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Mechanisms underlying the effects of inulin-type fructans on the intestinal calcium absorption

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meinen Eltern

Vladislav und Irene Raschka

für ihren Mut, alles zu verlassen und in ein fremdes Land zu ziehen,
um ihren Kindern eine freiere Entfaltung zu ermöglichen

Nicht die Wahrheit, in deren Besitz ein Mensch ist oder vermeint zu sein, macht den Wert des Menschen, sondern die aufrichtige Mühe, die er angewandt hat, hinter die Wahrheit zu kommen. Denn nicht durch Besitz, sondern durch Nachforschung erweitern sich seine Kräfte. Der Besitz macht ruhig, träge, stolz.

Gotthold Ephraim Lessing (1729 - 1781)

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ZUSAMMENFASSUNG

Polymere der Fruktose (Inulin und Oligofruktosen) als Nahrungskomponenten werden selektiv von der Mikroflora im Dickdarm fermentiert und dies wird mit einer Reihe von positiven Wirkungen auf die menschliche Gesundheit und das Wohlbefinden in Zusammenhang gebracht. Einer der beschriebenen Effekte besteht in der Erhöhung der intestinalen Calciumresorption, die in vielen tierexperimentellen Studien, aber auch am Menschen dokumentiert wurde. Da die zugrundeliegenden Mechanismen bisher jedoch nicht hinreichend geklärt sind, dienten hier *in vivo*- und *in vitro*-Studien im Modelltier Ratte dazu, die Vorgänge aufzuklären, über die Fruktane auf die Mineralhomöostase Einfluß nehmen.

Die Gabe von inulin-ähnlichen Fruktanen an Ratten führte auch in den vorliegenden Studien zu einer erhöhten scheinbaren intestinalen Calciumresorption, einer erhöhten Calciumretention (-bilanz) und einer erhöhten Akkumulation im Femur. Auch für Magnesium und Zink konnten ähnliche Wirkungen beobachtet werden. Die Zufuhr von den Fruktanen bewirkte auch eine Vergrößerung der Caecumoberfläche, eine Erhöhung der Gewebemasse von Caecum- und Colonwand sowie der intraluminalen Masse im Dickdarm. Die Analyse des Caecuminhalts zeigte einen starken Anstieg des gelösten und ionisierten Calciums bei Tieren nach Fruktangabe. In Studien zum transepithelialen Calciumtransport im Dickdarm mit Hilfe der Ussing-Kammertechnik zeigte sich, daß ein Calciumkonzentrationsgradient (mukosal > serosal), die Anwesenheit von kurzkettigen Fettsäuren und die Anwesenheit von intakten Fruktanen die Calciumaufnahme erhöhen. Im Gegensatz zu diesen akuten Effekten führte die Verabreichung der Fruktane an Ratten über drei Wochen jedoch zu keinen signifikanten Veränderungen des transepithelialen Calciumtransports in den Ussing-Kammerstudien. Wurden die transepithelialen Fluxe jedoch auf die gesamte – und nach Fruktangabe vergrößerte – Caecumoberfläche bezogen, ergab sich nahezu eine Verdoppelung der transepithelialen Netto-Calciumresorption. Die Transkriptomanalyse des Dickdarmgewebes mittels DNA-Mikroarrays identifizierte vor allem eine Reihe von regulierten Genen, die mit der Zellzykluskontrolle in Verbindung stehen. Ausgewählte Gene, deren Proteine am trans- und parazellulären Calciumtransport beteiligt sind, wurden mit real-time RT-PCR analysiert und zeigten vor allem erhöhte mRNA-Spiegel in zumindest einem der Dickdarmsegmente bei Tieren nach Fruktangabe.

Diese Befunde belegen, daß inulin-ähnliche Fruktane die intestinale Calciumresorption im Dickdarm erhöhen, primär aufgrund a) einer erhöhten Calciumverfügbarkeit, b) einer Vergrößerung der absorptiven Oberfläche, c) erhöhter transepithelialer Calciumtransportraten in Anwesenheit von Fruktanen und ihren Metaboliten, den kurzkettigen Fettsäuren, und d) erhöhter mRNA-Spiegel von Proteinen, die mit Calciumtransportwegen in Verbindung stehen. Da die Wirkungen der Fruktane auf die Gewebearchitektur und die Mineralverfügbarkeit nicht spezifisch für Calcium sind, kann auch die Resorption von anderen Mineralstoffen – wie hier für Magnesium und Zink gezeigt – erhöht werden.

SUMMARY

Inulin-type fructans in a diet are selectively fermented by the large intestinal microflora which causes a multitude of effects that are considered as beneficial for human health and well-being. One of these well documented actions is an increased intestinal calcium absorption, similarly observed in experimental animals and in humans. Since the underlying mechanisms are not yet understood, various *in vivo* and *in vitro* experiments with rats were conducted to elucidate the molecular actions of these fructans on mineral homeostasis.

The ingestion of inulin-type fructans to rats in the presented studies resulted in an increased intestinal apparent absorption, whole body retention (balance), and accumulation of calcium in femur. Similar effects were also observed for magnesium and zinc. Feeding these fructans increased also markedly the caecal absorptive surface, enhanced caecal and colonic mucosal mass, and increased caecal and colonic intraluminal contents. Analysis of the contents revealed huge increases in soluble and ionised calcium pools in rats fed the fructans. Studies on the transepithelial calcium transport in the large intestine conducted in the Ussing chambers revealed that a calcium concentration gradient (mucosal > serosal), the presence of short-chain fatty acids, and the presence of intact inulin-type fructans increased the transepithelial calcium transport. Though, feeding these fructans to rats for three weeks showed no significant alterations of the transepithelial calcium transport in the Ussing chamber experiments. However, when based on the total absorptive caecal surface – which was enlarged by the fructan feeding – net calcium absorption was doubled. Transcriptome analysis performed in large intestinal tissue samples by DNA microarrays, identified mostly regulated genes that are linked to cell cycle control. Selected genes encoding proteins involved in transcellular and paracellular calcium transport routes were analysed by real-time RT-PCR and revealed mostly increased mRNA levels in at least one of the large intestinal segments from fructan-fed animals.

The present findings strongly suggest that inulin-type fructans increase the large intestinal calcium absorption primarily by a) an increased calcium availability, b) enlarging the apparent absorptive surface area, c) increasing transepithelial calcium transport directly in the presence of inulin-type fructans and their metabolites (short-chain fatty acids), and d) increased mRNA levels of proteins related to calcium transport pathways. Since some of the effects like the intestinal tissue architecture and mineral availability are not calcium specific, inulin-type fructans can increase intestinal absorption of other minerals, too, as shown here for magnesium and zinc.

Der Anfang ist der wichtigste Teil der Arbeit.

Plato (427 - 348 od. 347 v. Chr.)

1. INTRODUCTION

1.1. INULIN-TYPE FRUCTANS

Inulin-type fructans have been found in more than 36 000 plant species and are after starch the most plentiful carbohydrates occurring in nature (Carpita *et al.* 1989). Inulin is a polydisperse substance with linear chains of β(2→1) bound fructose monomers with a terminal glucose molecule. The number of the fructose monomers is typical for the plant in which inulin occurs. In the chicory root the chain length of inulin varies between 3 and 65 molecules with an average degree of polymerisation (DP) of 10. Short-chain fructans are termed fructooligosaccharides or oligofructose whereby the latter was introduced as a synonym for fructooligosaccharides by Orafti in 1989 (Coussement 1999). In most cases oligofructose refers to a product obtained by partial enzymatic hydrolysis of chicory inulin and comprises chains with 2 to 8 fructose moieties and average DP of 4, whereas fructooligosaccharides describes structures produced from a sucrose molecule elongated with one to three fructose units.

As inulin-type fructans are widely distributed in nature they have been constituents of human diet as long as man eats plants. Particularly rich in these fructans (g/100 g) are Jerusalem artichoke (31.5), dandelion greens (24.3), garlic (17.5), leeks (11.7), onions (8.6), asparagus (5.0), wheat (4.8), and globe artichoke (4.8) (van Loo *et al.* 1995). In Western Europe the daily intake of inulin-type fructans is estimated between 2 and 12 g per person (Roberfroid *et al.* 1993). American diets provide on average 5 g per day and person with wheat (70%) and onions (23%) as the main food sources (Moshfegh *et al.* 1999). Historically, the fructan intake was much higher than in the industrial nations nowadays because people consumed more tuberous plants. The fructan-rich Jerusalem artichoke was the main source of dietary carbohydrates in Western Europe up to the 18th century when it was superseded by the fructan-low potato. Australian aborigines ate predominantly the fructan-rich murnong, *Microseris lanceolata*, as the main vegetable and thus reached fructan intakes of 200 to 300 g per day (Boeckner *et al.* 2001).

Inulin was discovered by Rose in 1804 in a boiling water extract from the roots of *Inula helenium* (Boeckner *et al.* 2001). The large industrial production of inulin started not until the late 20th century. Chicory root (*Cichorium intybus*, fig. 1) which contains around 70% inulin

in dry matter is the preferred source for industrial extraction of inulin. The production process involves hot water extraction, refining, evaporation, and spray-drying and is similar to the technology used in sugar and starch industries. Inulin and oligofructose are legally classified as food or food ingredients in all EU countries and are used in a variety of foods and drinks. Their technological advantages are a slight sweetness without any off-flavour or aftertaste and a water binding capacity with an impact on viscosity. For their technological and nutritional advantages, inulin and oligofructose are widely used as sugar and fat replacers, fibres and prebiotics, in synergy with sweeteners, and for foam stability, texture and melting, emulsion stability, and moisture retention in foods like dairy products, ice-creams, table spreads, baked goods, and others (Franck 2002).

Fig. 1 Chicory leaves and root (*Cichorium intybus*).

In the human body inulin-type fructans are not hydrolysed by the small intestinal enzymes (Ellegard *et al.* 1997) and are not recovered in faeces (Molis *et al.* 1996). Thus, they are totally fermented by the large intestinal microflora to their major products short-chain fatty acids (acetate, propionate, butyrate) (Campbell *et al.* 1997), lactate (Le Blay *et al.* 1999), and gasses (CO_2, H_2, in some cases CH_4) (Roland *et al.* 1995, Brighenti *et al.* 1999). The short-chain fatty acids are in turn utilised by the mucosa as energy substrates or are delivered into the blood. This fermentation provides an energy yield of around 6 kJ/g (Roberfroid 1999) which is considerably lower than from digestible carbohydrates. The fructans were shown to stimulate the growth of bifidobacteria (Gibson *et al.* 1995) increasing therefore the bacterial biomass which in turn increases faecal water contents and stool frequency (Castiglia-Delavaud *et al.* 1998). This fermentation in the large intestine leads to a multitude of physiological effects in animals and humans. Due to the bifidogenic effects, inulin-type fructans are also used in infant formulas which mimics the effects of human milk oligosaccharides (Vandenplas 2002). The administration of inulin-type fructans was shown to decrease serum triglycerides in animals and also in some but not all human studies (Williams and Jackson 2002). The number of mammary tumours in female rats fed these fructans was decreased (Taper and Roberfroid 1999) and anticarcinogenic properties in colon were also reported (Pool-Zobel *et al.* 2002). Local and systemic immunomodulatory effects have been described, too (Roller *et al.* 2004). Fairly consistent observations were made on an increased mineral intestinal absorption, increased bone mineral content, and improved bone architecture

after the administration of non-digestible oligosaccharides (Scholz-Ahrens and Schrezenmeir 2002). Although the fructan fermentation may cause some discomfort in sensible persons depending on the dosage, fructan ingestion appears to be beneficial for intestinal health and is considered as a feel-good factor in human well-being.

1.2. CALCIUM METABOLISM

Calcium is the 5th most abundant element in the earth's crust (Dixon 2002) and is found mostly as limestone ($CaCO_3$), gypsum ($CaSO_4 \cdot 2H_2O$), anhydrite ($CaSO_4$), fluorspar (CaF_2), apatite ($Ca_5(PO_4)_3(F, Cl, OH)$), and dolomite ($CaMg(CO_3)_2$) (Gutmann and Hengge 1990). During raining, water reacts with the carbon dioxide from the air and the carbonic acid solubilises calcium making it available for absorption by plants, animals, and humans. Thus, calcium is ubiquitous in human diet and plays important roles in many physiologic functions. The adult human body contains about 1200 g calcium where 99% is found in mineralised tissues, such as bones and teeth, mainly as calcium phosphate together with a small component of calcium carbonate. The remaining 1% is found in intra- and extracellular fluids (Cashman 2002). Intracellular calcium, particularly the cytosolic free calcium concentration, is an important second messenger and cofactor for proteins and enzymes regulating key cellular processes, i.e. neurotransmission, motility, hormone secretion, and cellular proliferation. Extracellular calcium serves as a cofactor for adhesion molecules, clotting factors, and other proteins, regulates neuronal excitability, and is an essential part of the mineral phase of bone (Brown 2000).

During the first years of human life the deposition rate of calcium in bone is very high and the bone calcium mass reaches its maximum in the range of 20 and 30 years of life. In the following years the bone calcium mass decreases gradually in men and abruptly in women after menopause (Bronner and Pansu 1999). The skeleton provides thus a large reservoir of mineral ions (e.g. calcium and phosphate) that can be mobilised when needed. The increasing recognition of the importance of dietary calcium as the ultimate source for the manifold biological roles of calcium led some to call it a "superstar of nutrients" (Brown 2000).

High calcium contents in foods (mg/100 g) are found in hard cheeses like Emmental (1100), sesame seeds (783), soft cheeses like Camembert (490), hazelnut (226), kale (212), yoghurt (120), milk (120), cream cheese (79), leek (63), broccoli (58), given as an example (Souci *et*

al. 2000). According to the German Nutrition Survey in 1998, the daily mean calcium intake accounts for 1250 mg in men (n = 1763) and 1150 mg in women (n = 2267) both aged 18 to 79 years (Mensink and Beitz 2004) and is thus higher than the recommended daily allowance of the German Society of Nutrition with 1000 mg for adult men and women (DGE 2000). Similar data were obtained in the United Kingdom from the EPIC-Oxford survey in the 1990s which is part of the European Prospective Investigation into Cancer and Nutrition (EPIC). Men and women aged 20 to 97 years had a daily mean calcium intake of 1042 mg for men (n = 12 969) and 988 mg for women (n = 43 582) (Davey *et al.* 2003).

In the human body, calcium from foods and beverages is absorbed in the intestine via two different pathways: a transcellular and a paracellular route (fig. 2). The transcellular pathway comprises a calcium entry at the apical membrane of intestinal epithelial cells, calcium movement through the cytosol from the apical to the basolateral membrane, and finally an extrusion step at the basolateral membrane into the extracellular fluid. The transient receptor potential vanilloid subfamily member 6 (TRPV6), also named calcium transport protein-1 (CaT1) or epithelial calcium channel-2 (ECaC2), appears to represent the major apical calcium entry step in the intestine and is most abundantly expressed in the duodenum. This channel is constitutively active, is not voltage- or ligand-gated, and has a K_m value in millimolar or submillimolar range. In the intestinal lumen, the calcium concentration is in the millimolar range but inside the absorptive cell it is only around 50 to 100 nM which results in more than a 10 000-fold transmembrane concentration gradient. In addition, with the cell interior negative, a resulting membrane potential of around 30 mV allows calcium to move down its electrochemical gradient through the apical channel protein into the cell without a direct energy expenditure. Since a sustained increase of intracellular calcium may cause cell death, the channel activity is regulated by the local calcium concentration beneath the apical membrane which causes a calmodulin binding to TRPV6 at higher Ca^{2+} levels and a closure of the channel. In human TRPV6, protein kinase C is involved as well and phosphorylates the calmodulin binding site of TRPV6 and prevents thus calmodulin binding. Another possibility to prevent toxic calcium concentration

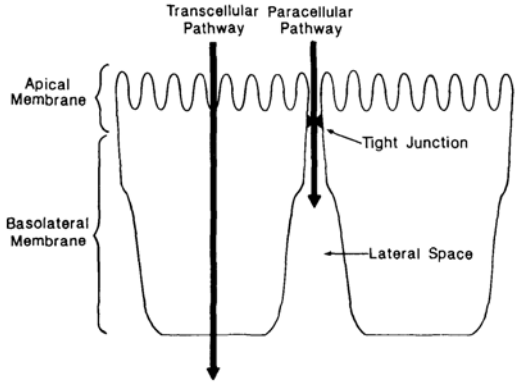

Fig. 2 Schematic representation of the transcellular and paracellular calcium transport pathways across the intestinal epithelium (from Ballard *et al.* 1995).

inside the cell is to bind calcium to proteins like the vitamin D-dependent calbindin-D9k (CaBP9k). CaBP9k functions also as a transport protein for calcium through the cytosol from the apical to the basolateral membrane. Calmodulin is another soluble calcium-binding protein, is not vitamin D-dependent, and contributes to the cytosolic calcium transport with around 10% of that of CaBP9k. Diffusion of free calcium ions through the cytosol is also possible but is very low as compared to its transport via CaBP9k. The intracellular calcium concentration near the basolateral membrane is in the nanomolar range but is around 1.2 mM (free calcium) in the extracellular fluid. Thus, calcium has to be extruded from the cell against a huge concentration gradient and an unfavourable electrical gradient and this is accomplished by the plasma membrane calcium ATPase (PMCA1b) (Bronner 1987, Peng *et al.* 2003). Another extrusion process may be represented by the exchange with sodium by the Na^+/Ca^{2+} exchanger while the sodium gradient is maintained by the Na^+/K^+ ATPase. The transport capacity of the Na^+/Ca^{2+} exchanger was shown to be 20% of that of the PMCA1b (Wasserman *et al.* 1992). In summarising the transcellular route: calcium enters the epithelial cell via an apical channel, is transported trough the cytosol by calcium binding proteins, and is extruded by an energy-requiring process across the basolateral membrane. An important question is which of these three steps is rate-limiting in overall calcium movement. As shown in vitamin D receptor-null mice, the mRNA level of TRPV6 was strongly decreased while the mRNA levels for CaBP9k and PMCA1b were slightly decreased and not altered, respectively. This suggested that the apical entry is the rate-limiting step in calcium transport (Peng *et al.* 2003). In addition to the active transcellular route there was a vesicular calcium transport observed with a calcium release at the basolateral membrane via exocytosis. However, the contribution of this vesicular transport route to the total transcellular calcium transport could not be evaluated (Wasserman *et al.* 1992).

The second pathway of calcium movement across the intestinal epithelium is the paracellular route. Calcium here enters the epithelium through the tight junctions into the intercellular space and reaches thus the extracellular fluid. Tight junctions are narrow belts that circumferentially surround the upper (apical) part of the lateral surfaces of adjacent epithelial cells to create fusion points or "kisses" (Gasbarrini and Montalto 1999). They have integral membrane components and membrane-associated proteins. Occludins, claudins, and junctional adhesion molecules (JAM) are integral membrane proteins involved in the formation of tight junction strands while the most representative membrane-associated proteins are the zonula occludens proteins ZO-1, ZO-2, and ZO-3. Tight junctions play

essential roles in the maintenance of a physical barrier between external and internal environments in the body (Hirabayashi *et al.* 2003). The postulated character of the tight junction complex consists of size- and charge-limiting pores within the junctional strands which enables a specific permeability. Thus an increase in the number of small pores would increase the electrical conductance which does not necessarily mean that the overall permeability is enhanced because large molecules cannot pass the pores. Protein kinase A may increase the ionic conductance of tight junctions without changing the barrier function for large molecules whereas protein kinase C may increase the permeability for large molecules (Karczewski and Groot 2000). Immediately below the tight junctions, the adherens or intermediate junctions are coupled to a circumferential actin-myosin ring. This perijunctional actin ring is a dynamic structure and may transmit cytoskeletal changes to the junctional complex. Further constituents of the junctional complex are desmosomes and gap junctions but the most important component of the junctional complex for restricting the passage of small solutes through the paracellular pathway are the tight junctions (Ballard *et al.* 1995).

The transcellular calcium pathway is predominant in the duodenum, is regulated by vitamin D, and is a saturable process. The paracellular calcium pathway, however, is a concentration dependent diffusional process throughout the length of the intestine. At low calcium concentrations in the chyme, much of the calcium in the duodenum is absorbed by the active, transcellular route while relatively little calcium will be available for paracellular absorption in the distal jejunum and ileum. On the other hand, if the calcium concentration in the chyme is high, most of the calcium is absorbed through the paracellular route, largely in the distal portions of the small intestine due to the sojourn time of the chyme in these intestinal segments. The active, transcellular calcium pathway is down-regulated in such cases but plays an important role when only low dietary calcium is provided (Bronner 2003).

When calcium is taken up across the intestinal epithelium it is present in the extracellular fluid and is available for all target cells. In the blood, calcium is found in ionised form (1.1 – 1.3 mM), bound to proteins (0.9 – 1.1 mM), and in other complexes (0.2 – 0.3 mM) (Weaver 1990). Because of the diverse extra- and intracellular roles of calcium, the calcium concentration in extra- and intracellular fluids is highly regulated. The parathyroid gland is the key organ in regulating extracellular calcium homeostasis. Parathyroid cells sense a small fall in the plasma calcium ion concentration from the normal value mediated by the calcium-

sensing receptor (CaR or CaSR) and secrete the parathyroid hormone (PTH). Secreted PTH then acts on the kidney to activate the vitamin D system. PTH also enhances tubular calcium reabsorption and inhibits phosphate reabsorption in the kidney, and acts in concert with vitamin D in bone to enhance bone resorption, allowing more calcium and phosphate ions to appear in plasma. This results in a normalised calcium concentration in blood which exerts a negative feedback on the parathyroid cells with a suppression of PTH secretion to a basal level. Nevertheless, PTH alone may not be sufficient for the maintenance of the extracellular calcium ion concentration as it was shown that PTH alone failed to increase reabsorption of calcium in the kidney in vitamin D-deficient rats (Fukugawa and Kurokawa 2002). Thus, PTH acts together with a second calcium regulating hormone, the 1,25-dihydroxychole-calciferol (calcitriol) which is produced from 25-hydroxycholecalciferol by the mitochondrial enzyme 1α-hydroxylase in the kidney. The genomic actions of calcitriol involve its binding to the vitamin D receptor (VDR) which then heterodimerises with the retinoid X receptor (RXR) and this dimer binds to response elements on target genes. Also non-genomic rapid effects of calcitriol have been described including a stimulation of protein kinase C, an increase in intracellular calcium, and an activation of mitogen activated protein kinase (MAP-kinase). Calcitriol enhances the intestinal calcium absorption, increases bone turnover, and seems to inhibit PTH synthesis and secretion from the parathyroid gland (Goltzman *et al.* 2004). VDR-null mice showed a 10-fold reduction in the mRNA levels of the intestinal apical calcium channel TRPV6 in the duodenum on a normal-calcium diet and CaBP9k was reduced by 3-fold whereas the calcium pump PMCA1b mRNA level remained unchanged (Peng *et al.* 2003). This suggests that the vitamin D system is essential for regulating transcellular calcium transport pathways in the intestine. Besides PTH and calcitriol, a third calcium regulating hormone is involved which is calcitonin secreted from the thyroid gland. Calcitonin inhibits the osteoclastic resorption of bone, enables bone mineralisation in synergism with calcitriol, and decreases calcium and phosphate reabsorption in the kidney. Thyreocalcitonin acts thus on a fine-tuning level with a very short half-time in blood of less than 1 h in order to prevent hypercalcaemia (Kurbel *et al.* 2003).

To assess the human calcium status, several approaches can be utilised. Since 99% of total body calcium resides in the skeleton and the turnover rate of the skeleton is low (i.e. several years), bone measurements are best to evaluate adequate long-term calcium status. The mineral content of specific bones or of the total body can be measured, the latter correlates well with the total body calcium content. The calcium concentration in blood is not an

appropriate calcium status indicator because this concentration is generally highly regulated. The urinary calcium could be a better indicator but the calcium output varies with the calcium intake, dietary protein, urine output, calcium requirement, and hormonal status. These factors complicate the interpretation of urinary calcium analysis. Thus, appropriate methods to determine calcium absorption and the short-term retention in humans are balance studies, *in vivo* perfusion techniques, and isotopic tracer techniques. During a balance study, the dietary calcium intake is measured as well as the calcium output with faeces and urine. This enables to calculate the apparent absorbed calcium, i.e. true absorption minus calcium secretion into the intestinal lumen, and the total body retention of calcium (Weaver 1990).

1.3. AIM OF THE STUDY

Since the early 1990s, the inulin-type fructans have been under extensive investigations with respect to many local and systemic effects on health in mammals (see overview in 1.1. Inulin-type fructans). One prominent effect was an increased intestinal apparent calcium absorption in rats that was independent of the type of applied fructan preparation like fructo-oligosaccharides (Ohta *et al.* 1994, Ohta *et al.* 1995b, Suzuki *et al.* 1998, Takahara *et al.* 2000), oligofructose (Brommage *et al.* 1993, Delzenne *et al.* 1995, Scholz-Ahrens *et al.* 2002), inulin (Levrat *et al.* 1991, Delzenne *et al.* 1995, Lopez *et al.* 2000, Younes *et al.* 2001, Coudray *et al.* 2004), or a blend of inulin and oligofructose (Coudray *et al.* 2003, Raschka and Daniel 2004). Interestingly, in 1975 it had already been shown that slowly absorbed saccharides like mannose, sorbose, lactose, fructose, and inulin resulted in higher intestinal calcium absorption than when free glucose or glucose easily released by intestine enzymatic activity were given (Lorinet 1975). Despite these early observations, the increasing effect of inulin-type fructans on calcium absorption was not expected because it was assumed that dietary fibres in general inhibit intestinal mineral absorption as several studies had shown such negative effects: carrageenan and agar-agar reduced the mineral absorption (Ca, Fe, Zn, Cu, Cr, Co) in rats (Harmuth-Hoene and Schelenz 1980); cellulose and pectin ingestion decreased both sodium and chloride net fluxes in rat jejunum (Schwartz *et al.* 1982); iron absorption was reduced by high-fibre meals in humans (Cook *et al.* 1983); different dietary fibres lowered serum and tibia zinc levels in chicks (van der Aar *et al.* 1983); and wheat bran and barley husk depressed absorption of zinc and calcium in rats (Donangelo and Eggum 1986). However, no major impact of dietary fibres on the mineral balance (Ca, Mg, Mn, Fe, Co, Zn) in men was reported (Behall *et al.* 1987) and it was stated that the observed inhibitory

effect in particular on iron absorption may be due to the phytate rather than the fibre content (Brune *et al.* 1992). Nevertheless, the increase of intestinal calcium absorption by inulin-type fructans was consistently found in various studies in rats and this effect is also confirmed in humans (Coudray *et al.* 1997, van den Heuvel *et al.* 1999, Griffin *et al.* 2002). Some of the studies in humans, however, failed to show a significant difference (van den Heuvel *et al.* 1998, Griffin *et al.* 2002, Tahiri *et al.* 2003) which may mainly be caused by the basic diet and its intrinsic fructan content as proposed by Raschka and Daniel (2004).

It is generally accepted that the highest proportion of calcium is absorbed in the ileum (Escoffier *et al.* 2000, Bronner 2003). Though, during the first experiments with inulin-type fructans investigating the effects on intestinal calcium absorption, a hypothesis was formulated that included the large intestine into the overall intestinal calcium absorption (Ohta *et al.* 1995a, Trinidad *et al.* 1996). This was a new concept and was supported by the following findings: Inulin did not affect the calcium absorption in the small intestine as shown in ileostomy patients with almost all inulin (90%) recovered in the ileal effluent (Bach Knudsen and Hessov 1995, Ellegard *et al.* 1997). Inulin-type fructans thus reaches the large intestine in intact form and were shown to be fermented by the large intestinal microflora resulting in an increased number of bifidobacteria and fermentation products such as short-chain fatty acids (Campbell *et al.* 1997) with no fructans recovered in faeces (Molis *et al.* 1996). This suggested that inulin-type fructans act specifically on the calcium absorption in the large intestine and that the large intestine may contribute to the overall intestinal calcium absorption. This increased intestinal calcium absorption caused by fructans is thought to be beneficial as it prolongs the period of gradual bone resorption during adulthood and thus contributes to the prevention of early onset osteoporosis.

As the increasing effect of inulin-type fructans on the calcium absorption was observed, the question arose how these fructans may act on the intestine to allow more calcium to be absorbed. Several hypothesis were formulated. The most plausible is that fructans are metabolised by the bacteria in the large intestine leading to an increased production of short-chain fatty acids, mainly acetate, propionate and butyrate (Campbell *et al.* 1997) which in turn lowers the luminal pH which increases mineral solubility (Lopez *et al.* 1998) and which raises the mineral gradient between the luminal and serosal side allowing passive mineral transport to increase. Additionally, short-chain fatty acids, especially butyrate, serve as a fuel for mucosal cells and stimulate cell proliferation (Blottiere *et al.* 2003) which in turn could

increase the absorptive surface area of the large intestine. A direct coupling of calcium transport with colonic uptake of short-chain fatty acids has also been proposed and experimental data support this coupling theory because the luminal presence of short-chain fatty acids increased the transepithelial calcium transport in rat caecum and colon (Mineo *et al.* 2001b). In addition fermentation of fructans could increase intestinal calcium absorption also by effects on gene transcription of proteins involved in mucosal calcium binding and sequestration. This is supported by findings of Ohta *et al.* (1998) reporting an increased level of CaBP9k in the large intestine of rats receiving fructans in their diet.

To investigate the mechanisms underlying the action of inulin-type fructans on the intestinal calcium absorption, several *in vivo* and *in vitro* approaches were combined. As the experimental model the male rat was chosen although the gastrointestinal tract differs in some respect when compared to that of humans. For the fructan administration *in vivo*, oligofructose (Raftilose®P95) was used to enable comparisons with published data but in the majority of the experiments a 1/1 blend of oligofructose and long-chain inulin (Raftilose® Synergy1) was used. This combination was shown to be physiologically more efficient as the shorter fructan chains are fermented more rapidly in the caecum and proximal colon whereas the longer chains reaches also the distal parts of the large intestine (van Loo 2004). In a first feeding experiment with rats, the effect of inulin-type fructans on the intestinal apparent calcium absorption was studied to allow a comparison with findings reported in literature. Beside calcium, the intestinal apparent absorption, body retention, and femur contents were also determined for the minerals magnesium and zinc. Selected macroscopic tissue parameters of various large intestinal segments were determined in animals receiving fructans for three weeks as compared to controls and caecal contents were analysed with respect to total, soluble, and ionised calcium. Moreover, *in vitro* and *ex vivo* transepithelial calcium transport measurements in large intestine were conducted using the Ussing chamber technique. The impact of several variables on net calcium flux was systematically investigated in view of acute and chronic effects of inulin-type fructans. Gene expression analysis were performed in large intestinal tissue samples after the fructan administration to rats using DNA microarrays to obtain a first insight into the genetic response and real-time RT-PCR for specific genes encoding proteins that relate to transepithelial calcium transport.

Freude an der Arbeit läßt das Werk trefflich geraten.

Aristoteles (384 - 322 v. Chr.)

2. MATERIALS AND METHODS

This chapter is organised in the same way as the Results section to enable a faster link of the experimental design and the obtained results. For all experiments, animals were maintained in accordance with the national guidelines for the care and use of laboratory animals.

2.1. INTESTINAL MINERAL ABSORPTION AND BODY MINERAL RETENTION

To determine intestinal apparent mineral absorption and body mineral retention as well as other parameters as described below, a series of different feeding experiments was conducted with male Sprague-Dawley rats (Charles River, Sulzfeld). Rats had an initial body weight of approx. 200 g, in the later experiments only 100 g, and were housed individually in metabolic cages in a temperature- (22 °C) and humidity-controlled (50%) room. Animals received 15 g/d of given diets to minimise variations in body weight gain between groups but they had free access to deionised water. For the first two feeding experiments (FE-2 and FE-3), a standard diet was used (No. 1321, powder, Altromin, Lage) composed of shredded wheat, barley and corn containing 19% protein, 4% fat, 50% carbohydrate, 6% dietary fibre, 0.9% Ca, 0.7% P, 0.2% Mg and 0.007% Zn according to the manufacturer. The basal diet for the later feeding experiments (FE-4 to FE-7) was a semi-synthetic diet (No. C 1034 mod., powder, Altromin, Lage) containing 17% casein, 6% corn oil, 60% corn starch, 5% cellulose, 0.5% Ca, 0.4% P, 0.05% Mg and 0.003% Zn according to the manufacturer. To both basal diets 10% (w/w) Raftilose®P95 (oligofructose) or Raftilose®Synergy1 (a 1:1 blend of oligofructose and long-chain inulin) as supplied by Orafti (Tienen, Belgium) was added for the fructan groups. Since fermentation of fructans yields around 20 to 25% of energy of digestible carbohydrates (mainly in form of short-chain fatty acids), the control groups received on top of the basic diet 2% maltodextrine (Orafti, Tienen) for isoenergetic substitution. In the adaptation period of around 6 days the fructan content in diet was gradually increased and thereafter animals were fed a 10% fructan diet for 15 to 28 days depending on the experimental protocol (see legend to table 1). Metabolic cages were metal-free and had mesh bottoms to enable the collection and separation of faeces and urine by a plastic mesh (fig. 3) as good as possible. The 24 h excretions of faeces and urine were collected separately either for the whole experimental period or only parts of it, they were pooled (as 3 day samples) and kept frozen until analysis. To assess mineral retention in bone,

rats from feeding experiment FE-5 were sacrificed after the feeding period and femora were removed and frozen until analysis.

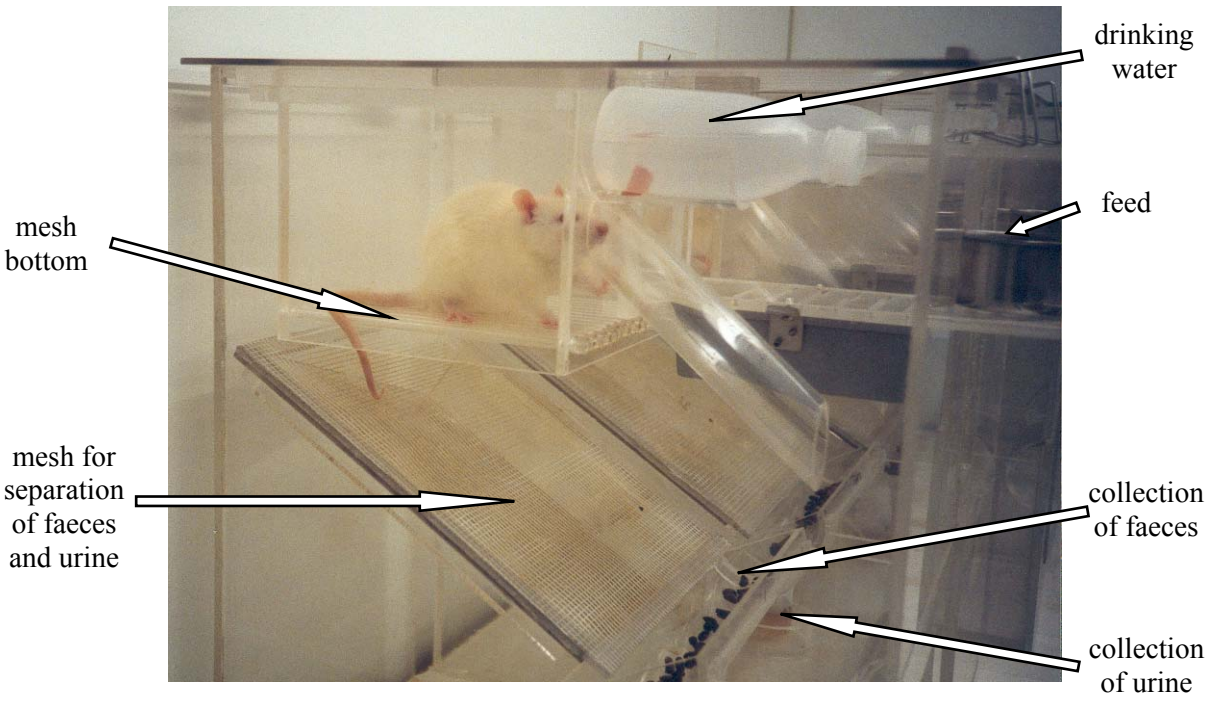

Fig. 3 Metabolic cage design enabling the quantitative determination of feed intake and the collection and separation of faeces and urine.

Diet and biological samples (i.e. faeces and total femur) were ashed at 580 °C for 48 h, the ash was then dissolved in 6 M HCl (Sigma-Aldrich, Seelze) and filtered through filter paper (No. 1507, Schleicher & Schuell, Dassel). These ashed samples and aliquots of urine were measured after appropriate dilution by flame atomic absorption spectrometry (Model 5100, Perkin-Elmer, Rodgau). For calcium and magnesium, samples were diluted with 0.5% lanthanum ($LaCl_3 \cdot 7H_2O$, Merck, Darmstadt) in 0.6 M HCl. Samples for assaying zinc were diluted only with 0.6 M HCl. Calcium was measured at 422.7 nm, magnesium at 285.2 nm, and zinc at 219.9 nm. Apparent mineral absorption was calculated as the dietary mineral intake minus faecal mineral excretion. Mineral balance is defined as the dietary mineral intake minus faecal and urinary mineral excretion.

2.2. MACROSCOPIC CHANGES IN INTESTINAL TISSUES AND ANALYSIS OF CAECAL CONTENTS

Male Sprague-Dawley rats with an initial body weight of 139 ± 8 g were fed a semi-synthetic diet over 21 days containing 10% (w/w) Raftilose®Synergy1 for the fructan group and 2% maltodextrine for the control group. They were housed in metabolic cages as described above

and had a final body weight of 239 ± 15 g in control and 244 ± 14 g in Synergy1 group. On day 22, rats were sacrificed 3 h after the usual feeding time to obtain sufficient amounts of caecal contents for further analysis. After opening the abdominal cavity, the mean pH of caecal contents was determined *in situ* with a pH microelectrode (InLab 423, Mettler Toledo, Giessen) through a small incision in the caecal wall. Then the entire intestine was removed and the lengths, total weights, and wall weights of small intestine, caecum, and colon were determined. Samples of caecal content were frozen at –20 °C. Tissue samples from caecum were cut open longitudinally and plotted onto a sheet of paper. The area was cut out and weighed, and based on a calibration the macroscopical (serosal) tissue surface area was determined.

Caecal contents were thawed and centrifuged at 15 000 x g at 4 °C for 10 minutes. The supernatant was then used to determine ionised calcium by a calcium sensitive electrode (Mettler Toledo, Giessen) whereas soluble calcium in supernatant and total calcium in ashed caecal contents were determined by flame atomic absorption spectrometry as described above.

2.3. TRANSEPITHELIAL CALCIUM TRANSPORT IN LARGE INTESTINE

For this series of experiments on transepithelial calcium transport employing the Ussing chamber technique (fig. 4), male Sprague-Dawley rats were sacrificed, caecum and colon were removed, cut open along the mesenteric line, rinsed with ice-cold serosal buffer solution and sections of caecum, proximal and distal colon were mounted into Ussing chambers (Physiologic Instruments, San Diego, CA, USA, modified in house). The exposed epithelial surface area was 1.03 cm². The chambers were filled with 5 ml of a mucosal and serosal buffer solution, gassed with carbogen (95% O_2 and 5% CO_2) and maintained at 37 °C. Via KCl-agar bridges on both sides of the mounted tissue the transepithelial potential difference (PD) was determined and clamped at 0 mV using current (I) electrodes with platinum tips. In definite time intervals the short-circuit was interrupted by the operator

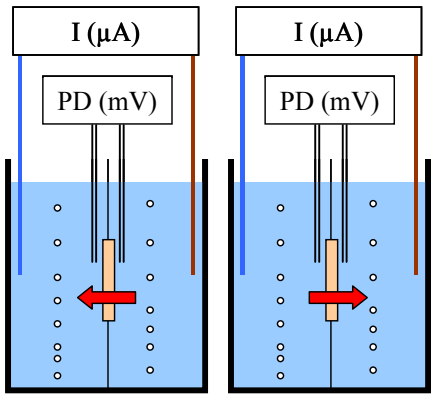

Fig. 4 Schematic illustration of the calcium flux measurement in Ussing chambers. Intestinal tissue is mounted between the mucosal and serosal chamber compartment enabling the determination of the calcium flux in the direction from mucosa to serosa and in a second chamber from serosa to mucosa. (For more details see text beside).

(model VCC MC6, Physiologic Instruments, San Diego, CA, USA) and the tissues were clamped to +2 and –2 mV with the required current enabling the calculation of tissue conductance. ^{45}Ca (ICN Biomedicals, Eschwege) was added to the mucosal or serosal side and samples were taken from this compartment to determine the specific activity. After 30 minutes for equilibration, the appearance of ^{45}Ca on the other tissue side was determined by taking samples every 15 minutes for a total of 45 minutes and replacing them with unlabelled buffer solution. The samples were mixed with a scintillation cocktail (Rotiszint eco plus, Roth, Karlsruhe) and measured in a β-counter (Wallac, Model 1450 Microbeta, PerkinElmer, Cologne). All experiments were performed with paired tissues (for mucosal to serosal, ms, and serosal to mucosal, sm fluxes) from the same intestinal segment – caecum, proximal colon, distal colon – allowing unidirectional calcium fluxes to be determined from the same animal and tissue segment. Net calcium fluxes ($J_{net} = J_{ms} – J_{sm}$) were calculated using standard equations (Schultz and Zalusky 1964) and are presented as the mean of three consecutive 15 minutes periods.

2.3.1. ACUTE EFFECTS OF CALCIUM CONCENTRATION, PH, SCFAS, AND INTACT FRUCTANS

To determine the impact of various luminal factors on net calcium transport in large intestine, the composition of the mucosal medium for Ussing chambers was changed and bidirectional calcium fluxes were determined as described above. For this series of experiments male Sprague-Dawley rats on a standard diet (No. 1324, pellets, Altromin, Lage) with a mean body weight of 291 ± 16 g were used. First, the effects of three different mucosal calcium concentrations were analysed by increasing calcium from 0.1 to 1.25 and 10 mM in the mucosal buffer solutions (composed of (mM) 25 $NaHCO_3$, 1.8 $Na_2HPO_4 \cdot 2H_2O$, 0.2 $NaH_2PO_4 \cdot H_2O$, 4.5 KCl, 1.0 $MgSO_4 \cdot 7H_2O$, 12 glucose$\cdot H_2O$, pH 7.4, and with 0.1/1.25/10 $CaCl_2 \cdot 2H_2O$ and 14.85/13.125/0 NaCl) while maintaining serosal calcium concentration at 1.25 mM (serosal buffer solution composed of (mM) 25 $NaHCO_3$, 1.8 $Na_2HPO_4 \cdot 2H_2O$, 0.2 $NaH_2PO_4 \cdot H_2O$, 13.125 NaCl, 4.5 KCl, 1.0 $MgSO_4 \cdot 7H_2O$, 1.25 $CaCl_2 \cdot 2H_2O$, 12 glucose$\cdot H_2O$, pH 7.4). Osmolarities in mucosal media were adjusted to isotonicity by changing NaCl concentrations. The effects of SCFAs were determined by the addition of 100 mM SCFAs to the mucosal medium (acetate, propionate and butyrate as sodium salts with the molar ratio 50:30:20 obtained from Merck, Darmstadt; Sigma-Aldrich, Munich; and Merck, Darmstadt) in the presence of 10 mM of mucosal calcium. In the serosal medium and in controls 100 mM sodium isethionate was used to isotonically replace SCFAs. In another experiment SCFAs

were given in their acidic form (Merck, Darmstadt) to the mucosal buffer solution while sodium isethionate was applied at both tissue sides increasing thus the osmolarity at the mucosal side. For assessing the role of mucosal pH, the mucosal medium with a pH of 6.0 (composed of (mM) 12 $NaHCO_3$, 1.8 $Na_2HPO_4 \cdot 2H_2O$, 0.2 $NaH_2PO_4 \cdot H_2O$, 4.5 KCl, 1.0 $MgSO_4 \cdot 7H_2O$, 10 $CaCl_2 \cdot 2H_2O$, 12 glucose$\cdot H_2O$, pH 6.0 adjusted by the addition of HCl) was used in the absence or presence of SCFAs. Finally, possible direct effects of inulin and oligofructose were studied by adding 10 g/l Raftilose®Synergy1 to the mucosal buffer solution (composed of (mM) 107 NaCl, 25 $NaHCO_3$, 1.8 $Na_2HPO_4 \cdot 2H_2O$, 0.2 $NaH_2PO_4 \cdot H_2O$, 4.5 KCl, 1.0 $MgSO_4 \cdot 7H_2O$, 10 $CaCl_2 \cdot 2H_2O$, 12 glucose$\cdot H_2O$, pH 7.4, with or without 10 g/l Raftilose®Synergy1). Since this addition did not cause considerable changes in osmolarity, no adjustment of tonicity was necessary for control and at the serosal side (equal to the mucosal buffer solution but with 1.25 $CaCl_2 \cdot 2H_2O$ and without Raftilose®Synergy1).

2.3.2. CHRONIC DIETARY EFFECTS OF INULIN-TYPE FRUCTANS

The effects of inulin and oligofructose administration on transepithelial calcium transport were investigated in male Sprague-Dawley rats in two experiments. Animals were housed individually and received a semi-synthetic diet for 21 days with 10% Raftilose®Synergy1 for the fructan group and 2% maltodextrine for control group as described above. In the first experiment rats had a mean initial body weight of 101 ± 5 g and reached a final mean body weight of 232 ± 27 g. After the feeding period rats were sacrificed, caecum and colon were removed and mounted into Ussing chambers as described above. The chambers were filled with a buffer solution at both tissue sides (composed of (mM) 107 NaCl, 25 $NaHCO_3$, 1.8 $Na_2HPO_4 \cdot 2H_2O$, 0.2 $NaH_2PO_4 \cdot H_2O$, 4.5 KCl, 1.0 $MgSO_4 \cdot 7H_2O$, 1.25 $CaCl_2 \cdot 2H_2O$, 12 glucose$\cdot H_2O$, pH 7.4). In the second experiment rats had a mean initial body weight of 140 ± 6 g and reached a final body weight of 273 ± 10 g. In the Ussing chamber different buffer solutions were used for the mucosal (composed of (mM) 25 $NaHCO_3$, 1.8 $Na_2HPO_4 \cdot 2H_2O$, 0.2 $NaH_2PO_4 \cdot H_2O$, 4.5 KCl, 1.0 $MgSO_4 \cdot 7H_2O$, 10 $CaCl_2 \cdot 2H_2O$, 12 glucose$\cdot H_2O$, pH 7.4) and serosal side (as mucosal but with 1.25 $CaCl_2 \cdot 2H_2O$ and 13.125 NaCl). 100 mM SCFAs were added into the mucosal compartment of tissues from both, control and Synergy1-fed rats. Serosal substitution was done by sodium isethionate as described above. After the calcium flux measurement the tissue from the Ussing chamber was rinsed twice with 15 mM ethylenediaminetetraacetic acid (EDTA), dried at 50 °C for 24 h, weighed, rehydrated with deionised water, and solubilised with Solvable (PerkinElmer, Cologne) at 50 °C for 21 h. In

these solubilised tissue samples, ^{45}Ca was detected in a β-counter after the addition of scintillation cocktail as described above. For these samples a quench curve correction was applied using phenolsulfonephthalein (Sigma-Aldrich, Taufkirchen) as the quench agent.

2.4. GENE EXPRESSION ANALYSIS OF INTESTINAL MUCOSA

2.4.1. CHANGES IN TRANSCRIPT LEVELS DETERMINED BY DNA MICROARRAYS

Male Sprague-Dawley rats with an initial body weight of 107 ± 7 g were housed in metabolic cages and received a semi-synthetic diet for 28 days with 10% Raftilose®P95 for the fructan group and 2% maltodextrine for control group as described above. Their final body weights were 243 ± 25 g and 245 ± 21 g for control and P95 group, respectively. After the feeding period rats were sacrificed and caecum and colon were removed and rinsed with 0.9% NaCl solution. Mucosa was scraped from everted intestinal segments (caecum, proximal and distal colon) by glass slides and frozen in liquid nitrogen. From these mucosa scrapings total RNA was isolated by RNA-wiz (Ambion, Wiesbaden), samples of three animals were pooled for each group and the RNA pools were digested by DNase I (Invitrogen, Groningen, Netherlands). The DNA-free RNA pools were submitted to reversed transcription by Superscript II (Invitrogen, Groningen, Netherlands) at 39 °C for 2 h using oligo d(T) primers and a dNTP mix where the control sample contained Cy5-labeled dCTPs and the P95 group Cy3-labeled dCTPs (Amersham, Freiburg). The remaining oligo-nucleotides were removed by the QIAquick PCR purification kit (Qiagen, Hilden). Prior to the hybridisation, the array was blocked by bovine serum albumin and sodium dodecyl sulphate (Roth, Karlsruhe) in order to reduce background caused by non-specific binding of nucleic acids to the surface of the array. Finally, the obtained cDNA pools from control as well as from P95 group were applied together to the array (Pan® rat liver array with 1166 genes, MWG Biotech, Ebersberg) and hybridised in a wet hybridisation chamber at 42 °C for approx. 20 h. The arrays were washed with sodium dodecyl sulfate in sodium citrate-sodium chloride buffer solution, dried, and scanned (Affymetrix GMS 418 Array Scanner, High Wycombe, United Kingdom). Using ImaGene software 4.2 (BioDiscovery, El Segundo, CA, USA), fluorescence of each spot was normalised for the total array fluorescence and the ratios for Cy3/Cy5 of each gene spot were calculated which are presented as the regulation factors. A regulation factor > 1 indicates an up-regulation, i.e. a higher mRNA level in samples from the Raftilose®P95 group, whereas a

regulation factor < 1 similarly indicates a down-regulation as compared to controls. Genes with regulation factors > 0.6 and < 1.6 were excluded and are considered as background.

2.4.2. CHANGES IN TRANSCRIPT LEVELS DETERMINED BY REAL-TIME RT-PCR

Selected genes were determined in their expression at mRNA levels using the real-time RT-PCR. To verify the regulation factors of selected genes obtained from DNA microarrays, the same RNA was used for real-time RT-PCR as had been used for the DNA microarrays. For further investigations, a feeding experiment was conducted with male Sprague-Dawley rats weighing initially 142 ± 6 g. They were housed individually and received a semi-synthetic diet with 10% Raftilose®Synergy1 for the fructan group and 2% maltodextrine for control group as described above. After the feeding period of 21 days – rats reached a body weight of 253 ± 12 g in control and 248 ± 24 g in the Synergy1 group – mucosa scrapings from caecum, proximal and distal colon were obtained and total RNA isolated as described above.

0.5 µg of total RNA from each animal or pool (as used for DNA microarrays) were reverse transcribed by MMLV reverse transcriptase (Promega, Mannheim) at 37 °C for 1 h using random hexamer primers and a dNTP mix (Fermentas, St. Leon-Rot). 12.5 ng generated cDNA were used for each real-time RT-PCR run in the LightCycler (Roche Diagnostics, Mannheim) as described previously (Pfaffl *et al.* 2002a). The temperature setting was: 95/60/72/79 °C for 15/10/20/5 seconds (except for occludin and NCX with 95/63/72/79 °C). The relative amount of target mRNA normalised to 18S rRNA (X01117) was calculated according to Pfaffl *et al.* (2002b) and is expressed as the regulation factor indicating an increased or decreased transcription in Synergy1 at factors > 1 or < 1 compared to control. Primer design was done with regard to primer dimer formation, self-priming formation and primer melting temperature using the LightCycler Probe Design Software Version 1.0 (Roche Diagnostics, Mannheim) and a 'blast search' in the published sequence database GenBank (www.ncbi.nlm.nih.gov/BLAST) revealed that primers are gene-specific. Based on these criteria the following primers were used: ApoA-I forward 5'-AGC GGC AGA GAC TAT G-3' and reverse 5'-CCT CCT CGT TCC ACT T-3' (position in the open reading frame: forward 142 – 157 and reverse 403 – 388), Aqp8 5'-AGA CGC CGA TGT GTA G-3' and 5'-CCA CCG CTG ATG TTC C-3' (14 – 29, 272 – 257), Cldn3 5'-GGG AGT GCT TTT CCT GT-3' and 5'-GTC CTT ACG GTC ATA GGC-3' (360 – 376, 651 – 634), CA-III 5'-TGG

ACC CTA CCG ACT T-3' and 5'-GCC TTT CTC CCG TCC TA-3' (255 – 270, 462 – 446), CaBP9k forward 5'-GCG CTA AGA AAT CTC CCG-3' and 5'-GCT AAC TTC TCC ATC ACC G-3' (5 – 22, 198 – 180), TRPV6 5'-GTG GCT ACT ACC GTG A-3' and 5'-CCC GGT TGA TTA TCC CT-3' (1789 – 1804, 2146 – 2130), NHE 5'-ACC GCA ATT TGA CCA AC-3' and 5'-GTT CTC CGT GAA CTG CC-3' (221 – 237, 567 – 551), NCX 5'-AGA TGC GTC CAT AGG C-3' and 5'-GCC GAT ACA GCA GCA C-3' (2583 – 2598, 2779 – 2764), Ocln 5'-CAC GTT CGA CCA ATG C-3' and 5'-CCC GTT CCA TAG GCT C-3' (100 – 115, 287 – 272), 18S 5'-AAG TCT TTG GGT TCC GGG-3' and 5'-GGA CAT CTA AGG GCA TCA CA-3' (1153 – 1170, 1517 – 1498).

2.5. CALCULATIONS AND STATISTICS

Data were calculated as described in each section and are expressed as mean ± standard deviation (SD), standard error of means (SE) or with the 95% confidence interval. Differences in means between groups were considered as significant when p was < 0.05 by using several statistic tests as indicated in the table and figure legends including unpaired and two-tailed Student's t test, one-way and two-way analysis of variances (ANOVA) with the Tukey test for homogeneous variances and Games-Howell test for inhomogeneous variances as post-hoc tests.

Der aus Büchern erworbene Reichtum fremder Erfahrung heißt Gelehrsamkeit.
Eigene Erfahrung ist Weisheit.

Gotthold Ephraim Lessing (1729 - 1781)

3. RESULTS

3.1. INTESTINAL MINERAL ABSORPTION AND BODY MINERAL RETENTION

A first feeding experiment (FE-2) with adult male rats in metabolic cages was conducted in which a standard diet enriched with 10% Raftilose®P95 or Raftilose®Synergy1 was provided (table 1). The calcium intake in the Synergy1 group was significantly lower as compared to control group because rats had a lower dietary intake. Neither in the P95 nor the Synergy1 groups any significant differences in intestinal apparent calcium absorption and in whole body calcium balance, i.e. retention, were observed when compared to the control group. In a repetitive experiment (FE-3) with only Raftilose®P95, a significant increase of 15% in apparent calcium absorption was observed in the fructan group but with no difference in calcium balance. These inconsistent results were also not in line with data reported in literature and questioned the design. As a consequence, a new feeding trial was carried out employing a semi-synthetic basal diet which was fed to younger (adolescent) rats to maximise the calcium demand. From this feeding experiment (FE-4), a significant increase of around 21% in apparent calcium absorption was observed for Raftilose®P95 and Raftilose®Synergy1 while the retained calcium (calcium balance) was significantly increased by 16 and 20%, respectively. These effects of Raftilose®Synergy1 in a semi-synthetic diet on apparent calcium absorption and calcium balance were consistently found in three further feeding experiments differing in length of feeding and balance periods.

Data from one of the conducted feeding experiments (FE-5) are presented more detailed in table 2 showing the obtained parameters for calcium as well as for magnesium and zinc. Mean dietary mineral intakes did not differ between Synergy1 and control groups for each mineral but mineral excretions with faeces were significantly lower in the group receiving Synergy1. Consequently, apparent absorption of calcium, magnesium, and zinc increased significantly by feeding fructans. Urinary mineral excretions were also increased in case of calcium and magnesium but not in case of zinc when Synergy1 was fed. Finally, the absolute mineral balance was significantly increased only for zinc whereas relative mineral balances showed significant increases for all minerals (Ca, Mg and Zn). Beside the whole body retention (balance), the content of calcium, magnesium, and zinc in femur was determined and revealed significantly increased levels in animals fed Raftilose®Synergy1.

Table 1 Feeding experiments (FE) investigating the apparent calcium absorption in rats fed 10% inulin-type fructans under different experimental conditions.

ID		control	Raftilose®P95	Raftilose®Synergy1
FE-2	Ca intake (mg/d)	171.3 ± 12.4	166.8 ± 12.5	150.4 ± 21.7*
	app. Ca absorption (%)	24.5 ± 1.2	26.5 ± 2.8	26.1 ± 2.7
	Ca balance (%)	23.4 ± 1.4	25.4 ± 2.7	24.5 ± 2.6
FE-3	Ca intake (mg/d)	138.8 ± 17.5	149.4 ± 14.4	nd
	app. Ca absorption (%)	27.3 ± 1.9	31.4 ± 2.2*	nd
	Ca balance (%)	26.1 ± 2.2	28.5 ± 2.8	nd
FE-4	Ca intake (mg/d)	58.5 ± 5.6	56.3 ± 5.7	53.4 ± 5.3
	app. Ca absorption (%)	60.4 ± 5.5	72.7 ± 6.9*	73.5 ± 3.1*
	Ca balance (%)	58.5 ± 5.9	67.6 ± 8.0*	70.3 ± 3.1*
FE-5	Ca intake (mg/d)	53.1 ± 5.3	nd	50.1 ± 6.7
	app. Ca absorption (%)	69.8 ± 5.1	nd	84.9 ± 3.5*
	Ca balance (%)	68.1 ± 5.0	nd	80.3 ± 2.3*
FE-6	Ca intake (mg/d)	55.8 ± 8.5	nd	58.2 ± 5.0
	app. Ca absorption (%)	69.1 ± 5.4	nd	83.7 ± 3.2*
	Ca balance (%)	67.2 ± 5.5	nd	79.1 ± 4.6*
FE-7	Ca intake (mg/d)	58.1 ± 2.4	nd	55.6 ± 3.1
	app. Ca absorption (%)	60.0 ± 2.0	nd	68.8 ± 3.4*
	Ca balance (%)	58.1 ± 1.5	nd	65.6 ± 3.0*

app. Ca absorption = apparent calcium absorption; nd = not determined
experimental design:
FE-2 200 g initial body weight, n = 8, standard diet for 28 d, final 27 d balanced
FE-3 200 g initial body weight, n = 8, standard diet for 28 d, final 9 d balanced
FE-4 100 g initial body weight, n = 8/8/7, semi-synthetic diet for 28 d, final 15 d balanced
FE-5 100 g initial body weight, n = 12, semi-synthetic diet for 15 d, 15 d balanced
FE-6 100 g initial body weight, n = 12, semi-synthetic diet for 21 d, 21 d balanced
FE-7 140 g initial body weight, n = 6, semi-synthetic diet for 21 d, final 6 d balanced
mean ± SD; * $p < 0.05$ compared to control as tested by ANOVA and Tukey test if appropriate

Table 2 Apparent mineral absorption, balance, and content in femur of calcium, magnesium, and zinc in rats fed a semi-synthetic diet with 10% Raftilose®Synergy1 for 15 days.

		control	Synergy1
	n	12	12
	initial body weight (g)	97 ± 9	101 ± 11
	final body weight (g)	166 ± 16	172 ± 21
	body weight gain (g/d)	4.6 ± 0.9	4.7 ± 0.9
	diet intake (g/d)	11.8 ± 1.2	12.2 ± 1.6
Ca	mineral intake (mg/d)	53.1 ± 5.3	50.1 ± 6.7
	faecal mineral excretion (mg/d)	15.9 ± 2.7	7.7 ± 2.3*
	apparent mineral absorption (mg/d)	37.1 ± 5.0	42.3 ± 4.9*
	apparent mineral absorption (%)	69.8 ± 5.1	84.9 ± 3.5*
	urinary mineral excretion (mg/d)	0.9 ± 0.3	2.2 ± 0.9*
	mineral balance (mg/d)	36.3 ± 4.9	40.1 ± 5.2
	mineral balance (%)	68.1 ± 5.0	80.3 ± 2.3*
	femoral mineral content (mg/g DM)	208.7 ± 4.9	213.1 ± 4.3*
Mg	mineral intake (mg/d)	7.6 ± 0.8	7.1 ± 0.9
	faecal mineral excretion (mg/d)	2.6 ± 0.5	0.9 ± 0.2*
	apparent mineral absorption (mg/d)	5.0 ± 0.5	6.2 ± 0.8*
	apparent mineral absorption (%)	66.1 ± 4.3	86.9 ± 2.6*
	urinary mineral excretion (mg/d)	3.0 ± 0.4	3.8 ± 0.4*
	mineral balance (mg/d)	2.1 ± 0.3	2.4 ± 0.5
	mineral balance (%)	26.8 ± 2.2	33.1 ± 3.8*
	femoral mineral content (mg/g DM)	4.5 ± 0.2	4.7 ± 0.1*
Zn	mineral intake (mg/d)	0.512 ± 0.052	0.486 ± 0.065
	faecal mineral excretion (mg/d)	0.356 ± 0.035	0.302 ± 0.043*
	apparent mineral absorption (mg/d)	0.156 ± 0.023	0.184 ± 0.026*
	apparent mineral absorption (%)	30.4 ± 2.7	37.9 ± 2.6*
	urinary mineral excretion (mg/d)	0.005 ± 0.001	0.005 ± 0.002
	mineral balance (mg/d)	0.151 ± 0.023	0.178 ± 0.026*
	mineral balance (%)	29.4 ± 2.8	36.7 ± 2.3*
	femoral mineral content (mg/g DM)	0.247 ± 0.018	0.269 ± 0.017*

DM = dry matter; mean ± SD; * $p < 0.05$ (t test)

To gain an insight into the time-dependency of the fructan effects on apparent calcium absorption, the feeding experiment FE-6 is split into balance units (of 3 days each) for which faecal and urinary outputs were collected and pooled. The faecal wet weight showed a continuous increase during the feeding period for control and Synergy1 groups whereas the Synergy1-fed rats had significantly elevated amounts of faeces (fig. 5A). Interestingly, the relative faecal moisture was not different in the first six days and in the final three days of the feeding period but between day 7 and day 18 it was significantly increased in the Synergy1 group (fig. 5B). The urinary volume did not differ between both groups during the entire experimental period (fig. 5C). For the apparent calcium absorption and calcium balance ratios, figures 6A and 6B show a distinct increase for the Synergy1 group throughout the experimental period with a greater difference between both groups in the first 12 days.

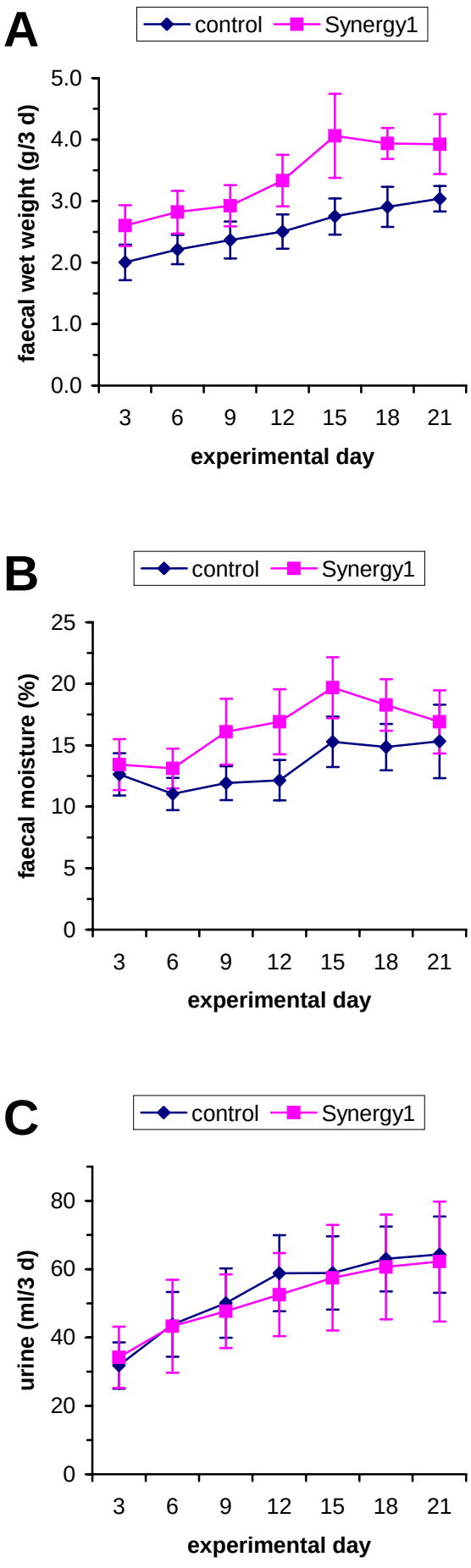

Fig. 5 **A** Faecal wet weight, **B** faecal moisture, and **C** urine volume of rats during the feeding trial with 10% Raftilose®Synergy1 over 21 days. Faecal and urinary excretions were pooled to 3-day-samples and are expressed as means ± 95% confidence interval (n = 12).

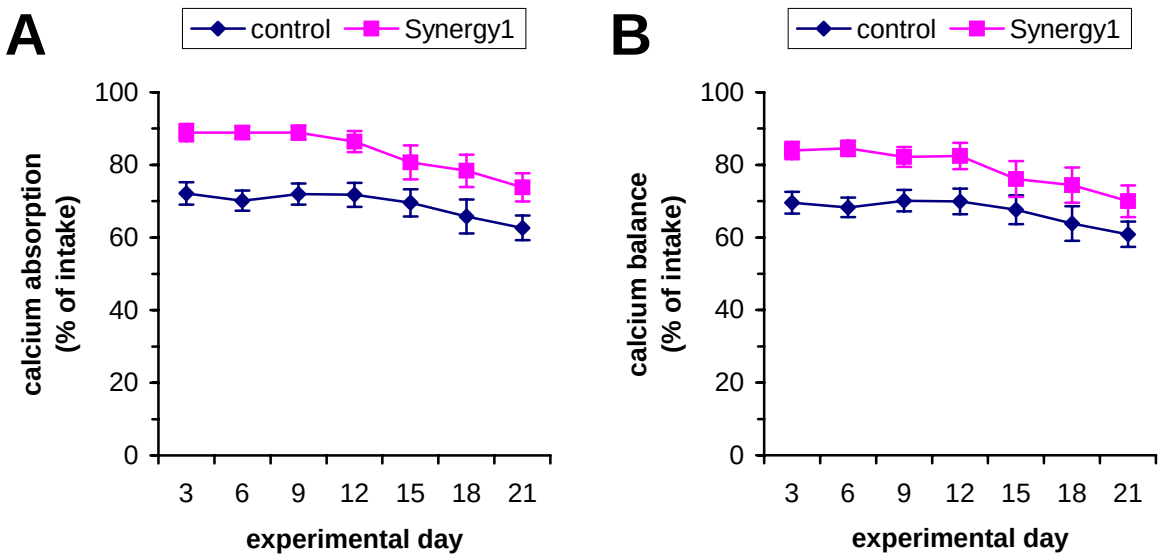

Fig. 6 **A** Intestinal apparent calcium absorption and **B** calcium balance (whole body retention) in % of calcium intake in rats during the feeding with 10% Raftilose®Synergy1 over 21 days. Data are determined for each 3-day-pool and are expressed as means ± 95% confidence interval (n = 12).

3.2. MACROSCOPIC CHANGES IN INTESTINAL TISSUES AND ANALYSIS OF CAECAL CONTENTS

To assess possible alterations in intestinal tissue architecture caused by inulin and oligofructose feeding, various macroscopic parameters were determined in animals from the feeding experiment FE-7 receiving 10% Raftilose®Synergy1 (table 3). The small intestine did not show any differences between both groups regarding the length, total weight, i.e. intestinal segment inclusive content, and intestinal wall weight. However, in case of caecum, the total surface area was more than doubled in the Synergy1 treated group, caecal total weight was nearly tripled and caecal wall weight more than doubled. The colon length did not differ significantly between both groups but colonic total and wall weights were increased in the Synergy1-fed rats by factors of 1.6 and 1.3, respectively.

Due to the visually obvious changes in caecal size and colour (fig. 7), caecal contents were analysed with respect to pH and calcium content (table 4). The weight of caecal contents tripled in Synergy1-fed rats and the pH decreased from a mean of 6.8 in controls to a mean of 5.3 in the Synergy1 group. The concentration of total calcium was lower in the Synergy1 group but when based on the entire content in caecum, total caecal calcium was increased.

After centrifugation of caecal contents, the amount of caecal water was found to be 4-times higher in the Synergy1 group. The contents of soluble and ionised calcium were strongly increased by 13- and nearly 40-fold, respectively, during feeding of Synergy1 and the concentrations of soluble and ionised calcium were increased, too.

Table 3 Morphological changes in the intestine of rats fed a diet with 10% Raftilose® Synergy1 for 21 days.

	control n = 6	**Synergy1** n = 6	**factor**	**p**
small intestine				
length (cm)	99.6 ± 5.2	100.4 ± 3.5	1.0	ns
total weight (g)	6.19 ± 0.97	6.73 ± 1.08	1.1	ns
wall weight (g)	5.90 ± 0.59	5.79 ± 0.29	1.0	ns
caecum				
surface (cm²)	14.3 ± 2.1	32.0 ± 7.3	2.2	< 0.001
total weight (g)	2.39 ± 0.68	6.98 ± 0.80	2.9	< 0.001
wall weight (g)	0.63 ± 0.06	1.49 ± 0.18	2.4	< 0.001
colon				
length (cm)	11.3 ± 1.9	12.9 ± 0.8	1.1	ns
total weight (g)	1.37 ± 0.30	2.17 ± 0.39	1.6	< 0.01
wall weight (g)	0.85 ± 0.10	1.10 ± 0.14	1.3	< 0.01

mean ± SD, $p < 0.05$ recognised as significant (t test), ns = not significant

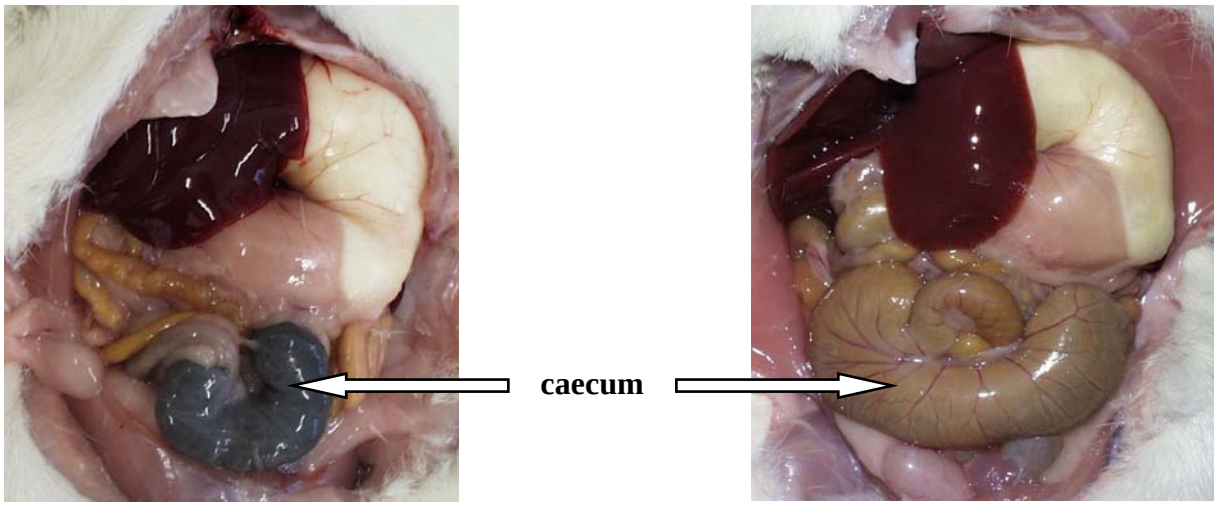

Fig. 7 Morphological changes of rat caecum after the feeding animals with 10% Raftilose®Synergy1 for 21 days (right picture) as compared to controls (left picture).

Table 4 Effect of 10% dietary Raftilose®Synergy1 fed to rats for 21 days on total, soluble, and ionised calcium in caecal contents.

	control n = 6	**Synergy1** n = 6	**factor**	**p**
caecal content				
weight (g)	1.76 ± 0.65	5.49 ± 0.67	3.1	< 0.001
pH	6.8 ± 0.1	5.3 ± 0.1	0.8	< 0.001
Ca_{total} (mg/g)	6.3 ± 0.8	3.6 ± 0.5	0.6	< 0.001
Ca_{total} (mg/caecum)	10.8 ± 3.6	19.6 ± 3.6	1.8	< 0.01
caecal water				
weight (g)	0.61 ± 0.32	2.44 ± 0.56	4.0	< 0.001
weight (% of content)	33.3 ± 6.7	44.3 ± 6.4	1.3	< 0.05
$Ca_{soluble}$ (mg/g)	0.341 ± 0.233	1.390 ± 0.357	4.1	< 0.001
$Ca_{soluble}$ (mg/caecum)	0.258 ± 0.273	3.433 ± 1.298	13.3	< 0.001
$Ca_{soluble}$ (% of Ca_{total})	2.1 ± 2.1	18.0 ± 7.0	8.4	< 0.001
Ca^{2+} (mg/g)	0.018 ± 0.011	0.207 ± 0.064	11.5	< 0.001
Ca^{2+} (mg/caecum)	0.014 ± 0.013	0.529 ± 0.291	39.1	< 0.01
Ca^{2+} (% of $Ca_{soluble}$)	5.3 ± 0.9	15.0 ± 2.8	2.8	< 0.001

mean ± SD, $p < 0.05$ recognised as significant (t test)

3.3. TRANSEPITHELIAL CALCIUM TRANSPORT IN LARGE INTESTINE

After determining the effects of inulin-type fructans on apparent calcium absorption, calcium balance, and some gut parameters in rats *in vivo*, the next step was to focus on the transepithelial calcium transport mechanisms in rat large intestine by applying the Ussing chamber technique. Several variables were assessed with respect to their effects on bidirectional calcium movements and which may be separated into acute and chronic effects of these fructans.

3.3.1. ACUTE EFFECTS OF CALCIUM CONCENTRATION, PH, SCFAS, AND INTACT FRUCTANS

In the studies with Ussing chambers, different calcium concentrations, the presence or absence of SCFAs, different pH values, and the presence or absence of Raftilose®Synergy1

were systematically varied in the mucosal chamber compartment and assessed with respect to net calcium transport effects. During the course of the experiments, changes in tissue conductance were observed with decreased conductances in the presence of SCFAs and increased conductances in the presence of Synergy1. A correlation between tissue conductance and net calcium flux was obtained and is shown in figure 8 for experiments with 10 mM calcium and for variables such as SCFAs and mucosal luminal pH. The strong correlations especially for the mucosa to serosa fluxes in various large intestinal segments were taken into account by expressing the net calcium fluxes as the flux over conductance ratios.

Increasing mucosal calcium concentrations increased the transepithelial calcium transport in caecum, proximal and distal colon (fig. 9). Whereas 0.1 mM of mucosal calcium resulted in a negative net calcium flux over conductance ratio, equal calcium concentrations at both tissue sides hardly showed a net calcium movement and only 10 mM of mucosal calcium led to a considerable net absorptive calcium flux. Furthermore, the presence of 100 mM SCFAs at the mucosal side did not alter the calcium transport at 0.1 and 1.25 mM mucosal calcium concentrations throughout the large intestine but at 10 mM mucosal calcium, the SCFAs significantly increased the calcium transport in caecum.

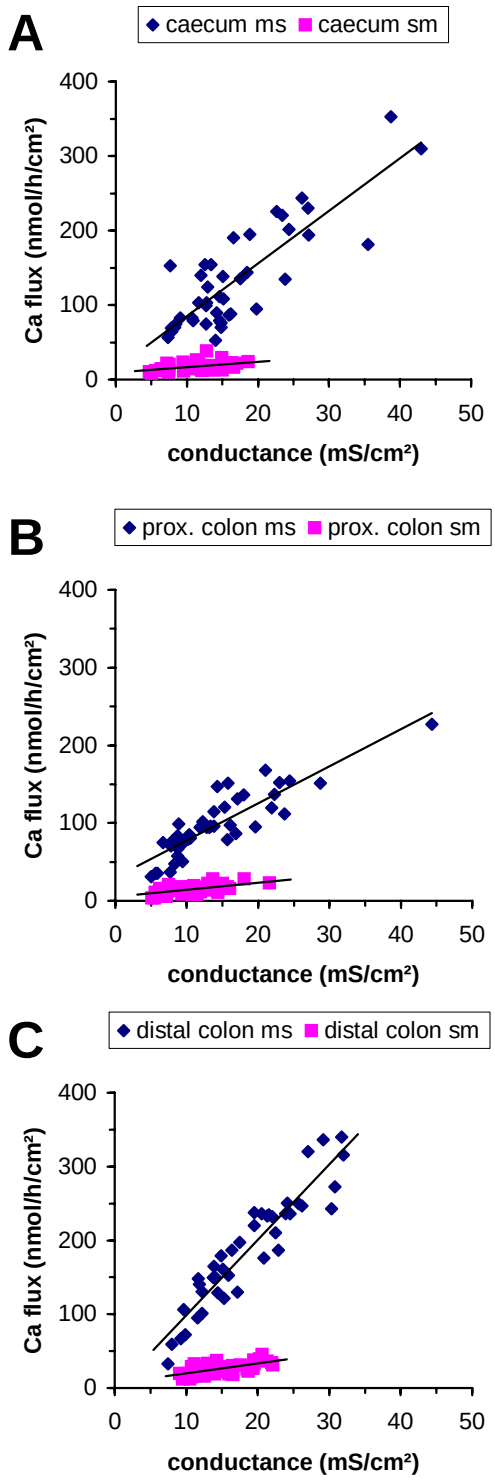

Fig. 8 Correlation between calcium flux and tissue conductance in rat **A** caecum, **B** proximal colon, and **C** distal colon. In each segment mucosal to serosal (ms) and serosal to mucosal (sm) fluxes are presented as a function of the tissue conductance recorded for the same intestinal segment in Ussing chambers. Data are from experiments with 0 and 100 mM mucosal SCFAs at two different mucosal pH levels (7.4 and 6.0). Pearson's correlation coefficient: caecum ms = 0.84, sm = 0.42; proximal colon ms = 0.87, sm = 0.61; distal colon ms = 0.94, sm = 0.64; $p < 0.01$, $n = 42$ (tissues from each flux direction from 42 animals).

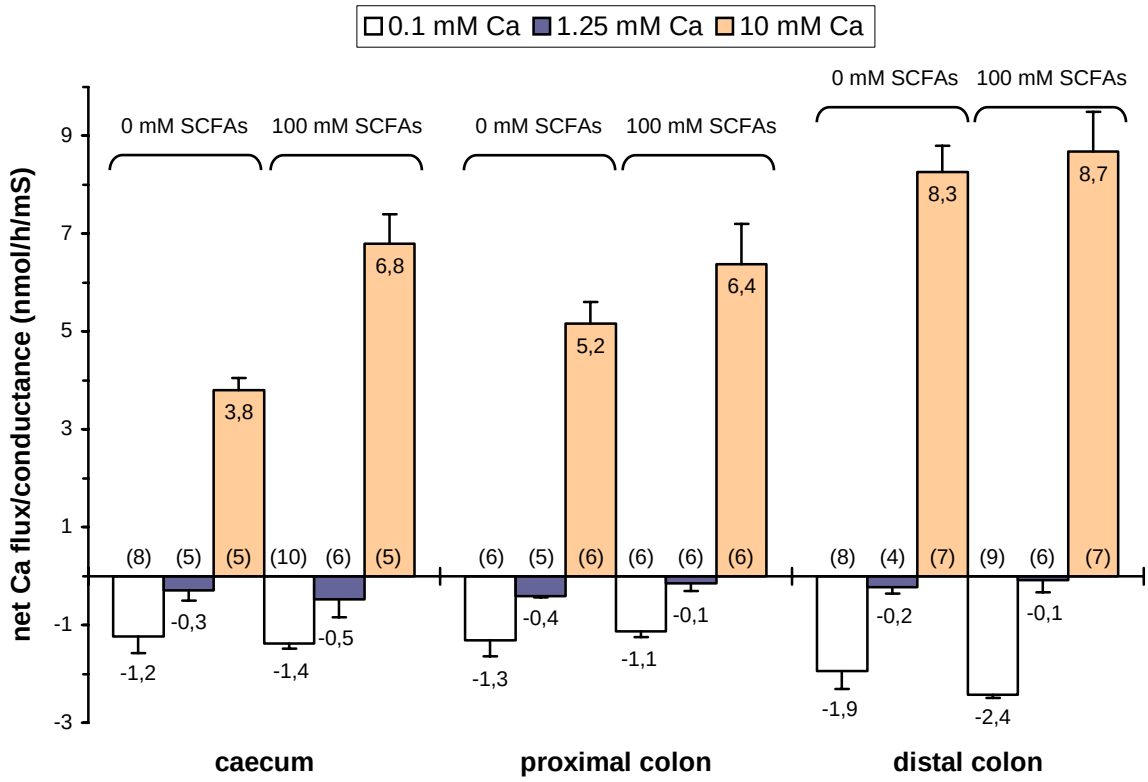

Fig. 9 Effects of different mucosal calcium concentrations (0.1, 1.25, and 10 mM) in the presence or absence of SCFAs on net calcium flux expressed as the flux/conductance ratio in rat caecum, proximal and distal colon. The pH was maintained at 7.4 for all variables. Mean ± SE, figures in parentheses = n. Two-way ANOVA: [Ca] $p < 0.001$ for caecum, proximal and distal colon; [SCFAs] $p < 0.01$ for caecum only; [Ca] x [SCFAs] $p < 0.001$ for caecum and only for the 10 mM calcium concentration (Games-Howell post-hoc test).

In the next series of experiments, the calcium gradient between mucosal (10 mM) and serosal (1.25 mM) tissue side was retained and the effects of mucosally provided SCFAs at two different pH levels were tested (fig. 10). A reduction of mucosal pH from 7.4 to 6.0 did not affect net transepithelial calcium transport in either intestinal segment whereas the presence of 100 mM mucosal SCFAs significantly increased the calcium movement in caecum by 53% independently of the mucosal pH. In colon, no significant effect was observed. Finally, the addition of SCFAs given as sodium salts at a pH of 7.4 were compared with their acidic form without maintaining mucosal pH (fig. 11). No significant differences were obtained between both forms of SCFAs but again a significant increase of 54% was observed in proximal colon comparing the acidic form of SCFAs with the absence of SCFAs. Beside the SCFAs, intact inulin and oligofructose provided by Raftilose®Synergy1 was added to the mucosal buffer solution (fig. 12). This resulted in significant enhanced net calcium transport rates in caecum and distal colon of 26 and 50%, respectively.

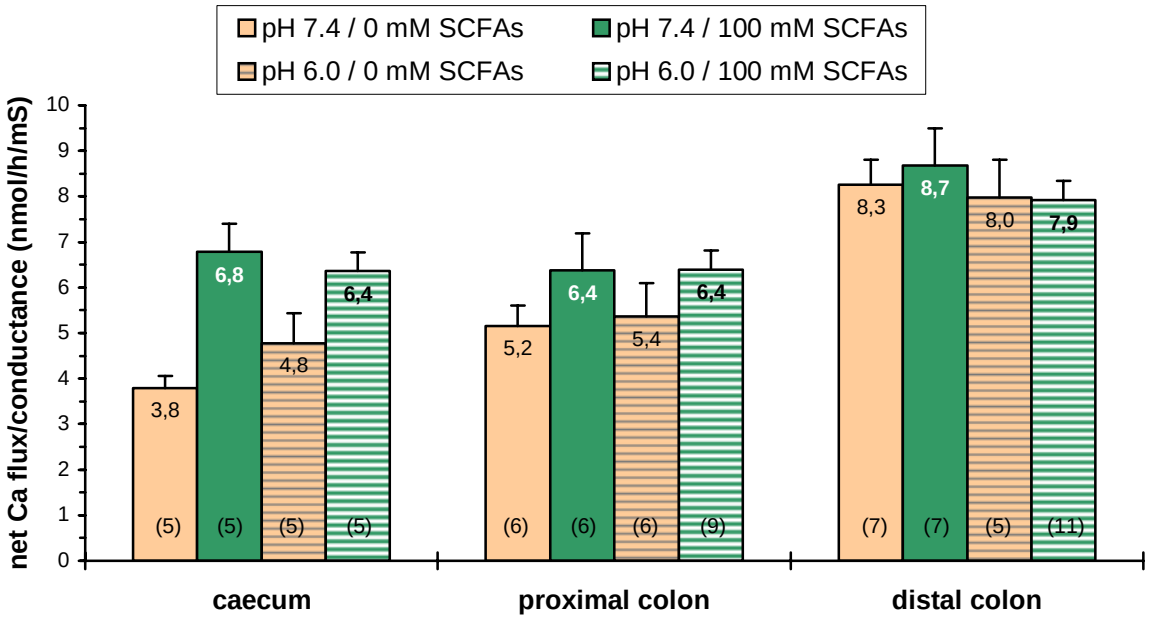

Fig. 10 Effects of SCFAs and pH on net calcium flux expressed as the flux/conductance ratio in rat caecum, proximal and distal colon. The mucosal calcium concentration remained at 10 mM for all variables. Mean ± SE, figures in parentheses = n. Two-way ANOVA: pH: $p > 0.05$ for all intestinal segments; SCFA: $p < 0.001$ for caecum only; pH x SCFA: $p > 0.05$ for all intestinal segments.

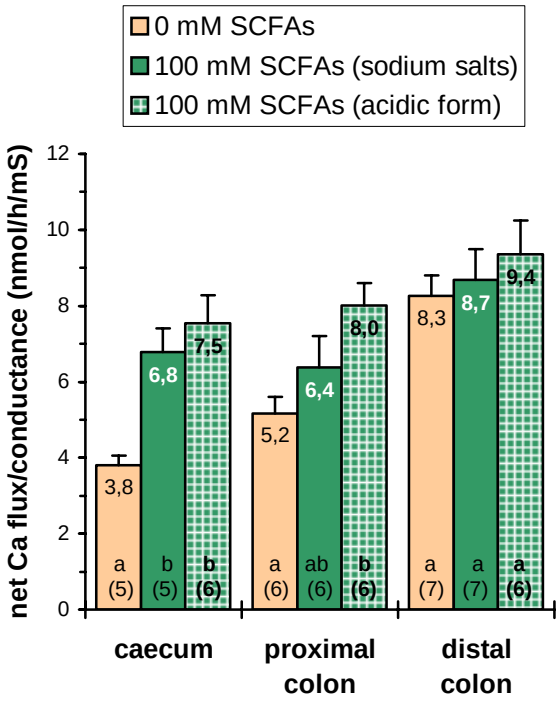

Fig. 11 Effects of SCFAs provided as sodium salts or in acidic form on the net calcium flux expressed as the flux/conductance ratio in rat caecum, proximal and distal colon. The mucosal calcium concentration remained at 10 mM for all variables. Mean ± SE, figures in parentheses = n, bars not sharing the same letter within an intestinal segment are significantly different (one-way ANOVA with Tukey test).

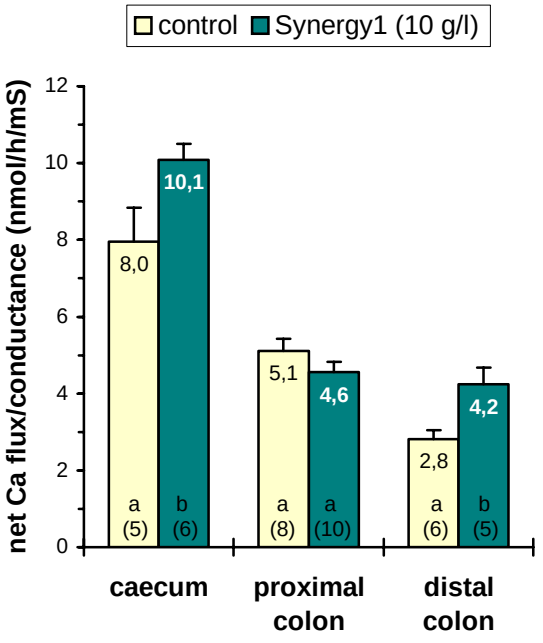

Fig. 12 Effect of Raftilose®Synergy1 in mucosal buffer solution (10 g/l) on net calcium flux expressed as the flux/conductance ratio in rat caecum, proximal and distal colon. The mucosal calcium concentration and pH were constant at 10 mM and 7.4, respectively. Mean ± SE, figures in parentheses = n, bars not sharing the same letter within an intestinal segment are significantly different (t test).

3.3.2. CHRONIC DIETARY EFFECTS OF INULIN-TYPE FRUCTANS

To investigate the chronic effects of orally administered inulin and oligofructose, transepithelial calcium transport was determined in Ussing chambers with large intestinal tissues obtained from rats fed 10% Raftilose®Synergy1 for 21 days. The first experiment without a calcium gradient between mucosal and serosal tissue sides revealed a decreased net calcium flux in caecal tissue of Synergy1-fed rats whereas net calcium fluxes in colon were low and did not differ significantly from controls (fig. 13).

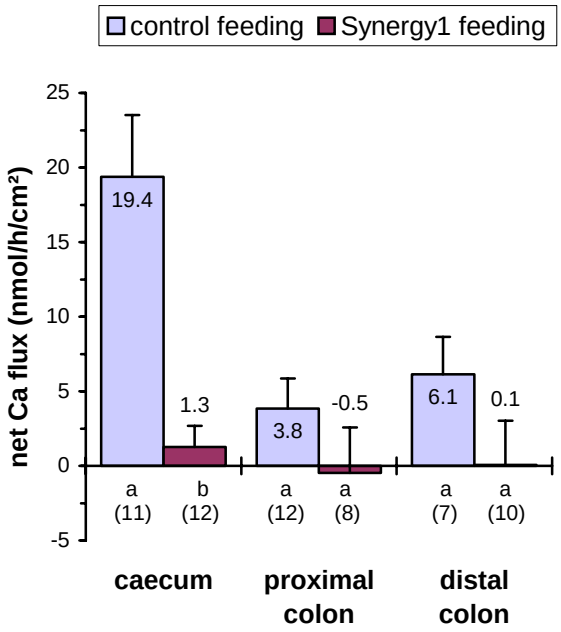

Fig. 13 Effects of feeding rats with 10% Raftilose® Synergy1 for 21 days on net calcium flux in caecum, proximal and distal colon with 1.25 mM calcium in the buffer solutions (without any electro-chemical gradient). Mean ± SE, figures in parentheses = n, bars not sharing a common letter within an intestinal segment are significantly different (t test).

When 10 mM calcium and 100 mM SCFAs were provided at the mucosal side, much higher net calcium fluxes were observed but without any significant differences between both feeding groups in either of the large intestinal segments (fig. 14A). Neither the weight of the exposed tissue in the Ussing chamber differed between both groups nor did the accumulation of ^{45}Ca in this tissue (table 5). Most interestingly, when the flux data as obtained from the Ussing chamber studies were based on the entire caecal surface area, the apparent net calcium transport was almost doubled in Synergy1-fed rats (fig. 14B).

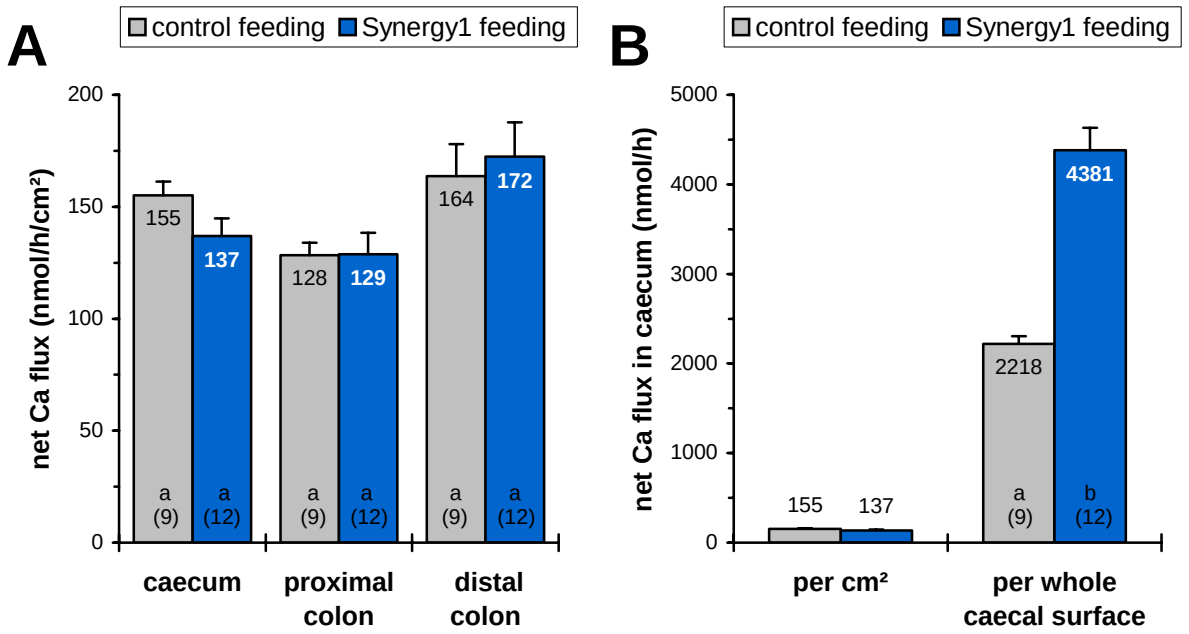

Fig. 14 Effects of feeding rats with 10% Raftilose®Synergy1 for 21 days on net calcium fluxes in caecum, proximal and distal colon with 10 mM calcium and 100 mM SCFAs in the mucosal buffer solution. **A** net calcium flux expressed per cm², **B** net calcium flux in caecum expressed for the whole caecal surface compared to the flux per cm². Mean ± SE, figures in parentheses = n, bars not sharing a common letter within an intestinal segment are significantly different (t test).

Table 5 Tissue weight and ^{45}Ca content in large intestinal segments after determining trans-epithelial calcium transport in tissues from rats fed 10% Raftilose®Synergy1 for 21 days.

	control n = 9	**Synergy1** n = 12
tissue weight (mg dry matter)		
caecum, ms	16.9 ± 1.4	18.9 ± 3.3
caecum, sm	20.1 ± 3.3	20.7 ± 4.4
proximal colon, ms	23.8 ± 2.9	22.1 ± 2.7
proximal colon, sm	19.5 ± 3.0	19.6 ± 3.7
distal colon, ms	23.0 ± 5.0	22.7 ± 3.1
distal colon, sm	15.3 ± 3.1	16.6 ± 2.4
tissue content of ^{45}Ca (pmol)		
caecum, ms	48.2 ± 4.0	58.6 ± 19.8
caecum, sm	56.6 ± 12.5	52.0 ± 14.9
proximal colon, ms	87.7 ± 12.7	77.5 ± 11.6
proximal colon, sm	51.8 ± 10.3	53.3 ± 9.0
distal colon, ms	92.4 ± 16.3	109.1 ± 33.9
distal colon, sm	51.9 ± 10.7	50.8 ± 9.1

ms = flux direction from mucosa to serosa; sm = flux direction from serosa to mucosa
mean ± SD, differences of means are not significant ($p > 0.05$, t test)

3.4. GENE EXPRESSION ANALYSIS OF INTESTINAL MUCOSA

3.4.1. CHANGES IN TRANSCRIPT LEVELS DETERMINED BY DNA MICROARRAYS

To obtain evidence that feeding fructans also affects gene expression in large intestinal segments, mRNA samples obtained from rats fed 0 or 10% Raftilose®P95 for 28 days were submitted to expression analysis by DNA microarrays. This resulted in a large number of regulated genes. Steady state transcript levels differed between control and P95 groups, in case of caecum with 37 regulated genes (3.2%), in proximal colon with 61 (5.2%), and in distal colon with 42 (3.6%) genes (see appendix table 1). These identified genes were classified into subgroups with respect to their known or suggested functions (fig. 15). The majority of regulated genes as identified in caecum, proximal and distal colon could be linked to cell homeostasis and cell stability. As examples of each subgroup few selected genes are presented in table 6.

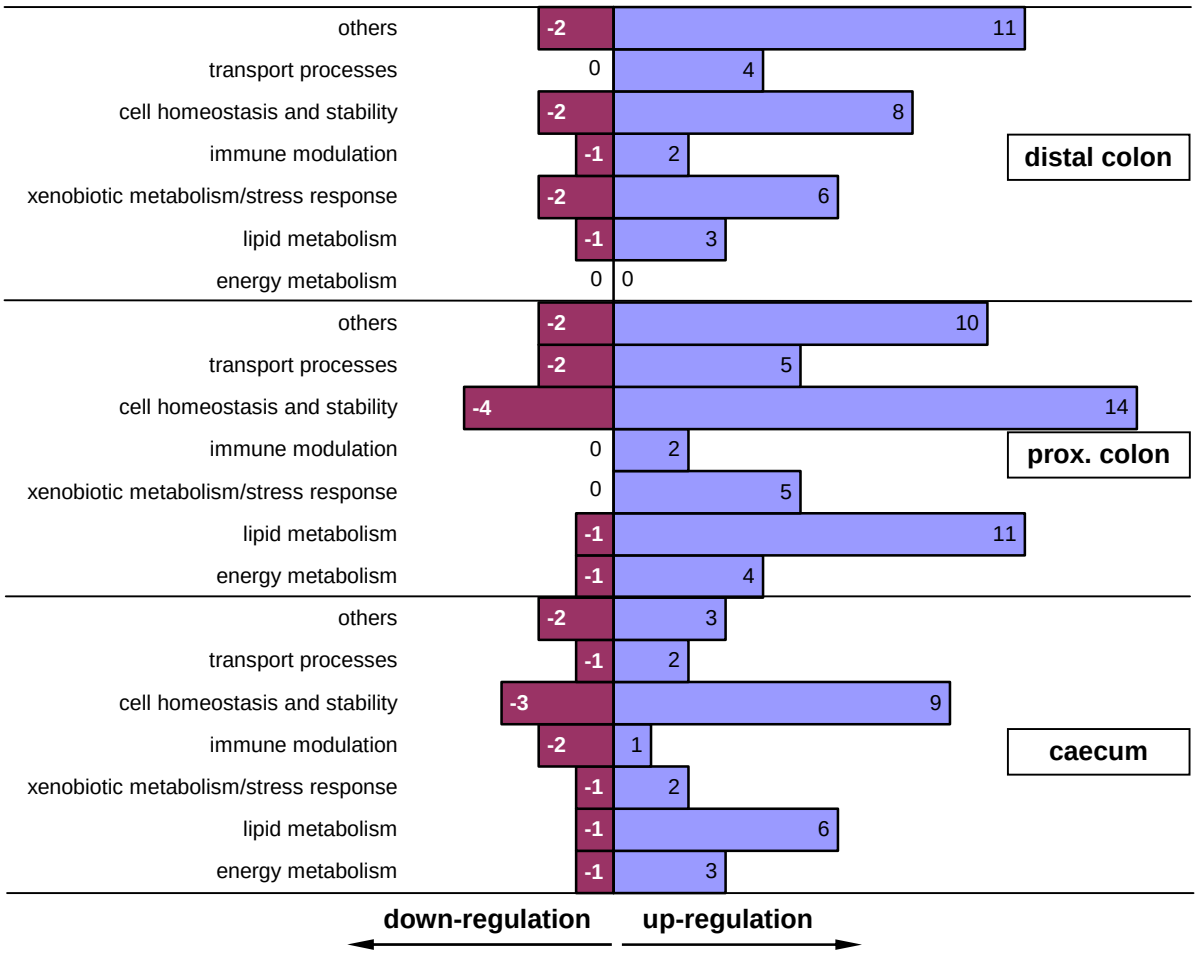

Fig. 15 Number of regulated genes with changes in steady state mRNA levels with up-regulation (towards the right) and down-regulation (towards the left) as identified in the large intestinal mucosa (caecum, proximal and distal colon) of rats after the feeding of 0 or 10% Raftilose®P95 for 28 days. Genes are grouped by their known or proposed functions.

Table 6 A selection of regulated genes at the mRNA level as identified in the mucosa of caecum (CA), proximal colon (PC), and distal colon (DC) of rats by DNA microarrays. Intestinal mucosa were obtained after feeding rats with 0 or 10% Raftilose®P95 for 28 days.

ID	gene	suggested function	regulation*		
			CA	PC	DC
energy metabolism					
M31805	NAD(P)H:quinone reductase	complex I of respiratory chain	3.7		
M25301	mitochondrial ATP synthase beta-subunit	ATP synthesis in most tissues	2.3		
U00926	F1F0 ATPase delta subunit	ATP hydrolysis and synthesis		2.0	
lipid metabolism					
M88592	peroxisome proliferator activated receptor (PPAR)	degradation of fatty and amino acids	3.4		
M00001	apolipoprotein A-I	constituent of chylomicrons and HDL		10.3	1.8
U02096	fatty acid binding protein	cytoplasmic transport of lipophilic ligands		6.0	
xenobiotic metabolism and stress response					
L15079	p-glycoprotein	multidrug resistance	2.0		
AF106945	peroxiredoxin PRx IV	antioxidant		2.3	
D25221	selenoprotein P	antioxidant and selenium transport			1.6
immune modulation					
AF151982	secretory leukocyte protease inhibitor (SLPI)	regulator of inflammatory response; suppresses the activation of NF-kappaB	2.6		
Z50051	bovine C4b-binding protein (C4BP) alpha chain	degradation of activated complement factors		4.5	
M83177	amyloid P component (SAP)	binding of pathogens such as influenza A virus and malfolded proteins			2.5
cell homeostasis and cell stability					
AF136583	protein kinase SNK	control of cell cycle progression	2.9		
AF030050	replication factor C	replication of DNA	2.1		
D90265	proteasome subunit RC2	basic cellular processes		2.7	
Y07504	elongation factor 2 (EF-2)	protein biosynthesis in the cell		2.0	1.6
D28875	osteonectin (SPARC)	cellular interaction with the extracellular matrix		1.9	
M15481	insulin-like growth factor I (IGF-I)	control of cell growth, differentiation, and protection against apoptosis		1.9	
AF178669	p34 (Cdk1/Cdc2)	cell proliferation and apoptosis induction		0.4	
M55022	nucleolin	ribosome biogenesis and maturation			1.7
transport processes					
AF007775	aquaporin (AQP8)	water channel	2.6		
AF033027	prenylated SNARE protein (Ykt6)	intracellular membrane fusion	1.9		
AJ011656	claudin-3 (Cldn3)	protein of tight junctions	0.6		
AF250142	NEFA (DNA binding, EF-hand, acidic region protein) precursor	calcium binding		2.1	
AB030829	carbonic anhydrase III	cellular ion transport and pH homeostasis		1.9	
L19031	organic anion transporter (Oatp)	absorption of anionic compounds including short-chain fatty acids			1.8
U75409	nucleobindin	calcium binding			1.7

* regulation factor < 1 = down-regulation; > 1 = up-regulation as compared to the control group

3.4.2. CHANGES IN TRANSCRIPT LEVELS DETERMINED BY REAL-TIME RT-PCR

From the regulated genes identified by the microarray analysis, four genes were chosen and altered mRNA levels in intestinal mucosa were verified by using real-time RT-PCR (table 7). The regulation factors were strengthened for all genes and in all segments except for claudin-3 in caecum where the regulation factor was 0.6 based on the microarray analysis indicating a down-regulation whereas by real-time RT-PCR no regulation was found (factor 1.1).

Table 7 Verification of results from DNA microarray analysis by real-time RT-PCR for a set of selected genes. Large intestinal mucosa samples were obtained from rats after the feeding with 0 or 10% Raftilose®P95 for 28 days.

gene	gene ID	method	regulation*		
			caecum	prox. colon	distal colon
ApoA-I (apolipoprotein A-I)	NM_012738	DNA-array	0.8	10.3	1.8
		RT-PCR	1.5	17.2	5.8
Aqp8 (aquaporin-8)	NM_019158	DNA-array	2.6	1.4	1.3
		RT-PCR	3.5	1.1	1.6
Cldn3 (claudin-3)	NM_031700	DNA-array	0.6	0.9	0.7
		RT-PCR	1.1	0.9	1.5
CA-III (carbonic anhydrase-3)	NM_019292	DNA-array	0.7	1.9	0.8
		RT-PCR	0.4	2.3	0.7

* regulation factor < 1 = down-regulation; > 1 = up-regulation compared to the control group

In the next series, transcript levels of the above listed four genes were amplified from tissue samples of rats fed 10% Raftilose®Synergy1 for 21 days to be able to compare the effects with those caused by Raftilose®P95 (table 8). Both preparations of inulin and oligofructose caused mainly different regulation factors. Apolipoprotein A-I in proximal colon displayed a 17.2-fold increase in steady state mRNA levels by Raftilose®P95 whereas Raftilose®Synergy1 resulted in only a 3.4-fold up-regulation. Carbonic anhydrase-3 in proximal colon was much stronger regulated (13.6-fold) by Synergy1 than by P95 (2.3-fold). Aquaporin-8 showed much higher regulation factors in all intestinal segments by Synergy1 administration and claudin-3 remained unchanged in caecum and distal colon independent of the fructan preparation but was up-regulated by Synergy1 in proximal colon as compared to no change by P95 feeding.

Table 8 Comparison of the ingestion of Raftilose®P95 and Raftilose®Synergy1 (both 0 or 10%) on changes in transcript levels of four selected genes identified in rat large intestinal mucosa samples by real-time RT-PCR.

gene	gene ID	fructan	regulation*		
			caecum	prox. colon	distal colon
ApoA-I (apolipoprotein A-I)	NM_012738	P95	1.5 ± 0.7	17.2 ± 14.9	5.8 ± 1.0
		Synergy1	2.9 ± 0.6	3.4 ± 0.8	2.7 ± 0.5
Aqp8 (aquaporin-8)	NM_019158	P95	3.5 ± 0.9	1.1 ± 0.6	1.6 ± 0.2
		Synergy1	6.4 ± 1.5	5.7 ± 1.9	4.0 ± 1.6
Cldn3 (claudin-3)	NM_031700	P95	1.1 ± 0.2	0.9 ± 0.2	1.5 ± 0.4
		Synergy1	1.3 ± 0.2	2.2 ± 0.3	1.5 ± 0.1
CA-III (carbonic anhydrase-3)	NM_019292	P95	0.4 ± 0.2	2.3 ± 0.7	0.7 ± 0.3
		Synergy1	0.1 ± 0.0	13.6 ± 11.9	0.6 ± 0.2

* regulation factor < 1 = down-regulation; > 1 = up-regulation compared to the control group
mean ± SE, for Raftilose®P95 n = 3, for Raftilose®Synergy1 n = 6

Finally, the transcript levels of selected genes reported or suggested to be involved in intestinal calcium transport were determined in the mucosa of Synergy1-fed rats (table 9). In general, only modest changes for the majority of genes were observed. Though, aquaporin-8 showed 4- to 6-fold increases in steady state mRNA levels in the Synergy1 group throughout all segments and carbonic anhydrase-3 was strongly down-regulated in caecum (16.7-fold) but strongly up-regulated in proximal colon (13.6-fold).

Table 9 Regulation of selected genes associated with the intestinal calcium transport. The mRNA levels were determined by real-time RT-PCR in large intestinal mucosa samples obtained from rats fed 0 or 10% Raftilose®Synergy1 for 21 days.

gene	gene ID	regulation*		
		caecum	prox. colon	distal colon
CaBP9k (calbindin)	NM_012521	2.1 ± 0.4	1.8 ± 0.3	1.6 ± 0.6
TRPV6 / CaT1 / ECaC2 (transient receptor potential cation channel 6)	NM_053686	0.9 ± 0.1	0.7 ± 0.1	0.6 ± 0.2
NHE (sodium/hydrogen exchanger)	NM_012652	1.7 ± 0.3	1.7 ± 0.1	1.1 ± 0.1
NCX (sodium/calcium exchanger)	NM_019268	1.2 ± 0.2	2.3 ± 0.1	0.6 ± 0.1
CA-III (carbonic anhydrase-3)	NM_019292	0.1 ± 0.0	13.6 ± 11.9	0.6 ± 0.2
Ocln (occludin)	NM_031329	1.4 ± 0.1	1.5 ± 0.1	1.4 ± 0.2
Cldn3 (claudin-3)	NM_031700	1.3 ± 0.2	2.2 ± 0.3	1.5 ± 0.1
Aqp8 (aquaporin-8)	NM_019158	6.4 ± 1.5	5.7 ± 1.9	4.0 ± 1.6

mean ± SE, n = 6; * regulation factor < 1 = down-regulation; > 1 = up-regulation compared to the control group

Eigentlich weiß man nur, wenn man wenig weiß. Mit dem Wissen wächst der Zweifel.

Johann Wolfgang von Goethe (1749 - 1832)

4. DISCUSSION

4.1. INTESTINAL MINERAL ABSORPTION AND BODY MINERAL RETENTION

Several studies in experimental animals (Levrat *et al.* 1991, Ohta *et al.* 1994, Delzenne *et al.* 1995, Takahara *et al.* 2000, Younes *et al.* 2001, Coudray *et al.* 2003, Raschka and Daniel 2004) and in humans (Coudray *et al.* 1997, van den Heuvel *et al.* 1999, Griffin *et al.* 2002) demonstrated that supplementing a diet with inulin-type fructans increases the intestinal absorption of calcium and of other minerals. A striking finding of the here presented first feeding experiment (FE-2) was that it failed to show an increase in apparent calcium absorption when animals were fed with either Raftilose®P95 (oligofructose) or Raftilose®Synergy1 (inulin and oligofructose) (table 1). As a possible explanation it was hypothesised that the basal diet (standard diet) was not appropriate for supplementation with fructans because this diet was composed of shredded cereals which could have contained inulin and oligofructose. Analysis of this diet indeed revealed that it possessed a natural fructan content of 1.5% (w/w). This intrinsic content of inulin and oligofructose in the background diet is obviously sufficient to induce effects on mineral absorption and to elevate calcium absorption but at the same time it prevents any additional effect of extra added inulin-type fructans to further enhance calcium uptake. That this low quantity of fructans already affects mineral absorption appears reasonable since it was shown that 3% of inulin-type fructans in the diet could significantly increase apparent calcium absorption in adolescent rats (Suzuki *et al.* 1998). Moreover, Levrat *et al.* (1991) showed a linear relation ($r = 0.99$) between the inulin content in diet (0, 5, 10 and 20%) and the caecal calcium absorption in growing rats. From these findings, one would have expected that increasing the fructan content in the diet from 1.5% to 11.5% by Raftilose®Synergy1 or Raftilose®P95 should have further increased calcium absorption. However, this was not the case in the first feeding experiment (FE-2) presented here. The reason might be that Levrat *et al.* measured specifically caecal calcium absorption whereas the apparent calcium absorption for the whole intestine was determined here. The second feeding experiment (FE-3) resulted in an increased apparent calcium absorption which could be contributed to the extra load of fructans despite the intrinsic fructan content in controls. However, an explanation for these inconsistent results cannot be given.

As a consequence of these observations, for the further feeding studies a semi-synthetic diet devoid of intrinsic fructans was implemented and adolescent rats with a higher calcium demand were used which could boost the effect of fructans on calcium absorption. This resulted in a significant increase in apparent calcium absorption in Raftilose®P95 and Raftilose®Synergy1 groups (FE-4) and was confirmed for Synergy1 by the three following feeding experiments (FE-5 to FE-7). In the case of Synergy1, a recent study has confirmed this increasing effect on apparent calcium absorption but for oligofructose (comparable to Raftilose®P95) only a modest and not significant increase was observed (Coudray *et al.* 2003).

To assess the time-dependence of the fructan effects on calcium absorption *in vivo*, the feeding experiment FE-6 is displayed more detailed over the experimental period of 21 days. The differences in apparent calcium absorption ratios as well as calcium balance ratios between control and Synergy1 groups were greater in the first 12 days but remained significantly different throughout the entire feeding period (fig. 6A and 6B). In a very recent study, it was shown that the increasing effect of inulin on calcium absorption persisted even over 40 days in adult rats with an initial body weight of 275 g but low calcium intakes (Coudray *et al.* 2004).

A comparison of the animals fed the standard or the semi-synthetic diet (FE-2 and FE-4) reveals almost identical absolute apparent calcium absorptions in the P95 and Synergy1 groups (44.6 ± 5.3 and 39.2 ± 8.0 mg/d on a standard diet versus 40.9 ± 5.5 and 39.2 ± 3.2 mg/d on a semi-synthetic diet) despite a three times higher calcium intake of rats on a standard diet by the higher food intake and the higher dietary calcium content in the standard diet. In the control group fed the semi-synthetic diet, a significantly lower calcium absorption was observed as compared to animals kept on the standard diet (35.2 ± 3.8 versus 41.9 ± 4.6). As already addressed, this may be attributed to the intrinsic natural fructan content of 1.5% in the basal standard diet which obviously promotes calcium absorption efficiently but prevents a further increase when additional fructans are added to this diet.

The administration of 10% Raftilose®Synergy1 via the semi-synthetic diet led to an increased apparent calcium absorption and similarly increased magnesium and zinc absorptions despite almost identical intakes of the minerals in control and Synergy1 groups (table 2). The higher intestinal absorptions were partly compensated by an elevated urinary excretion of calcium

and magnesium but not of zinc which is essentially not cleared through the kidneys. However, the balance ratios for calcium, magnesium, and zinc were all increased by the fructan supplementation and an increased mineral accumulation in bone (femur) was observed in case of all minerals (calcium, magnesium, zinc). These findings are consistent with data reported by Zafar *et al.* (2004) with a higher calcium content in femur in ovariectomised rats fed a diet with 5.5% inulin and oligofructose and those of Scholz-Ahrens *et al.* (2002) with an increase in bone mineral content (femur) in ovariectomised rats on an oligofructose diet. Kruger *et al.* (2003) reported no differences in femur bone mineral content after fructan administration but an increase in that of spine in male rats and Roberfroid *et al.* (2002a) demonstrated that whole-body bone mineral content of rats fed inulin was increased as well. Bone architecture was also altered as the trabecular bone area in the tibia increased by oligofructose administration in ovariectomised rats (Scholz-Ahrens *et al.* 2002). An increased trabecular bone volume was observed in femur of male rats fed fructooligosaccharides, too (Takahara *et al.* 2000).

In summary, various studies in experimental animals (mainly rats) have shown that a higher concentration of fructans in the diet increases significantly intestinal calcium absorption and whole body calcium retention and that of other minerals as well and thereby affects also bone density and bone architecture. This uniform activity of fructans, however, also suggests that a common mechanism mediates these effects in the gut despite the quite different routes, regulation and extent of absorption of the different minerals.

4.2. MACROSCOPIC CHANGES IN INTESTINAL TISSUES AND ANALYSIS OF CAECAL CONTENTS

Inulin and oligofructose administration to rats resulted in major changes of morphometric parameters of caecum and colon tissues but not at all in small intestine (table 3). Markedly changed was the caecum in its total organ weight, wall weight, and in (serosal) surface area. The macroscopical total caecal surface area (based on serosal surface) increased 2.2-fold and similarly, tissue wall wet and dry weight increased 2.4-fold (table 3) and 2.2-fold (data not shown), respectively. This suggests a net enlargement in total tissue surface but not in the thickness of the tissue wall. Colonic tissue differed in its total and wall weight, too. These findings confirm previous studies that also observed an enlarged caecum (caecum weight) (Levrat *et al.* 1991, Lopez *et al.* 2000, Younes *et al.* 2001) with increased weight of caecal

content (Delzenne *et al.* 1995, Suzuki *et al.* 1998, Lopez *et al.* 2000) and caecal wall weight (Levrat *et al.* 1991, Campbell *et al.* 1997, Younes *et al.* 2001) in fructan-fed rats. However, an increase in colonic wall weight was not observed in other studies (Delzenne *et al.* 1995, Campbell *et al.* 1997). On the microscopic level, an enlarged crypt length (Remesy *et al.* 1993, Kleessen *et al.* 2003) and a thicker mucus layer (Kleessen *et al.* 2003) was determined. A recent study performed in piglets fed fructans also reported a larger crypt length and an increased number of epithelial, mitotic, and mucin-containing cells (Tsukahara *et al.* 2003). Taken together, these macro- and microscopic parameters describe an enlarged absorptive area especially in caecum but also in colon which may represent an adaptive mechanism of the large intestine to cope with a larger load of fermentable substrates. At the same time, the enlarged surface to which the intestinal contents are exposed may provide an increased mineral absorption capacity.

The analysis of caecal contents revealed a decrease of intraluminal pH and an increase in total, soluble, and ionised calcium in Synergy1-fed rats as compared to controls (table 4). The pH decline corresponds well with data obtained in other studies showing a pH range from 6.8 (Campbell *et al.* 1997) to 7.4 (Suzuki *et al.* 1998) in control rats and pH values of 5.6 (Younes *et al.* 2001) and 6.3 (Ohta *et al.* 1995b) in fructan-fed rats. Together with increased caecal contents of calcium, the low pH could cause the major changes in the concentration as well as the amount in the entire caecum of soluble and ionised calcium which was found to be increased 13-fold and almost 40-fold for soluble and ionised calcium pools, respectively. For caecal soluble calcium pools, similar findings were reported previously with an enhancement of 4-fold (Younes *et al.* 2001), 13-fold (Levrat *et al.* 1991), and even 21-fold (Ohta *et al.* 1995b) in fructan-fed rats. The total caecal calcium in Synergy1-fed animals was increased but the mean calcium concentration was reduced as compared to controls. This indicates a dilution effect and indeed, total caecal water was increased 4.0-fold after Synergy1 administration. Although not reaching statistical significance, such decreased concentrations but increased pools of total calcium in caecum of fructan-fed rats were also observed previously (Ohta *et al.* 1995b, Younes *et al.* 2001). In conclusion, these data strongly suggest a huge increase in intracaecal calcium availability by pH changes and higher concentrations of non-complexed calcium that together with the enlarged surface area may explain the higher calcium absorption in the large intestine when fructans are fermented. These processes similarly could increase the availability of other minerals as well.

4.3. TRANSEPITHELIAL CALCIUM TRANSPORT IN LARGE INTESTINE

Inulin-type fructans increase the intestinal calcium absorption as shown in studies with experimental animals (Levrat *et al.* 1991, Ohta *et al.* 1994, Delzenne *et al.* 1995, Takahara *et al.* 2000, Younes *et al.* 2001, Coudray *et al.* 2003, Raschka and Daniel 2004) and in humans (Coudray *et al.* 1997, van den Heuvel *et al.* 1999, Griffin *et al.* 2002). However, in ileostomy patients, these fructans failed to increase calcium absorption and retention and the majority of the ingested fructans (around 88%) was found in the ileal effluent (Ellegard *et al.* 1997). This strongly suggests that the large intestine rather than duodenum, jejunum or ileum is the site of fructan action on calcium absorption. Moreover, inulin-type fructans were shown to be fermented efficiently by the large intestinal flora with an increase in the concentrations of short-chain fatty acids (Campbell *et al.* 1997). Whether SCFA production increases calcium absorption directly or via pH-induced increases in soluble calcium is not known. Therefore, calcium transport processes were studied in different segments of the rat large intestine using the Ussing chamber technique with special emphasis on effects of luminal pH and SCFAs. In addition, tissues from rats fed inulin and oligofructose were submitted to Ussing chamber studies to assess whether fructan feeding increases the capability of the large intestine for calcium absorption.

4.3.1. ACUTE EFFECTS OF CALCIUM CONCENTRATION, PH, SCFAS, AND INTACT FRUCTANS

The effectors tested in the Ussing chamber studies were chosen based on *in vivo* data reported in literature. A mean SCFA concentration of 100 mM composed of acetate, propionate, and butyrate in molar ratios 50:30:20 as well as a pH value of 6.0 were selected on basis of findings in rat caecum after feeding inulin-type fructans with reported concentrations of 156 mM SCFAs (42:38:20) and a luminal pH of 6.0 (Levrat *et al.* 1991), 61 mM SCFAs (74:8:18) and a pH of 6.2 (Campbell *et al.* 1997), or 77 mM SCFAs (45:44:10) and a pH of 6.3 (Suzuki *et al.* 1998).

Experiments in Ussing chambers revealed that the addition of SCFAs or lowering mucosal pH caused a decrease in tissue conductance in all large intestinal segments studied. This in turn lowered net calcium fluxes in proximal and distal colon. The changes in tissue conductance correlated well with the calcium fluxes especially with the mucosal to serosal flux (fig. 8) suggesting that tissue conductance is a major determinant for the transepithelial calcium flux.

The results are therefore presented as the ratio of net calcium flux over tissue conductance to allow only the effect of one variable to be detected. Why the tissue conductance decreased in the presence of SCFAs or a reduced pH is not clear. Butyrate was shown to restore mucosal barrier function in rat distal colon after heat-induced epithelial damage and thus the tissue conductance decreased (Venkatraman *et al.* 1999). However, the preparation and mounting of the tissue in the Ussing chamber may have caused a release of prostaglandins in the muscle layer as a stress response which could alter the tissue conductance via effects on chloride secretion. This may be counteracted by SCFAs or pH and thus lowering tissue conductance. In non-stripped tissue preparations as used here, the muscularis propria (= muscularis tunica) contributes to the subepithelial conductance by 68% as shown e.g. for rat rectum whereas the epithelial conductance remained essentially unchanged in stripped and non-stripped tissues (Schulzke *et al.* 1986).

When different calcium concentrations either in the presence or absence of 100 mM SCFAs were applied at the mucosal side, net calcium transport in caecum, proximal and distal colon was only observed in the presence of a downhill calcium concentration gradient (fig. 9). This suggests that increased calcium concentrations as observed in caecal contents for soluble and ionised calcium *in vivo* after fructan feeding (table 4) may be essential for the increased calcium absorption *in vivo*. The observed increased transepithelial net calcium movement when increasing mucosal calcium concentrations is in agreement with findings of a study with rat caecum and colon tissues perfused with 1.7, 3.4, and 5 mM calcium *in vivo* (Urban *et al.* 1978). Since SCFAs affected calcium transport only at 10 mM mucosal calcium and only in caecum (fig. 9), all further experiments were conducted with a mucosal concentration of 10 mM calcium.

When 100 mM SCFAs were provided at either pH 7.4 or pH 6.0, caecal net calcium absorption increased in the presence of SCFAs but pH changes alone had no significant effect (fig. 10). Although there was evidence reported that an increase in luminal proton concentration opens the paracellular pathway (Capurro and Parisi 1992), an increase in calcium movement could not be observed in the present experiment. Mineo *et al.* (2001b) observed a strong increase in transepithelial calcium absorption in caecum but in contrast to the presented findings also in colon when 130 mM SCFAs were provided in the mucosal solution. However, the lack of a voltage clamp and possibly different osmolarities at both tissue sides in these experiments makes a direct comparison with the here presented data

difficult. Moreover, these authors did not maintain pH after SCFA addition to the buffer solutions which reduced pH to 3.0. When the effects of SCFAs added either in their acidic or salt form were compared (fig. 11), their action on calcium movement were similar in caecum and distal colon whereas in proximal colon the SCFAs in acidic form but not as sodium salts significantly increased net calcium transport. The luminal pH change from 7.4 to 6.0 was without an significant effect on calcium transport (fig. 10) but lowering pH further to 3.0 – although unphysiologically – may have an additive effect, mainly in colon (fig. 11). Taken these different experiments together, the presence of around 100 mM SCFAs provided at the mucosal side consistently increased transepithelial calcium transport in large intestine.

The effect of SCFAs on transepithelial calcium transport was shown in a variety of epithelia under quite different experimental conditions. Net calcium flux in rat colon for example significantly increased in an *in vivo* perfusion when 30 mM butyrate was provided (Lutz and Scharrer 1991). Moreover, 60 mM mixed SCFAs in ovine rumen *in vitro* (Schroder *et al.* 1999) and 50 mM mixed SCFAs in ovine reticulo-rumen *in vitro* and *in vivo* increased calcium transport (Wadhwa and Care 2000). Beside calcium, net fluxes of sodium and chloride in rat distal colon (Binder and Mehta 1989, Vidyasagar and Ramakrishna 2002) as well as magnesium transport in ovine rumen (Leonhard-Marek *et al.* 1998) and iron uptake in rat proximal colon (Bougle *et al.* 2002) were increased in the presence of luminal SCFAs. In the human distal colon, 50 mM propionate increased net sodium flux, too (Zaharia *et al.* 2001).

The underlying mechanism that cause the increase in net calcium transport (and obviously also of that of other minerals) in the presence of SCFAs is not yet understood. In general, SCFAs can enter colonocytes by simple diffusion of the non-ionised protonated form and by carrier-mediated uptake of the ionised form (Topping and Clifton 2001). Von Engelhardt *et al.* (1993) concluded from their SCFA flux measurements in guinea-pig large intestine that SCFA absorption is linked to H^+ secreting systems in the apical membrane. In caecum and proximal colon these appear mainly as Na^+/H^+ exchanger mechanism whereas in distal colon a K^+/H^+-ATPase could dominate in proton secretion. A low luminal pH in the vicinity of the apical membrane favours the protonation of SCFAs which in turn can permeate via non-ionic diffusion into the epithelium. SCFA effects on calcium flux in ovine rumen also showed a stimulation of calcium uptake that was not affected by the calcium channel blocker verapamil nor by vanadate as a calcium pump inhibitor (Schroder *et al.* 1999). Surprisingly, here the

Na^+/H^+ exchange inhibitor amiloride enhanced further the SCFA-stimulated calcium flux and Schröder *et al.* took this as evidence for the presence of a Ca^{2+}/H^+ exchange mechanism coupled to SCFA uptake. Wadhwa *et al.* (2000) observed a stimulation of net calcium and magnesium fluxes in the ovine reticulo-rumen *in vivo* by SCFAs. Acetazolamide, an inhibitor of carbonic anhydrase, reduced the SCFA-stimulated calcium and magnesium fluxes whereas in the absence of SCFAs acetazolamide failed to alter ion fluxes. The authors concluded that SCFAs could stimulate the calcium flux through an activation of a $Ca^{2+}/2H^+$ antiporter system. Assuming similar mechanisms in place of rat caecum, protonated SCFAs entering epithelial cells via non-ionic diffusion would – after intracellular dissociation by a higher intracellular pH – release protons for exchange with luminal calcium and thereby increase net calcium uptake. Alternatively, SCFA anions could be absorbed in exchange with HCO_3^- from H_2CO_3 as produced by carbonic anhydrase in epithelial cells with an apical proton secretion in exchange with calcium.

Beside the SCFAs as fermentation products of inulin-type fructans, these fructans themselves increased net transepithelial calcium transport in caecum and distal colon when presented in the mucosal buffer solution of Ussing chambers (fig. 12). This also led to an increased tissue conductance in proximal and distal colon. Mineo *et al.* (2001a) similarly observed an increase in transepithelial calcium transport in rat large intestine in the presence of six different non-digestible saccharides added to mucosal buffer solutions. Fructooligosaccharides in concentrations of 1 and 10 mM (corresponding to 0.6 and 6.0 g/l) increased significantly calcium uptake in caecum and colon, respectively. When simultaneously the permeability for lucifer yellow, as a paracellular marker, and transepithelial electrical resistance were recorded, mucosal addition of oligosaccharides such as difructose anhydride-III increased lucifer yellow permeability and decreased electrical resistance in both large intestinal segments (Mineo *et al.* 2002a). Furthermore, sugar alcohols such as erythritol, xylitol, maltitol, and lactitol increased net calcium absorption in rat jejunum, ileum, caecum, and colon (Mineo *et al.* 2002b) and mannitol was shown to enhance calcium absorption in rat ileum (Escoffier *et al.* 2000). The presence of fructooligosaccharides and other non-digestible saccharides at the apical side of Caco-2 cell monolayers did not alter transcellular net calcium transport but significantly increased the paracellular route and decreased transepithelial resistance (Suzuki and Hara 2004). Madara and Pappenheimer (1987) showed that glucose induced an expansion of lateral intercellular spaces and dilatations within occluding junctions in hamster small intestine. They also observed a condensation of microfilaments in the zone

of the perijunctional actomyosin ring in a glucose containing solution indicating an overall increased junctional permeability. Although glucose is efficiently absorbed in the small intestine and may in turn or via its effects on metabolism alter tight junction permeability, inulin-type fructans are not absorbed in intact form at least not in considerable amounts in the small intestine. How the carbohydrates directly or indirectly interact with the tight junction complex and increase the paracellular permeability for calcium and possibly for other minerals, remains to be determined. It needs also further proof whether these observations made *in vitro* also contribute to the fructan effects on mineral absorption *in vivo*.

4.3.2. CHRONIC DIETARY EFFECTS OF INULIN-TYPE FRUCTANS

To assess whether chronic feeding of inulin and oligofructose affects intestinal calcium pathways also *in vitro*, bidirectional calcium fluxes in Ussing chambers with tissues from rats fed fructans were determined. The first experiment, no transmucosal calcium concentration gradient was applied (fig. 13). This resulted in a significantly reduced net calcium flux in caecum in Synergy1-fed rats while the colonic segments did not show any significant differences. In the next experiment, a downhill calcium concentration gradient was applied with 100 mM SCFAs added to the mucosal side (fig. 14A). The obtained net calcium fluxes were markedly higher than without a calcium concentration gradient but again, there was no significant difference between control- and Synergy1-fed rats in either large intestinal segment. There were also no significant differences in the quantity of ^{45}Ca retained in tissues during the Ussing chamber experiment between both feeding groups (table 5). In addition, Mineo *et al.* (2003) observed no effect of feeding another non-digestible saccharide (difructose anhydride-III) to rats on net calcium absorption in caecum and colon except when this saccharide was added into the mucosal buffer solution (100 mM). This all argues for a lack of significant and sustained effect of long-term fructan feeding on the tissues with respect to their ability to absorb calcium from the lumen based on the exposed tissue surface area in the Ussing chamber. When, however, the transport data were recalculated to include the increased caecal tissue surface area, the entire caecum of rats fed fructans showed an almost twofold increase in overall calcium absorption capacity and this may explain best the *in vivo* findings together with the increased caecal calcium load and its increased solubility.

4.4. GENE EXPRESSION ANALYSIS OF INTESTINAL MUCOSA

4.4.1. CHANGES IN TRANSCRIPT LEVELS DETERMINED BY DNA MICROARRAYS

The administration of 10% dietary Raftilose®P95 to rats over 28 days had an distinct effect on the genetic response of the large intestinal mucosa as determined by DNA microarray hybridisations. Around 3 to 5% of spotted genes displayed changes in transcript levels with the majority responding with increased message levels in the oligofructose group. When regulated genes were classified into subgroups according to their known or suggested functions it became obvious that most of the regulated genes were linked to processes that control cell homeostasis and cell stability (fig. 15). This corresponds well with the morphometric measurements of caecum and colon that showed major alterations in tissue architecture which may be submitted by changes in gene expression by fermented fructans. A very recent study reported similar changes in gene expression involved in cell growth when tissue samples from colorectum of rats fed 10% dietary fructooligosaccharides were analysed (Fukushima *et al.* 2004). However, in contrast to the present experiment, these authors observed the majority of genes down-regulated (20 genes, 3.4%) compared to the up-regulated (6 genes, 1.0%).

An inspection of the list of regulated genes with respect to cellular transport processes (appendix table1) identified for example the SNARE protein (soluble N-ethylmaleimide-sensitive-factor attachment protein receptor) which is part of the protein complexes that are required for membrane fusion, for intercompartmental transport and exocytosis (Ungar and Hughson 2003). Proteins involved in this membrane fusion machinery are encoded by genes such as the prenylated SNARE protein (AF033027), alpha-soluble NSF attachment protein (X89968), and endobrevin (AF132812) which all showed 1.5 to 2.0-fold increased mRNA steady state expression levels in tissues from oligofructose-fed rats. Cellular interactions with the extracellular matrix are modulated by osteonectin/SPARC (secreted protein acidic and rich in cysteine) (D28875) which displayed a 1.9-fold elevated mRNA level. The expression of SPARC in adult animals is limited largely to remodelling tissue such as bone, gut mucosa, and healing wounds (Brekken and Sage 2001). A decreased mRNA level (1.7-fold) in the oligofructose group was observed in case of claudin-3 (AJ011656) as an integral protein of tight junction complexes (Gonzalez-Mariscal *et al.* 2003). The nonmuscle caldesmon (U18419) with a 1.8-fold increased mRNA level binds to a number of structural or contractile

proteins including actin, myosin, tropomyosin, and calmodulin (McMartin *et al.* 2003) and was suggested to be involved in contraction of the perijunctional actomyosin ring regulating junctional permeability. Responsible for intracellular calcium binding, NEFA (DNA binding, EF-hand, acidic region protein) precursor (AF250142) and nucleobindin (U75409) were found to be increased in mRNA expression 2.1- and 1.7-fold, respectively. The organic anion transporter (L19031) mRNA was 1.8-fold increased and the encoded protein is involved in pH-dependent and carrier-mediated absorption of various anions including short-chain fatty acids (Nozawa *et al.* 2004).

Although an increased or decreased mRNA level not necessarily means that the protein level shows corresponding changes and that the physiological function of the protein is similarly changed, such a first screening for molecular changes in colonic tissues in response to feeding of fructans appears a useful tool to help to understand how fructan fermentation transmits the signals into changes in tissue structure and functional impairments.

4.4.2. CHANGES IN TRANSCRIPT LEVELS DETERMINED BY REAL-TIME RT-PCR

To verify mRNA regulation as found by DNA microarray analysis, four regulated genes (at least in one segment) were selected and their mRNA expression level determined using the real-time RT-PCR (table 7). The up-regulation of the selected genes was confirmed with even more pronounced regulation factors by real-time RT-PCR but the down-regulation of claudin-3 could not be confirmed. Furthermore, six of eight non-regulated genes analysed additionally were confirmed as non-regulated by real-time RT-PCR when the same threshold values for regulation were applied as used for array analysis (> 0.6 and < 1.6). Even if the number of selected genes for verification was low as compared to total number of spotted genes on the array, it may be stated that both methods overall showed a fair agreement and this matches with previous analysis where DNA microarrays were verified by Northern blots and real-time RT-PCR (tom Dieck *et al.* 2005).

To obtain first information if different inulin preparations have different effects on the transcriptome of large intestinal tissues, four regulated genes (at least in one segment) from the microarrays were selected and amplified also from tissues of rats fed Raftilose®Synergy1 rather than Raftilose®P95. For the majority of the regulation factors determined, major differences were found between the two fructan preparations (table 8). This may be due to

differences in the course of fermentation because oligofructose is already fermented in more proximal parts of large intestine whereas inulin with its longer fructan chains reaches in larger quantities also more distal parts of the colon.

Finally, the real-time RT-PCR analysis were used to determine changes in mRNA levels of genes related to transcellular and paracellular calcium transport pathways (table 9). Observed increased transcript levels for CaBP9k and Na^+/Ca^{2+} exchanger suggest an increased intracellular calcium binding capacity and increased calcium extrusion rate at the basolateral membrane. At the protein level, it was shown previously that fructooligosaccharides feeding increased CaBP9k levels in mucosa of rat caecum and colorectum (Ohta *et al.* 1998) and this was independent of the dietary calcium level and the action of 1,25-dihydroxycholecalciferol (Takasaki *et al.* 2000). However, TRPV6 at the apical membrane considered as the rate-limiting step in transcellular calcium absorption (van Abel *et al.* 2003) did not show altered transcript levels. In support of the coupling theory of SCFAs movement and calcium flux, a strong increase of carbonic anhydrase transcript levels in proximal colon of Synergy1-fed animals was found. Carbonic anhydrase could provide more intracellular HCO_3^- for exchange with SCFA anions while the remaining H^+ could be extruded by $Ca^{2+}/2H^+$ and Na^+/H^+ exchangers, the latter consists of several isoforms expressed in the apical or basolateral membrane (Zachos *et al.* 2005). The decreased mRNA level of carbonic anhydrase in caecum could indicate that here a diffusion of protonated SCFAs without a coupled HCO_3^- exchange predominates over other mechanisms. Beside transcellular calcium movement, the paracellular pathway might also be involved. In Synergy1-fed animals increased transcript levels of the tight junction protein claudin-3 were found. Most interestingly, a 6-fold increase in the mRNA of aquaporin-8 identified previously in rat colon (Calamita *et al.* 2001) suggests that water movement in large intestine could be markedly altered by inulin and oligofructose feeding.

As already alluded, these changes in mRNA levels do not proof any functional alterations but they are tempting to speculate that the corresponding gene products may be involved in the overall response of the large intestine to fructan administration and fermentation. In the context of these findings, those from literature, and certain plausibilities, a model was drawn that may explain how inulin-type fructans – mainly via the SCFA produced during bacterial fermentation – could increase transcellular and paracellular calcium transport in the large intestine (fig. 16). This model serves in hypothesis building and needs experimental proof.

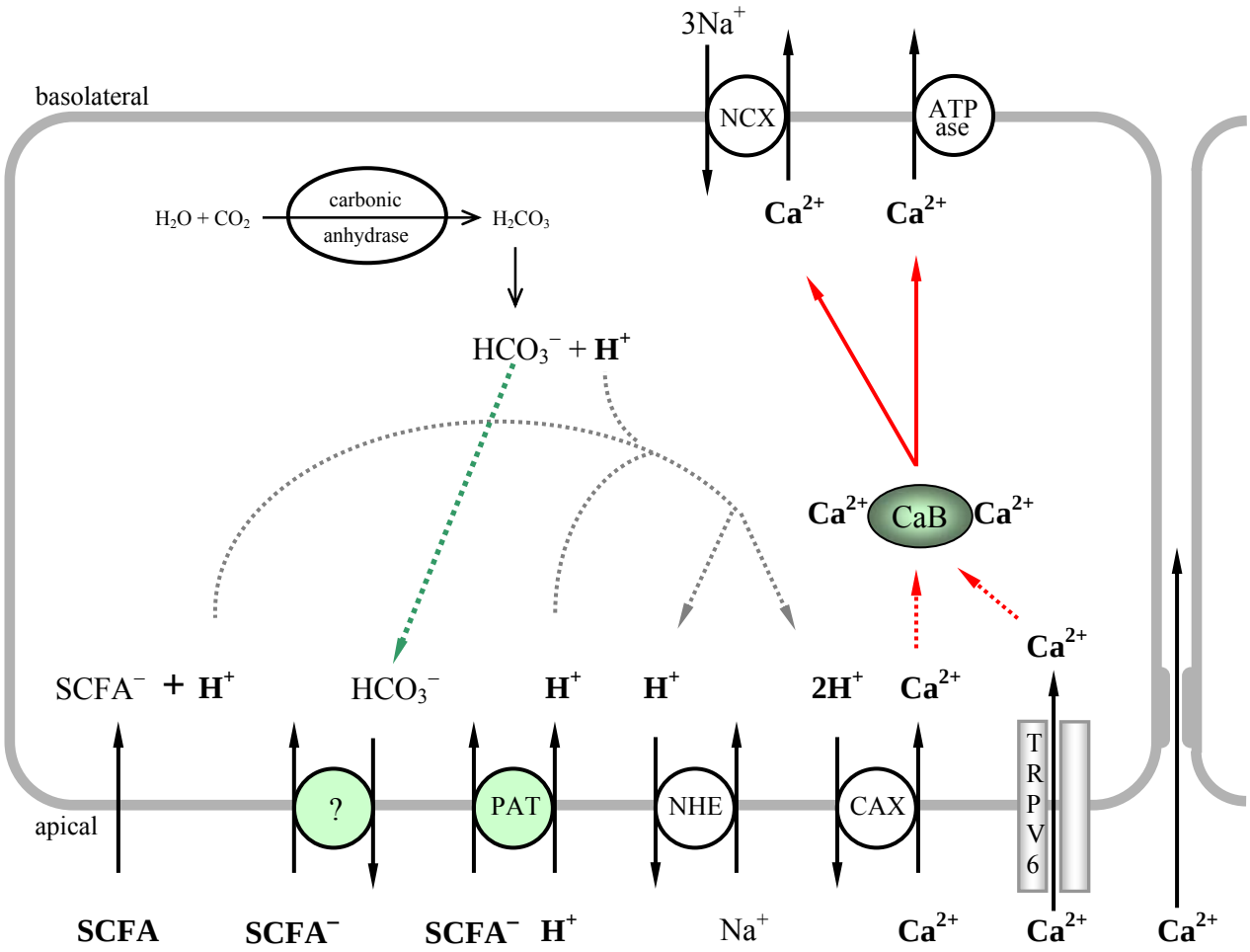

Fig. 16 Suggested model for the effect of inulin-type fructans on the transcellular and paracellular calcium transport pathway in the large intestine to increase net calcium movement. SCFAs as the metabolites of the fructans may be taken up into the cell by non-ionic diffusion, in exchange with bicarbonate (transporter is unknown) (Zaharia *et al.* 2001) or in symport with protons by PAT (Foltz *et al.* 2004). Bicarbonate is provided by the carbonic anhydrase. Protons from intracellularly dissociated SCFAs or from the symport of SCFA anions with protons may be extruded back into the lumen via an antiport with sodium (NHE) (Schroder *et al.* 1999) and/or with calcium (CAX) (Wadhwa and Care 2000). CAX is a suggested electroneutral exchanger yet only identified in plants and bacteria (Waditee *et al.* 2004). Calcium is also taken up through the calcium channel TRPV6 (Peng *et al.* 2003). Calbindin (CaBP9k) binds the intracellular calcium and shuttles it to the basolateral membrane where it is extruded by the calcium ATPase and in exchange with sodium via the NCX protein (Wasserman *et al.* 1992). Calcium is also transported through the tight junctions as a function of the calcium concentration gradient (Bronner 2003).

4.5. CONCLUSION

Dietary inulin and oligofructose administration (10% w/w) in rats leads to an increased intestinal apparent calcium absorption, increased whole body calcium retention, and increased calcium accumulation in femur. As inulin-type fructans are fermented to SCFAs in the large intestine, the pH in caecum is reduced which in turn raises the intraluminal quantities of soluble and ionised calcium. Moreover, fructan-fed rats display a marked enlargement of absorptive surface area of caecum and colon enabling more calcium to be absorbed. These changes in tissue morphology and in mineral availability are considered to provide the main mechanisms for the observed increased calcium absorption *in vivo*. At the epithelial level, the presence of SCFAs and intact inulin-type fructans were shown to increase net calcium absorption in large intestine, most likely by interconnected solute transport processes driven by the larger amount of SCFAs produced and absorbed. Although no sustained effect of fructan feeding on the tissue capability was observed to absorb calcium in course of changes in gene/protein expression, a variety of genes associated with the solute transport processes and tissue dynamics showed major changes in transcript levels. This suggests fructans to possess a variety of other effects in caecal and colonic tissues.

To translate the observed findings into human physiology, it should be considered that the anatomy of rat and human large intestine and especially of the caecum differs markedly between the two species. In the rat, the caecum appears as a separate organ in size comparable to the stomach whereas humans have a poorly defined caecal region which is continuous with the colon (Kararli 1995). Feeding inulin and oligofructose to rats causes marked changes in caecum which is probably not the case in humans. However, as calcium absorption in humans *in vivo* is also increased by inulin-type fructans it can be anticipated that similar mechanisms are responsible for the effects – although their magnitude may be smaller and they may not be as pronounced to the caecal region as found in rats.

4.6. FUTURE RESEARCH PERSPECTIVES

From the experiments presented here, a number of interesting scientific questions arise regarding the physiological consequences of the ingestion of inulin-type fructans. It could be valuable for example to study the effects of these fructans on the intestinal calcium transport at low dietary calcium intakes. As shown by Coudray *et al.* (2004), a low calcium diet (0.25% Ca) provided together with inulin led to lower concentrations of total and soluble calcium in rat caecum than a diet with adequate calcium content (0.5%) without inulin. Despite the differences in intraluminal calcium, the intestinal apparent calcium absorption did not significantly differ between both groups. This suggests that fructans are even more important than a high calcium intake in providing sufficient body calcium. This is of particular importance in explaining the fairly good bone mineral status in humans on a plant-based diet with low calcium intakes but by its nature high in fermentable substrates.

The number and character of the genes identified as regulated in caecal and colonic tissues in response to fructan feeding is striking. It suggests not only major effects on tissue dynamics, i.e. cell cycle control and tissue integrity, but also on a variety of processes associated with water and electrolyte transport and metabolism. Fluid movement – critical with respect to gut motility and constipation – could be modified and studies in humans have already shown that theses parameters change upon ingestion of sufficient quantities of fructans. A diet rich in fructans – as that of our ancestors – therefore appears to modify gut physiology by various mechanisms including changes in gene expression. These pleiotrope effects deserve future studies.

Es werden viel zu viele Bücher geschrieben und das viele Grübeln kann dich bis zur Erschöpfung ermüden.

Das Buch des Predigers 12,12

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Der Abschied schmerzt immer, auch wenn man sich schon lange auf ihn freut.
Arthur Schnitzler (1862 - 1931)

7. APPENDIX

7.1. ABBREVIATIONS

ApoA-I	apolipoprotein A-I
Aqp8	aquaporin-8
CaBP9k	calcium binding protein, 9 kD
CA-III	carbonic anhydrase-3
CaT1	calcium transport protein-1
Cldn3	claudin-3
Cy3	cyanine dye, fluorescence emission at 570 nm
Cy5	cyanine dye, fluorescence emission at 670 nm
dCTP	deoxycytidine triphosphate
dNTP	deoxynucleotide triphosphate
DP	degree of polymerisation
ECaC2	epithelial calcium channel-2
FE	feeding experiment
MMLV	moloney murine leukaemia virus
NCX	sodium/calcium exchanger
NHE	sodium/proton exchanger
Ocln	occludin
PMCA1b	plasma membrane calcium ATPase-1b
PTH	parathyroid hormone
SCFA	short-chain fatty acid
TRPV6	transient receptor potential vanilloid subfamily, member 6
VDR	vitamin D receptor

7.2. TABLE 1 COMPLETE LIST OF REGULATED GENES DETERMINED BY DNA MICROARRAYS.

The transcription of genes in large intestinal epithelium (CA = caecum, PC = proximal colon, DC = distal colon) was analysed in rat tissues after the feeding of 0 or 10% Raftilose®P95 for 28 days. *Regulation > 1 means increased transcript levels, regulation < 1 means reduced transcript levels, compared to controls. References cited here are attached at the end of the table.

ID	gene	suggested function	regulation*		
			CA	PC	DC
energy metabolism					
M31805	NAD(P)H:quinone reductase	complex I of respiratory chain (Bayney *et al.* 1989)	3.7		
S70011	tricarboxylate carrier	transport protein between intra- and extra-mitochondrial space, e.g. citrate carrier (Palmieri *et al.* 1996)	3.6		
M25301	mitochondrial ATP synthase beta-subunit	ATP synthesis in most tissues (Das 2003)	2.3		
J01435	mitochondrial cytochrome oxidase subunits I, II, III	complex IV of respiratory chain (Löffler and Petrides 1998)	0.6		
AF067728	transactivating protein BRIDGE	coactivation and regulation of transcription of the insulin gene (Thomas *et al.* 1999)		2.6	
U00926	F1F0 ATPase delta subunit	ATP synthase (F1F0-ATPase); F1 hydrolyses ATP, F0 synthesises ATP (Pan *et al.* 1998)		2.0	
D13124	P2 for ATP synthase subunit c	ATP synthase (F1F0-ATPase); F0 membrane domain in its simplest form consists of three different polypeptides (Arechaga *et al.* 2002)		1.8	
X14044	glutamate dehydrogenase (GLDH)	metabolisation of amino acids for energy production in mucosa; nitrogen fixation for biosynthesis or excretion (Löffler and Petrides 1998); urea synthesis occurs not only in hepatocytes but also in enterocytes (Bush *et al.* 2002)		1.7	
K00750	cytochrome c	respiratory chain		0.6	
lipid metabolism					
M88592	peroxisome proliferator activated receptor (PPAR)	peroxisomes contain enzymes for fatty acid and amino acid degradation (Löffler and Petrides 1998); transcription factor involved in regulation of gene expression (Pegorier *et al.* 2004)	3.4		
J02585	stearyl-CoA desaturase	desaturation of fatty acids; the produced mono-unsaturated fatty acids are used for synthesis of triglycerides, wax esters, cholesteryl esters and membrane phospholipids (Miyazaki and Ntambi 2003)	2.6		
X15551	beta-2 glycoprotein I	(= apolipoprotein H) constituent of chylomicrons, VLDL and HDL; suggested in fatty acid metabolism and blood coagulation (Wang and Chiang 2004)	2.1	1.9	
J02582	apolipoprotein E	constituent of chylomicrons, VLDL, LDL and HDL (Löffler and Petrides 1998)	2.1		
AF119667	lipolysis-stimulated remnant receptor (LSR) alpha subunit	could be a rate-limiting step for the clearance of dietary triglycerides (Yen *et al.* 1999)	2.0		
X95189	trihydroxycoprostanoyl-CoA oxidase	shortening of the side chain of the bile acid intermediates di- and trihydroxycoprostanic acids	2.0		

ID	gene	suggested function	regulation*		
			CA	PC	DC
		via one cycle of beta-oxidation (Mannaerts *et al.* 2000)			
M00001	apolipoprotein A-I	constituent of chylomicrons and HDL (Löffler and Petrides 1998); reduced levels of apo A-I, a component of anti-atherogenic HDL, are associated with increased cardiac events (Walldius and Jungner 2004)		10.3	1.8
U02096	fatty acid binding protein	implicated in cytoplasmic transport of lipophilic ligands, such as long-chain fatty acids and retinoids (Schaap *et al.* 2002)		6.0	
M00002	apolipoprotein A-IV	constituent of chylomicrons and HDL		2.1	
D90109	long-chain acyl-CoA synthetase	activation of long-chain fatty acids on the mitochondrial outer membrane, first step in beta-oxidation (Kerner and Hoppel 2000)		2.0	
K03046	retinol-binding protein (RBP)	retinol is complexed with cellular retinol-binding protein type 2 (CRBP2) and serves as a substrate for reesterification; the retinyl esters are incorporated into chylomicrons and intestinal lipoproteins and secreted into the lymph (Harrison and Hussain 2001)		1.8	
M33648	mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase	involved in ketogenesis (Miliar *et al.* 2001) and in biosynthesis and regulation of systemic and cellular cholesterol metabolism (Broitman *et al.* 1993)		1.7	2.2
AF095449	L-3-hydroxyacyl-CoA dehydrogenase precursor (HAD)	NAD-dependent conversion of L-3OH-acyl-CoA to 3-ketoacyl-CoA, part of beta-oxidation (Cosgrove *et al.* 2004)		1.7	1.5
AB009686	CYP8B for sterol 12alpha-hydroxylase P450	key enzyme in cholic acid biosynthesis (Lundell and Wikvall 2003)		1.7	
K01934	hepatic product spot 14 (S14)	key role in the tissue-specific regulation of lipid metabolism; S14 antisense oligonucleotides inhibit both the intracellular production of lipids and their export as VLDL particles (Cunningham *et al.* 1998)		1.7	
D90058	peroxisomal 3-ketoacyl-CoA thiolase A	beta-oxidation of straight-chain fatty acyl-CoAs (Chevillard *et al.* 2004)		1.6	
D00362	carboxyesterase E1	hydrolysis of several lipids and cholesterol esters, cholesterol absorption in the gut (Schwer *et al.* 1997); may also act synergistically with efflux transporters of organic anions, affecting the intestinal availability of many xenobiotics (Suzuki and Sugiyama 2000)	0.6		
D45252	2,3-oxidosqualene: lanosterol cyclase	conversion of (3S)-2,3-oxidosqualene to lanosterol, key step in the biosynthesis of sterols (Abe *et al.* 2001)		0.6	
U89905	alpha-methylacyl-CoA racemase (AMACR)	beta-oxidation of branched fatty acids, shown to be elevated in prostate cancer (Kumar-Sinha *et al.* 2004); significant up-regulation of AMACR, also known as P504S, mRNA was found in colon carcinomas (Jiang *et al.* 2003)			0.6
xenobiotic metabolism and stress response					
AB008423	CYP2D2 (cytochrome P450 isoform)	several functions, e.g. hydroxylation of xenobiotics or synthesis of bile acids (Löffler and Petrides 1998)	3.0		
L15079	p-glycoprotein	multidrug resistance genes encode for p-glycoprotein; may also function as chloride channel (Brown *et al.* 1993); actively transports drugs back into the gut lumen and limits their oral bioavailability (Kharasch *et al.* 2003)	2.0		

ID	gene	suggested function	regulation*		
			CA	PC	DC
M11794	metallothionein-2 and metallothionein-1	binds and eliminates Cd, Zn, Cu, Ag; elevated levels of this protein during stress (Löffler and Petrides 1998)	0.6		
AF106945	peroxiredoxin PRx IV	antioxidant; may protect the vascular system from reactive oxygen species (ROS) (Matsumoto *et al.* 1999)		2.3	
D86086	canalicular multispecific organic anion transporter (cMOAT)	ATP-dependent efflux transport pump for a variety of drugs (Arimori *et al.* 2003); MRP2 = cMOAT = cMRP (Payen *et al.* 2002)		2.2	
M15345	microsomal xenobiotic epoxide hydrolase	bioactivation and detoxification of polycyclic aromatic hydrocarbons derived from tobacco smoke and charred meat intake (Tranah *et al.* 2004)		1.8	
AB010466	multidrug resistance-associated protein (MRP)-like protein-1 (MLP-1)	might be a novel member of the MRP family responsible for the excretion of organic anions (Hirohashi *et al.* 1998)		1.7	
X12367	glutathione peroxidase I	primary protection against acute oxidative stress (De Haan *et al.* 2003)		1.7	
AF045464	aflatoxin B1 aldehyde reductase (AFAR)	metabolises the environmental carcinogen aflatoxin B(1); the expression of this enzyme is markedly increased in rat liver by cancer chemopreventive agents (Ellis *et al.* 2003)			1.6
D16339	alpha-tocopherol transfer protein	scavenger of reactive metabolites of oxygen and inhibitor of lipid peroxidation in membranes; also a regulator of gene transcription (Gohil *et al.* 2003)			1.6
D25221	selenoprotein P	antioxidant and selenium transport (Burk *et al.* 2003); a protective role in colon carcinogenesis is discussed (Mork *et al.* 2000)			1.6
K02422	cytochrome P-450d methylcholanthrene-inducible	oxidative degradation as well as biosynthesis of many compounds (Jung 2002); oxidation of foreign compounds (Gonzalez and Kimura 2001)			1.6
M13711	cytochrome P450 PB1	oxidative degradation as well as biosynthesis of many compounds (Jung 2002); oxidation of foreign compounds (Gonzalez and Kimura 2001)			1.5
M73714	microsomal aldehyde dehydrogenase	ethanol is metabolised by ADH to acetaldehyde and finally to acetate (Brennan *et al.* 2004); microbes may produce acetaldehyde endogenously without alcohol administration (Salaspuro 2003)			1.5
AJ238391	sulfotransferase K1	excretion of drugs and xenobiotics; in some cases biological activation of such compounds to be carcinogenic or pharmacologically active (Xiangrong *et al.* 2000)			0.6
J03752	glutathione S-transferase	cell protection from toxic xenobiotics and oxidant stress, both directly and as a cofactor of glutathione peroxidases (Pompella *et al.* 2003)			0.6
immune modulation					
AF151982	secretory leukocyte protease inhibitor (SLPI)	important regulator of inflammatory response; suppresses the activation of NF-kappaB (Ward and Lentsch 2002)	2.6		
D00675	alpha-1-protease inhibitor	(also referred to as alpha-1-antitrypsin) increased level in plasma in the acute phase of a number of disorders including inflammation and bacterial infection (Miyamoto *et al.* 2000)	0.6		
Y08531	beta 2 microglobulin	forms the single beta chain of the human leukocyte antigen (HLA) class I molecule (Winchester *et al.* 2003); elevated serum levels are observed in several autoimmune and neoplastic diseases (e.g. multiple sclerosis) (Bagnato *et al.* 2003)	0.6		
Z50051	bovine C4b-binding	inhibits all pathways of complement activation,		4.5	

ID	gene	suggested function	regulation*		
			CA	PC	DC
	protein (C4BP) alpha chain	acting as a cofactor to the serine protease factor I (FI) in the degradation of activated complement factors C4b and C3b (Blom 2002)			
AB042598	pre-procarboxypeptidase R	control of the inflammatory response and cleaving C-terminal lysin residues from partially degraded fibrin (Shimomura *et al.* 2003)		3.2	0.6
M83177	amyloid P component (SAP)	candidate protein for the clearance of nuclear debris in the blood; binds pathogens such as influenza A virus and Shiga toxin 2 and also malfolded proteins (Sen *et al.* 2002)			2.5
M83176	C-reactive protein	suppressive effect on the expression of nitric oxide synthase (NOS) (Ikeda *et al.* 2003); opsonisation of ligands with complement (Diaz *et al.* 2003); blood levels of CRP, haptoglobin (Hp) and fibrinogen (Fbgn) increase during acute inflammation (Giffen *et al.* 2003)			1.7
cell homeostasis and stability					
AF136583	protein kinase SNK	SNK (serum-inducible kinase) = PLK2 (polo-like kinase); PLKs control cell cycle progression through the regulation of centrosome maturation and separation, mitotic entry, metaphase to anaphase transition, mitotic exit and cytokinesis (Leung *et al.* 2002); PLK2 deficient mice showed retarded growth and skeletal development late in gestation (Ma *et al.* 2003a); activity of SNK is inhibited by calcium- and integrin-binding protein (CIB) (Ma *et al.* 2003b)	2.9		
L14441	phosphotidylethanolamine N-methyltransferase (PEMT)	conversion of phosphatidylethanolamine to phosphatidylcholine in hepatocytes; required for normal growth and liver function when mice have insufficient choline in diet (Vance *et al.* 1997); PEMT activity in liver 1238, lung 104, intestine 25 pmol/min/mg protein (Cui et al., 1993); PEMT activity is depressed in alcoholic liver disease (Lieber 2001)	2.8		
S69315	endoplasmin (GRP94)	(glucose regulated protein) (or GP96) showed activity in prophylactic and therapeutic cancer immunotherapy models (Castelli *et al.* 2004)	2.4		
AF030050	replication factor C	replication of DNA (Henneke *et al.* 2003)	2.1		
L11587	leukocyte common antigen-related phosphatase (LAR-PTP2)	protein tyrosine phosphatases (PTPs) are critical regulators of cellular phosphorylation functioning in processes such as cell growth, differentiation, and adhesion (Wheeler *et al.* 2002)	2.1		
AF140031	activin beta C	activins are members of the transforming growth factor-beta (TGF-beta) superfamily of growth factors; involved in differentiation and control of proliferation (Vejda *et al.* 2002); activin C is thought to be involved in the regulatory mechanism of activins (Mellor *et al.* 2003)	2.0		
M86341	ADP-ribosylarginine hydrolase	posttranslational modification of proteins (Okazaki and Moss 1999)	2.0		
S68589	SET alpha isoform	oncogenic (tumorogenic) regulator; SET negatively regulated cell-proliferative activities (Miyamoto *et al.* 2003); potent inhibitor of phosphatase 2A which is involved in the regulation of cell cycle progression (Canela *et al.* 2003)	1.9		
X15906	fibronectin	a extracellular matrix (ECM) protein, controls both cell proliferation and survival in thyroid cells; after	1.9		

ID	gene	suggested function	regulation*		
			CA	PC	DC
		integrin (transmembrane receptor for ECM proteins) activation, increase of intracellular calcium concentration $[Ca^{2+}]_i$ has been observed which mediates important components of the integrin signaling pathway by controlling cell functions (Illario *et al.* 2003)			
L10336	guanine nucleotide-releasing protein (MSS4)	encodes for phosphatidylinositol 4,5-bisphosphate which plays a role in cellular processes; mutated MSS4 causes disorganisation of the actin cytoskeleton, aberrant cell morphology and loss of cell integrity (Desrivieres *et al.* 2002)	0.6		
J04503	phosphatase 2c	protein phosphorylation, regulation of cellular functions (Tamura *et al.* 1989)	0.5		0.6
D10706	ornithine decarboxylase antizyme	inhibitor of ornithine decarboxylase which decarboxylises ornithine to putrescine; from this spermidine and spermine is formed which are essential growth factors; they may stabilise DNA and stimulate RNA synthesis (Michal 1999); widely distributed in living organisms, related to cell growth (Goda *et al.* 2004)	0.4		
D90265	proteasome subunit RC2	ubiquitin-proteasome pathway functions as intracellular protein breakdown; the pathway is involved in a vast field of basic cellular processes, such as cell cycle and division, differentiation and development, the response to stress and extra-cellular modulators, morphogenesis of neuronal networks, modulation of cell surface receptors, ion channels, secretory process, DNA repair, immune and inflammatory responses regulation, biogenesis of organelles, and apoptosis (Chai *et al.* 2003)		2.7	
AF146044	aminopeptidase A	hydrolysis of amino acids from the N-termini of peptides and proteins; key role in maturation, activation, modulation and degradation of proteins, signal transduction, cell differentiation and cell adhesion; aminopeptidase A, an integral membrane protein, specifically cleaves N-terminal acidic amino acid residues such as Glu and Asp (Lee *et al.* 2000)		2.3	
AF014009	acidic calcium-independent phospholipase A2 (aiPLA2)	metabolism of internalised surfactant phospholipids (Fisher *et al.* 1994)		2.1	2.0
M81681	biliverdin reductase	degradation of haem; converts biliverdin to bilirubin (Fakhrai and Maines 1992)		2.1	
Y07504	elongation factor 2 (EF-2)	protein biosynthesis in the cell, i.e. peptide chain elongation (Parrado *et al.* 2003)		2.0	1.6
AF058791	G10 protein homolog (Edg2)	Lpa1/Edg-2/Gpcr26 encodes for a G protein-coupled receptor which mediates the cellular effects of lysophosphatidic acid (LPA); LPA induces a variety of cellular responses in most cell types, including intracellular calcium mobilisation, stress fiber formation, cell rounding, neurite retraction, proliferation, and survival (Contos *et al.* 2002)		2.0	
X70900	hepsin	integral membrane protein, may participate in cell growth and in maintaining proper cell morphology (Somoza *et al.* 2003)		2.0	
D28875	osteonectin	osteonectin/secreted protein acidic and rich in cysteine (ON/SPARC) modulates cellular		1.9	

ID	gene	suggested function	regulation*		
			CA	PC	DC
		interaction with the extracellular matrix by its binding to structural matrix proteins, such as collagen and vitronectin; SPARC inhibits cellular proliferation by an arrest of cells in the G1 phase of the cell cycle; it also regulates the activity of growth factors, such as platelet-derived growth factor, fibroblast growth factor, and vascular endothelial growth factor; the expression of SPARC in adult animals is limited largely to remodeling tissue, such as bone, gut mucosa, and healing wounds (Brekken and Sage 2001)			
M15481	insulin-like growth factor I (IGF-I)	is a polypeptide hormone, structurally homologous to insulin; insulin generally regulates metabolism, IGFI controls cell growth, differentiation, and protects cells against apoptosis (Toyoshima *et al.* 2004)		1.9	
X62160	150K dynein-associated polypeptide	intracellular movement of membrane-bound organelles (Dillman and Pfister 1994)		1.9	
AF000942	inhibitor of DNA binding (Id3a)	expression of various Id genes is downregulated when cells terminally differentiate, and overexpression of Id impairs differentiation (Matsumura *et al.* 2001)		1.8	
D25224	40kDa ribosomal protein	protein from the rat 40 S ribosomal subunit (Tohgo *et al.* 1994)		1.7	
M13011	c-ras-H-1	ras familiy is thought to play a role in the normal cell cycle (Ruta *et al.* 1986); H-Ras and other components of the MAP (mitogen-activated protein) kinase cascade have been found in caveolae which may provide a microenvironment that allows efficient signal transduction (Kranenburg *et al.* 2001)		1.7	
X62145	ribosomal protein L8	protein synthesis; all ribosomal protein components have not yet been fully characterised (Ruhf and Meister 1999)		1.7	
D10755	proteasome subunit R-IOTA	ubiquitin-proteasome pathway functions as intracellular protein breakdown; the pathway is involved in a vast field of basic cellular processes, such as cell cycle and division, differentiation and development, the response to stress and extracellular modulators, morphogenesis of neuronal networks, modulation of cell surface receptors, ion channels, secretory process, DNA repair, immune and inflammatory responses regulation, biogenesis of organelles, and apoptosis (Chai *et al.* 2003)		0.6	
L12383	ADP-ribosylation factor 4	interacts with the intracellular part of epidermal growth factor receptor which plays a critical role in cell development, proliferation, and differentiation (Kim *et al.* 2003)		0.6	
U75391	B-cell receptor associated protein 37 (BAP-37)	prohibitin interacts with the structurally related protein BAP-37 (Nijtmans *et al.* 2000); prohibitin inhibits cell proliferation and is associated with chaperone-like functions (Bacher *et al.* 2002)		0.6	
AF178669	p34	cyclin-dependent kinase 1 (Cdk1), formerly Cdc2 or p34; contribution to the mitotic prophase and metaphase and probably to apoptosis induction (Castedo *et al.* 2002)		0.4	
AF041066	ribonuclease 4	degradation of RNA; RNase 4 only cleaves RNA			1.7

ID	gene	suggested function	regulation*		
			CA	PC	DC
		after pyrimidine bases (Hofsteenge *et al.* 1998)			
M55022	nucleolin	directly involved in the regulation of ribosome biogenesis and maturation; nucleolin is fundamental to the survival and proliferation of cells (Srivastava and Pollard 1999)			1.7
L14462	R-esp1	suggested role in the post-injury response of the adult mammalian brain; overexpression of R-esp1 promotes cell survival in PC12 (pheochromocytoma) cells (Arndt *et al.* 1999)			1.6
M64300	extracellular signal-related kinase (ERK2)	is activated by diverse extracellular stimuli and by proto-oncogene products that induce proliferation or enhance differentiation; is also linked to the small heat-shock protein HSP27 which modulates contraction of intestinal smooth muscle (Harnett and Biancani 2003)			1.6
AF121265	beta-catenin	beta-catenin signaling, key component in regulating epithelial cell proliferation (Sun *et al.* 2004)			1.5
M24092	alpha-B-crystallin	involved in the stabilisation and the regulation of cytoskeleton, such as intermediate filaments and actin (Fujita *et al.* 2004); is a member of the small heat-shock protein family possessing chaperone-like function (Horwitz 2003)			1.5
AF290194	hypertension-related calcium-regulated gene (HCaRG)	is negatively regulated by extracellular calcium with higher mRNA levels in spontaneously hypertensive rats; HEK293 cells stably transfected with HCaRG exhibited much lower proliferation (Solban *et al.* 2000)			0.3
transport processes					
AF007775	aquaporin-pancreas and liver (AQP8)	water channel, integral membrane protein; expression of the cRNA in Xenopus oocytes markedly enhanced osmotic water permeability; (Koyama *et al.* 1997); AQP8 mRNA was detected in duodenum, proximal jejunum, proximal colon, rectum, pancreas and liver and, to a lesser extent, in stomach and distal colon; physiological role is suggested for absorption of water in the intestine and bile secretion in liver (Calamita *et al.* 2001)	2.6		
AF033027	prenylated SNARE protein (Ykt6)	intracellular membrane fusion requires SNARE (soluble N-ethylmaleimide-sensitive-factor attachment protein receptor) proteins that form complexes bridging the two membranes (Ungar and Hughson 2003)	1.9		
AJ011656	claudin-3 (Cldn3)	integral protein of tight junctions (together with occludin and JAM) (Gonzalez-Mariscal *et al.* 2003)	0.6		
J00732	fatty acid binding protein (FABP)	maintenance of cellular membrane fatty acid levels, intracellular trafficking of these substrates, modulation of specific enzymes of lipid metabolic pathways, modulation of cell growth and differentiation (Massolini and Calleri 2003)		4.0	
AF250142	NEFA (DNA binding, EF-hand, acidic region protein) precursor	may be involved in autoimmunity, apoptosis and calcium homeostasis in the Golgi apparatus and bone matrix (Otte et al. 1999); NEFA protein binds Ca^{2+}-ions via its EF-hand domain at a ratio of 2 mol Ca^{2+}/mol protein (Nesselhut *et al.* 2001)		2.1	
X89968	alpha-soluble NSF attachment protein	membrane fusion for intercompartmental transport and exocytosis; this fusion requires membrane-associated helical proteins known as soluble N-ethylmaleimide-sensitive factor (NSF) attachment		2.0	

ID	gene	suggested function	regulation*		
			CA	PC	DC
		protein receptors (SNAREs), which coil together into SNARE complexes (Marz *et al.* 2003)			
AB030829	carbonic anhydrase III	hydration of CO_2 to generate protons and bicarbonate ions for cellular ion transport and pH homeostasis (Sowden *et al.* 1998)		1.9	
AF132812	endobrevin	endobrevin/VAMP-8 is a member of the SNARE membrane fusion machinery, syntaxin 2 and endobrevin, specifically localise to the midbody during cytokinesis in mammalian cells (Low *et al.* 2003)		1.7	1.5
AF041373	clathrin assembly protein short form (CALM)	clathrin-coated vesicles are involved in pathways of receptor-mediated intracellular transport and transfer of proteins from trans-Golgi network to pre-lysosomal compartment and recycling of synaptic vesicles (Kim and Lee 1999)		0.6	
J04022	brain Ca^{2+}-ATPase	calcium pump		0.6	
L19031	organic anion transporter (Oatp)	apical bile acid transport in small intestine (Walters *et al.* 2000); involved in pH-dependent and carrier-mediated absorption for anionic compounds including short-chain fatty acids (Nozawa *et al.* 2004)			1.8
U75409	nucleobindin	Ca^{2+}-binding protein, identified within the nucleus and endoplasmic reticulum, and in association with the Golgi membrane; also a minor constituent of bone extracellular matrix playing a role in mineralisation (Somogyi *et al.* 2004)			1.7
J02997	bile canaliculus domain-specific membrane glycoprotein	transport and secretion of organic anions, including bile acids, into bile in liver (Hong and Doyle 1987); also found in other epithelial brush borders (small intestine, kidney, colon, pancreatic duct) (McCaughan *et al.* 1990)			1.5
others					
NM_012589	interleukin 6 (interferon, beta 2) (Il-6)	is released by muscle contraction and activates processes to maintain metabolic homeostasis; Il-6 production is activated by intracellular calcium (Febbraio 2003)	2.4		
AF107727	sertolin	cell-cell interactions in the testis (Mruk and Cheng 1999)	1.9		
X55995	dimethylglycine dehydrogenase	oxidative degradation of choline to glycine; converts dimethylglycine into sarcosine (which is converted into glycine by sarcosine dehydrogenase) (Bergeron *et al.* 1998)	1.9		
J00751	cardiac myosin heavy chain insert 21/26	myosin heavy chain is the molecular motor driving muscle contraction (Haddad *et al.* 2003)	0.6		
S73894	endogenous vascular elastase = serine proteinase adipsin homolog	increased activity precedes the development of sustained pulmonary hypertension and vascular abnormalities (Rabinovitch 1995)	0.4		2.9
U97667	round spermatid protein RSP29	is expressed in many tissues and in small intestine and colon; is thought to have a fundamental role in all tissues but important role in spermatogenesis (Ji *et al.* 1997)		10.1	
L00094	angiotensinogen	renin-angiotensin system (RAS), important for regulation of blood pressure, electrolyte balance and vascular growth (Stanton 2003)		2.1	
L04796	glucagon receptor	highly homologous to glucagon-like peptide-1, parathyroid hormone, calcitonin (Hansen *et al.* 1995); activation of glucagon-like peptide-2		2.1	

ID	gene	suggested function	regulation*		
			CA	PC	DC
		receptor signaling maintains the integrity of the intestinal epithelial mucosa via regulation of crypt cell proliferation (Yusta *et al.* 2000)			
M17083	major alpha-globin	constituent of hemoglobin (heterotetramer of two alpha-globin and two beta-globin polypeptides) (Hardison 1998)		1.8	1.6
U18419	nonmuscle caldesmon	binds to a number of structural or contractile proteins including actin, myosin, tropomyosin, and calmodulin; is proposed to regulate contractile tone in smooth muscle cells (McMartin *et al.* 2003)		1.8	
U62315	alpha-globin (GloA)	constituent of hemoglobin (heterotetramer of two alpha-globin and two beta-globin polypeptides) (Hardison 1998)		1.8	
U17603	rS-Rex-s	belong to the family of neuroendocrine-specific protein/reticulons which are localised in the endoplasmic reticulum and identified as markers for neuroendocrine differentiation (Huang *et al.* 2004)		1.7	2.3
D00753	contrapsin-like protease inhibitor related protein (CPi-26)	contrapsin is a member of the serpin superfamily and inhibits trypsin; mouse and rat contrapsins have similarity in sequence to human alpha1-antichymotrypsin (Yoshida *et al.* 2001)		1.7	1.7
AF097723	hematopoietic lineage switch 2 related protein (Hls2-rp)	100% homologous to glutamate carboxypeptidase (NM_031640) which serves as a high-affinity folate hydrolase in the gut cleaving the polyglutamate chain to permit the absorption of folate (Tsai *et al.* 2003)		1.7	1.5
S37709	thyrotropin receptor	binds thyrotropin which regulates growth and function of the thyroid (Wonerow *et al.* 2001)		1.7	
U66461	protein inhibitor of neuronal nitric oxide synthase (PIN)	nitric oxide is a molecule with cytotoxic properties and a modulator of signal transduction pathways (Yu *et al.* 2002); NHE3 (sodium proton exchanger) and Na-K-2Cl cotransporter (NKCC2) protein abundances in kidney are positively regulated by nitric oxide (Turban *et al.* 2003)		0.6	
X05566	myosin regulatory light chain (RLC)	major regulatory subunit of smooth-muscle and non-muscle myosins (Szczesna-Cordary *et al.* 2004)		0.6	
K03248	phosphoenolpyruvate carboxykinase (GTP)	initial step in hepatic gluconeogenesis (Herzog *et al.* 2004); mRNA encoding PEPCK is detectable in rat intestinal mucosa and the relative abundance increases markedly (3- to 8-fold) during starvation (Watford and Tatro 1989)			1.6
M63482	cytokeratin 8 polypeptide	structural protein, has a broad range of expression patterns for the columnar epithelium (Baek *et al.* 2004)			1.6
X67215	CE9 transmembrane glycoprotein	may be correlated in a positive fashion with metabolic activity in a diverse array of cell types (Nehme *et al.* 1995)			1.6
AF248548	syntenin (Sdcbp)	syndecan-1, E-cadherin, beta-catenin, and alpha-catenin colocalise with syntenin at cell-cell contacts in epithelial cells (Zimmermann *et al.* 2001)			1.5
M81687	core protein (HSPG)	interacts with many proteins including growth factors, chemokines and structural proteins of the extracellular matrix to influence cell growth, differentiation, and the cellular response to the environment (Blackhall *et al.* 2001)			1.5
X65295	carboxylesterase	conversion of a carboxylic ester to an alcohol and a			1.5

ID	gene	suggested function	regulation*		
			CA	PC	DC
		carboxylic acid; also hydrolysis of amides, thioesters, phosphoric acid esters, and acid anhydrides (Rooseboom *et al.* 2004)			
U19181	rabin3	involved in the regulation of vesicle traffic in cells (Coppola *et al.* 2002)			0.6
X67156	(S)-2-hydroxy acid oxidase	oxidation of a variety of hydroxy acids into keto acids (Belmouden and Lederer 1996)			0.6

References to 7.2. Table 1

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