect the same trend (Stevens and Hume 1998). Propionic acid, the next most important SCFA we report, is released particularly during hind-gut fermentation, including that of sugars such as the methylpentose L-rhamnose by humans (Vogt et al. 2004).

We have shown that xylose utilization by the bacterial and fungal species isolated from *A. namaquensis* caecum results in the production of SCFA, particularly acetic, propionic and butyric acids, but also iso-butyric, valeric and iso-valeric acids. Also, molar concentrations of the first three and the last of these acids together explain more than 95% of all inter-specific (inter-isolate) pH variation under the culture conditions that we used. Bacterial gut symbionts of *A. namaquensis* appear to be stronger fatty acid producers than are the fungal species. The proportions of fatty acids produced vary between microbial species studied, with acetic acid predominating. The fatty acid concentrations we report are for in vitro cultures of individually grown bacterial species. In the gut lumen, these bacteria would most likely experience interspecific competition that might affect the proportions of SCFA produced by each. Other carbon sources in the rumen fluid medium such as peptones may have contributed to fatty acid production, but our use of a single batch of rumen fluid ensured that differences in fatty acid production between the two media solely reflected different concentrations of xylose in the two media, rather than variation in concentration of other carbon sources. Although medium pH decreased after incubation as a result of acid production, the magnitude of this change in most cases indicates only weak acid production (Holdeman et al. 1977). It is important to note that the culture medium used in this experiment was used to maximize colony growth and detection of xylose use rather than acid production. An alternative medium coupled with manipulation of growth conditions might result in stronger acid production. Nonetheless, we have shown that the caecal microflora of rock mice, and even possibly its pathogenic elements, produce the SCFA typical of gut microbial fermentation when given xylose as a substrate.

*Protea* nectar may be merely a dietary supplement for rock mice (Wiens et al. 1983; Fleming and Nicolson 2002), but as it is available in winter and early spring when abundance of other resources such as seeds may be low, xylose is likely a "junk food" of some energetic value to these rodents. The breeding season of rock mice coincides with the end of the *P. humiflora* flowering season and continues for approximately 2 months afterwards. Rock mice may make use of nectar and pollen to improve their body condition in preparation for breeding (Fleming and Nicolson 2002). In this context, the symbiotic fermentation of xylose we describe permits utilization of a carbon source that otherwise would be entirely lost to the mice, given their tissues' apparently inefficient metabolism of this sugar. Although *A. namaquensis* makes metabolic use of xylose (Johnson et al. 2006), this remains its least preferred sugar (Johnson et al. 1999). The rodent caecum, with its resident microflora capable of fermenting xylose to SCFA, may be a pre-adaptation that has enabled Namaqua rock mice to benefit from the energy in this intriguing nectar sugar as they pollinate the Proteaceae.

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