

**Physiological and microbiological studies of nectar xylose  
metabolism in the Namaqua rock mouse, *Aethomys namaquensis***

**(A. Smith, 1834)**

by

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**Declaration**

I declare that the work contained in this thesis is my own original work and I have not previously submitted this thesis or any part of it for degree purposes at any other university.

Shelley Johnson

30 November 2005

## Thesis Summary

### **Physiological and microbiological studies of nectar xylose metabolism in the Namaqua rock mouse, *Aethomys namaquensis* (A. Smith, 1834)**

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Xylose is an unusual nectar sugar found in the nectar of *Protea* and *Faurea* (Proteaceae). Since nectar composition is an important floral characteristic in plant strategies for ensuring reproductive success, the unexplained presence of xylose in *Protea* nectar prompted this study of the interaction between pollinators and *Protea* species. Among pollinators that visit *Protea* flowers in the south-western Cape Floral Kingdom, South Africa, insects and birds show an aversion to, and are poor assimilators of, xylose, whereas rodents such as Namaqua rock mice *Aethomys namaquensis* are the only pollinators so far shown to consume xylose willingly, and are able to obtain metabolic energy from this sugar. Mammalian tissues are not capable of catabolizing xylose efficiently, but certain gastrointestinal microflora are, through a process of fermentation which produces short-chain fatty acids used by host animals in oxidative metabolism. I explored mechanisms enabling *Aethomys namaquensis* to utilize xylose, in particular, the role of resident intestinal microflora in this process.

Chapter One discusses pollination syndromes and the definitions thereof, mammal pollination, with particular reference to Australia and South Africa, and explains the rationale behind the questions addressed in this thesis.

To assess xylose utilization in wild-caught mice with and without their natural gastrointestinal microflora, an antibiotic treatment was developed (Chapter Two). The veterinary antimicrobial agent, Baytril 10% oral solution, was found to be effective in significantly reducing gut microflora in animals on a four-day treatment protocol. The protocol developed here reduced the gut microflora sufficiently for subsequent experiments comparing xylose utilization in mice with and without intact microflora.

Xylose utilization was assessed using  $^{14}\text{C}$ -labelled xylose (Chapter Three). Rock mice were caught during *Protea humiflora* flowering and non-flowering seasons, and fed  $^{14}\text{C}$ -labelled xylose. Exhaled  $\text{CO}_2$  and excreted urine and faeces were collected, and label recovery determined. These experiments showed that xylose-utilizing bacteria in the rock mouse gut are very important for xylose utilization. More efficient xylose utilization during the flowering season suggests that this component of the gut microflora is inducible.

Culturable gut microflora were then isolated from rock mouse faecal and caecal samples, assessed for xylose utilization to identify positive xylose-fermenters and classified by 16S rRNA based taxonomy (Chapter Four). Faecal isolates were *Lactobacillus murinus* and *Enterococcus faecium*, and caecal isolates were three *Bacillus* species, *Shigella boydii*, one *Arthrobacter* species and two fungal isolates from *Aspergillus* and *Penicillium* genera. The types and concentrations of short-chain fatty acids arising from xylose fermentation by caecal microflora were measured using gas chromatography. The fatty acid profile produced by rock mouse gut microflora is similar to that for other animals that rely on gut microbial fermentation to produce fatty acids then used in oxidative metabolism.

Chapter Five concludes with a discussion of possible explanations for the presence of xylose as a nectar sugar, its ecological significance, and the relevance of the fermentative capacity of pollinator digestive systems for xylose utilization in animals.

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## **Chapter 1**

### **General Introduction**

## **General Introduction**

Pollination systems are enormously diverse worldwide, and study of these systems has led to debate about how these should be described and classified. While this thesis focuses on a particular ecophysiological aspect of rodent pollination of some of the Proteaceae of the south-western Cape, South Africa, the context for this is the broader debate about the definition of pollination systems/syndromes, and requires some discussion before concentrating on mammal pollination and the mice that pollinate *Protea* flowers.

### **Pollination syndromes**

In the early studies of pollination biology the notion that flowers are specialized for pollination by particular animal types, for instance birds or bees, led to the description of pollination syndromes. These syndromes were characterized by a set of floral traits that biologists believed were strongly associated with the flower visitor. A variety of syndromes has been described to date, in which pollinators are grouped by taxonomic order (eg. beetles), and even by class (eg. birds), and within these syndromes there may also be subsyndromes to account for the variation among animals within the orders or classes (Faegri and van der Pijl 1966; Buchmann and Nabhan 1996). Certain flowers became known as “beetle flowers” or “hummingbird flowers”, and such defined pollination syndromes became the paradigm for studying interactions between flowers and their pollinators. However, observational data has shown that flowers attract a broader spectrum of visitors than one would expect based on these syndromes. Flowers originally described as fly blossoms were later considered “open house” flowers as they were seen to be visited by many other insect species as well, such as small hymenopterans, lepidopterans and coleopterans. These observations led to the view that

generalization is widespread in pollination systems, and that in the interest of reproductive success for most plants, the generalized approach to attracting pollinators may be more suitable than being specialized (Waser *et al.* 1996). Although it is now widely understood that the traditional idea of pollination syndromes should be applied with caution, as it underestimates the complexity and variability of interactions in nature, specialization cannot be ignored, as plants occupy every point of the continuum from extreme generalization to extreme specialization in their pollination systems (Johnson and Steiner 2000).

A recent review on the subject of pollination syndromes and floral specialization (Fenster *et al.* 2004) explains the importance of redefining pollination syndromes with pollinators organized into functional groups. Pollinators are clustered into functional groups according to the presumed similar selection pressures they exert, due to similarities in how they behave when visiting flowers, which in turn influences the floral traits of the plant species they visit. These authors define a pollination syndrome as a suite of floral traits, including rewards, associated with the attraction and utilization of a specific group of animals as pollinators. These functional groups can contain many species or only one species, and any particular species of pollinator can belong to multiple functional groups. Organizing pollinators into functional groups is more relevant to pollination biology in general, and to floral specialization, than the more commonly used species lists. For example, *Collinsia heterophylla* (Scrophulariaceae) is pollinated by at least 14 species of animals, and thus some would describe it as generalized, yet it can more accurately be viewed as specialized to a functional group of large-bodied, long-tongued bees in a community of potential pollinators (Fenster *et al.* 2004).

Within a pollination syndrome, an important floral trait in the plant-pollinator relationship is the nutritional reward being offered in the form of nectar and pollen. The quantities of nectar and pollen produced, as well as the timing of nectar production and anthesis (pollen presentation) have been related to pollinator type. Plants that rely on vertebrate pollinators, such as birds and mammals, have to produce substantially more pollen and nectar to meet the energy requirements of their pollinators than do plants that rely on insects (Paton 1986). The ‘pollen presentation theory’, which states that pollen release that is controlled and timed to visitors reflects selection for successful pollination was examined in bee- and bird-adapted species of *Penstemon* and *Keckiella* (Scrophulariaceae) (Thomson *et al.* 2000). Here, high visitation rates and high removal of pollen by bees favoured for pollen presented in small doses, by anthers opening more gradually and less completely than in bird-pollinated species. Diurnal nectar production is considered as a trait favoured by diurnal nectarivorous birds, while nocturnal nectar production and anthesis are favoured by mammals, as most mammal pollinators are crepuscular or nocturnal (Carthew and Goldingay 1997). However, within the Australian Proteaceae, there appears to be no clear dichotomy in bird and mammal pollination systems when associating patterns of nectar production and anthesis with other floral traits eg. diurnal nectar production by bird-pollinated flowers, and nocturnal nectar production by mammal-pollinated flowers (Saffer 2004). This particular study also showed no clear relationship between nectar production and duration of anthesis, suggesting that the systems are in fact more generalized than specialized (Saffer 2004).

In addition to the variation in quantity and timing of nectar according to pollinator type, plants with different pollinators also produce nectar with different sugar compositions and concentrations. Such correlations have led to suggestions of coevolutionary relationships between nectar sugar composition and pollinator type. Three sugars commonly dominate in

nectar – the disaccharide sucrose and the hexose monosaccharides, glucose and fructose. Studies of nectar chemistry have shown that hummingbird-pollinated flowers produce nectar rich in sucrose, while passerine bird-pollinated flowers produce nectar rich in hexose sugars (Baker and Baker 1982; 1983; Stiles and Freeman 1993; Baker *et al.* 1998). Analyses of nectar within the Proteaceae have shown that *Protea* species pollinated by birds produce hexose-rich nectars, while the species pollinated by non-flying mammals produce nectar with a more balanced combination of nectar sugars (Nicolson and van Wyk 1998). In addition, bird-pollinated flowers are considered to produce more dilute nectars (concentrations averaging 20-25% w/w), than do insect-pollinated flowers (averaging 36% w/w in a sample of bee-pollinated flowers) (Pyke and Waser 1981). Among bird-pollinated flowers, those pollinated by passerine birds produced extremely dilute nectar (10-15% w/w) (Nicolson 2002).

Reciprocal modification in floral specialization and specialization of pollinators to particular flowers is considered to be evidence of a coevolutionary relationship. *Banksia* species in Australia possess characteristics that are equally suited to bird and mammal (more specifically, marsupial) pollination, and are visited by both birds and marsupials, as well as insects (Collins and Rebelo 1987). Consequently, there have been conflicting theories regarding the coevolution of *Banksia* species and their pollinators (Turner 1982). Among an array of more generalist and non-coevolved pollination systems involving *Banksia* species, a high degree of specialization in the honey possum, *Tarsipes rostratus*, for a diet of nectar and pollen attests to a long evolutionary association between this marsupial and some *Banksia* species (Wooller *et al.* 1993). Specialized systems and pairwise coevolution between plants and their pollinators appear to be more common in the tropics than in temperate regions (Hingston and McQuillan 2000). In Madagascar, lemurs share a long evolutionary history

with some of the plant species they pollinate and act as the sole pollinators, implying a coevolved system (Kress *et al.* 1994).

### **Mammal pollination**

Within mammal pollination there are two categories of pollination, namely, bat pollination (chiroterophily) and non-flying mammal pollination (therophily). Bat pollination is characterized by flowers that open only at night, with creamy-white colours or shades of green and purple, and a nocturnal scent that is strong and fruity or musty, often resembling the stale odour of fermentation (Buchmann and Nabhan 1996). The flowers produce large quantities of nectar and pollen, and are robust and held in exposed positions making them accessible to bats. Flowers pollinated by mega-chiropteran bats (Old World bats) produce sucrose-rich nectar, while microchiropteran bats (New World bats) visit flowers with more hexose-rich nectar (Baker and Baker 1982; 1983).

The general floral traits of the non-flying mammal pollination syndrome are in many respects similar to those for pollination by bats, and include dull colour, robust structure, musky odours, nocturnal nectar production and abundant nectar and pollen (Carthew and Goldingay 1997). Flowers are usually borne on short robust stems to facilitate visitation by a non-flying mammal and are much less conspicuous than bat flowers. In addition to these general traits, specific traits have been described for nocturnal terrestrial mammals, nocturnal arboreal mammals, diurnal monkeys and other non-flying mammals that have resulted in more sharply defined subsyndromes (Proctor *et al.* 1996, Carthew and Goldingay 1997). It is therefore impossible to describe one specific non-flying mammal pollination syndrome that includes

all such interactions, and these subsyndromes suggest some degree of specialization within the non-flying mammal pollination syndrome.

Studies of the interactions between non-flying mammals and the plants they visit, are less advanced than those of insect, bird and bat pollination. Although pollination by non-flying mammals was first mentioned in the 1930s by Porsch (cited by Rourke and Wiens 1977), it did not receive much attention again until the late 1970s. Non-flying mammals were initially considered to be primarily destructive, consuming flowers rather than pollinating them, but since renewed interest in the 1970s a significant amount of research has been done on terrestrial and arboreal mammals. We now know that rodents, marsupials and primates serve as pollinators in biological systems in Africa, Australia, Madagascar, South and Central America and India (eg. Rourke and Wiens 1977; Janson *et al.* 1981; Steiner 1981; Turner 1982; Goldingay *et al.* 1991; Gautier-Hion and Maisels 1994; Carthew and Goldingay 1997; Mora *et al.* 1999; Johnson *et al.* 2001; Fleming and Nicolson 2002a; Tandon *et al.* 2003).

Mammal pollination in Australia and South Africa has received considerable attention because both continents have members of the family Proteaceae that are pollinated by non-flying mammals. The supercontinent Gondwana was the centre of origin of the family Proteaceae, one of the most prominent flowering plant families in the southern hemisphere (Johnson and Briggs 1975, 1983). When Gondwana split some 140 million years ago, primitive representatives of the Proteaceae were spread across the continents and today most species are found in Australia, followed by Africa, then South and Central America, Pacific islands east of New Guinea, New Caledonia, and a few species in Madagascar, Southeast Asia, New Guinea and New Zealand, with a single species occurring as far north as southern Japan (Matthews 1993; Paterson-Jones 2000; Rebelo 2001). Convergent floral evolution in

Australian and South African Proteaceae for cryptic flowers and geoflorous (ground-flowering) shrubs has been considered an adaptation for non-flying mammal pollination (Rourke and Wiens 1977).

#### *Mammal pollination in Australia*

Of the approximately 60 genera of Proteaceae, 45 are found in Australia and they contain more than 800 species (George 1984; Wrigley and Fagg 1989). In this group the geoflorous trait is most highly developed in the genus *Dryandra*, which has cup-shaped inflorescence heads, and to a lesser extent in its close relative, the genus *Banksia*, which has spiked inflorescences (Rourke and Wiens 1977). Pollination by diminutive marsupials such as honey possums and pygmy-possums is important in Australian Proteaceae, especially *Banksia* (Cunningham 1991; Goldingay *et al.* 1991; Goldingay 2000).

The most widely studied marsupial pollinator in Australia is the honey possum, *Tarsipes rostratus*, which feeds on flowers in the families Proteaceae, Myrtaceae and Epacridaceae (Russell and Renfree 1989). The honey possum is unique among non-flying mammal pollinators in that it feeds exclusively on nectar and pollen (Bradshaw and Bradshaw 1999). Special structures (long filiform papillae) on the surface of its tongue assist in collecting nectar and pollen (Richardson *et al.* 1986). *Banksia* is its primary food source and the abundance of honey possums fluctuates in concert with *Banksia* flower production (Wooller *et al.* 1993). This close association between *T. rostratus* and *Banksia* species strongly suggests that this is a coevolved relationship.

Other species of marsupials involved in pollination of Australian Proteaceae include the eastern pygmy-possum *Cercartetus nanus*, western pygmy-possums *Cercartetus concinnus*,

sugar gliders *Petaurus breviceps*, as well as several dasyurid marsupial species belonging to the genus *Antechinus* (Turner 1984; Landwehr *et al.* 1990; Carthew 1994; Goldingay 2000). Although much work has been done on other marsupials, the dasyurid marsupials are more widespread and abundant and may be more reliable as pollinators (Goldingay 2000).

#### *Mammal pollination in South Africa*

Africa harbours about 400 species of Proteaceae, of which more than 360 species are found in southern Africa, and 330 of those (in 14 genera) are confined to the Cape Floral Kingdom (an area from Nieuwoudtville in the northwest to Grahamstown in the east of the Cape Province) (Cowling and Richardson 1995; Paterson-Jones 2000; Rebelo 2001). Rourke and Wiens (1977) listed 22 South African *Protea* species that were ground or near-ground flowering, and observed evidence of rodent activity on the flowers of eight of these. Wiens *et al.* (1983) later published a list of 31 *Protea* species with morphology and floral rewards consistent with non-flying mammal pollination. In South Africa, the interaction between mammals (particularly rodents) and the *Protea* species they visit is described as a non-coevolved system, based on the small overlap in their geographical distributions, the short flowering season when the animals have access to nectar and pollen, and the contrast between specialized floral traits and generalist mammal visitors (Wiens *et al.* 1983; Rebelo and Breytenbach 1987). *Protea amplexicaulis*, *P. cordata*, *P. humiflora*, *P. decurrens* and *P. subulifolia* are referred to as the rodent sugarbushes in Rebelo (2001).

Among these geoflorous South African Proteaceae, *P. humiflora* has been frequently included in studies concerning the relationship between *Protea* and its mammal pollinators (Wiens and Rourke 1978; Wiens *et al.* 1983; Fleming and Nicolson 2002a). Mammals trapped near flowering *P. humiflora* plants commonly include rodents such as Namaqua rock

mice *Aethomys namaquensis*, striped mice *Rhabdomys pumilio* and Cape spiny mice *Acomys subspinosus*, as well as elephant shrews *Elephantulus edwardsii* (Fleming and Nicolson 2002a). When the effect of mammal visitors on seed set was quantified as a measure their effectiveness as pollinators, *A. subspinosus* was highlighted as a significant pollinator of *P. humiflora* (Fleming and Nicolson 2002a). This small rodent (body mass  $\pm 18$ g) utilizes the nutrient-rich food resource of pollen and nectar to facilitate its winter breeding (Fleming and Nicolson 2002b). *Acomys subspinosus* feeds exclusively on *P. humiflora* pollen and nectar during the winter flowering months of *P. humiflora*, and is thus highly dependent on this species and in turn, is a very effective pollen vector. *Aethomys namaquensis* is less closely associated with *P. humiflora* as it is not dependent on the flowers as a food resource, but forages from flowers and carries enough pollen on its nose to be a pollen vector and effect pollination (Fleming and Nicolson 2002a). Both *A. subspinosus* and *A. namaquensis* have also been recorded as visitors to *P. amplexicaulis* and *Massonia depressa*, an African lily (Wiens and Rourke 1978; Johnson *et al.* 2001). Pollination by these two rodent species has been demonstrated for *M. depressa* (Johnson *et al.* 2001). Insectivorous shrews like *Elephantulus edwardsii* seem unlikely pollinators, but appear to visit *P. humiflora* flowers to feed on the insects, particularly ants, in the inflorescence, and in doing so facilitate pollination (Fleming and Nicolson 2002a; Fleming and Nicolson 2003). Small mammals like *A. subspinosus*, *A. namaquensis* and *E. edwardsii* are responsible for about 56% of seed set in *P. humiflora* (Fleming and Nicolson 2002a).

The present study is based on the interaction between the Namaqua rock mouse, *Aethomys namaquensis*, and the rodent sugarbush, *Protea humiflora*. It has been shown that *A. namaquensis* occurs in high abundance at the sites previously used in studies of rodent-*Protea* interaction, comprising 60% of the number of individuals captured and 67% of the

number of captures at these sites (Fleming and Nicolson 2002a). *Aethomys namaquensis* was the subject of sugar preference tests and xylose absorption efficiency studies on a rodent pollinator of Proteaceae (Johnson *et al.* 1999). Although it has subsequently been shown that *A. subspinosus* has a stronger relationship with *P. humiflora*, results from those studies on *A. namaquensis* prompted the questions addressed in this thesis.

### ***Aethomys namaquensis* (Rodentia: Muridae)**

*Aethomys namaquensis* (A. Smith, 1834), commonly known as Namaqua rock mice, are widely distributed in South Africa, except in central and northern parts of the Cape Province and coastal Kwazulu-Natal (Skinner and Smithers 1990). They show a preference for rocky habitats, using rock crevices for shelter, and are to some extent arboreal. Although rock mice are not dependent on *Protea* flowers for food, from the plant perspective the abundance of these mice in areas where *P. humiflora* grows makes them valuable as pollinators (Fleming and Nicolson 2002a). Both rock mice and *P. humiflora* show a preference for rocky, dry, north-facing slopes. As herbivores/granivores (Perrin and Curtis 1980), rock mice may be responsible for some of the damage observed on geoflorous *Protea* flowers, but they are nevertheless effective as pollinators of *P. humiflora* when visiting the flowers to feed on nectar and pollen (Fleming and Nicolson 2002a). Due to the winter flowering period of geoflorous *Protea* species in the southwestern Cape, rock mice have access to a valuable energy-rich food resource in the form of floral nectar and pollen when other resources, such as seeds, are low. Like Australian marsupial pollinators that also feed on Proteaceae flowers, rock mice are capable of extracting and digesting the protoplast from pollen grains (Richardson *et al.* 1986; van Tets and Whelan 1997; van Tets 1997). The mechanism employed by these mammals to deal with the highly resistant outer wall (exine) of pollen

grains is unclear. Without the enzyme exinase, which is only secreted by certain Collembola pollen-feeders (Scott and Stojanovich 1963), animals gain access to pollen protoplasm through pseudo-germination and exudation of the protoplasm in the gut, osmotically or chemically induced bursting of pollen grains, or direct enzymatic action through the pores of the pollen wall (Turner 1984; Richardson *et al.* 1986). The most likely method occurring in rock mice consuming *P. humiflora* pollen is enzymatic digestion through the pores, as no evidence of pollen tubes or burst pollen grains in excreted faeces was found, just empty or partially empty grains with intact outer walls (van Tets 1997). In the Australian marsupial *T. rostratus*, an obligate flower-feeder, 95-100% of pollen grains excreted in faeces were empty (Richardson *et al.* 1986). For rock mice, the mean percentage of empty or partially digested pollen grains in faeces was 60% (van Tets 1997). Pollen provides rock mice with a nitrogen source, and has greater biological value than does the purified protein casein, in that nitrogen obtained from pollen is utilized more efficiently (van Tets *et al.* 2000). A diet of *Eucalyptus calophylla* pollen, which has similar proportions of amino acids to *P. humiflora* pollen, is capable of meeting the maintenance nitrogen requirements of rock mice (van Tets *et al.* 2000).

### **Nectar sugar composition**

The constituents of floral nectar include amino acids, proteins, lipids, antioxidants, phenolics, alkaloids and ions (Baker and Baker 1982; 1983), but the most abundant components of nectar are the sugars. The proportions of the dominant sugars in nectar, namely sucrose and the hexoses glucose and fructose, vary in different plant species, and nectar of a particular species generally can be characterized by the proportions of sucrose and hexose sugars. Sugar proportions of plants with the same pollinator type are frequently similar, even if the plant

species are taxonomically unrelated. Consequently, it is believed that the sugar proportions of a particular plant species may reflect the sugar preferences of the pollinator (Baker and Baker 1982; 1983).

The pentose monosaccharide xylose has been reported as a fourth major nectar sugar in two genera of Proteaceae (van Wyk and Nicolson 1995; Nicolson and van Wyk 1998). *Protea* and *Faurea* species produce nectar in which xylose comprises as much as 39% of the total nectar sugars. Prior to the analysis of nectar composition in the Proteaceae, xylose had never been reported as a nectar sugar, except in the extrafloral nectar of a grass (Bowden 1970). Xylose has subsequently been found in the nectar of the rodent-pollinated African lily, *Massonia depressa* (Johnson *et al.* 2001). Following the discovery of substantial xylose in Proteaceae nectar, a number of studies have been conducted to investigate the responses of *Protea* pollinators to xylose. These studies included sugar preference and absorption efficiencies tests of the different sugars in nectar. A review of these studies and other aspects of xylose metabolism proposes possible explanations for the unusual occurrence of xylose in nectar (Jackson and Nicolson 2002).

### **Pollinator responses to xylose**

Among the potential insect pollinators of *Protea* species, the responses of Cape honeybees *Apis mellifera* and green protea beetles *Trichostetha fascicularis* to xylose have been tested. Honeybees are potentially important pollinators of both *Protea* and *Faurea* species (Hepburn and Radloff 1995). They showed an aversion to xylose when provided with a range of glucose/xylose mixtures, and their survival time decreased as the proportion of xylose in the diet increased (Allsopp *et al.* 1998). Green protea beetles were also averse to drinking

solutions of xylose, but not mixtures of xylose with other nectar sugars (S.W. Nicolson and S. Jackson, unpublished data).

Representatives of three major families of nectarivorous birds in southern Africa have been examined. The Cape sugarbird *Promerops cafer*, has no preference for sucrose, glucose or fructose when offered a choice, while the lesser double-collared sunbird *Nectarinia chalybea*, and the Cape white-eye *Zosterops pallida*, both prefer sucrose to glucose and fructose, and all three sugars were digested with almost 100% efficiency by all three bird species (Lotz and Nicolson 1996; Franke *et al.* 1998; Jackson *et al.* 1998a; 1998b). However, the birds all strongly rejected pure xylose solutions and would only ingest sugar mixtures containing small amounts of xylose. When ingested, xylose was very poorly assimilated. Apparent absorption efficiencies in sugarbirds and white-eyes that did consume xylose were 53 and 61% respectively, but in sugarbirds high concentrations of xylose in cloacal fluid suggested very poor utilization of the sugar (Franke *et al.* 1998; Jackson *et al.* 1998b).

In contrast to the insect and bird pollinators, Namaqua rock mice willingly consumed pure xylose solutions (Johnson *et al.* 1999). The sugar preferences of the rodents were consistent with the mixed sugar composition of the nectar in the flowers they pollinate. Also, ingested xylose was efficiently metabolized, with an apparent absorption efficiency of 97%, and very low concentrations of xylose were measured in blood and urine. Together with the fact that there is a higher percentage of xylose in rodent pollinated *Protea* species than in bird-pollinated ones (Nicolson and van Wyk 1998), these findings suggest that xylose is of some importance as an energy source to rodent pollinators.

## **Xylose metabolism**

Xylose has nutritional value only if it can be absorbed and utilized: the first criterion is met for many mammals, but the second is not. Intestinal absorption of xylose has been studied in a number of animals such as hamsters, rats, frogs, rabbits and cows (Salem *et al.* 1965; Alvarado 1966; Lassen and Csàky 1966; Heyman *et al.* 1980; Miyamoto *et al.* 1993; Scharrer and Grenacher 2000). In these animals, xylose transport is active, Na-dependent, inhibited by phlorizin, and occurs by a mechanism similar to that of active glucose transport. Xylose absorption in humans differs from that in other mammals (Heyman *et al.* 1980) in that it occurs by diffusion, is Na-independent, and phlorizin insensitive (Ohkohchi *et al.* 1986; Fine *et al.* 1993). Cell lines cultured from hamster ovaries and human fibroblasts are able to survive with xylose as their sole energy source (Demetrakopoulos and Amos 1976; 1978). However, cells from metabolically active mammalian tissues, such as liver, muscle and kidney, are very poor xylose metabolizers (Blatherwick *et al.* 1936; Krebs and Lund 1966; Metzger *et al.* 1972). The slow passive absorption of xylose in humans, and persistence of xylose in the blood, form the basis of two biomedical tests: 1) the xylose absorption or tolerance test, which assesses gastrointestinal mucosa functioning (Zilva and Pannall 1984; <http://www.labcorp.com/datasets/labcorp/html/chapter/mono/sc032800.htm>), and 2) the intestinal bacterial overgrowth test which is used to assess the population size of enteric bacteria, which may be affected by alterations in intestinal anatomy, gastrointestinal motility or lack of gastric acid secretion (Saltzman and Russell 1994). In the xylose absorption test, a patient is given a weight-based oral dose of D-xylose and analysis of the concentrations of xylose in blood and urine at specific times after ingestion are used to detect intestinal malabsorption, blind loop syndrome and thyroid disease. The bacterial overgrowth test is based on the efficiency with which enteric bacteria are able to metabolize xylose and release

CO<sub>2</sub> as a by-product. Patients are given an oral dose of <sup>14</sup>C-D-xylose and exhaled air is tested for <sup>14</sup>CO<sub>2</sub>. Patients with intestinal bacterial overgrowth exhale <sup>14</sup>CO<sub>2</sub> within one hour of dosage. Bacterial overgrowth can cause vitamin deficiencies, fat malabsorption or malnutrition, and antibiotics are used to treat enteric bacterial overgrowth to create normal enteric bacterial population sizes and correct nutritional deficiencies (Saltzman and Russell 1994). Xylose-metabolizing bacteria are thus present in human intestines. Much of what we know about xylose metabolism is because of these medical implications of xylose metabolism in humans.

Vertebrate digestion of plant material is accomplished by gastro-intestinal microflora (Stevens and Hume 1995) and is a well-studied area of vertebrate digestive physiology. The efficiency with which enteric bacteria are able to metabolize xylose is well understood as D-xylose is a monomer of xylan, the hemicellulose component of plant cell walls.

Hemicellulose is the second most abundant polysaccharide in plant cell walls and comprises up to 35% dry weight of plant material (Gong *et al.* 1981). The degradation of xylan and the microbial utilization of xylose have been well researched in ruminants (Turner and Robertson 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). The predominant end-products of microbial fermentation in the gut are short-chain fatty acids (SCFA) that diffuse out of the bacterial cells into the gut lumen. The host animals are then able to access the energy of ingested plant material by absorbing SCFA across the gut wall and utilizing them in oxidative metabolism.

The contribution of xylose to metabolizable energy in birds and pigs was found to be between 38 and 64% of xylose dietary energy (Schutte 1990; Verstegen *et al.* 1997). The percentage of metabolizable energy decreased as the concentration of D-xylose in the diet of broiler chicks increased (Schutte 1990; Schutte *et al.* 1992). The percentage of ingested xylose that is excreted in the urine is usually reported: in roosters and pigs fed a diet containing 10% xylose it was on average 20% and 37% respectively (Schutte *et al.* 1991; Verstegen *et al.* 1997), leading to speculation that the remaining fraction is metabolized to CO<sub>2</sub> by the animal or fermented by its intestinal microbiota.

The possible catabolism of xylose by intestinal bacteria of *A. namaquensis* was previously highlighted in laboratory experiments (Johnson *et al.* 1999), in which the sugar preferences and absorption efficiency of xylose were investigated. Although control animals used in that study were not fed xylose, but given water instead, small amounts of xylose were detected in their blood, urine and faeces. This xylose could only have been a result of hydrolysis of xylans in the food pellets, which consisted primarily of plant material. The results of that study, as well as our current knowledge of xylose metabolism by vertebrates and microbes, led to the questions addressed in this thesis.

**Key questions and thesis outline:**

The key questions of this thesis, given the background discussed above, are:

- Does *Aethomys namaquensis* have an indigenous gut microflora that is able to metabolize the pentose sugar, xylose or do they have the ability to do so themselves, through their own metabolic processes?
- Does the ability of *A. namaquensis* to metabolize xylose help explain why xylose is present as a nectar sugar in Proteaceae? Is the presence of xylose of ecological significance?

**Chapter 2 – The effect of different oral antibiotics on the gastrointestinal microflora of a wild rodent (*Aethomys namaquensis*)**

Aim: To determine what antibiotics can be used to significantly reduce the natural gut microflora of *Aethomys namaquensis*.

This chapter describes the establishment of an antibiotic treatment for *A. namaquensis*, and how non-absorbable antibiotics can be used to eliminate large portions of the indigenous population of gut microflora in this rodent. The antibiotic treatment protocol was developed for implementation in a study in which animals could be tested with their natural gut flora intact, and then after antibiotic treatment, with a reduced gut microflora population.

**Chapter 3 – Nectar xylose metabolism in a rodent pollinator (*Aethomys namaquensis*): defining the role of gastrointestinal microflora using <sup>14</sup>C-labelled xylose**

Aim: To determine to what extent, if any, are the indigenous gut microflora of *Aethomys namaquensis* responsible for xylose metabolism in the gut.

This chapter describes a study in which the antibiotic treatment protocol, developed in Chapter 2, was implemented. Mice were fed radiolabelled xylose and the recovery of the

label in exhaled CO<sub>2</sub>, urine and faeces, from mice in the two states (with natural gut flora and with antibiotic-treated gut flora), was used to evaluate the involvement of gut microflora in xylose metabolism. This <sup>14</sup>C-xylose whole-animal metabolism study is the most important component of this thesis, and its results determined the direction of the research done in the following chapter.

**Chapter 4 - Xylose utilization and short-chain fatty acid production by intestinal microflora of a rodent pollinator (*Aethomys namaquensis*)**

Aim: To determine what species of gut microflora are involved in xylose metabolism in *Aethomys namaquensis*, and what short-chain fatty acids these species produce during xylose metabolism.

This chapter describes the isolation and identification of rock mouse intestinal microflora that were identified as xylose utilizers by pH, growth and substrate utilization assays. Here I also describe how caecal microbes identified as positive xylose utilizers were analyzed in order to identify the SCFA they produce that ultimately contribute to the energy requirements of *A. namaquensis*.

**Chapter 5 – Concluding comments**

In conclusion I discuss the ecological significance of xylose in nectar, with possible explanations for the presence of xylose as a nectar sugar, and consider how the fermentative capacity of an animals' digestive system determines its ability to benefit nutritionally from xylose.

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## Chapter 2

### **The effect of different oral antibiotics on the gastrointestinal microflora of a wild rodent (*Aethomys namaquensis*)**

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## Abstract

Gut sterilization via the oral administration of antibiotics facilitates physiological studies of the nutritionally important relationship between intestinal microflora and the host. However, the composition of gut flora is extremely variable, and as a result the efficacy of antibiotics in achieving gut sterilization varies considerably between species. I tested the effectiveness of three antibiotic cocktails in sterilizing the gut of a rodent pollinator, the Namaqua rock mouse (*Aethomys namaquensis*). The cocktails were (1) streptomycin sulfate and bacitracin (previously used with domestic mice and rats), (2) chloramphenicol and bacitracin (based on antibiotic screening tests performed on faecal flora) and (3) Baytril 10% oral solution (a veterinary antimicrobial agent containing enrofloxacin). I tested for antibiotic inactivation by determining bacterial viability through fluorescence staining of faecal samples. I also tested techniques to maintain sterility during antibiotic treatment without the benefit of a laminar flow cabinet. Antibiotics were administered orally in food and water consumed *ad libitum* over four consecutive days. Antibiotic effectiveness was assessed by culturing anaerobic bacteria from faecal samples collected before and after each antibiotic treatment. Treatment with Baytril 10% oral solution eliminated or significantly reduced faecal flora, whereas other antibiotics did not. This study clearly demonstrates the importance of testing the effectiveness of antibiotics before their use in studies that involve antibiotic-treated subjects, particularly if these are species previously untested.

Key words: Antibiotics; Bacterial viability; Baytril; Gastrointestinal microflora; Gut sterilization; Wild rodents; Xylose

## Introduction

Shortly after birth the mammalian digestive tract becomes colonized with bacteria acquired from the mother and the immediate environment. The many bacterial species that establish populations in the variety of habitats offered by the digestive tract make up the indigenous gastrointestinal flora of an animal, and contribute to a gastrointestinal ecosystem that is necessary for the efficient digestion and absorption of nutrients from the alimentary canal. The average human being comprises  $10^{13}$  cells (Dobzhansky 1970) and the alimentary tract alone is colonized by an additional  $10^{14}$  bacterial cells (Luckey 1972). A similar animal to bacterium cell ratio exists in the mouse (Schaedler and Orcutt 1983). The gastrointestinal microflora inhabiting the digestive tract of mammals has a huge influence on the host's physiology, intestinal anatomy and resistance to infectious diseases (Tannock 1999). Study of this relationship between gastrointestinal microflora and host physiology is facilitated by the use of germ-free (GF) and specific-pathogen free (SPF) animals in the field of gnotobiology (Gordon and Pesti 1971), and temporary gut sterilization achieved via the administration of antibiotics (van der Waaij and Strum 1968). The development and maintenance of GF and SPF animals is laborious and expensive, and in some cases it is not necessary to develop GF or SPF colonies or to breed such animals from one generation to the next. Study of the relationship between the gastrointestinal microflora and the host in the case of both laboratory and wild animals, especially in a laboratory not equipped with germ-free isolators, is possible using temporary gut sterilization.

Temporary gut sterilization may involve absolute or selective elimination of microflora. Van der Waaij and Strum (1968) described technical procedures whereby the bacterial flora of the digestive tract of mice can be completely eliminated and the digestive tract maintained free

from bacteria by the oral administration of the antibiotics: bacitracin, kanamycin, neomycin and streptomycin. They reported that a combination of 4 mg/ml of bacitracin and 4 mg/ml of any one of the other three antibiotics in the drinking water effectively decontaminated the digestive tract. Subsequent studies used a combination of streptomycin sulfate and bacitracin (Berg 1980; Maejima *et al.* 1984; Deitch *et al.* 1985), and later streptomycin sulfate and penicillin (Deitch *et al.* 1990; Guo *et al.* 1995; Shimizu *et al.* 2001). Various antibiotic cocktails have been shown to completely or selectively sterilize the gastrointestinal tracts of mice and rats (Satoh *et al.* 1983; Toorop-Bouma and van der Waaij 1987; Wells *et al.* 1990; Adachi *et al.* 1995; Pulverer *et al.* 1997; Enomoto *et al.* 1999; Schlegel *et al.* 1999; Schlegel *et al.* 2000; Weisner *et al.* 2001), but it is evident that the composition of indigenous gastrointestinal microflora is very variable between and within species, and any antibiotic cocktail should be tested for its effectiveness before it is implemented in experiments involving antibiotic-treated subjects.

Antibiotics may be inactivated enzymatically and non-enzymatically by intestinal contents, reducing the effective dose of the antibiotic to varying degrees, depending on individual differences in microflora and on the pharmacokinetic properties of each antibiotic (see van der Waaij and Nord 2000 for review). Non-enzymatic inactivation by binding of antibiotics to faecal material in the gut may be reversed when the antibiotics become unbound and reactivated when faecal material is suspended in water or saline, such as occurs during preparation for culture. Microbial culturing of faecal material to test the efficacy of antibiotics may overestimate the *in vivo* efficacy of such reactivated antibiotics. It is therefore necessary to consider antibiotic inactivation if culture counts are to be reliable indicators of antibiotic effectiveness against gut microflora.

The context for this paper is an investigation of the possibility that intestinal bacteria play a role in xylose metabolism in a rodent pollinator, the Namaqua rock mouse, *Aethomys namaquensis*. The pentose sugar xylose constitutes 1 - 39% of the total nectar sugar of *Protea* and *Faurea* species (Proteaceae; van Wyk and Nicolson 1995; Nicolson and van Wyk 1998), and the nectar produced by rodent-pollinated *Protea* species in the southwestern Cape of South Africa generally contains larger quantities of xylose than does the nectar produced by bird-pollinated species (Nicolson and van Wyk 1998). Moreover, sugar preference and absorption efficiency tests on rodent, bird and insect pollinators (reviewed by Jackson and Nicolson 2002) suggest a possible connection between the presence of xylose in protea nectar and rodent pollination of proteas. *A. namaquensis*, one of the important pollinators of ground-flowering proteas (Rourke and Wiens 1977; Wiens and Rourke 1978; Fleming and Nicolson 2002), willingly consumes pure xylose solutions and shows a very high apparent absorption efficiency for the sugar (97%) (Johnson *et al.* 1999). Although xylose metabolism by mammalian tissue is very slow, bacteria, yeasts and filamentous fungi are known to readily convert this sugar to D-xylulose, which is then converted via the pentose phosphate pathway to fructose-6-phosphate (Demetrakopolous and Amos 1978; Chandrakant and Bisaria 1998; Ho *et al.* 1999; Jeffries and Shi 1999; Aristidou and Penttilä 2000). With an effective gastrointestinal sterilizing antibiotic treatment in place, the recovery of <sup>14</sup>C-labelled xylose fed to normal and antibiotic-treated mice could help determine the degree to which the mice depend on intestinal bacteria for metabolism of nectar xylose.

Here I describe tests of procedures to effectively sterilize the mouse digestive tracts. I wanted to develop a non-invasive, inexpensive and fairly simple means of sterilizing the gut, testing for sterility and maintaining sterility for further experiments.

## Materials and Methods

### *Animals*

*Aethomys namaquensis* individuals were caught under permit (numbers 245/2001 and 180/2003, Western Cape Nature Conservation Board) in Sherman live traps at Wolfieskop, near Villiersdorp in the Riviersonderend Mountains, South Africa. This site was chosen because of the presence of dense populations of *Protea humiflora*, a ground-flowering protea which is pollinated by *A. namaquensis* (Fleming and Nicolson 2002). Animals were caught during the night and taken to the laboratory where they were housed individually in conventional rodent cages at a constant temperature of 22°C with *ad libitum* access to water and food pellets (Prestige Products, Crispy Rat Muesli: 18% protein, 3% oil, 5.5% fiber and 7.5% ash, with a maximum of 88% total digestible nutrients). The period between animal capture and the start of experiments ranged from 1 - 5 weeks. During antibiotic treatment individuals were housed in filter-topped cages (Tecniplast, Labotec (Pty) Ltd, South Africa). Each filter-topped cage was placed within a plastic sheet-enclosed compartment on a shelf for the duration of the antibiotic treatment. Ethical approval for the study was obtained from the Sub-Committee B of the Research Committee of the University of Stellenbosch.

### *Culture of faecal samples*

To assess the effectiveness of the antibiotics administered and the procedures employed faecal pellets were collected before and after antibiotic treatment. Within two hours of collection, samples from the core of each pellet (Trial 1) or whole pellets (Trials 2 – 4) were processed for anaerobic growth and enumeration of culturable bacteria. Since anaerobic bacteria predominate the digestive tract of mice (Gordon and Dubos 1970; Lee *et al.* 1971) and other mammals (Harris *et al.* 1976; Schaedler and Orcutt 1983), we decided to culture

only bacteria that would grow under anaerobic conditions. Serial 10-fold dilutions of each sample resuspended in sterile pre-reduced buffer solution (2.25 g NaCl, 0.105 g KCl, 0.12 g CaCl<sub>2</sub> and 0.05 g NaHCO<sub>3</sub> in 1L distilled water) were plated out in triplicate on supplemented Brain-Heart Infusion (BHI) agar (Holdeman *et al.* 1977). Plates were incubated at 37°C for 48 h in an anaerobic chamber (Forma Scientific, atmosphere: 10% H<sub>2</sub>, 10% CO<sub>2</sub>, 80% N<sub>2</sub>) and numbers of visible colony forming units (CFU) per gram of faecal material were calculated.

### ***Antibiotic screening tests***

The antibiotics fed to mice in Trial 3 (described below) were chosen from antibiotics screened beforehand to determine the sensitivity of the culturable faecal flora to a variety of antibiotics, and to identify potentially effective antibiotics for sterilization of *A. namaquensis* guts. These antibiotic screening tests were done on total bacterial cultures from *A. namaquensis* faecal pellets collected in the field. Culture plates were prepared as described above for faecal samples from four individuals caught in the field. Antibiotics were applied to the plates in two ways: as discs placed on the surface of the medium, or as 50 µl of a stock solution of antibiotic placed into a well in the medium. Discs contained one of the following antibiotics: streptomycin (10 µg/ml), neomycin (5 µg/ml), kanamycin (5 µg/ml), ampicillin (10 µg/ml), chloramphenicol (10 µg/ml), nalidixic acid (30 µg/ml), erythromycin (5 µg/ml) and tetracycline (50 µg/ml). Bacitracin was used as a stock solution and tested at the following concentrations: 4000 µg/ml, 2000 µg/ml, 1000 µg/ml, 500 µg/ml, 50 µg/ml and 10 µg/ml. Culture plates were incubated overnight at 37°C in an anaerobic chamber, after which each antibiotic's effectiveness in inhibiting bacterial growth was visually assessed by the size of the bacteria-free zone around the disc or well. Those antibiotics producing the largest

zones were considered the most effective at inhibiting growth of *A. namaquensis* faecal flora on the culture plate.

#### ***Antibiotic treatment (Trials 1 - 4)***

Animals were randomly assigned to the different trials. Each antibiotic trial lasted four consecutive days and was carried out on four different individuals, all of which were females (average body mass 61.52 g  $\pm$  SD 14.0g). A high vacuum autoclave was used to sterilize entire filter-topped cages, sawdust and water bottles at 121°C for 15 min plus 6 min drying time, and food pellets at 134°C for 4 min plus 5 min drying time. Drinking water, 30% aqueous sucrose solutions and agar-feed mixes (an agar block containing crushed food pellets, sucrose and peanuts) were autoclaved at 121°C for 15 min. Antibiotics were offered *ad libitum* in the sucrose solution to improve their palatability. Of the four nectar sugars (sucrose, glucose, fructose and xylose), sucrose is preferred by *A. namaquensis* in cafeteria-type tests (Johnson *et al.* 1999). All materials and gloves used when handling mice were disinfected with 0.5% chlorhexidine in 70% alcohol. Animals were weighed at the beginning and the end of each trial (Sauter R3000 balance). Fresh antibiotic cocktails were given daily and food and water consumption was monitored daily by determining volumes of solutions drunk and/or percentage of agar eaten, to obtain estimates of the doses consumed. Pre-trial experiments showed that individuals ate large amounts of the agar-feed mix in the first day it was offered to them, but progressively less each day thereafter. This decline did not reverse after five days, indicating that they had developed an aversion that was unlikely to be reversed by continued feeding. When the proportions of each food component were varied to produce slightly different tastes each day, mice maintained their food intake at higher levels that were nonetheless lower than their intake of food free of antibiotics. When feeding antibiotics for the experiments, I therefore varied the proportions of each component of the

agar mix on a daily basis. I also chose not to administer antibiotics for more than four days, as this would have resulted in unacceptably high levels of weight loss in these wild mice, which experience stress during captivity.

#### *Trial 1*

Each individual was offered 4 mg/ml streptomycin sulfate (Amersham Biosciences, South Africa) and 4 mg/ml bacitracin (Amersham Biosciences, South Africa) in a sterile 30% sucrose solution and sterile food pellets *ad libitum*. A fresh antibiotic solution was given each day. At the end of the third day the sawdust in each cage was replaced with fresh autoclaved sawdust to facilitate a fresh faecal pellet collection the following morning for culturing the post-trial samples. The pre- and post-treatment faecal pellets were cut in half using a sterile scalpel and a core sample for culturing was scraped out from the center with a sterile needle.

#### *Trials 2, 3 and 4*

In trial 2 each individual was offered a 4 mg/ml streptomycin sulfate and 4 mg/ml bacitracin in a sterile 30% sucrose solution and sterile food pellets *ad libitum*, as in trial 1. Based on the preliminary antibiotic screening tests, chloramphenicol (Parke-Davis(Pty) Ltd.) and bacitracin were offered to the mice in trial 3 at concentrations of 0.1 mg/g body mass/day and 0.5 mg/g body mass/day respectively, in a sterile agar-feed-mix. In trial 4 each individual was offered 0.3 µl/g body mass/day of Baytril 10% oral solution (Bayer (Pty) Ltd.) in 30% aqueous sucrose solution as well as in a sterile agar-feed-mix. Baytril is a veterinary antimicrobial agent containing enrofloxacin. In trials 2, 3 and 4, mice were confined in an inner wire cage that fitted inside the filter-topped cage. No sawdust was added and each day the inner cage with the mouse was removed and placed on a chlorhexidine-disinfected surface. The cage bottom was then washed with distilled water and wiped with chlorhexidine.

Whole pellets collected at the beginning and the end of the trial on a sterile surface were used for processing pre- and post-treatment total culturable counts.

***Efficacy of antibiotics: estimation of bacterial viability in faecal samples***

To determine whether reversible antibiotic inactivation occurred in trial 4, and whether re-suspension of faecal samples for microbial culturing resulted in reactivation of antibiotics bound to faecal material, 2 mice were treated with Baytril 10% oral solution as in trial 4, and their faecal pellets tested for bacterial viability. Two fresh faecal pellets were collected from each mouse before and after antibiotic treatment. Each pellet was immediately and separately re-suspended in 300 µl sterile buffer solution (see above), by breaking it up in buffer with a sterile needle. 100 µl of this suspension was stained with 100 µl *BacLight*<sup>TM</sup> LIVE/DEAD viability probe (Molecular Probes Inc., Oregon, USA) and left in the dark for one hour at room temperature.

The *BacLight*<sup>TM</sup> LIVE/DEAD viability kit contains two fluorescent nucleic acid stain solutions: SYTO 9 and propidium iodide, which exploit cell membrane integrity to distinguish between live and dead bacterial cells. Cell membranes are permeable to SYTO 9, which stains the cell green. Propidium iodide only crosses compromised membranes of non-viable cells, staining these with red fluorescence which overwhelms the green fluorescence of the SYTO 9 stain. This combination thus stains viable bacteria green, while non-viable cells appear red.

After 1 hour incubation in the dark, 100 µl of the stained suspension was vacuum filtered through 0.22 µm, 47 mm Micron-PES, Polysulfone membrane filters (Osmonics Inc.) and mounted on glass slides in *BacLight*<sup>TM</sup> mounting oil. A Nikon Eclipse E400 epifluorescence

microscope equipped with a multipass filter set appropriate for viewing DAPI, and with excitation/barrier filter sets of 465-495/515-555 nm (Texas Red), and 540-580/600-660 nm (FITC), was used. Fluorescence emission images were captured with a Nikon (Coolpix 9909) digital camera. Separate green and red fluorescence emission images of the same microscopic field were captured randomly across the entire surface area of each sample viewed. Total numbers of viable (green) and non-viable (red) bacteria were determined for each image and the number of viable cells expressed as a percentage of all cells present in each field (Korber *et al.*, 1996). Twenty-four images were captured for each pellet. Thus for each of the two mice, 48 images from two faecal pellets were generated for the “before antibiotic treatment” percentages, and a further 48 for the “after antibiotic treatment.

### ***Statistics***

Single factor ANOVA was used to test for significant differences in weight loss between trials. Paired two-tailed *t*-tests were performed on the log-transformed data of the CFU/g faecal sample cultured in each trial, to test for significant differences between counts before and after antibiotic treatments. *t*-tests for independent samples were performed on percentage viability data obtained from each pellet from each mouse, to test for differences between the two pellets from one particular mouse. *t*-tests for dependent samples were performed on the pooled percentage viability data for each mouse to test for differences in bacterial viability before and after treatment.

## Results

The weight lost by the animals during each trial is shown in Fig. 2.1. On average individual animals lost 11.15% of their initial body mass during the four day antibiotic treatment. One animal died during trial 2. No significant differences were found in weight loss between the four trials ( $P > 0.05$ ), suggesting that food consumption did not differ between antibiotics and implying that no one of the antibiotics offered was more unpalatable than any other. Six days after the end of each trial animals were weighed again, by which time all had recovered their body mass. Some animals showed signs of diarrhea after antibiotic treatment, but this only lasted a day or two, after which normal faeces were formed once again.

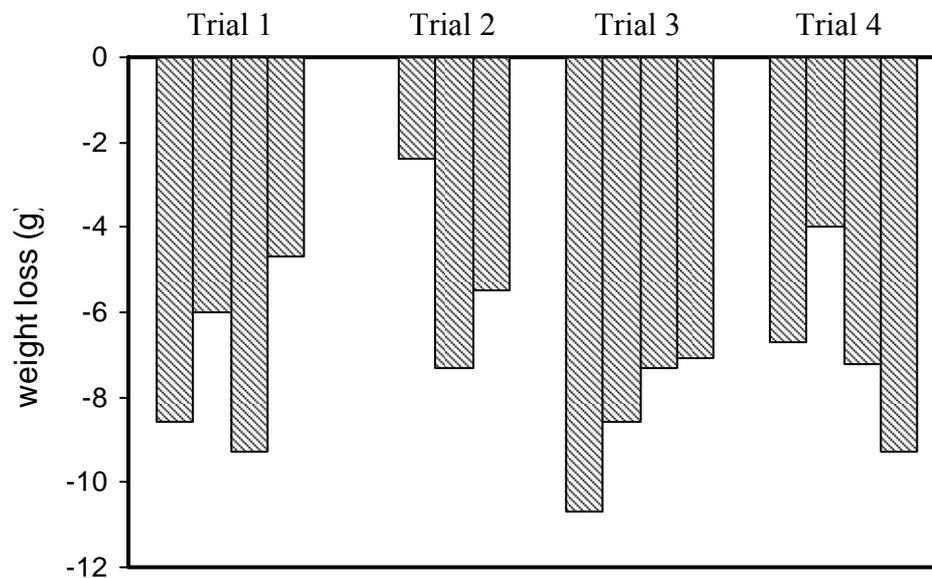


Fig 2.1. Weight loss (g) of animals in each trial. Note that four different animals were used in each antibiotic trial. Single factor ANOVA showed no significant differences in the weight losses between the different trials ( $P > 0.05$ ).

The methods used to sub-sample faecal material did not appear to greatly influence culturable bacterial counts. In trial 1, the pre- and post-treatment faecal samples were taken from the insides of whole pellets. In trials 2, 3 and 4 the faecal pellets were collected on a sterile surface and the whole pellet was processed for culturing bacteria. The masses of the faecal samples used for culturing ranged from 18.6 mg - 3.2 mg (average = 9.4 mg). Internal sampling of pellets is difficult if pellets are too small to permit removal of the core, as was the case for one animal in trial 1 from which no post-treatment data could be obtained.

### *Efficacy of different antibiotics*

Of the four trials performed, trial 4 was successful in eliminating gut flora (Fig. 2.2). In trial 1, for the three animals for which post-treatment data was obtained, the antibiotic treatment and techniques used did not result in a significant reduction in the number of CFU cultured from the faeces ( $P > 0.05$ ). In trial 2, one animal died on day 2 of the trial and for the remaining three animals antibiotic treatment did not significantly reduce the number of CFU cultured from the faeces ( $P > 0.05$ ). The antibiotic screening tests showed that of the antibiotics tested, chloramphenicol and bacitracin, in all the concentrations applied, were the most effective in resisting bacterial growth on the plate. However, in trial 3, no significant reduction in faecal bacteria was observed after treating the animals with an antibiotic cocktail containing chloramphenicol and bacitracin ( $P > 0.05$ ). In trial 4 the number of CFU from post-treatment faecal samples from all four animals was significantly reduced compared to pre-treatment samples ( $t_{(2,3)} = 5.22, P < 0.05$ ). Three animals produced no culturable bacteria under anaerobic conditions after 48 h of incubation, and the fourth animal showed a greater reduction in culturable counts than that observed by any animal in any of the other trials.

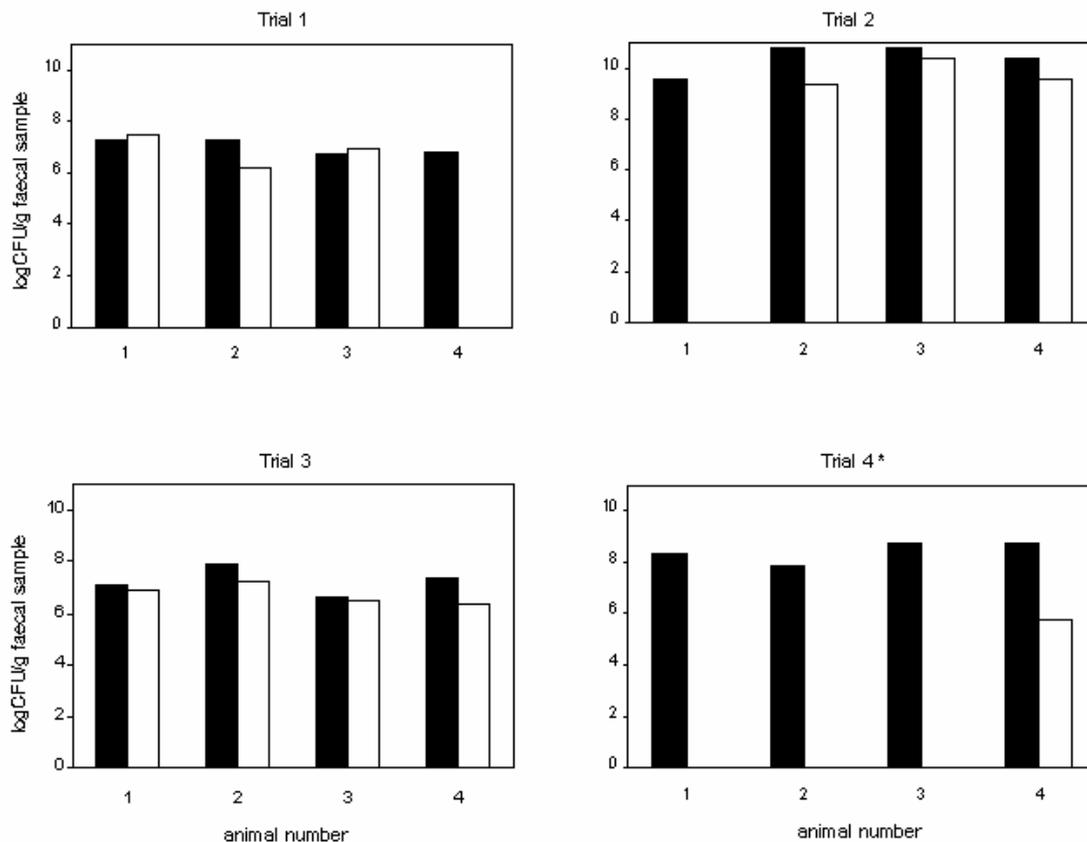


Fig 2.2. Logarithmic transformed data of the CFU/g faecal sample cultured for each animal in each trial before (■) and after (□) antibiotic treatment. Antibiotic cocktails used: Trials 1 and 2 – streptomycin sulfate and bacitracin; Trial 3 – chloramphenicol and bacitracin; Trial 4 – Baytril 10% oral solution. \* Only Trial 4 produced significantly less culturable bacteria after antibiotic treatment ( $P < 0.05$ ).

The percentage of the administered antibiotic dose consumed on a daily basis was very variable, ranging from 20-70% for the streptomycin sulfate-bacitracin cocktail in trials 1 and 2, 20 - 90% for the chloramphenicol-bacitracin cocktail in trial 3 and 15 - 80% for Baytril 10% solution in trial 4 (Table 2.1). For the most part individuals in each trial consumed fairly large and consistent amounts of the antibiotic cocktails each day. In trial 4, one animal

consumed much less of the antibiotic, and was the only animal in that trial to produce culturable bacteria after antibiotic treatment.

Table 2.1. Percentage consumption by each mouse of daily doses of antibiotic cocktail.

<b><i>Trial 1</i></b>	<b>Day 1</b>	<b>Day 2</b>	<b>Day 3</b>	<b>Day 4</b>	<b>Mean <math>\pm</math>SD</b>
#1	55	50	60	70	58.75 $\pm$ 8.54
#2	40	50	40	60	47.50 $\pm$ 9.57
#3	60	50	50	45	51.25 $\pm$ 6.29
#4	60	60	55	50	56.25 $\pm$ 4.79
<b><i>Trial 2</i></b>					
#1	50	20	-	-	35 $\pm$ 21.21
#2	60	40	45	50	48.75 $\pm$ 8.54
#3	60	40	55	30	46.25 $\pm$ 13.77
#4	50	60	45	60	53.75 $\pm$ 7.50
<b><i>Trial 3</i></b>					
#1	60	60	50	70	60 $\pm$ 8.16
#2	80	60	70	80	72.5 $\pm$ 9.57
#3	90	60	90	80	80 $\pm$ 14.14
#4	80	60	80	20	60 $\pm$ 28.28
<b><i>Trial 4</i></b>					
#1	75	55	50	60	60 $\pm$ 10.80
#2	65	70	53	50	59.5 $\pm$ 9.54
#3	50	60	50	50	52.5 $\pm$ 5.00
#4	80	30	15	25	36.25 $\pm$ 29.83

***Bacterial viability in faecal samples***

Statistical tests showed that there was no significant difference in percentage viability data obtained from two different pellets from one mouse. I therefore pooled the data from the two pellets for each mouse. My validation using the *BacLight*<sup>TM</sup> LIVE/DEAD viability probe showed that for one mouse, the mean proportion of live bacteria remaining in the faeces before antibiotic treatment, expressed as a percentage of the total bacterial cells present, was  $13.86 \pm 7.17\%$ , whereas after antibiotic this proportion was reduced to  $0.17 \pm 0.37\%$ . Corresponding values for the second mouse were  $13.37 \pm 5.0\%$  before and  $0.15 \pm 0.35\%$  after treatment. In both cases the percentage viability of bacterial cells in faecal material was significantly reduced to less than 1% of the original values ( $t_{(2,47)} = 13.22, P < 0.05$  and  $t_{(2,47)} = 18.52, P < 0.05$  respectively) after treatment with Baytril.

## Discussion

Each animal that completed a four day trial lost weight (Fig. 2.1). This weight loss can be, to some degree, attributed to the stress due to handling, confinement and the change of environment (Steyermark and Mueller 2002). The filter-topped cages are approximately half the size of the conventional ones and the inner wire cage imposes even more restriction and does not allow space for sawdust. These stresses may have caused mice to eat less of the available food. Moreover, decreased bacterial populations due to antibiotic treatment may have temporarily compromised nutrient absorption from the gut, particularly by reducing bacterial fiber breakdown. However, the fiber component (5.5% dry weight) does not represent a large fraction of the available energy of the food, hence it is unlikely that overall energy absorption efficiency was reduced enough to account for the observed weight loss. Antibiotic treatment was kept to a maximum of 4 days in order to minimise these stresses and weight loss, and to ensure that animals survived treatment. If the antibiotics offered required a longer period of administration before decontamination could be achieved, the extended time in the confines of the filter-topped cages would have been detrimental for the mice. In the broader context of this study, the purpose of gut sterilization was to facilitate further experiments on the relationship between host and microflora, taking into account the fact that the host in this case is a wild rodent unaccustomed to captivity and handling. I therefore aimed to keep treatments as short as possible.

Of the four trials performed, sawdust was included in the cages during antibiotic treatment only for trial 1. To improve sterility maintenance during treatment, sawdust was not included in trials 2, 3 and 4, and the mice were confined in an inner cage within the filter-topped cage. This facilitated daily cleaning and disinfecting of the cage without touching the mice.

Therefore, in trials 1 and 2, the same antibiotics were administered but the cage set-up was different and in trials 2, 3 and 4, different antibiotics were tested but the same cage set-up was used. Also, in trial 1 a sample of a whole faecal pellet was taken for culturing, whereas in trials 2, 3 and 4 whole pellets collected on a sterile surface were used for culturing bacteria. The latter method ensures that entire faecal pellets can be used, even if they are small and reduces the risk of contamination by reducing handling of faecal samples.

In trials 1 and 2 mice were given streptomycin sulfate and bacitracin at dosages recommended by van der Waaij and Strum (1968) for domestic mice. Streptomycin sulfate is an aminoglycoside isolated from *Streptomyces griseus*, and its antimicrobial mode of action involves interference with bacterial protein synthesis and RNA metabolism through binding to the 30S ribosomal subunit, causing misreading of the genetic code (Jacoby and Gorini 1967; Franklin and Snow 1998). Bacitracin is a polypeptide synthesised by *Bacillus licheniformis* and acts as an antimicrobial by 1) suppression of protein synthesis, 2) inhibition of cell wall synthesis and 3) interference with cell membrane function (Weinberg 1967; Franklin and Snow 1998). However, trials 1 and 2 did not reduce the numbers of microbes in the gut significantly (Fig. 2.2). Chloramphenicol is an antibiotic isolated from *Streptomyces venezuelae* and *S. phleochromogenes* var. *chloromyceticus* which inhibits protein biosynthesis by binding to the 70S ribosomes and their 50S subunits (Hahn 1967; Franklin and Snow 1998). The dose of chloramphenicol administered here was that recommended for veterinary use (Dr I. Gersbach, personal communication). Although antibiotic screening tests highlighted chloramphenicol and bacitracin as effective antibiotics against the faecal flora of *A. namaquensis*, these antibiotics were not effective when fed to the mice in trial 3. Chloramphenicol may not have been effective in the mouse digestive tract since it is an antibiotic that can be absorbed from the gut (Roy *et al.* 1983), unlike streptomycin sulfate

and bacitracin, which are non-absorbable antibiotics (van der Waaij and Strum 1968).

Antibiotic treatment of *A. namaquensis* with Baytril 10% oral solution in trial 4 produced the desired results, in that after administration of the antibiotic solution for four consecutive days, no bacteria could be cultured from post-treatment faecal samples of three of the four animals offered the antibiotic. Baytril is an antimicrobial registered for veterinary use. It contains enrofloxacin, the first member of the class of modern fluoroquinolones exclusively developed for veterinary medicine. Fluoroquinolones interfere with bacterial DNA metabolism by the inhibition of two enzymes, topoisomerase II (DNA gyrase) and topoisomerase IV (Hooper and Wolfson 1993). These two enzymes act to rejoin DNA strands during the bacterial replication process. In Gram-negative organisms DNA gyrase is the primary target, whereas in Gram-positive bacteria topoisomerase IV is the most affected by Baytril. The recommended dose of Baytril 10% oral solution for infections is 1 ml per 10 kg body mass per day for three consecutive days (Baytril 10% oral solution package insert). Since I wanted to target the indigenous gastrointestinal microflora and not just an infection, I tripled the recommended dose and administered it for four consecutive days. Although fluoroquinolones are absorbable antimicrobial agents, the rate of absorption is variable in different species (Sárközy 2001), and the rate may have been slow enough in the case of these rodents to allow for antimicrobial action to take place in the gut lumen. Consumption of between 50% and 75% of the dose given here, on each day for four days, was effective in producing sterile faeces (Fig. 2.2 and Table 2.1). Thus, consumption of 1.5 to 2.5 times the recommended veterinary dose of Baytril 10% oral solution was effective here.

It is important to note that during the trials the animals were fed the dose of antibiotics in their food and/or water. A significant reduction in the gastrointestinal microflora could only be achieved if they ate and drank sufficient food and water each day, and if the antibiotic was

effective in its antimicrobial action in their system. Observation of food and water consumption during antibiotic treatment is important in order to monitor daily intake of the antibiotics. The maximum total number of CFU previously cultured, under the conditions described here, from faecal samples of a wild population of *A. namaquensis* was  $4.9 \times 10^{11}$  CFU per gram of faecal material. Across 20 individuals the inter-individual coefficient of variation for the total number of CFU cultured from faecal samples was high (80%). This high variance would not, however, influence the statistical comparisons in my experiments, in which each animal acted as its own control and I compared pre- and post-treatment CFU to ascertain the effect of each antibiotic regimen. Although animals consumed large amounts, on average  $55.83\% \pm 11.97$ , of the daily dose of antibiotics in trials 1, 2 and 3 (Table 2.1), the total number of CFU produced from post-treatment faecal samples was very close to the total number of CFU produced before antibiotic treatment (Fig. 2.2). In trial 4, three of the animals consumed enough of the effective antibiotic each day to result in post-treatment samples from which no culturable bacteria could be produced. The one animal in trial 4 that did produce culturable bacteria post-treatment did not consume sufficient amounts of the antibiotic for it to be effective enough to eliminate faecal flora as in the other three animals, but still showed a dramatic reduction in the culturable counts (Fig. 2.2). Baytril was also offered in both food and water in trial 4, so animals were more likely to take in enough antibiotic each day.

More than 99.9% of gastrointestinal flora are anaerobes (Scheidler and Orcutt 1983). Fastidious obligate anaerobes may not have survived the non-invasive sampling methods employed here, and as a result the culturable bacteria would consist largely of aerotolerant obligate and facultative anaerobes. Thus, the pre-treatment counts are not true representations of the actual numbers of gastrointestinal microflora present in the mice. As little as a 2 h

delay in processing faecal samples can result in a reduction of 24% in the counts, compared to samples processed immediately after collection (Moore and Holdeman 1974). Also, microbial types isolated and their population levels vary to some extent depending on the microbiological methods used to culture bacteria. Here, all samples were processed by the same person using the same culture media and techniques to allow for comparable results from each trial. The only differences between trials were the techniques used to maintain sterility during treatment and the antibiotics offered. In the study presented here it is the comparison between counts obtained before and after antibiotic treatment which is important. Up to 40% of faecal volume consists of wet microbial cells shed from all the habitats they occupy along the gut (Savage 1977). Many of these bacteria are dead by the time they are excreted, having left the preferred environment of the caecum. This explains the low bacterial viability that we report in *BacLight*<sup>TM</sup> stained faecal samples from mice before antibiotic treatment. Although the faecal flora does not exactly represent the gut flora, comparison of faecal culturable counts before and after antibiotic treatment indirectly reflects the effect of the antibiotic on bacteria in the gut.

Faecal samples are a good reflection of changes in gut microflora in rats (Campbell *et al.* 1997). The zero culturable counts that we report from post-treatment faecal samples suggest that the Baytril that we used dramatically reduced gastrointestinal microflora. Two reasons suggest that these counts probably reflect true reduction of the bacterial populations within the mouse guts, rather than being an artifact created by deactivation of Baytril within mouse guts and subsequent re-activation after collection and treatment of faecal samples. First, direct fluorescent counts such as those we report above detect both viable culturable and non-culturable bacterial cells (Kepner and Pratt 1994), permitting estimation of relative proportions of dead and living bacteria in samples before culture. Had deactivation of Baytril

occurred within mouse guts, proportions of green-stained, viable bacteria would have been higher in the treated mice. Second, a similar fluoroquinolone antibiotic, norfloxacin, shows inactivation (it is up to 75% bound to faeces in pigs; van der Waaij *et al.* 1989) that is slow to reverse: after 2 hours of re-suspension in buffer at room temperature, less than 15% of bound antibiotic had dissociated from human faecal material (Edlund *et al.* 1988). I processed faecal samples for both fluorescence staining and culturing within 2 hours of collection, before antibiotic re-activation to the degree necessary to explain the low culturable counts I report with Baytril, would have occurred.

It is evident that there is great variety in the composition of gastrointestinal microflora among animals, as well as varying degrees of sensitivity among these microbes to the variety of antibiotics that can be used to inhibit their growth. Previous studies involving temporary gut sterilization of rodent digestive tracts have been carried out on laboratory rats (Adachi *et al.* 1995; Guo *et al.* 1995; Enomoto *et al.* 1999; Shimizu *et al.* 2001) and mice (Huycke *et al.* 1995; Wells *et al.* 1990; Wiesner *et al.* 2001). The study presented here was carried out on wild rodents. Without the advantage of a laminar flow cabinet, sterility was successfully maintained by disinfecting cages with chlorhexidine daily and keeping individual filter-topped cages within a plastic-sheet enclosed compartment. These techniques, together with the administration of an increased dose of Baytril 10% oral solution in food and drinking water successfully reduced the numbers of gastrointestinal microflora in *A. namaquensis* individuals to reflect dramatically reduced growth from faecal samples. These findings will be implemented in future studies of the intestinal microflora-host relationship in Namaqua rock mice (Chapter 3), investigating the possibility of bacterial involvement in metabolism of the nectar sugar xylose.

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## Chapter 3

### **Nectar xylose metabolism in a rodent pollinator (*Aethomys namaquensis*): defining the role of gastrointestinal microflora using <sup>14</sup>C-labelled xylose**

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## Abstract

The Namaqua rock mouse *Aethomys namaquensis*, a rodent pollinator of certain geoflorous *Protea* species, consumes nectar containing xylose. Xylose is not known to be efficiently utilized by mammals. However, it is fermented by certain bacteria, yeasts and fungi, particularly gastrointestinal bacteria. The end products of microbial fermentation are utilized by the host in oxidative metabolism. Here I investigate the degree to which intestinal bacteria of *A. namaquensis* contribute to xylose metabolism. Mice were caught during *Protea humiflora* flowering and non-flowering seasons and given an oral dose of  $^{14}\text{C}$ -labelled xylose. Exhaled  $\text{CO}_2$  and excreted urine and faeces were continuously collected for 30 hours thereafter and label recovery determined. Each mouse was then treated with antibiotics to reduce gut microflora, and the experiment repeated. With their natural gut flora population intact, mice caught during the flowering season exhaled significantly more  $^{14}\text{CO}_2$  than did mice caught during the non-flowering season. Also, during both seasons, mice exhaled significantly more  $^{14}\text{CO}_2$  before antibiotic treatment than after. Antibiotic treatment caused a significant increase in the proportion of  $^{14}\text{C}$ -labelled xylose that was excreted in the urine. The mouse diet likely influences the composition of the gastrointestinal community. *A. namaquensis* relies on its gut microflora to ferment xylose, thereby converting it into end products that are used by the mice for metabolism.

Key words:  $^{14}\text{C}$ -xylose;  $^{14}\text{CO}_2$ ; Gastrointestinal bacteria; Antibiotics; Xylose metabolism; Rodents; *Aethomys namaquensis*; *Protea humiflora*; Microbial fermentation

## Introduction

Floral nectars contain three major sugars: the disaccharide sucrose, and its component hexoses, fructose and glucose, with other minor sugars present in trace amounts (Baker and Baker 1982). However, analysis of nectar sugars of the Proteaceae has highlighted the presence of the pentose sugar xylose (van Wyk and Nicolson 1995; Nicolson and van Wyk 1998). Before these studies, xylose had not been reported in nectar except in the extrafloral nectar of grasses (Bowden 1970). Subsequently, xylose was reported in the nectar of the African lily, *Massonia depressa* (Johnson *et al.* 2001). In two South African genera of Proteaceae, *Protea* and *Faurea*, xylose comprises up to 39% of the total nectar sugars (Nicolson and van Wyk 1998). This finding prompted studies of sugar preference and sugar absorption efficiencies among insect, bird and rodent pollinators of the Proteaceae. Insect and bird pollinators are strongly averse to xylose (Lotz and Nicolson 1996; Allsopp *et al.* 1998; Franke *et al.* 1998; Jackson *et al.* 1998a; 1998b), and show very poor intestinal absorption of this sugar (Allsopp *et al.* 1998; Franke *et al.* 1998; Jackson *et al.* 1998b). In contrast, a rodent pollinator of the Proteaceae, the Namaqua rock mouse *Aethomys namaquensis* (A. Smith 1834), consumed pure xylose solutions, showed a very high (97%) apparent absorption efficiency for xylose, and exhibited extremely low concentrations of this sugar in blood, urine and feces after a xylose feeding trial (Johnson *et al.* 1999). This suggested that the sugar was not merely absorbed, but was being utilized. Moreover, there is a higher mean percentage of xylose in the nectar produced by rodent-pollinated *Protea* species (mean = 7.1% of total nectar sugars; n = 10 species) than in that of bird-pollinated species (mean = 2.8%; n = 10 species) (Nicolson and van Wyk 1998), suggesting that xylose is of some importance in the diet of rodent pollinators such as *A. namaquensis*.

Xylose has nutritional value only if it can be absorbed and utilized. Mammals are well equipped to absorb simple sugars like glucose and galactose, they can hydrolyse the disaccharides sucrose, lactose and maltose to their constituent monomers, and they can degrade starch to glucose monomers, but are limited in their capacity to hydrolyse and utilize other polysaccharides, such as cellulose and hemicellulose (Ferraris 2001). Intestinal absorption of xylose has been studied in a number of animals such as hamsters, rats, frogs, rabbits and cows (Salem *et al.* 1965; Alvarado 1966; Lassen and Csàky 1966; Heyman *et al.* 1980; Miyamoto *et al.* 1993; Scharrer and Grenacher 2000). In these animals xylose transport across the gut epithelium is active, Na-dependent, inhibited by phlorizin, and occurs by means of a mechanism similar to that of active hexose transport. However, xylose is not known to be efficiently utilized by mammals. Although cultured cell lines from hamster ovaries and human fibroblasts are able to survive with xylose as their sole energy source (Demetrakopoulos and Amos 1976; 1978), cells from metabolically active mammalian tissues, such as liver, muscle and kidney, are very poor xylose metabolizers (Blatherwick *et al.* 1936; Krebs and Lund 1966; Metzger *et al.* 1972).

Xylose metabolism by bacteria, yeasts and fungi, however, is well-documented. D-xylose is a monomer of xylan, which is the hemicellulose component of plant cell walls. Hemicellulose is the second most abundant polysaccharide in plant cell walls and comprises up to 35% of plant material (Gong *et al.* 1981). Vertebrate utilization of plant polysaccharides can only be accomplished through the initial catabolism of the polysaccharides by gastrointestinal microflora (Stevens and Hume 1995). The degradation of xylan and the microbial utilization of xylose have been well studied in ruminants (eg. Turner and Robertson 1979; Hespell *et al.* 1987; Matte *et al.* 1992; Thomson 1993; 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; 1998).

The possible utilization of xylose by intestinal bacteria of *A. namaquensis* was previously suggested in laboratory experiments (Johnson *et al.* 1999). I wished to investigate the degree to which intestinal bacteria contribute to xylose metabolism in this rodent pollinator.

Recovery of the radio-isotopic label after oral dosing with  $^{14}\text{C}$ -labelled xylose was compared in mice with their natural wild gut flora and then with reduced gut flora after antibiotic treatment (Johnson *et al.* 2004, Chapter 2). Since xylose-containing nectar is only available in winter, I also compared xylose utilization in mice trapped during and after the flowering season of *Protea humiflora*.

## Materials and Methods

### *Animals*

*Aethomys namaquensis* individuals were caught under permit (numbers 180/2003 and 001-201-0002, Western Cape Nature Conservation) at Wolfieskop, near Villiersdorp in the Riviersonderend Mountains, South Africa. This site was chosen because of the presence of dense populations of *Protea humiflora*, a ground-flowering protea which is pollinated by *A. namaquensis* (Fleming and Nicolson, 2002) and in which xylose comprises 5-9% of total nectar sugars (Nicolson and van Wyk 1998). *Protea humiflora* flowers during July – September (Rebello 2001). Animals were caught during the night in Sherman live traps baited with oats and peanut butter, and taken to the laboratory where they were housed individually in conventional rodent cages (44 cm L x 28 cm B x 23 cm H) at a constant temperature of 22°C with *ad libitum* access to water and food pellets (Prestige Products, Crispy Rat Muesli: 18% protein, 3% oil, 5.5% fibre and 7.5% ash, with a maximum of 88% total digestible nutrients). Animals remained in these on the night following their capture, and <sup>14</sup>C-xylose experiments commenced at 06:00 the following morning. Three or four individuals were caught and used as experimental animals during each month of August, October, November and December 2003 and February, August and September 2004. Ethical approval for the study was obtained from Sub-Committee B of the Research Committee of the University of Stellenbosch.

### *<sup>14</sup>C-xylose experiment*

#### *Administration of the label and sample collection*

Before each <sup>14</sup>C-xylose experiment, two or three experimental mice and a single control mouse were each weighed. At time zero (06:00) experimental animals were given an oral dose of 0.5 µCi of <sup>14</sup>C-xylose (Amersham Life Science). Labelled xylose (2.5 µl of an

aqueous solution of  $^{14}\text{C}$ -xylose,  $0.2 \mu\text{Ci} / \mu\text{l}$ ) was drawn up into a micropipette along with 0.25 ml distilled water, and the mixture carefully introduced directly into each mouse's mouth with a micropipette, while the mouse was restrained in the hand. Control mice were given the same volume of distilled water with no label, in exactly the same way. Each animal was then placed individually into a respiration chamber for 30 h for collection of exhaled  $\text{CO}_2$  and the separation and collection of urine and faeces (Figure 3.1). This pre-determined collection time was based on a trial experiment in which mice ( $n = 3$ ) were given an oral dose of  $0.5 \mu\text{Ci}$  of  $^{14}\text{C}$ - xylose, and exhaled  $\text{CO}_2$  was collected until  $^{14}\text{C}$  counts returned to background levels. The respiration chamber consisted of an airtight glass chamber (12 cm diameter x 25 cm length) with air inlet and outlet tubes. Airflow through the chamber was regulated using a Sable Systems TR-FC1 mass flow controller (Henderson, Nevada) connected to a Series 830 Side-Trak mass flow meter (Sierra Instruments Inc., Monterey, California). Before entering the chamber at 1L/min, room air was pumped (RE-9500 Regent air pump) through two containers of soda lime to remove incoming  $\text{CO}_2$ . Air exiting the chamber bubbled through 80 ml of 1.0 M sodium hydroxide (NaOH) contained in a glass cylinder (2.5 cm diameter x 40 cm length), in order to trap the expired  $\text{CO}_2$  in the form of a sodium hydrogen carbonate solution ( $\text{NaHCO}_3$ ). The trapping solution was replaced at 1-hour intervals during the 30-hour collection period. The effectiveness of the soda lime in removing incoming  $\text{CO}_2$  was tested using a Li 6262  $\text{CO}_2/\text{H}_2\text{O}$  infrared gas analyser (LiCor, Lincoln, Nebraska, USA). The same gas analyzer was also used to test the system before the experiments, using non-radioactive mice, to ensure that no exhaled  $\text{CO}_2$  escaped into the room air.

Inside the chamber the animal rested on a double wire mesh floor above a funnel-shaped base which allowed for the collection of all faeces and urine excreted during the experiment. Urine

was collected under liquid paraffin to prevent evaporation. At the end of the 30-hour collection period (12:00 the following day), the mouse was removed from the chamber and the chamber was washed twice with 50 ml distilled water to collect any label dissolved in urine or water adhering to the surface of the glass, where it came in contact with urine or where condensation occurred. These water samples are hereafter referred to as “cage-washings”. The mice were weighed and returned to the conventional rodent cages, where they were given food pellets and water *ad libitum*.

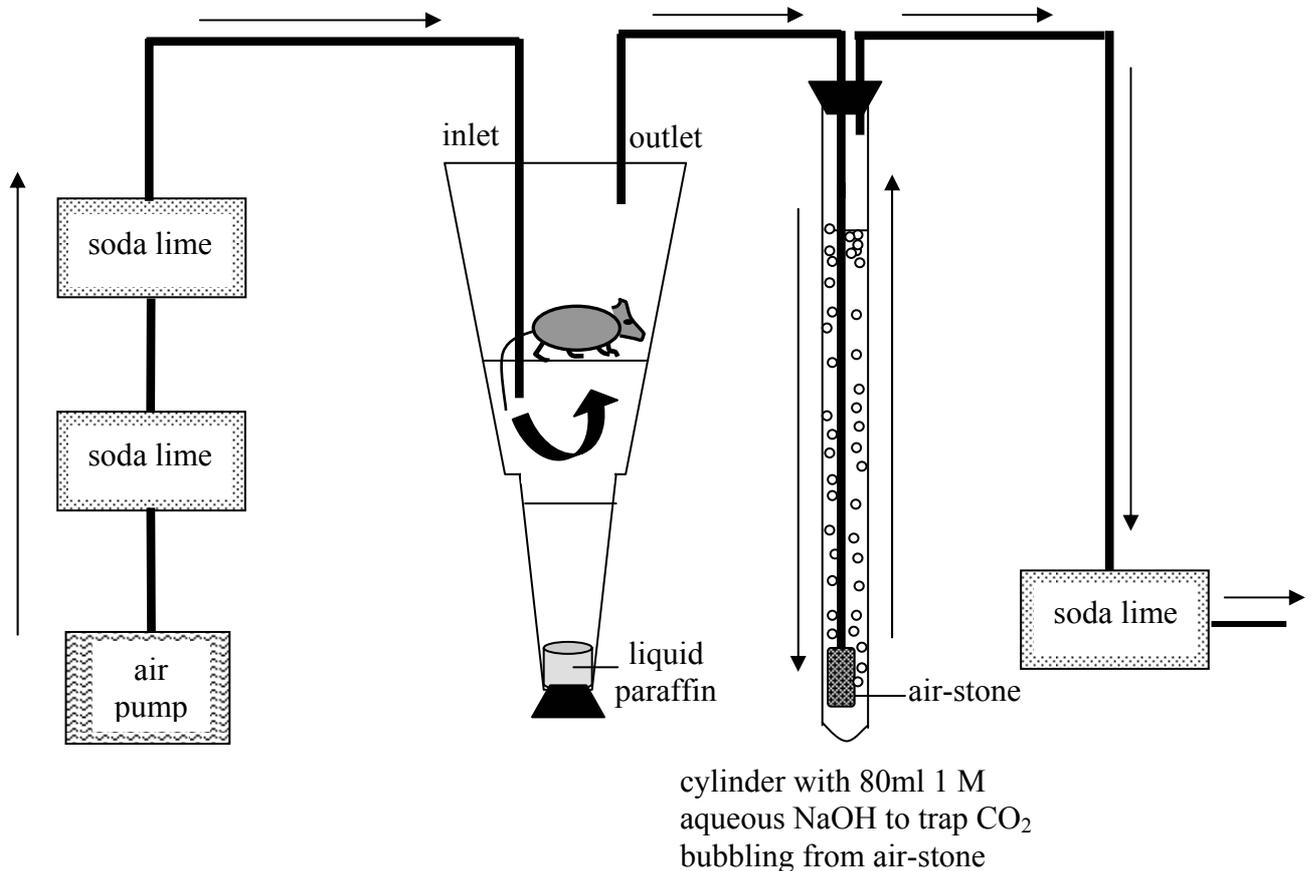


Figure 3.1. Closed system for the collection of expired CO<sub>2</sub>, urine and faeces. Solid black line represents the air line, and arrows indicate the direction of air flow through the system.

I controlled for interindividual variation in the responses of natural gut microflora populations to antibiotics (Johnson *et al.* 2004, Chapter 2) by using a repeated-measures design to compare recovery of the  $^{14}\text{C}$  label in the same mouse before and after antibiotic treatment. After 1 week of rest in conventional rodent cages, mice were weighed again and placed individually into sterilized filter-topped cages (33 cm L x 15 cm B x 13 cm H) (Tecniplast, Labotec (Pty) Ltd, South Africa). Within these cages, mice were contained in an inner cage and rested on a wire floor raised 2 cm to facilitate the removal of urine and faeces. These cages were placed on a heating pad inside a laminar flow cabinet. To eliminate bacteria, an antibiotic treatment developed by Johnson *et al.* (2004, Chapter 2) was used. Mice were fed Baytril 10% oral antibiotic solution, a veterinary antimicrobial agent containing enrofloxacin (Bayer (Pty) Ltd.), at a dose of  $0.3\mu\text{l/g}$  body weight/day in sterilized food and water for 4 days. Fresh sterilized food and water were given each day, and the outer cages cleaned and disinfected daily with 0.5% chlorhexidine in 70% alcohol. After 4 days on the antibiotic treatment, mice were returned to the respiration chambers for a repeat of the  $^{14}\text{C}$ -xylose experiment described above. On completion of experiments, mice were released at the site of capture.

#### *Recovery of the label*

$^{14}\text{C}$  recovery was estimated by summing the radioactivity of exhaled  $\text{CO}_2$  samples in the NaOH trap and in cage-washings, faecal and urine samples. Exhaled  $\text{CO}_2$  and cage-washing samples were analysed immediately after each experiment. Faecal and urine samples were stored at  $-20\text{ }^\circ\text{C}$  until analysis. Aliquots of exhaled  $\text{CO}_2$ , cage washing and faecal samples were counted using a liquid scintillation counter (LS 5000 TD, Beckman Instruments, Fullerton, California, USA). Urine samples were analyzed on a high performance liquid chromatograph (HPLC) (10AVP, Shimadzu, Kyoto, Japan) fitted with a radio-

chromatography detector (Radiomatic, Meriden, Connecticut, USA) to count the label and to ascertain whether any label present in the urine was still in its original form of  $^{14}\text{C}$ -labelled xylose. Labelled xylose was identified using  $0.0045\ \mu\text{Ci}\ ^{14}\text{C}$ -xylose as a standard. Any label that was not xylose was further analysed by liquid chromatography-mass spectrometry (LCMS) to identify the labelled compound.

To determine the radioactivity of the  $\text{CO}_2$  samples, 2 ml of each one-hour collection of exhaled  $\text{CO}_2$  trapped in NaOH was mixed with 14 ml Hionic Fluor (Perkin Elmer, Boston, Massachusetts, USA) for liquid scintillation counting. One ml of each 50 ml cage-washing was mixed with 10 ml Ultima Gold (Packard, Meriden, Connecticut, USA) for scintillation counting. The counting protocol programmed into the scintillation counter for exhaled  $\text{CO}_2$  and cage-washing samples counted radioactivity in the full  $^{14}\text{C}$  window (0 to 154 KeV) to ensure the highest counting efficiency.

Faecal pellets were solubilized by heating samples at  $80^\circ\text{C}$  for 1 hour with 0.5 ml 12 % sodium hypochlorite. When the solution turned clear, samples were cooled to room temperature and air in the sample vials was flushed out by pumping room air into the vials. 15 ml Hionic Fluor was added to solutions for counting. Solubilization of faecal pellets with sodium hypochlorite resulted in chemiluminescence quenching of faecal samples. To correct for this quenching, a different counting protocol to that used for  $\text{CO}_2$  and cage-washing samples was programmed into the counter, in which samples were counted in the  $^{14}\text{C}$  above  $^3\text{H}$  window (18 to 154 KeV).

Urine samples were centrifuged for 15 min at 10500 g. The supernatant underneath the paraffin layer was removed and 750  $\mu\text{l}$  was loaded on a tC18 cartridge (Sep-Pak Waters,

Milford, Massachusetts, USA). The eluted sample was concentrated 4 times in a Speed Vac, and filtered through a 0.45 µm filter (Millex-HV Millipore, Billerica, Massachusetts, USA). 10 µl of the filtrate was injected into the HPLC column. The mobile phase was 60 mM NaOH (2 ml/min) at 30 °C. The column used was a Hamilton RCX 10 with guard column. The label was detected and counted with a radio-chromatography detector using Ultima Flo M scintillation fluid (Packard, Meriden, Connecticut, USA).

One urine sample was randomly chosen for further analysis by LCMS to identify a labelled compound revealed by the HPLC analyses that was known not to be xylose. A Waters API QTOF Ultima (Waters Corp., Massachusetts, USA) quadrupole-time of flight mass spectrometer (TOF MS) with electrospray ionization was used. The capillary voltage was set to -2.7 kV and the cone voltage was set to 40 V. The desolvation temperature was 350°C and the source temperature was 120°C. Desolvation gas was supplied at 400 L/h and cone gas at 50 L/h. The TOF MS was calibrated using clusters of sodium iodide. The quadrupole was set to transfer all ions and the TOF MS monitored ions from 100 to 2000 m/z. Chromatographic separation was achieved with a 30 mm x 7.8 mm Supelcogel Pb HPLC column. The column was kept at 80°C in a Shimadzu CTO-10ASvp column heater (Shimadzu, Kyoto, Japan). An Agilent 1100 HPLC (Agilent Technologies, Paolo Alto, California, USA) was used to supply 18 mΩ water at 0.5 ml/min.

### ***Statistics***

Recovery of the <sup>14</sup>C label, expressed as percentages for exhaled CO<sub>2</sub> and urine, proved to be normally distributed (Shapiro-Wilk's test:  $W = 0.797$  to  $0.968$ , in all cases  $P > 0.05$ ). I therefore used a *t*-test for independent samples to compare percentage label recovery as <sup>14</sup>CO<sub>2</sub> in mice with their natural gut flora during the flowering season and non-flowering

seasons. Paired *t*-tests were used to compare label recovery in exhaled CO<sub>2</sub> and urine in mice before and after antibiotic treatment. Data for percentage label recovery in faeces and cage-washings was not normally distributed (Shapiro-Wilk's test:  $W = 0.491$  to  $0.971$ , in all cases  $P < 0.05$ ). I therefore used the non-parametric Wilcoxon's matched pairs test to compare percentage label recovery in faeces and cage-washings for mice before and after antibiotic treatment. All data are expressed as means  $\pm$  SD.

## Results

### *Total recovery of <sup>14</sup>C label*

For all 32 repetitions of the experiment, the total recovery of <sup>14</sup>C label expressed as a percentage of the oral dose, ranged from 58.4 to 89.6% (mean = 78.2% ± 11.6 for data from mice before antibiotic treatment and mean = 71.9% ± 12.3 after antibiotic treatment) (Table 3.1). Counting efficiencies of exhaled CO<sub>2</sub>, cage-washings and urine samples were higher than 90%, while the quench-corrected counting protocol resulted in lower counting efficiencies (73.3%) for fecal samples (Table 3.1).

Table 3.1: Long-term label recovery: mean percentages of total <sup>14</sup>C dose recovered in 30 h from mice with their natural gut flora and then after antibiotic treatment.

Microbial state of gastrointestinal tract	Exhaled CO <sub>2</sub> (CE=92.8%)	Cage washings (CE=95.3%)	Urine (CE=93.7%)	Feces (CE=73.3%)	Total
Natural	41.5 ± 11.8	0.6 ± 0.4	34.2 ± 13.4	1.8 ± 0.7	78.2 ± 11.6
Antibiotic-treated	23.9 ± 7.7	1.7 ± 1.3	46.1 ± 12.5	0.1 ± 0.2	71.9 ± 12.3

Note. *N* = 16. Values are expressed as the mean percentage of the 0.5 μCi dose ± SD.

CE = mean counting efficiencies for each type of sample.

### *Recovery as <sup>14</sup>CO<sub>2</sub> in mice with natural gut flora in flowering vs non-flowering season*

Consistently, during the full 30-hour collection period of each experiment, the amount of label exhaled as <sup>14</sup>CO<sub>2</sub> peaked during the first 7 h of collection, followed by a much slower release during the remaining 23 h (Figure 3.2). Consequently, I calculated the percentage of

the dose exhaled as  $^{14}\text{CO}_2$  during those first 7 h as a fraction of the total recovery of  $^{14}\text{CO}_2$  for the full 30 h, as a measure of the animals' ability to metabolize the  $^{14}\text{C}$ -xylose with the natural population of gut microflora. This calculation is hereafter referred to as "short-term label recovery". These values were determined for mice with their natural gut flora during the flowering season ( $n = 8$  individuals) as well as during the non-flowering season ( $n = 8$ ). Mice caught during the flowering season exhaled a significantly higher percentage of labelled  $\text{CO}_2$  during the first 7 h of collection ( $78.8\% \pm 6.3$ ) than did mice caught during the non-flowering season ( $47.4\% \pm 13.4$ ) ( $t_{14} = 5.99$ ,  $P < 0.05$ ).

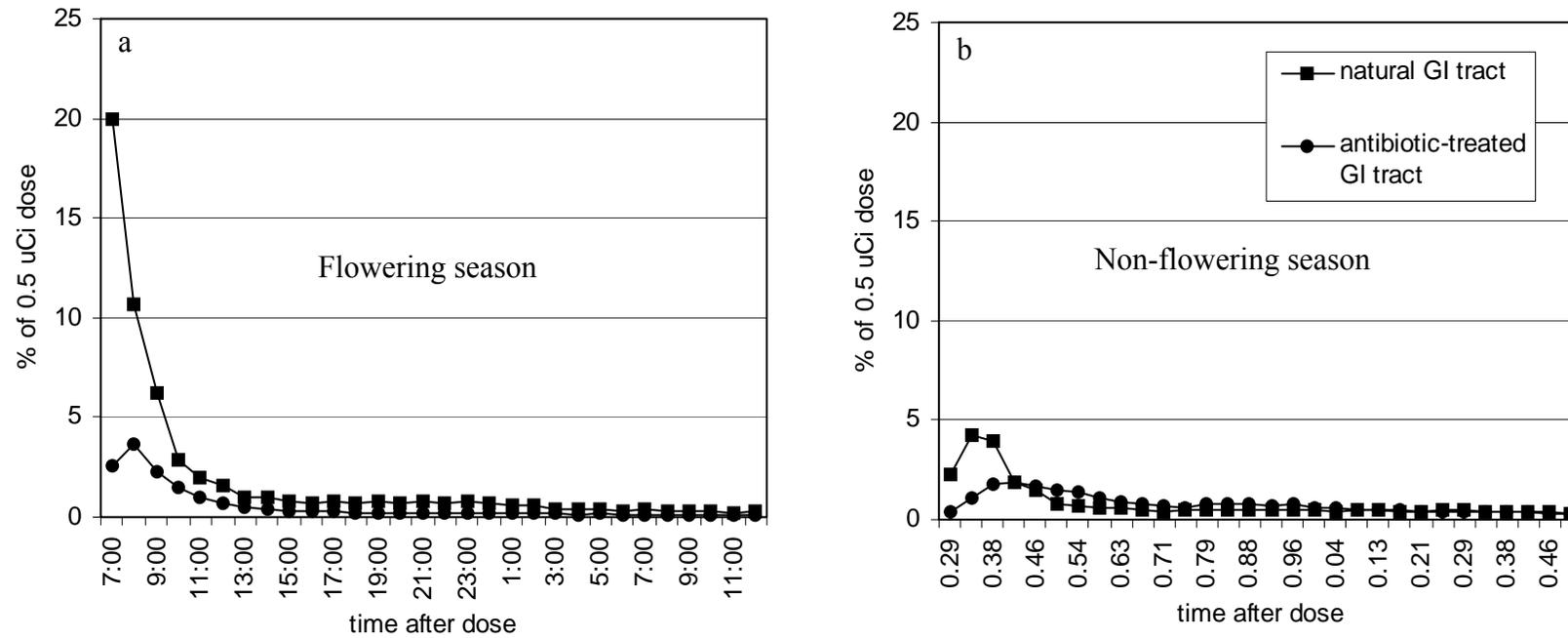


Figure 3.2. a) Exhaled  $^{14}\text{CO}_2$ , expressed as a percentage of the  $^{14}\text{C}$ -xylose dose exhaled per hour after oral administration to a mouse captured during the flowering season, with the gastrointestinal (GI) tract in the natural state and after antibiotic treatment. b) The same information obtained from a mouse captured during the non-flowering season.

***Recovery of the label in mice before and after antibiotic treatment***

As another measure of the animals' ability to metabolize  $^{14}\text{C}$ -xylose and to compare the recovery of the label in mice before and after antibiotic treatment, I calculated the percentage of the dose exhaled as  $^{14}\text{CO}_2$  and recovered in the NaOH trap, and excreted in urine and faeces during the full 30-hour collection period. These calculations are hereafter referred to as "long-term label recovery". Due to the observed difference in label recovery between mice with their natural gut flora during flowering (July - September) and non-flowering seasons (October - June), I separated the data for the two seasons. For both the flowering and non-flowering seasons, significantly higher fractions of  $^{14}\text{C}$  were exhaled as  $^{14}\text{CO}_2$  when mice had their natural population of gut microflora before antibiotic treatment (Table 3.2; Flowering season  $t_7 = 3.31$ ,  $P < 0.05$ ; Non-flowering season  $t_7 = 4.46$ ,  $P < 0.05$ ). After antibiotic treatment the result was reversed, and significantly more label was excreted in the urine (Table 3.2; Flowering season  $t_7 = -3.08$ ,  $P < 0.05$ ; Non-flowering season  $t_7 = -2.78$ ,  $P < 0.05$ ).

HPLC analysis of urine samples showed that of the total radioactivity present in urine, on average 82% was in the form of  $^{14}\text{C}$ -xylose. An unknown labelled compound, which was consistently present in all the samples, constituted an average of 12% of the total radioactivity in urine. LCMS analysis of one urine sample suggested that the unknown labelled compound was xylulose-5-phosphate, produced by phosphorylation of xylulose by xylulokinase during the second step in bacterial catabolism of xylose (Figure 3.3).

Less than 3.5% of the original label dose was recovered from faeces (Table 3.2). For both flowering and non-flowering seasons, significantly more label was recovered in faecal samples before antibiotic treatment (Table 3.2; Flowering season  $Z = 2.521$ ,  $P < 0.05$ ; Non-flowering season  $Z = 2.571$ ,  $P < 0.05$ ). Faecal samples could not be counted and analysed by

HPLC to ascertain whether the label in faeces was still in the form of  $^{14}\text{C}$ -labelled xylose, since the radioactivity of the samples, as determined by liquid scintillation counting, was far below the minimum required for detection by the radio-chromatograph fitted to the HPLC ( $0.0027\mu\text{Ci} / 10\mu\text{l}$  sample).

Table 3.2: Long-term label recovery: mean percentages of total  $^{14}\text{C}$  dose exhaled as  $^{14}\text{CO}_2$  and excreted in urine and faeces over 30 h from mice caught during the flowering and non-flowering seasons, with natural gut flora and then after antibiotic treatment.

Label		Natural bacterial state	Antibiotic-treated state
Exhaled $^{14}\text{CO}_2$	FS	$41.6 \pm 12.8$	$24.5 \pm 10.7$
	NFS	$41.5 \pm 11.7$	$23.4 \pm 3.6$
Urine	FS	$29.3 \pm 11.4$	$42.0 \pm 14.6$
	NFS	$39.1 \pm 14.3$	$50.1 \pm 9.2$
Faeces	FS	$2.1 \pm 0.8$	$0.2 \pm 0.3$
	NFS	$1.6 \pm 0.5$	$0.1 \pm 0.2$

Note.  $N = 8$  for both groups. Values are expressed as the mean percentage of the  $0.5 \mu\text{Ci}$  dose  $\pm$  SD. FS = flowering season; NFS = non-flowering season

Significantly more label was recovered in cage-washings after antibiotic treatment (Table 3.1;  $Z = 3.516$ ,  $P < 0.05$ ). This is probably due to the fact that urine contained more label after antibiotic treatment. When urine ran down the inside of the chamber and was collected under liquid paraffin the residue left on the chamber wall was washed out and was incorporated into the cage-washing counts.

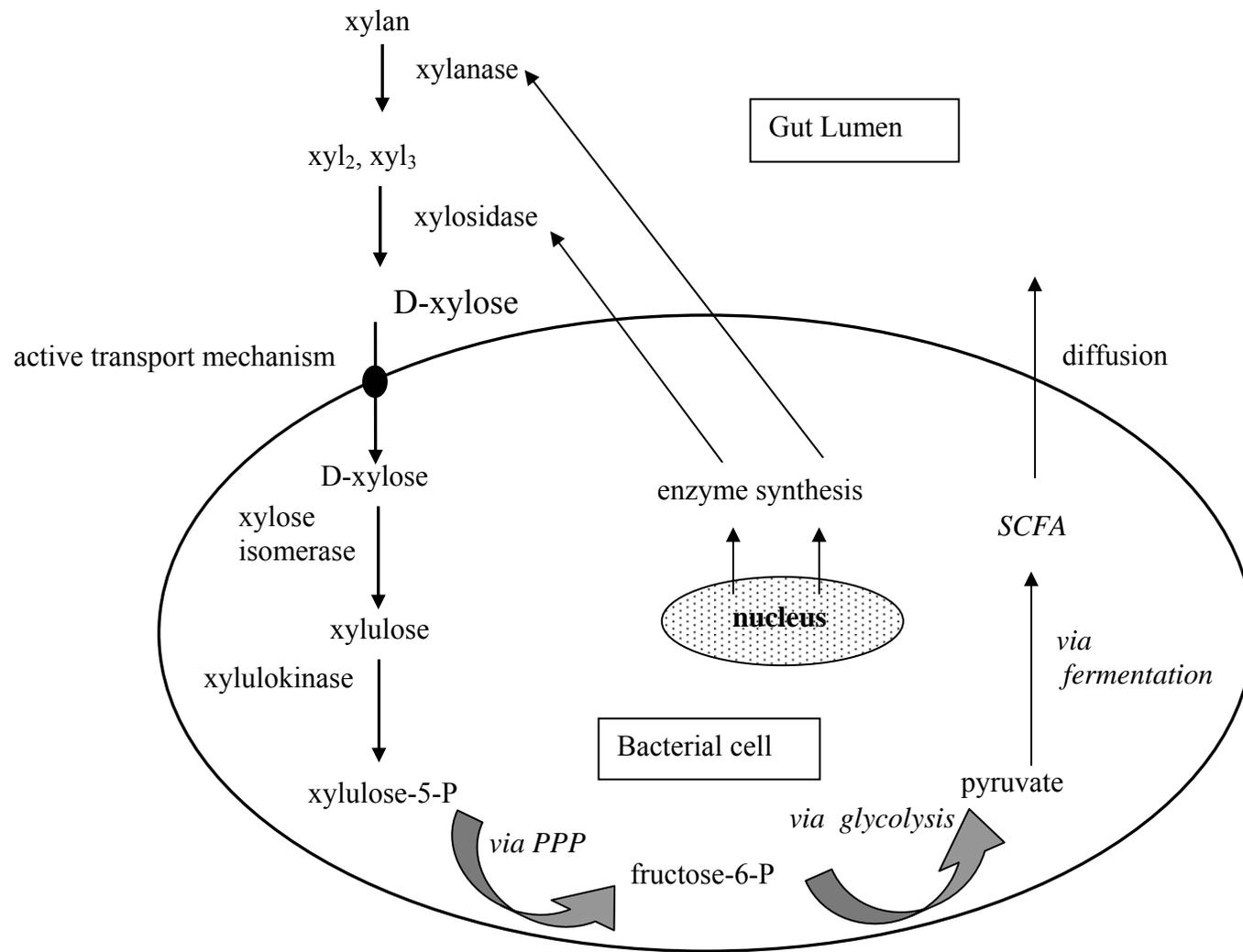


Figure 3.3. The pathway of xylan degradation and xylose utilization by a bacterial cell in the lumen of the vertebrate gastrointestinal tract.

## Discussion

### *Seasonal changes in xylose metabolism among wild-caught mice*

It is clear from the results that intestinal bacteria of *A. namaquensis* are largely responsible for the catabolism of xylose in the mouse diet. Apart from the results of the experiments in which the gastrointestinal tract is exposed to antibiotic treatment in the laboratory to reduce indigenous gut microflora, the first piece of evidence for the importance of the intestinal bacteria came from mice with their indigenous gut microflora undisturbed, but caught at different times of the year. The significant difference between total fractions of xylose metabolized by mice caught during the flowering season and those caught during the non-flowering season suggests that the indigenous population of xylose-metabolizing bacteria in the mouse gut is an inducible population, with higher numbers of those bacteria present when xylose is available in the diet in the form of *Protea humiflora* nectar. During the flowering season significantly more of the  $^{14}\text{C}$  label was exhaled as  $^{14}\text{CO}_2$  than during the non-flowering season, particularly during short-term label recovery, the first 7 hours of the collection period (Figure 3.2).

In the laboratory, substrate utilization studies are performed over different time periods, and if the bacteria under investigation are efficient users of the substrate, initial utilization occurs rapidly during the first few hours of incubation. Xylose utilization by different ruminal strains of *Butyrivibrio fibrisolvens*, a ubiquitous bacterial species in the gastrointestinal tract of mammals (Brown and Moore 1960), occurred most rapidly during the first 2-9 hours of incubation (Marounek and Kopečný 1994). Although in the present study substrate utilization is measured at the whole animal level, rather than by particular bacterial species inoculated into a growth medium containing the substrate, a similar pattern of utilization of the substrate by the bacterial community in the intestine is evident here.

The gastrointestinal tract constitutes an ecosystem of microbial habitats and communities. Its functioning is influenced by numerous factors: the external environment and exposure of individuals to microbes able to establish themselves in the gut; how microbial communities themselves influence each other with respect to competition for nutrients or the production of end products that create an environment unsuitable for certain microbes to grow and survive; and the diet consumed by the host. Standard microbiological cultivation-based techniques, as well as the more recent advanced molecular techniques used in studies of the associations between diet and intestinal microflora composition, consistently indicate that diet directly influences microflora composition. The effect of diet on the mammalian gut flora was reviewed by Rowland *et al.* (1985). Humans consume specific foods and supplements that are known to increase the proportions of beneficial intestinal bacteria such as bifidobacteria and lactic acid bacteria (Blaut 2002). Diet-dependent changes in intestinal bacterial communities have been shown in chickens, mice, pigs and cows (Leser *et al.* 2000; McCracken *et al.* 2001; Tajima *et al.* 2001; Knarreborg *et al.* 2002; Mai *et al.* 2003). In the wild, seasonal changes in the quantity of xylose in the mouse diet, prompted by the availability of *P. humiflora* nectar, apparently induce changes in the intestinal bacterial community that facilitate metabolism of a greater proportion of diet xylose.

#### ***Antibiotic treatment confirms importance of bacteria***

Oral administration of the antibiotic Baytril to *A. namaquensis* also clearly demonstrated the importance of intestinal bacteria in xylose metabolism in these rodent pollinators. With respect to long-term label recovery, during both the flowering and non-flowering seasons, significantly more of the labelled xylose was converted to  $^{14}\text{CO}_2$  when mice had their gastrointestinal tract undisturbed, than when the same individuals had been treated with antibiotics (Table 3.2). After antibiotic treatment, the intestinal bacterial community was

reduced but not completely abolished (Johnson *et al.* 2004, Chapter 2). Mice with reduced gut flora were less capable of metabolizing the  $^{14}\text{C}$ -xylose, and significantly more labelled xylose was excreted in the urine.

Ruminant and non-ruminant vertebrates that eat diets high in fibrous plant material lack the enzymes necessary for the breakdown of polysaccharides from components of plant cell walls (eg. cellulose, xylan and pectin), and much dietary carbohydrate that passes through the mammalian gastrointestinal tract would be of no nutritional benefit to the animal without the ability of the intestinal microflora to degrade these biomolecules. Studies of an anaerobic bacterial species, *Bacteroides thetaiotaomicron*, prominent in the normal human and mouse gut, has led to much of our understanding of the mechanisms used by intestinal bacteria to degrade complex polysaccharides (Hooper *et al.* 2002). The degradation of xylan and the utilization of its component monomer (D-xylose) by ruminal bacteria have been extensively studied (eg. Turner and Robertson 1979; Hespell *et al.* 1987; Matte *et al.* 1992; Thomson 1993; Marounek and Kopečný 1994). The pathways of xylan degradation and xylose utilization by a bacterial cell are shown in Figure 3.3. Xylanase is secreted by the bacterial cell into the gut lumen, where it fragments the xylan polymer into xylo-oligosaccharides (eg.  $\text{xyl}_2$ ,  $\text{xyl}_3$ ). These oligosaccharides are cleaved into D-xylose monomers by xylosidase, and D-xylose is then actively transported into the bacterial cell. Inside the cell, xylose isomerase catalyses the conversion of xylose to xylulose, which is converted to xylulose-5-phosphate by xylulokinase. Xylulose-5-phosphate is converted to fructose-6-phosphate via the pentose phosphate pathway (PPP) and then to pyruvate via glycolysis. There are a number of possible routes that the resulting pyruvate molecule can follow, depending on the microbial species and the environment in which it exists (Aristidou and Penttilä 2000). In the anaerobic environment of the vertebrate gut, bacterial cells extract more carbon and energy from

pyruvate by microbial fermentation. The predominant end products of this process of fermentation in the gut are short chain fatty acids (SCFA), which diffuse out of the bacterial cell into the gut lumen, from whence they are absorbed and used in oxidative metabolism by the host animal.

Other by-products of microbial fermentation in the gut are CO<sub>2</sub>, H<sub>2</sub>, and CH<sub>4</sub> (in the case of methanogenic bacteria). In studies such as my own using small vertebrates confined in whole-animal metabolism chambers, the radio-labelled CO<sub>2</sub> recovered from the chambers has three components: firstly, CO<sub>2</sub> generated by the animals during their own oxidative metabolism of the SCFA produced by its gut microflora; secondly, CO<sub>2</sub> produced by gut bacteria during fermentation, absorbed from the gut lumen into the bloodstream, and exhaled by the animals together with the CO<sub>2</sub> generated by their own metabolism; and thirdly, CO<sub>2</sub> also produced by bacterial fermentation but directly released by flatulence rather than via the blood and then lungs of the vertebrate (Stevens and Hume 1995). Of these three components, the greatest fraction of labelled CO<sub>2</sub> follows first route, with SCFA making a major contribution to the energy requirements of the host animal. Partitioning of these three components of recovered CO<sub>2</sub> could be achieved by separation of experimental animals' mouths and noses from their anuses in two discrete chambers, which would necessitate restraining the animals. Such whole-animal studies could be coupled with *in vitro* cultures of bacterial isolates using labelled CO<sub>2</sub> as their sole carbon source, a potential subject for future research.

My <sup>14</sup>C recovery data showed that with their natural intestinal microflora intact, Namaqua rock mice were able to metabolize the SCFA produced by microflora that had utilized and fermented the <sup>14</sup>C-xylose fed to the mice. Most of the SCFA were probably absorbed from

the mouses' caeca, the principal site of microbial fermentation in small herbivores. Yang *et al.* (1970) introduced  $^{14}\text{C}$ -labelled SCFA into the caeca of rats and showed that SCFA were absorbed from this gut compartment, and metabolized, since the label was exhaled as  $^{14}\text{CO}_2$ . After *A. namaquensis* individuals had undergone antibiotic treatment, their intestinal microflora was compromised, and as a result more of the administered dose of  $^{14}\text{C}$ -xylose was lost to the mouse and simply absorbed into the bloodstream, filtered through the kidneys and excreted in the urine as unaltered xylose. Other studies of xylose metabolism in vertebrates, in which metabolizable energy contributions were determined, have also reported the percentage of ingested xylose that is excreted in the urine: in pigs and roosters fed a diet containing 10% xylose it was on average 37% and 20% respectively (Schutte *et al.* 1991; Verstegen *et al.* 1997), with speculation that the remaining fraction is metabolized to  $\text{CO}_2$  by the animal or fermented by its intestinal microbiota. Here I have shown that intestinal bacteria are indeed necessary for efficient use of xylose by a mammal, and that without the bacteria much of the nutritional benefit of this sugar is lost.

$^{14}\text{C}$ -xylose that was not metabolized by intestinal bacteria, or absorbed into the bloodstream and filtered by the kidneys, simply passed straight through the gastrointestinal tract and was excreted in the faeces. A very small fraction of the label (a maximum of 3.5% of the total oral dose) was excreted in faeces. I expected a greater fraction of label recovery from faeces after antibiotic treatment, when less of the total label dose was metabolized, and found this to be the case for urine. However, I recovered significantly more of the label from faeces before antibiotic treatment rather than after. This unexpected result is probably due to the amount of sample available for analysis, since after antibiotic treatment, mice produced very few, if any, faecal pellets while inside the respiration chambers. Their diet during the 4 days of antibiotic treatment did not result in the production of large amounts of faeces, in contrast to the many

pellets produced during the pre-antibiotic treatment experiment, when mice came directly from the field into the respiration chambers.

The presence of  $^{14}\text{C}$ -labelled-xylulose-5-phosphate in the urine provides evidence for the occurrence of the pathway whereby xylose is catabolized by microbes (Figure 3.3). A possible explanation for the presence of this compound in urine is that fecal bacteria entering urine during its passage through the collection chamber may have continued breaking down  $^{14}\text{C}$ -xylose while urine collected under liquid paraffin during the experiment.

Currently under way is a study coupling isolation of intestinal bacteria from *A. namaquensis*, identification of these bacteria to species level, testing of isolates for xylose utilization, and quantification of SCFA thus produced (Chapter 4). Although at this stage the contribution of SCFA to total energy requirements of these rodent pollinators is not known, the present study clearly shows that intestinal bacteria are important contributors to xylose metabolism in *Aethomys namaquensis*.

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## **Chapter 4**

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

## Abstract

The pentose sugar xylose is a component of the nectar of some Proteaceae. During the flowering season, Namaqua rock mice (*Aethomys namaquensis*), consume nectar xylose when visiting *Protea* flowers. Xylose is very poorly metabolised by mammals, but it is efficiently utilized by bacteria, yeasts and fungi, particularly gastrointestinal microbes that degrade ingested plant material and produce by-products that are then utilized by their host animals. Whole-animal metabolism studies suggest that gastrointestinal microflora play an important role in xylose metabolism in *A. namaquensis*. Here I describe two studies: first, a faecal microflora study in which aerotolerant anaerobic microflora were cultured, and their xylose utilization assessed according to three indicators: (1) pH values; (2) optical density measurements of xylose-free and xylose-containing culture media; and (3) xylose concentrations before and after incubation, determined by HPLC analysis. Second, I collected caecal contents under anaerobic conditions, and cultured caecal microflora both aerobically and anaerobically. Caecal microbial xylose utilization was assessed using a <sup>14</sup>C-xylose incubation assay. Two of the six animals used in the faecal microflora study appeared to have culturable intestinal bacteria that utilized xylose. Two randomly chosen colonies were identified using 16S rRNA based taxonomy to ensure that the faecal cultures being studied were from the intestine of the rodents. Both colonies identified, *Lactobacillus murinus* and *Enterococcus faecium*, are organisms typically found in the digestive tract of most animals. All four animals used in the caecal microflora study had culturable caecal microflora that tested positive for xylose utilization. They were also classified by 16S rRNA based taxonomy as: *Bacillus subtilis*, *B. pumilus*, *B. licheniformis*, *Shigella boydii*, *Arthrobacter* sp. and members of the fungal genera *Aspergillus* and *Penicillium*. Cultures of these isolates were then analysed 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.

Key words: Intestinal microflora; Xylose fermentation; <sup>14</sup>C-labelled xylose; Faecal; Caecal; 16S rRNA; Short-chain fatty acids; Rodents

## Introduction

In two South African genera of the Proteaceae, *Protea* and *Faurea*, xylose comprises as much as 39% of the total nectar sugars, together with the common nectar sugars, sucrose, glucose and fructose (Nicolson and van Wyk 1998). Before the analysis of nectar sugars of the Proteaceae, the pentose sugar xylose had not been reported as a sugar in floral nectar, except in the extrafloral nectar of grasses (Bowden 1970). The presence of xylose 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*, a rodent pollinator of certain geoflorous *Protea* species. Sugar preference and absorption efficiency tests showed that rock mice consumed pure xylose solutions and absorbed and metabolised the sugar fed to them in xylose feeding trials (Johnson *et al.* 1999). Xylose is not known to be efficiently used by mammals, but certain bacteria, yeasts and fungi are well described as strong 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 Robertson 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).

The possible utilization of xylose by intestinal bacteria of *A. namaquensis* was first highlighted in laboratory experiments (Johnson *et al.* 1999), and later confirmed in a study where mice, first with their natural gut flora intact and then after antibiotic treatment to reduce gut flora populations (Johnson *et al.* 2004, Chapter 2), were fed <sup>14</sup>C-labelled xylose, and recovery of the radio-isotopic label was monitored (Johnson *et al.* 2006, Chapter 3). As 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. The work presented here describes - 1) an exploratory study, in which I cultured aerotolerant obligate and facultative anaerobic microbes from faecal pellets, and 2) since faecal bacterial cultivation may be a poor reflection of the bacterial community present in the colon (Sarma-Rupavatham *et al.* 2004), in subsequent experiments I cultured obligate and facultatively anaerobic microbes isolated from the mouse caecum under strict anaerobic conditions. In the first series of experiments, pH values, optical densities and xylose concentrations of the culture media were used as indicators of xylose utilization. To ensure that the cultures under investigation were in fact from the intestines of the mice rather than environmental contaminants, randomly chosen

colonies were identified to the species level by DNA sequencing. In the latter set of experiments, in which samples were obtained anaerobically, bacterial xylose utilization was assessed using a  $^{14}\text{C}$ -xylose incubation assay, and positive xylose utilizers were also identified to species level by DNA sequencing.

## 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 to September) when xylose, in the form of *P. humiflora* nectar, was present in their diet. Ethical approval for animal capture and experimental methods used in the study was obtained from Sub-Committee B of the Research Committee of the University of Stellenbosch.

### *Faecal samples*

Six animals (Mouse 1f – Mouse 6f) were caught during the night and released from the traps the following morning. Faecal pellets left behind were collected with sterile equipment and taken to the laboratory within 3 h, where they were placed inside an anaerobic chamber (atmosphere: 85% N<sub>2</sub>, 10% CO<sub>2</sub> and 5% H<sub>2</sub>) (Forma Scientific, Ohio, USA). A single pellet was selected from each animal (n = 6). Pellets were cut in half using a sterile scalpel, and a sample ( $\pm$  0.01 g) was scraped out from the centre of each with a sterile needle, and placed into an Eppendorf tube containing 0.3 ml sterile pre-reduced buffer solution (2.25 g NaCl, 0.105 g KCl, 0.12 g CaCl<sub>2</sub> and 0.05 g NaHCO<sub>3</sub> in 1L distilled water). Samples were vortexed to resuspend bacteria from the faecal material.

### *Caecal samples*

Four animals (Mouse 1c – Mouse 4c) caught during the night were taken back to the laboratory and housed individually in standard rodent cages (44 cm L x 28 cm B x 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<sub>2</sub> inhalation and placed in an anaerobic chamber where the caecum 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.

### ***Cultivation of microflora***

#### *Faecal samples*

All procedures for the isolation, enumeration and growth of culturable faecal bacteria for xylose utilization assessment were carried out under strictly anaerobic conditions. For the single sample from each mouse, serial 10-fold dilutions ( $10^{-2}$  –  $10^{-7}$ ) of the suspension in buffer were plated out in duplicate on supplemented Brain-Heart Infusion (BHI) agar (Holdeman *et al.* 1977). Plates were incubated at 37°C for 48 h and the number of colony forming units per gram of faecal material calculated.

#### *Caecal samples*

Isolation of caecal samples was carried out under strict anaerobic conditions, but 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 faecal 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 per gram of caecal content was calculated for each plate.

### ***Xylose utilization***

#### *Faecal microflora*

From each of the six animals sampled, 36 representative colonies were chosen at random from the BHI plates and transferred to six separate xylose utilization selective plates (in total 216 bacterial isolates were screened). These selective plates contained a basal peptone-yeast extract medium with 1% xylose (w/v) as the sole carbohydrate (Holdeman *et al.* 1977). This basal medium also included the indicator, bromthymol blue (BMB) at 0.004 g/100 ml media. BMB is blue at pH 7.6, green at pH 7 and golden yellow at pH < 6. The pH was adjusted to pH 7 and inoculated plates were incubated anaerobically overnight at 37°C. Isolates were graded as strong utilizers, weak utilizers or non-utilizers according to colour and formation of a zone of changed colour around each colony. Isolates that formed large bright yellow zones were selected as the strongest potential xylose utilizers producing the most acid, a pale yellow zone indicated weak utilization, and no zone formation indicated no utilization of the sugar (no acid production). Six of the strongest potential xylose utilizers from each selective plate were transferred using a sterile loop into 10 ml sterilin tubes containing 8 ml peptone-yeast extract liquid medium with xylose (PYX) and, as a control, 8 ml peptone-yeast extract liquid medium without xylose (PY). The constituents of the PYX and PY media were the same as for the selective plates (Holdeman *et al.* 1977), except that they contained no agar or indicator and the PY medium lacked xylose.

For both media types, initial pH and optical densities (OD) at 600 nm (Beckman DU-64 Spectrophotometer, Beckman Instruments, California, USA) were recorded. After incubation for 7 h at 37 °C, 1 ml aliquots were removed from each tube for pH determination and OD measurements of the medium after modification by bacterial growth. Substrate utilization studies are performed over different time periods, and if the bacteria under investigation are efficient substrate fermenters, initial utilization occurs rapidly during exponential growth, the first few hours of incubation. The 7 h incubation time used here was also based on *in vivo* studies of xylose utilization by natural populations of gut microflora in Namaqua rock mice (Johnson *et al.* 2006, Chapter 3). Following these measurements, the same aliquots of PYX medium were prepared for high-performance liquid chromatography (HPLC) in order to determine their xylose concentration. Un-inoculated PYX medium was also prepared for HPLC analysis to determine the original xylose concentration. Each 1 ml aliquot was centrifuged at 12 000 rpm for 3 min and filter sterilized through a 0.22 µm filter before analysis. HPLC analysis was carried out on a high performance liquid chromatograph (10AVP, Shimadzu, Kyoto, Japan) linked to a Waters 464 Pulse Electrochemical Detector (PAD) (Waters Corp., Massachusetts, USA) fitted with a dual gold electrode. The sugars were separated at 27°C on a Hamilton RCX-10 column using 60 mM NaOH as the mobile phase at a flow rate of 2 ml min<sup>-1</sup>. Detection conditions were set at the following potentials (E<sub>1</sub> = +80 mV; E<sub>2</sub> = +730 mV; E<sub>3</sub> = -570 mV) and pulse durations of (t<sub>1</sub> = 0.4 s; t<sub>2</sub> = 0.4 s; t<sub>3</sub> = 0.2 s).

#### *Caecal microflora*

Single representative colonies from the total culturable plates obtained from each of the four mice were isolated on the basis of colony morphology, and re-streaked onto rumen fluid-based medium to produce pure cultures (Mouse 1c: 10 isolates, 4 aerobic and 6 anaerobic;

Mouse 2c: 13 isolates, 10 aerobic and 3 anaerobic; Mouse 3c: 16 isolates, 7 aerobic and 9 anaerobic; Mouse 4c: 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  $^{14}\text{C}$ -xylose radio-isotopic incubation assay. The  $^{14}\text{C}$ -assay was performed by placing a 2 ml microtube into a 20 ml glass vial. The vials and tubes were autoclaved and 1.25 ml aliquots of a 1 M NaOH solution were placed into the microtube to trap  $\text{CO}_2$  released by growing cultures during incubation. NaOH traps  $\text{CO}_2$  as sodium hydrogen carbonate ( $\text{NaHCO}_3$ ) 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, 7.5 ml solution A (g/L: 6.0  $\text{K}_2\text{PO}_4$  and 2.0  $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$ ) and 7.5 ml solution B (g/L: 12.0 NaCl, 12.0  $(\text{NH}_4)_2\text{SO}_4$ , 6.0  $\text{KH}_2\text{PO}_4$ , 1.2  $\text{CaCl}_2$ , 2.46  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  and 20.0  $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$ ) in 100 ml distilled water. Prior to inoculation, 0.1  $\mu\text{Ci}$   $^{14}\text{C}$ -labelled xylose (Amersham Life Science, Illinois, USA) was added to the assay medium in each vial. Isolated cultures were inoculated into the assay medium in 3 separate vials, which were sealed with a sterile rubber stopper and metal crimp seal. Vials inoculated with anaerobic cultures were flushed with  $\text{CO}_2$  before being crimp sealed. Vials were incubated at  $37^\circ\text{C}$  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 and dispensed into a glass scintillation vial, to which 14 ml Hionic Fluor scintillation cocktail (Perkin Elmer, Massachusetts, USA) was added; 0.25 ml aliquots of each sample's assay medium were dispensed into 4 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, California, USA).

The protocol counted radioactivity in the full  $^{14}\text{C}$  window (0 to 154 KeV) to ensure the highest counting efficiency. Xylose utilization was assessed by determining the percentage of the original  $0.1\mu\text{Ci }^{14}\text{C}$ -xylose dose converted to  $^{14}\text{CO}_2$  and trapped in the NaOH solution.

### ***Microbe identification***

#### *Faecal microflora*

To check that samples grown on the BHI plates were in fact from the intestines of the rodents and not contaminants from the external environment, two isolated colonies were chosen at random for DNA extraction and species identification. DNA was extracted using the Dneasy Tissue Kit (QIAGEN, Hilden, Germany, catalogue no. 69504) as per manufacturer's instructions. DNA encoding the 16S rRNA gene was amplified by the polymerase chain reaction (PCR) with 16S rRNA primers. Three forward (F1:S16,1; F3:S16,5 and F5:S16,9) and three reverse (R1:S16,4; R3:S16,8 and R5:S16,12) primers were used to cover the entire length of the 16S rRNA sequence (approximately 1423 base pairs) (Lane 1991; Wheeler *et al.* 1996). PCR products were purified following the exonuclease I, shrimp alkaline phosphatase PCR purification protocol (Amersham Pharmacia Biotech, New Jersey, USA). Nucleotide sequencing was performed on a MegaBACE 500 sequencer (Amersham Biosciences, New Jersey, USA), an automated capillary DNA sequencing system. The nucleotide sequence was determined by traditional dideoxynucleotide chain termination chemistry (Sanger *et al.* 1977) using the DYEnamic ET Dye Terminator Cycle Sequencing kit for MegaBACE 500 and MegaBACE 500 Sequence Analyzer vol. 2.4 software. All reactions were performed as per manufacturer's instructions and cycle sequenced on a GeneAmp PCR System 9700 (Perkin Elmer, Applied Biosystems, California, USA). Nucleotide sequences obtained were analyzed using DNAMAN sequence analysis software. Nucleotide homology searches were carried out against the databases held at the National

Center for Biotechnology Information (NCBI), Maryland, USA, using the BLAST program in GenBank (<http://www.ncbi.nlm.nih.gov>). Sequence identity matrices were constructed based on uncorrected pairwise sequence divergence values.

#### *Caecal microflora*

Positive bacterial xylose utilizers, identified by the  $^{14}\text{C}$ -xylose assay, were also processed for identification. As for faecal bacteria, DNA was extracted using the Dneasy Tissue Kit (QIAGEN catalogue no. 69504). DNA encoding the 16S rRNA gene was amplified by PCR with only two 16S rRNA primers, one forward (F1:S16,1) and one reverse (R5:S16,12) primer. Amplifications were carried out on an Applied Biosystems (California, USA) 27000 thermal cycler. PCR products were purified using the Wizard SV Gel and PCR Clean-up System (Promega Corporation, Wisconsin, USA). Strands were cycle sequenced using BigDye dye termination chemistry (Perkin Elmer, Connecticut, USA) and all unincorporated dye label was removed by Sephadex columns (strip of 8 columns, Princeton Separations, New Jersey, USA) according to the manufacturers' protocol, before the samples were run on an ABI Prism 3100 sequencer (Applied Biosystems, California, USA). Nucleotide sequences obtained were analyzed using Sequence Navigator software (ABI, version 1.01). As for faecal bacteria, 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.

### *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 short-chain fatty acids (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° 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 the concentration of xylose was either 1% or 2%. 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 000g 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 analysed 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 a 1 L

volumetric flask with 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  $\mu$ l of n-hexanol as internal standard. The injection volume was 1  $\mu$ l. 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  $\mu$ m film thickness). The column temperature was initially held at 105 °C for 2 min, increased at 10 °C min<sup>-1</sup> 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<sup>-1</sup>. Short-chain fatty acids 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***

To compare pre- and post-incubation values of the criteria used to assess xylose utilization in bacteria obtained from faecal cultures, two-tailed *t*-tests were used to compare average pH values and OD measurements between the two media for each animal. Since HPLC readings for the original xylose concentration of un-inoculated PYX medium varied between samples, one-sample *t*-tests were used to compare average final xylose concentrations for each animal to the lower limit (average – 1 SE = 7.23 mM) of the original xylose concentration (7.83  $\pm$  0.6 mM). Only significant differences from the lower end of the range of the original concentration were considered as positive indication of xylose utilization.

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. These analyses were performed using the statistical software package NCSS 2000.

## Results

### *Isolation and cultivation of microflora*

The total number of colony forming units (CFUs) cultured from faecal samples on BHI medium ranged from  $3.6 \times 10^{10}$  to  $4.9 \times 10^{11}$  per g faeces (coefficient of variation = 80%).

The total number of CFUs 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*

#### *Faecal microflora*

In the liquid PYX medium, utilization of xylose would result in a greater drop in pH after the 7 h incubation than that observed in the PY medium. Also, the presence of xylose as an additional carbon source in the medium would improve bacterial growth, reflected by higher OD readings. Such indicators of xylose utilization did not differ significantly between bacterial cultures from Mice 1f, 2f, 4f and 5f grown in PY or PYX medium (Table 4.1). In Mouse 3f, a single isolated colony (F) grew very poorly and was therefore excluded from the experiment: remaining colonies from this mouse showed significantly lower mean pH values in the PYX than in PY medium after growth ( $p < 0.05$ ). Mean OD measurements for colonies grown from Mouse 3f did not differ between the two media. For Mouse 6f, the mean pH value of cultures grown in PY medium was significantly lower than those in PYX medium ( $p < 0.05$ ), and mean OD measurements were significantly lower in the PY medium ( $p < 0.05$ ).

Xylose concentration did not decrease substantially in the medium containing any of the bacterial isolates from the six mice. Final xylose concentrations ranged from 5.86 – 7.62 mM, decreasing from an original concentration of  $7.83 \pm 0.6$  SE mM. Maximum decrease during the 7 h incubation period was 1.97 mM. Results from one-sample t-tests showed that culture media from Mice 1f, 2f, 3f and 6f each had final mean xylose concentrations that were significantly less ( $p < 0.05$ ) than the lower limit (7.23 mM) of the range for the original concentration. For Mouse 4f the mean final xylose concentration was significantly greater ( $p < 0.05$ ) than 7.23 mM, and for Mouse 5f the mean final xylose concentration did not differ from the lower limit.

Taken together, decreased pH and xylose concentrations and increased optical density in xylose-enriched culture medium relative to medium without xylose reflect the degree of xylose utilization by bacteria during the exponential growth phase of that particular isolate colony. Most colonies screened did not show consistency in all three of these indicators of xylose utilization. However, isolate E from Mouse 3f fulfilled all three criteria for xylose utilization, as did five out of six isolates from Mouse 6f, exhibiting differences between medium with and without xylose for all three indicators that were both more consistent and greater in magnitude than indicators for cultures from the other five mice.

Table 4.1. Faecal cultures: pH measurements, optical density (OD) readings at 600 nm and xylose concentrations (mM) after 7 h incubation at 37°C, for 6 isolate colonies (A-F) from 6 animals (1f-6f). PY – peptone-yeast medium, PYX – peptone-yeast-xylose medium. Initial values in uninoculated media: pH = 7, OD =  $\pm 0.04$ , original xylose concentration =  $7.83 \pm 0.60$  mM. For pH and optical density, isolate colonies which showed significant differences between the two media ( $p < 0.05$ ) are represented by bold values. For xylose concentration, bold values denote animals in which the average final xylose concentration produced by all six isolates, was significantly lower ( $p < 0.05$ ) than the lower limit (average – 1 SE, = 7.23 mM) of the original concentration.

Isolate	Mouse number												
	1f		2f		3f		4f		5f		6f		
	PY	PYX	PY	PYX	PY	PYX	PY	PYX	PY	PYX	PY	PYX	
pH													
A	5.5	5.5	5.8	5.8	<b>5.5</b>	<b>5.5</b>	5.6	5.6	5.5	5.5	<b>5.5</b>	<b>4.7</b>	
B	5.5	5.5	5.8	5.8	<b>5.5</b>	<b>5.5</b>	5.6	5.6	5.5	5.5	<b>5.7</b>	<b>4.5</b>	
C	5.5	5.5	5.4	5.4	<b>5.5</b>	<b>5.0</b>	5.5	5.5	5.5	5.5	<b>5.8</b>	<b>4.5</b>	
D	5.5	5.5	5.0	5.2	<b>5.5</b>	<b>5.0</b>	5.5	5.5	5.5	5.5	<b>5.3</b>	<b>5.3</b>	
E	5.5	5.5	5.0	5.0	<b>5.5</b>	<b>5.0</b>	5.6	5.5	5.7	5.2	<b>5.7</b>	<b>4.5</b>	
F	5.5	5.5	5.0	5.0			5.5	5.5	5.6	5.6	<b>5.8</b>	<b>4.5</b>	
mean	5.5	5.5	5.3	5.4	<b>5.5</b>	<b>5.2</b>	5.6	5.5	5.6	5.5	<b>5.6</b>	<b>4.7</b>	

Table 4.1 continued

Isolate	Mouse number											
	1f		2f		3f		4f		5f		6f	
	PY	PYX	PY	PYX	PY	PYX	PY	PYX	PY	PYX	PY	PYX
OD												
A	0.76	0.77	0.43	0.50	0.68	0.68	0.63	0.58	0.86	0.84	<b>0.69</b>	<b>0.87</b>
B	0.77	0.77	0.46	0.50	0.63	0.68	0.64	0.72	0.71	0.62	<b>0.80</b>	<b>0.95</b>
C	0.75	0.76	0.39	0.40	0.73	0.67	0.63	0.80	0.68	0.79	<b>0.71</b>	<b>0.93</b>
D	0.64	0.71	0.41	0.42	0.63	0.74	0.65	0.73	0.77	0.81	<b>0.91</b>	<b>0.93</b>
E	0.65	0.71	0.41	0.43	0.64	0.69	0.72	0.72	0.77	0.87	<b>0.77</b>	<b>0.94</b>
F	0.78	0.73	0.38	0.41			0.76	0.71	0.76	0.73	<b>0.73</b>	<b>0.82</b>
mean	0.73	0.74	0.41	0.44	0.66	0.69	0.67	0.71	0.76	0.78	<b>0.77</b>	<b>0.91</b>
xylose												
A		<b>5.86</b>		<b>7.36</b>		<b>6.52</b>		7.34		7.06		<b>5.88</b>
B		<b>6.82</b>		<b>6.44</b>		<b>6.06</b>		7.60		7.10		<b>6.18</b>
C		<b>6.98</b>		<b>6.46</b>		<b>6.64</b>		7.34		6.86		<b>6.06</b>
D		<b>6.76</b>		<b>6.46</b>		<b>7.20</b>		7.48		6.88		<b>6.40</b>
E		<b>6.68</b>		<b>6.46</b>		<b>6.16</b>		7.60		7.62		<b>6.32</b>
F		<b>7.10</b>		<b>6.46</b>				7.32		7.52		<b>6.26</b>
mean		<b>6.70</b>		<b>6.61</b>		<b>6.52</b>		7.45		7.17		6.18

*Caecal microflora*

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%  $\pm$  11.7 SE). Of the 45 isolates assayed, 24 showed evidence of xylose utilization by label being trapped in the NaOH solution. Of those 24 positive xylose utilizers, 4 were isolated from Mouse 1c, 6 from Mouse 2c, 11 from Mouse 3c and 3 from Mouse 4c (Table 4.2). 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.

Table 4.2. Caecal cultures: Mean percentage of  $^{14}\text{C}$  dose recovered from each isolate, assayed in triplicate, that showed evidence of xylose utilization. CE: mean counting efficiency for each type of sample.

Mouse number	Isolate number	NaOH trap (CE = 93.82%)	Culture medium (CE = 92.71%)	Vial wash (CE = 96.18%)	Total
2c	1	17.0	55.6	4.8	77.4
3c	2	19.3	55.8	4.8	79.9
1c	3	40.5	42.9	1.9	85.2
2c	4	36.1	60.6	2.0	98.8
3c	5	14.7	67.6	0.8	83.0
1c	6	6.7	64.5	7.7	78.8
2c	7	24.3	36.0	7.5	67.8
3c	8	19.1	41.3	7.7	68.2
2c	9	43.6	54.2	2.1	99.9
2c	10	17.1	75.2	4.1	96.4
4c	11	15.4	78.2	4.8	98.4
4c	12	8.5	79.1	4.3	92.0

Table 4.2 continued

Mouse number	Isolate number	NaOH trap (CE = 93.82%)	Culture medium (CE = 92.71%)	Vial wash (CE = 96.18%)	Total
1c	13	34.4	31.4	3.1	98.9
2c	14	6.4	81.4	5.0	92.8
1c	15	34.4	60.2	4.2	98.8
3c	16	35.6	62.2	1.1	98.9
3c	17	37.7	43.3	4.8	85.8
3c	18	27.9	39.0	1.2	68.2
3c	19	6.8	70.9	0.7	78.4
4c	20	6.5	78.8	0.7	86.0
3c	21	48.1	40.6	2.2	90.8
2c	22	24.0	36.6	2.8	63.4
3c	23	60.1	37.7	2.1	99.8
3c	24	34.4	40.9	3.6	79.0

Note: Isolates 1-21 are bacterial and 22-24 are fungal.

### ***Microbe identification***

#### *Faecal microflora*

The two isolates chosen for identification were identified as *Lactobacillus murinus* and *Enterococcus faecium*. Sequence identity matrices showed that the *L. murinus* sequence was 99.6% similar to *L. murinus* (AF157049) held in GenBank at NCBI and the *E. faecium* sequence 99.8% similar to *E. faecium* (AF145258).

#### *Caecal microflora*

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: *B. subtilis*, *B. pumilus* and *B. licheniformis*), four *Shigella boydii* isolates and one isolate belonging to the genus *Arthrobacter* (Table 4.3). Morphological analyses of the three fungal isolates identified one as an *Aspergillus* species and two as members of the genus *Penicillium*. Ultimately seven species of caecal microflora were identified as xylose utilizers.

Table 4.3. Caecal cultures: Taxonomic identification of the 21 bacterial isolates that were identified as positive xylose utilizers.

Isolate number	Growth conditions	Species	GenBank accession code for strains with >99% sequence match
1	aerobic	<i>B. subtilis</i>	AY304996
2	aerobic	<i>B. subtilis</i>	AY162127
3	aerobic	<i>B. licheniformis</i>	AY842874
4	anaerobic	<i>B. subtilis</i>	AY162127
5	anaerobic	<i>S. boydii</i>	AY696668
6	aerobic	<i>B. pumilus</i>	AY260861
7	aerobic	<i>B. licheniformis</i>	AY842874
8	aerobic	<i>B. licheniformis</i>	AY842874
9	aerobic	<i>B. licheniformis</i>	CP000002
10	aerobic	<i>B. subtilis</i>	AY030331
11	aerobic	<i>B. subtilis</i>	AY304996
12	aerobic	<i>B. subtilis</i>	AY162128
13	aerobic	<i>B. subtilis</i>	AY030331
14	aerobic	<i>B. pumilus</i>	AB098578
15	anaerobic	<i>B. licheniformis</i>	AY842874
16	anaerobic	<i>S. boydii</i>	AY696668
17	anaerobic	<i>S. boydii</i>	AY696681
18	anaerobic	<i>S. boydii</i>	AY696668
19	anaerobic	<i>Arthrobacter</i> sp.	AY864629
20	anaerobic	<i>B. licheniformis</i>	AJ880762
21	Anaerobic	<i>B. licheniformis</i>	AY842874

### ***Short-chain fatty acid production***

The concentrations of short-chain fatty acids 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 species of caecal microflora previously identified as xylose-utilizers produced the SCFA that are typically end-products of microbial fermentation, namely acetic, propionic and butyric acids. 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 I 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 from xylose fermentation. The production of acetic, propionic and butyric acids by the five bacterial species was greater than that by the two fungal species, which was less than 0.1 mg/ml culture medium, except for acetic acid production by the *Penicillium* isolate, which was 0.13 and 0.3 in the 1% and 2% xylose media respectively. Values for the two fungal species are not included in Table 4.4. The production of iso-butyric, valeric and iso-valeric acids was less than 0.1 mg/ml for all seven microflora species tested and was also excluded from Table 4.4.

All three *Bacillus* species produced more acetic acid when grown in the 2% xylose medium compared to 1% xylose. *Bacillus subtilis* produced six times as much acetic acid, while *B. pumilus* and *B. licheniformis* produced twice as much. *Shigella boydii* produced slightly more acetic acid in the 1% xylose medium, whereas there was not much difference in the amount of acetic acid produced by the *Arthrobacter* species in either media. *Bacillus subtilis* produced more propionic acid in the 1% medium than 2%, while *B. pumilus* produced twice

as much in the 2% medium compared to 1% medium. For *B. licheniformis*, propionic acid concentrations did not differ between the two media after incubation. *Shigella boydii* produced almost twice as much propionic acid in the 2% medium relative to 1%, whereas *Arthrobacter* produced twice as much propionic acid in the 1% medium. There was not much difference in the amounts of butyric acid produced in the two media, except for *S. boydii* which produced almost twice as much in the 2% medium and *Arthrobacter*, which produced three times more butyric acid in the 1% medium. The remaining acids, iso-butyric, valeric and iso-valeric, were all produced in much lower quantities (less than 0.1 mg/ml medium) than the other three acids. The quantities of these acids were, however, included in estimations of the total quantity of acid produced during incubation. 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.

The production of acid during incubation was also reflected in the decrease in pH from the initial medium 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 4.4). 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 more significant decrease in the 2% medium ( $p = 0.000002$ ) than the 1% medium ( $p = 0.019$ ).

Table 4.4. Amounts of acetic, propionic and butyric acids (mg/ml culture medium) produced by each species during 12 h incubation at 37°C in 1% and 2% xylose media, total SCFA acid production (mg/ml) and final pH after incubation. Values are means of two independent determinations. 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.

	Control	<i>Bacillus subtilis</i>		<i>Bacillus pumilus</i>		<i>Bacillus licheniformis</i>		<i>Shigella boydii</i>		<i>Arthrobacter</i> species	
		1%	2%	1%	2%	1%	2%	1%	2%	1%	2%
Acetic acid	0.13	0.11	0.66	0.22	0.41	0.35	0.75	0.36	0.28	0.17	0.21
Propionic acid	0.02	0.30	0.18	0.17	0.33	0.15	0.19	0.13	0.21	0.23	0.10
Butyric acid	0.04	0.09	0.10	0.09	0.12	0.10	0.14	0.08	0.15	0.16	0.05
Total acid	0.21	0.69	1.04	0.58	1.05	0.72	1.65	0.67	0.75	0.66	0.41
pH	7.2	7.12	5.89	7.04	5.94	7.03	5.55	5.57	5.47	6.39	5.86

## Discussion

### *Faecal microflora study*

Fastidious obligate anaerobes would probably not survive during the initial experimentation on faecal material from wild animals in their natural environment, as samples were partially exposed to air for up to 12 hours. Thus, faecal populations examined from the internal material of the pellets would consist largely of aerotolerant obligate and facultative anaerobes. This method of sampling faecal pellets of *A. namaquensis* did, however, result in the isolation and identification of two organisms that are typically found in the digestive tract of animals, *Lactobacillus murinus* and *Enterococcus faecium*.

Previously isolated from rat and mouse digestive tracts, *L. murinus* is a typical and dominant species in the intestine of rodents (Hemme *et al.* 1980). It is a Gram positive microaerophilic rod (Holt *et al.* 1994) that facultatively ferments hexoses and pentoses (Sneath *et al.* 1986). More than 90% of all strains of *L. murinus* are unable to ferment D-xylose (Sneath *et al.* 1986). The *L. murinus* strain that we isolated was recorded as a xylose-utilizer because the colony formed a zone of colour change on a xylose selective plate, but xylose utilization was not quantified in the faecal study.

The distribution of the gram-positive facultatively anaerobic coccus *E. faecium* (Holt *et al.* 1994) is widespread and this organism is found in the intestinal tract and faeces of humans and homeothermic and poikilothermic animals, in insects and on plant leaves, buds, shoots, fruits and seeds (Mundt 1963a; 1963b; Geldreich *et al.* 1964; Martin and Mundt 1972; Sneath *et al.* 1986). Enterococci on vegetation are probably a result of contamination by animal faeces or untreated sewage (Jett *et al.* 1994). As with *L. murinus*, 90% or more of the

strains of *E. faecium* do not produce acid from D-xylose (Holt *et al.* 1994). However, Chen *et al.* (2000) claimed that all enterococci ferment xylose, although at different rates. The strain that we isolated here did form a zone of colour change on a xylose selective plate, but utilization was not quantified.

Changes in medium pH are not conclusive evidence for sugar fermentation. I considered fulfillment of all three criteria (decreased pH and xylose concentration and increased optical density) (Table 4.1) as the most reliable indication of xylose utilization. A final pH between 5.5 and 6.0 is indicative of production of weak acid, and a final pH of 5.5 and below indicates strong acid production (Holdeman *et al.* 1977). All but five of the 35 isolates from all six animals produced strong acid in PYX, with pH values ranging from 4.5 – 5.5. The remaining five produced weak acid, with pH values at 5.6 or 5.8. It is, however, important to consider what happens in the PY medium. After inoculation, the pH of PY is usually lowered to 6.2 - 6.4, but may be lowered even more if acids are produced from the peptones in the medium (Holdeman *et al.* 1977). I found this to be the case: final pH of PY media ranged from 5.0 – 5.8, falling in both the weak and strong acid production categories. Medium pH changes attributable to sugar fermentation must thus only be inferred where control and xylose-containing media differ. Such a difference was seen in three isolates from Mouse 3f and one from Mouse 5f, but the biggest differences were observed in five isolates from Mouse 6f.

If the bacteria from all six faecal samples were able to make biologically significant use of the xylose present in the PYX medium, I expected a greater difference between colonies grown on PY and PYX media with respect to decrease in pH, growth rate (optical density) and drop in xylose concentration during exponential growth. All three of the xylose utilization criteria outlined above were consistently met by only two of the six animals

studied, Mice 3f and 6f (Table 4.1). From Mouse 3f one isolate (E) may be a xylose utilizer, but growth in the xylose-containing medium was only slightly stronger than growth in the control medium. The five isolates (A, B, C, E and F) from Mouse 6f, which exhibited the most marked and statistically significant differences in pH, OD and xylose concentration, suggested stronger xylose utilization in this mouse. Mouse 6f may have been feeding more consistently on nectar containing xylose before its capture than were the other five animals I used, exposing its intestinal microflora more to xylose and inducing an increase in the numbers of xylose-utilizing bacteria in its gut.

### ***Caecal microflora study***

Removal of the caecum under anaerobic conditions and exposure of caecal contents in an anaerobic environment improved the sampling methods, allowing obligate anaerobic microflora, not cultured in the faecal study, a better chance of surviving exposure to the external environment and being cultured in the laboratory. Also, a rumen-fluid based medium, previously used to obtain total culturable counts of rat caecal and faecal microflora (Campbell *et al.* 1997), was used to improve the growth conditions to obtain a variety of caecal microflora. This created conditions that allowed for the growth of positively identified xylose utilizers: *Bacillus subtilis*, *B. pumilus*, *B. licheniformis*, *Shigella boydii*, an *Arthrobacter* species and fungal isolates belonging to the genera *Aspergillus* and *Penicillium*. It is likely that these bacteria contribute to the maintenance of a beneficial microbial community in the digestive tract of Namaqua rock mice.

The genus *Bacillus* comprises Gram-positive endospore-forming rods, which are aerobic or facultatively anaerobic (Sneath *et al.* 1986). The type species of the genus is *Bacillus subtilis* and the primary habitat of *Bacillus* species is the soil. Due to the high resistance of *Bacillus*

endospores they have a wide distribution in nature. The spores of *B. subtilis* have been shown to be capable of germinating in the gastrointestinal tract of mice (Hoa *et al.* 2001). The ability of *B. subtilis* spores to germinate in the gastrointestinal tract has made the species a good organism for use as a beneficial bacterium (a probiotic) in human and animal consumption (Hoa *et al.* 2000; Duc *et al.* 2003). 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. *Bacillus 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. In *B. subtilis*, *B. pumilus* and *B. licheniformis*, more than 90% of strains are positive for acid production 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 – 36.1% for the seven *B. subtilis* isolates and from 6.5 – 48.1% for the seven *B. licheniformis* isolates. The two *B. pumilus* isolates showed very low xylose utilization (6.4 and 6.7%) (Tables 4.2 and 4.3).

Xylose utilization by bacteria involves the isomerization of xylose to xylulose, followed by phosphorylation to xylulose-5-phosphate. The enzymes of this pathway are encoded by *xyl* operons, and expression of these encoded pathways in *B. licheniformis* is induced in individual bacteria by xylose (Scheler and Hillen 1994). Also, based on the findings of the <sup>14</sup>C-xylose whole-animal metabolism study (Johnson *et al.* 2006, Chapter 3), the microflora of *Aethomys namaquensis* alters in response to xylose content of the diet, with populations of xylose utilizing bacteria fluctuating in response to the quantity of xylose ingested in the wild.

The pathogenic bacterium *Shigella 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 – 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 (*A. globiformis* and *A. 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). 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 <sup>14</sup>C-xylose dose was trapped as CO<sub>2</sub> in the NaOH solution).

Gastrointestinal fungi also have an important role degrading plant fibre during digestion in herbivores. However, the two fungal isolates that we cultured from caecal samples do not belong to the known group of beneficial fungi found in the gut, but are opportunistic pathogens. Fungi belonging to the *Aspergillus* and *Penicillium* genera are commonly found in the environment worldwide. They grow on plant material and are problematic in the production and storage of cereal grains and animal feeds, due to the secretion of mycotoxins which cause mycotic diseases when contaminated feeds are consumed (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; Jensen *et al.* 1991). *Penicillium* species also cause infections in humans that give rise to intestinal mycoses (Fantry 2001). Although the fungal genera isolated in this study are not known to be beneficial intestinal microbes, they nevertheless are xylose utilizers. Both *Aspergillus* and *Penicillium* species have xylan-degrading enzyme systems (Filho *et al.* 1991; Kormelink *et al.* 1993; McCrae *et al.* 1994). As a filamentous fungus used in fermentation biotechnology, *Aspergillus* and its xylose catabolism have been studied in order to develop methods of metabolic control of pathways to improve the yield of fermentation products (Prathumpai *et al.* 2003). Xylose utilization, as the percentage of the dose trapped in NaOH during the incubation assay, was 24.0% for the *Aspergillus* isolate and 34.4 and 60.1% for the two *Penicillium* isolates.

Infections of any kind reduce an animal's immune response, and immunocompromised individuals are more susceptible to infection. It is interesting that pathogenic microbes such as *S. boydii* and *Penicillium* fungi originated from the same mouse - an individual that also happened to be infected with an intestinal nematode. Some *Arthrobacter* species are also regarded as opportunistic pathogens (Funke *et al.* 1996), and the single *Arthrobacter* species

isolated here also came from that same mouse. Although all these pathogens were isolated from Mouse 3c, samples from its caecum still yielded some of the beneficial gut microbes, the *Bacillus* species. *Aspergillus* fungi were isolated from Mouse 2c. The pathogenicity of these microbes to wild mice, particularly the *Arthrobacter* species and the *Penicillium* and *Aspergillus* fungi, is not known, and they may not be detrimental to the health of these mice, but rather beneficial as xylose utilizers.

The isolation procedures and culture conditions used in the two studies yielded a variety of gut microbes. The two bacterial species identified in the faecal study, *L. murinus* and *E. faecium*, were not cultured as xylose utilizers in the caecal study. A possible reason for this is that these two species, if indeed present in the caecal samples, were not strong enough xylose utilizers when assessed by the radio-isotopic incubation assay to qualify as isolates for identification, or perhaps the culture conditions used in the caecal study were unable to support the growth of *L. murinus* and *E. faecium*. The primary objective of anaerobic sampling and preparation of caecal samples, and the use of a rumen-fluid based medium, was to create culture conditions favourable for most of the caecal bacteria, including obligate anaerobes. However, this approach may favour *Bacillus* species, as *Bacillus* species were isolated from the caecal samples of all four mice.

### ***Short-chain fatty acid production***

Xylose utilization by intestinal bacteria of *A. namaquensis* was first suggested by sugar absorption efficiency experiments in this rodent (Johnson *et al.* 1999). Further evidence to suggest xylose utilization and an ability to break down xylan (a plant polysaccharide made up of xylose monomers) was supplied by small amounts of xylose in the blood, urine and faeces of control animals, given water instead of xylose solutions. The only possible source of this

xylose is from hydrolysis of xylan in the plant-based food pellets that were the animals' sole food. Mammals do not possess the enzyme, xylanase, which is required to hydrolyse 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, short-chain fatty acids (SCFA), are absorbed by the host, providing energy for body functions (Stevens and Hume 1995). In Chapter 3, I supplied convincing evidence for xylose utilization by gut bacteria in *A. namaquensis* using whole-animal metabolism studies, in which individual mice were given an oral dose of <sup>14</sup>C-labelled xylose (Johnson *et al.* 2006). By-products of gut microbial fermentation are CO<sub>2</sub>, H<sub>2</sub>, and CH<sub>4</sub> (in the case of methanogenic bacteria). The radio-isotopic assay that was used here assesses utilization of a labelled substrate, in this case <sup>14</sup>C-xylose, by recovery of the label released as the CO<sub>2</sub> produced during fermentation. Label that was not recovered as CO<sub>2</sub> trapped in NaOH, would have remained in the assay culture as SCFA, other by-products of fermentation, and unutilized <sup>14</sup>C-xylose. Of the products of microbial fermentation in the gut, it is the SCFA that are the principal end products of fermentation and the major contributors to the energy requirements of the host animal. Such fermentation contributes to the energy balance of all herbivorous mammalian species (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 are acetic acid, propionic acid and butyric acid, and each is utilized by different tissues in the host's body (Bergman 1990).

Gut epithelial blood vessels rapidly absorb SCFA from the gut lumen by simple diffusion down a concentration gradient, preventing toxic levels of acids and excessive drops in pH in the gut (Stevens and Hume 1995). Acetic and propionic acids pass through the gut epithelium largely unchanged, but at this site almost all butyric acid is metabolized to the ketone beta-hydroxybutyric acid, which fuels the gut epithelial cells and protects against cellular differentiation and tumour growth (Young and Gibson 1994). Remaining acids are transported via the portal vein to the liver, where butyric acid is metabolized to ketone bodies. Acetic acid, minimally utilized in the liver in ruminants, continues on to peripheral tissues such as skeletal muscle and adipose tissue, where it is oxidized to generate ATP and provides a major source of acetyl CoA for lipid synthesis. In non-ruminants, such as rodents, some acetic acid remains in the liver for synthesis of lipids (Bergman 1990). Propionic acid is completely removed from portal blood by the liver, where it serves as a major substrate for gluconeogenesis. Pathways of SCFA metabolism differ slightly between species, but the paths described above are widespread. The types and proportions of SCFA produced are dependent on the bacterial species inhabiting the gastrointestinal tract, and the host's diet. In ruminants, rumenal molar proportions of acetate to propionate to butyrate are 70:20:10; in non-ruminants such as horses and pigs, the ratio in the hind-gut lumen is the same, but rabbit hind-guts contain more butyrate than propionate, leading to a ratio of 70:10:20 (Bergman 1990). Across all mammal species studied to date, acetic acid remains the SCFA produced in the greatest quantities (see Bergman, 1990 for a review of mammals), constituting as much as 98% of all SCFA in the rock hyrax stomach (Rübsamen *et al.* 1982). Tropical marine fish fed on microalgae show the same trend (Stevens and Hume 1998).

This study has 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 (Table 4.4). Bacterial gut symbionts of *Aethomys namaquensis* appear to be stronger fatty acid producers than are the fungal species. The proportions of acetic to propionic to butyric acids produced are similar to those determined in other animals that rely on gut microbial fermentation, with acetic acid as the predominant SCFA (Stevens and Hume 1998). The fatty acid concentrations reported here are for *in vitro* cultures of individually grown bacterial species. In the gut, these bacteria would most likely experience interspecific competition that might affect the proportions of SCFA that each produces. Other carbon sources, such as cellulose in the rumen fluid medium, may have contributed to fatty acid production, but a single batch of rumen fluid was used here so differences in fatty acid production between the two media reflect the different concentrations of xylose in the two media. Although there is a decrease in the pH of the medium after incubation as a result of acid production, the magnitude of this change in most cases indicates weak acid production (Holdeman *et al.* 1977). It is important to note that the culture medium used in this experiment was used to achieve colony growth and show that microbial species tested use xylose as a substrate to produce fatty acids, and was not chosen to achieve best acid production. An alternative to the rumen fluid-based medium used here, and different growth conditions, might together result in stronger acid production. Nonetheless, the caecal microflora isolated from rock mice, even possibly pathogenic species, produce the SCFA typical of gut microbial fermentation, when given xylose as a substrate. Thus, through the process of xylose metabolism by intestinal microflora, xylose in *Protea nectar* can be catabolized, producing SCFA that ultimately contribute to the energy requirements of rock mice.

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## **Chapter 5**

### **Concluding Comments**

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#### **Xylose as a nectar sugar: ecological significance**

The presence of xylose in *Protea* and *Faurea* nectar remains puzzling. Although I have shown that mice are ultimately able to obtain energy from xylose in nectar, xylose is nonetheless their least preferred sugar (Johnson *et al.* 1999). Also, xylose is present in the nectar of Proteaceae species that are not pollinated by mice but by avian pollinators that show a strong aversion to xylose solutions (Lotz and Nicolson 1996; Allsopp *et al.* 1998; Franke *et al.* 1998; Jackson *et al.* 1998). This leads to the question: why is xylose present in *Protea* and *Faurea* nectar?

#### *A coevolutionary perspective*

*Protea* and *Faurea* species share distinct characteristics that led to speculation that they are sister genera (Rourke 1973; Johnson and Briggs 1975). A molecular study of phylogenetic relationships within the southern African Proteaceae has now resolved *Protea* and *Faurea* as sister genera, and placed near the base of the family, they are distantly related to the rest of the Cape genera (Barker *et al.* 2002). As suggested by Nicolson and van Wyk (1998), xylose in *Protea* and *Faurea* nectar may be a synapomorphic characteristic of this relationship, (a character shared by these two genera which was derived from their last common ancestor). However, nectar composition is an important consideration in pollination ecology since the role of floral nectar is to attract pollinators and ensure reproduction. Studies of nectar chemistry and the interactions between plants and pollinators, a field pioneered by Herbert and Irene Baker (Kevan 2003), have revealed relationships between the proportions of different sugars in plants' nectar, and pollinator types attending those plants (Baker and

Baker 1982; 1983). Similar relationships may also exist for nectar amino acid content and pollinator type (Gardener and Gillman 2002). Since nectar sugar composition may be related to pollinator type, pollinators of *Protea* and *Faurea* species might be expected to have a preference for xylose. It is clear from sugar preference tests performed on these pollinators that this is not the case (Lotz and Nicolson 1996; Allsopp *et al.* 1998; Franke *et al.* 1998; Jackson *et al.* 1998, Johnson *et al.* 1999).

Previously described absorption efficiency studies and the results from this research show that only mammal pollinators are able to benefit from xylose in their diet. However, the pollinators that visit *Protea* species include insects and birds as well. Also, mammals are not known to pollinate *Faurea* species, members of the other Proteaceae genus that produces xylose-containing nectar. Even though xylose concentrations in the nectar of mammal-pollinated *Protea* species are higher than corresponding values for bird-pollinated species (Nicolson and van Wyk 1998), coevolution of nectar composition and pollinator physiology is unlikely to explain the presence of xylose in *Protea* and *Faurea* nectar. Nevertheless, it is interesting that an unrelated plant species, an African lily, *Massonia depressa*, which is also rodent-pollinated, produces nectar containing 3% xylose (Johnson *et al.* 2001).

#### *A plant physiology perspective*

If xylose is not in nectar for the attraction and benefit of pollinators, why is it there? Jackson and Nicolson (2002) suggested two possible explanations that do not invoke coevolution: (1) xylose in nectar results from the degradation of xylan in nectary walls by bacteria, yeasts and fungi in nectar, and (2) xylose in nectar originates from phloem sap.

Nectar-inhabiting microorganisms are abundant in flowers, and yeasts tend to predominate in this nutrient-rich medium (Kevan *et al.* 1988; Raguso 2004; Antonovics 2005). However, although nectar-secreting tissue is in direct contact with nectar, which potentially harbours microbes, there is little evidence for microbial breakdown of nectary walls (Carter and Thornburg 2004). While producing nectar that attracts pollinators, plants need to protect themselves against microbial attack. A number of toxic compounds in nectar are considered to have antimicrobial properties for this purpose (Adler 2000). Recent studies in the nectar biochemistry of ornamental tobacco plants have identified proteins that are directly involved in defense against microbial attack (Thornburg *et al.* 2003; Carter and Thornburg 2004). If microbial degradation of xylan in nectary walls does occur in *Protea* and *Faurea* species, it is very unlikely that it would be confined to just these two genera.

Sugar concentrations in nectar vary greatly depending on the type and location of nectaries (specialized glands that secrete nectar) (Roshchina and Roshchina 1993). The position and structure of nectaries varies among plant species. Nectaries can be located on every type of floral tissue (calyx, corolla, stamens or carpel) and comprises three parts: an epidermis with secretory cells, parenchymatous tissue and a vascular bundle (Pacini *et al.* 2003). Fine ramifications from the plant's vascular system enter the nectaries, allowing movement of phloem sap (pre-nectar) through the nectary parenchymatous cells and into secretory cells at the surface of the glands (Fahn 1988). Sugars in secreted nectar originate from phloem sap (Fahn 2000; Pacini *et al.* 2003; de la Barrera and Nobel 2004). High concentrations of xylose in phloem sap are unusual (Kandler and Hopf 1981), although Völkl *et al.* (1999) found that 33% of phloem sap sugars of *Tanacetum vulgare* consisted of xylose. However, the concentrations of sugars in *T. vulgare* nectar have not been determined. Xylose in nectar may not necessarily reflect phloem composition since the pre-nectar phloem exudate can be

modified by enzymatic transformations in nectariferous tissue (Fahn 2000). These enzymatic transformations can change the composition of pre-nectar when it is secreted as nectar. Baker *et al.* (1978) recorded the concentrations of constituents of pre-nectar and secreted nectar of the extrafloral nectaries of *Ricinus communis* plants. Although no glucose, fructose or galactose was present in the phloem exudates, there were high concentrations of these sugars in the secreted nectar. In some plant species, nectar sugars are produced in the nectary cells themselves during photosynthesis (Stpiczyńska *et al.* 2005). *Banksia prionotes* is the only member of the Proteaceae for which phloem sap composition has been analyzed, and it lacks xylose (Jeschke and Pate 1995), as does the nectar of the flowers (Nicolson and van Wyk 1998). Studies of *Protea* and *Faurea* phloem sap composition are desirable, since we do not know if this is where the xylose in the nectar originates. Nectar xylose may be a reflection of phloem composition in these two genera of Proteaceae, or may be the product of enzymatic transformations of pre-nectar phloem exudates. Species-specific variation in nectar composition may be due to both variation in the secretory processes in nectaries and variation in phloem composition between species (Adler 2000). Perhaps the presence of xylose in *Protea* and *Faurea* nectar is related to phylogeny, but despite the important role nectar plays in plant reproduction, very little is known about the heritability of nectar traits (Mitchell 2004). The most likely explanation for the presence of xylose in *Protea* and *Faurea* nectar may be one that includes plant genetics, biochemistry and physiology.

### **Fermentative gut microflora and xylose metabolism**

Whether or not we will eventually be able to understand the ecological significance of xylose in nectar, the results of this thesis have shown that Namaqua rock mice have the advantage of obtaining energy from xylose in nectar. The diet of rock mice includes plant material (floral

structures and seeds), and the rodents have a fermentation chamber in the form of a caecum where plant material is broken down by gut bacteria. Gut morphology and fermentative capacity may thus be a preadaptation *sensu* Shelley (1999) allowing mice consuming xylose-containing nectar to access the energy in this sugar.

In birds, large populations of intestinal bacteria and specialized caecal bacteria both contribute to the efficient functioning of the digestive system (Mead 1989; Lan *et al.* 2005). The avian caecum functions as a site for microbial fermentation, utilization and absorption of water and nitrogenous compounds, and production of immunoglobins and antibodies (Clench and Mathias 1995). Caeca tend to be large in herbivorous and omnivorous birds, and small, vestigial 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 appears to be linked to the fermentative capacity of the host animal's digestive system, one would not expect nectarivorous bird pollinators of the Proteaceae to have xylose-fermenting gut microflora.

Insects that ingest plant matter also rely on gut symbionts to degrade that cellulosic and hemicellulosic food source. Siricid wood wasps rely on gut fungi to degrade woody plant matter (Gilbertson 1984) and wood-ingesting passalid beetles harbour a number of gut yeasts for the same purpose (Suh *et al.* 2003). Studies of insect gut yeasts have highlighted the *Pichia stipitis* clade as xylose-fermenting gut yeasts associated with wood-ingesting beetles (Nardon and Grenier 1989; Suh *et al.* 2003). Since the diet of the green protea beetle (*Tricostetha fascicularis*), a pollinator of *Protea* species with xylose in their nectar, includes plant tissue and pollen (Johnson and Nicolson 2001), it is not unreasonable to expect these insects to have gut microbes that can metabolize xylose. However, protea beetles showed

strong xylose aversion in the tests (Nicolson and Jackson, unpublished data). Perhaps a more rigorous study of protea beetle sugar preferences, digestive efficiencies and gut microbial inhabitants would help to clarify a relationship between gut fermentative capacity and xylose metabolism.

It has been suggested that *Protea* nectar is merely a dietary supplement (“junk food”) for rock mice (Wiens *et al.* 1983, Fleming and Nicolson 2002). That may be true, but as a food resource that is available in winter, when abundance of other resources such as seeds may be low, such supplementation is of value to the animals’ energy budgets. The breeding season of rock mice coincides with the end of the *P. humiflora* flowering season and continues for approximately two months afterwards. Rock mice may make use of nectar and pollen to improve their body condition in preparation for breeding (Fleming and Nicolson 2002). The rodent caecum with its resident microflora, capable of fermenting xylose to short-chain fatty acids, may be a preadaptation that enables Namaqua rock mice to benefit from the energy in this intriguing nectar sugar of the Proteaceae.

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