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The Effect of Probiotic Bacteria on Transepithelial Calcium Transport and Calcium Uptake in Human Intestinal-like Caco-2 Cells

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Abstract
While prebiotic substances have attracted considerable attention in terms of their stimulatory effect on intestinal calcium absorption, the potential influence of probiotic bacteria on calcium absorption has received little research emphasis. Therefore, the objective of this study was to investigate the effect of well-characterized probiotics (*Lactobacillus salivarius* (UCC 118) and *Bifidobacterium infantis* (UCC 35624)) on calcium uptake and transepithelial calcium transport in human intestinal-like, Caco-2, cells in culture. Cells were seeded onto permeable transport membranes and allowed to differentiate, over 16 d, into intestinal-like cell monolayers. Monolayers (n=12–20/ treatment) were then exposed to *E. coli,* UCC 118, UCC 35624 (10^7 cfu/ml) or no bacteria (control) for 6 or 24 h prior to calcium transport studies. Calcium transport was unaffected by exposure of Caco-2 cells to *E. coli*, UCC 118 or UCC 35624 for 6 or 24 h. Calcium uptake into Caco-2 cell monolayers after 24 h was unaffected by UCC 35624, but was significantly (P<0.05) or tended (P=0.079) to be increased by UCC 118 and *E. coli*, respectively, relative to the control. In conclusion, the findings of this study which suggest that bacteria can enhance intestinal calcium uptake, if not calcium transport, highlights the need to undertake further studies in this, to date, vastly under-investigated area.

Introduction
The effect of dietary factors on calcium absorption is poorly understood and there is a need for detailed studies to define the ways in which food components/functional food ingredients influence calcium absorption in order to determine how calcium bioavailability from foods can be optimized (Kennefick and Cashman, 2000). Non-digestible oligosaccharides (also known as prebiotics) have attracted considerable attention in terms of their potential stimulatory effect on calcium absorption (for review, see Cashman, 2003). A prebiotic substance has been defined as “a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon” (Gibson and Roberfroid, 1995). There is relatively good evidence of a beneficial effect of prebiotics on calcium bioavailability in rats (see reviews by Scholz-Ahrens *et al*. 2001, Scholz-Ahrens and Schrezenmeir, 2002; Cashman, 2003). Moreover, while there have only been a few studies on the effect of prebiotics on calcium absorption in humans, so far, there would appear to be a stimulatory effect by these prebiotics on intestinal calcium absorption, at least in subgroups of the population which have increased calcium requirements (e.g. adolescents and postmenopausal women) (Coudray *et al*. 1997; van den Heuvel *et al*. 1999a,b, 2000; Griffins *et al*. 2002, and see review by Cashman, 2003). While the exact mechanism by which these substances stimulate intestinal calcium absorption is unclear, it is generally believed to be linked to their ability to escape digestion in the upper gut, by pancreatic and brush-border enzymes, and whereupon reaching the large bowel (especially, the cecum) they will be utilized selectively by a restricted group of micro-organisms, i.e., health-promoting probiotic bacteria (usually bifidobacteria and lactobacilli) (Macfarlane and Cummings, 1999; Cummings *et al*. 2001). These bacteria, in turn, produce fermentation by-products, including short-chain fatty acids (SCFA, essentially acetate, propionate, and butyrate) and other organic acids (e.g. lactate), which by contributing to a reduced luminal pH and increased amount of soluble calcium, may increase calcium absorption (van den Heuvel *et al*. 1999a). It is also possible that bacterially-produced SCFA directly influence calcium absorption by other means (Cashman, 2003).

While non-digestible oligosaccharides have attracted considerable attention, the potential direct influence of probiotic bacteria on intestinal calcium absorption has received much less research emphasis. Brassart and Vey (1998) filed a patent application which showed that several *Lactobacilli* stimulated transepithelial calcium transport across fully differentiated Caco-2 cell monolayers in culture, a suitable *in vitro* model for predicting calcium absorption in humans (Fleet and Wood, 1999). In the present study, we investigated the effect of a well-characterized probiotic *Lactobacillus* (*Lactobacillus salivarius* (UCC 118)) and *Bifidobacterium* (*Bifidobacterium infantis* (UCC 35624)) on calcium uptake and transepithelial calcium transport in fully differentiated human intestinal-like, Caco-2, cells in culture.

Results
Treatment of fully differentiated Caco-2 cell monolayers with *E. coli*, UCC 118 or UCC 35624 for 6 h had no effect on total transepithelial calcium transport or TEER across the monolayer (Table 1). Similarly, treatment of Caco-2 cell monolayers with *E. coli*, UCC 118 or UCC 35624 for 24 h had no effect on total transepithelial calcium transport or TEER across the monolayer (Table 2).

Calcium uptake into Caco-2 cell monolayers after 24 h was unaffected by UCC 35624 (P=0164), but was significantly (P=0.015) increased by UCC 118 and tended (P=0.079) to be increased by *E. coli*, relative to the control (Table 3).

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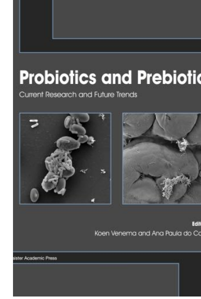

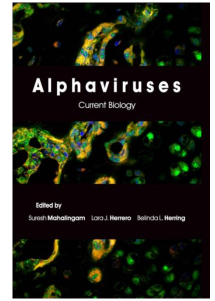

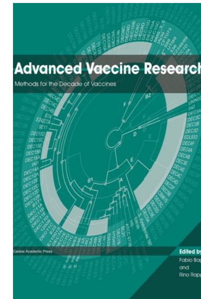

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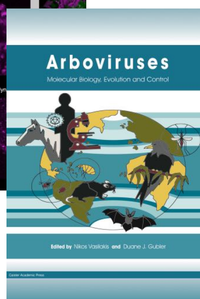

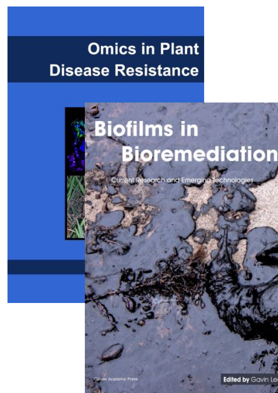

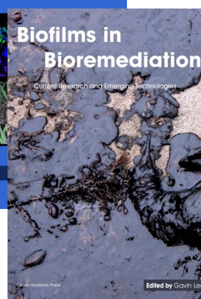

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Table 1. Effect of 6 hour exposure to non-pathogenic *E. coli*, *B. infantis* (UCC 35624) and *L. salivarius* (UCC 118) on total transepithelial calcium transport in Caco-2 cell monolayers in culture* (mean values with their standard errors).

		Total transepithelial calcium transport				TEER	
		nmol/well per min		%/h		(Ω.cm²)	
Treatment†	*n*	Mean	SE	Mean	SE	Mean	SE
Control	20	0.049	0.003	0.163	0.010	1671	42
E. coli	16	0.048	0.005	0.160	0.017	1668	45
B. infantis (UCC 35624)	17	0.045	0.003	0.150	0.010	1744	45
L. salivarius (UCC 118)	19	0.042	0.002	0.140	0.007	1724	40
ANOVA (one-way); *P* value		0.386		0.386		0.510	

TEER, transepithelial electrical resistance (after 6 hour exposure to the different treatments).
*For details of procedure, see pp 4–7.
†Treatments were given for 6 h before measurement of calcium transport.

Discussion

Caco-2 cells have been suggested to be a suitable model for predicting calcium absorption in humans (Giuliano and Wood, 1991; Fleet and Wood, 1999). In culture, Caco-2 cells spontaneously differentiate and form a polarized epithelial monolayer with tight junctions and express a differentiated cell phenotype consistent with absorptive small intestine-like enterocytes (Pinto *et al*. 1983; Yee, 1997). In particular, these cells have a functional vitamin D receptor (Giuliano *et al.* 1991) and have calcium transport kinetics that suggest the presence of both a saturable and nonsaturable calcium transport pathway, similar to what has been observed in the human and animal intestine (Fleet and Wood, 1999). Treatment with 1, 25 dihydroxyvitamin D_3 (1,25 $(OH)_2D_3$) induces the saturable, but not diffusional, component of calcium transport (Giuliano and Wood, 1991) and induces accumulation of calbindin D_{9K} and 24-hydroxylase mRNA in these cells (Fleet and Wood, 1994; Fleet *et al*. 1996). In addition, probiotic bacteria have been shown to adhere to Caco-2 cells (Bernet *et al*. 1993; Tuomola and Salminen, 1998; Gopal *et al*. 2001) as well as inducing changes in gene expression in Caco-2 cells (Mack *et al*. 2003). Therefore, this relatively simple *in vitro* method appears to be a good model for investigation of the possible effect of probiotic bacteria on calcium bioavailability in humans.

In the present study, exposure of fully differentiated Caco-2 cell monolayers to *E. coli* and two "probiotic" strains of bacteria, namely *L. salivarius* (UCC 118) and *B. infantis* (UCC 35624) had no effect on transepithelial calcium transport in fully differentiated 16-d-old Caco-2 cells. Calcium uptake into the Caco-2 cell monolayers after 24 h, however, was significantly higher in the cells exposed to UCC 118. Calcium uptake into the enterocyte is the first step of a multi-step process of moving calcium from the lumen to the general circulation (Cashman, 2003). The only other reported investigation of the effect of bacteria on intestinal calcium absorption was in the form of a patent application by Brassart and Yey (1998). They tested 11 different strains of *lactobacilli* and found that seven strains (*Lactobacillus johnsonii* strains La1 and La22, *Lactobacillus acidophilus* La 10 and La18, and *L. paracasei* (ST11), *L. gasseri* (LGA7) and *L. reuterii* (LR7)) (at 6.7×10^7 to 3.4×10^8 cfu/ml) significantly increased calcium transport in the Caco-2 cell model. The duration of the bacterial treatment in the studies by Brassart and Yey (1998) was not specified. We used a short-term (6 h) and longer-term (24 h) exposure time. However, in both the short-term and longer-term experiments in the present study, gentamycin was added to the bacterial cells after 1 h. This was required to prevent bacterial proliferative damage to the Caco-2 cells. If bacterial cells are allowed to proliferate excessively this can have adverse effects on Caco-2 cell monolayer integrity. Thus, knowledge of exposure times and the conditions under which cells are exposed to bacteria in these types of studies is an important consideration so as to discount the possibility of increased calcium transport arising due to leaky monolayers, in place of true physiological modulation of transport mechanisms. Despite being

Table 2. Effect of 24 hour exposure to non-pathogenic *E. coli*, *B. infantis* (UCC 35624) and *L. salivarius* (UCC 118) on total transepithelial calcium transport in Caco-2 cell monolayers in culture* (mean values with their standard errors).

		Total transepithelial calcium transport				TEER	
		nmol/well per min		%/h		(Ω.cm²)	
Treatment†	*n*	Mean	SE	Mean	SE	Mean	SE
Control	13	0.055	0.002	0.184	0.007	2095	47
E. coli	13	0.057	0.002	0.191	0.007	2127	34
B. infantis (UCC 35624)	12	0.059	0.002	0.198	0.007	2158	29
L. salivarius (UCC 118)	14	0.059	0.002	0.195	0.007	2185	64
Statistical significance of effect (one-way ANOVA): *P*		0.517		0.517		0.554	

TEER, transepithelial electrical resistance (after 24 h exposure to the different treatments).
*For details of procedure, see pp 4–7.
†Treatments were given for 24 hours before measurement of calcium transport.

Table 3. Effect of 24 hour exposure to non-pathogenic *E. coli*, *B. infantis* (UCC 35624) and *L. salivarius* (UCC 118) on calcium uptake in Caco-2 cell monolayers in culture* (mean values with their standard errors).

		Calcium uptake. (nmol/well).	
Treatment†	*n*	Mean	SE
Control	13	0.63[a]	0.04
E.coli	12	0.87[ab]	0.06
B.infantis (UCC 35624)	13	0.84[ab]	0.08
L.salivarius (UCC 118)	13	0.94[b]	0.09
Statistical significance of effect (one-way ANOVA): *P*		0.018	

a, b Mean values within a column with different superscript letters were significantly different (ANOVA followed by Tukey's Multiple Comparison Test; $P<0.05$).
*For details of procedure, see pp 4–7.
†Treatments were given for 24 hours before measurement of calcium uptake.

treated with antibiotics, the bacteria-host cell interaction/communication can still occur over subsequent hours inducing cellular and molecular changes within the host cell (L. O'Mahony, unpublished).

The mechanism by which some, but not other probiotic bacterial strains enhanced transepithelial calcium transport (as shown by Brassart and Vey, 1998) and calcium uptake (present study) is unclear. Brassart and Vey (1998) reported a decrease in pH in the microenvironment of the Caco-2 cells which appeared to be induced by the bacteria. While it is possible that this acidification could increase the solubility of calcium and promote intestinal absorption, the authors found no correlation between the increase in calcium absorption and the decrease in pH. The differences between bacterial strains may also be due to differences in capacity of the bacteria to adhere to the intestinal cells and thus, induce physiological changes. Interestingly, Dunne *et al*. (2001) reported that in their assessment, *Lactobacillus* strains possessed good adherence abilities, whereas *Bifidobacterium* strains performed poorly in terms of adherence to Caco-2 cells. This might underlie the stimulatory effect of UCC 118 (a *Lactobacillus*), but not UCC 35624 (a *Bifidobacterium*), on calcium uptake into Caco-2 cells in the present study. However, Brassart and Vey (1998) found that the capacity of the bacteria to adhere to the intestinal cells did not appear to correlate directly with their capacity to increase the absorption of calcium by these same cells.

Another possibility is that the bacteria directly influenced calcium absorption by modulating some other aspect of Caco-2 cell function. Recently, Mack *et al.* (2003) reported that *Lactobacillus* strains (5×10^5 cfu/ml) increased mucin (MUC3) mRNA transcription and translation, illustrating probiotics ability to induce host cell proteins. In the present study, although not investigated, UCC 118 may have increased the mRNA transcription and translation of proteins central to calcium uptake at the apical surface. However, the increase in calcium uptake by Caco-2 cells brought about by exposure to UCC 118 was not translated into an increase in overall transepithelial calcium transport. Twenty-four hours may not have been sufficient time to see the overall effect on transport. Alternatively, the extra calcium taken up into the cells may not have been ferried across the enterocytes or extruded any faster at the basolateral membrane.

The present *in vitro* study investigated whether probiotic or a commensal bacteria influenced intestinal calcium absorption. This is an important consideration in light of the fact that the number of bacterial cells far outnumber the number of intestinal cells (Teitelbaum and Walker, 2002). Therefore, the findings of the present study which suggest that bacteria can enhance intestinal uptake of calcium, if not calcium transport, highlights the need to undertake further studies in this, to date, vastly under-investigated area. Furthermore, special consideration needs to be given to the duration of exposure of intestinal cells to live bacteria as well as the bacterial load.

Experimental procedures

Materials

Tissue culture materials, including Dulbecco's modified Eagle's medium with L-glutamine and sodium bicarbonate, fetal bovine serum (FBS), minimum essential medium (MEM), non-essential amino acids, phosphate buffered saline (PBS) and gentamycin were purchased from Sigma-Aldrich Ireland Ltd, Dublin, Ireland. ^{45}Ca (as ^{45}Ca in an aqueous solution of $CaCl_2$, with a specific activity of 1.85 MBq/mg Ca) was purchased from Nensure™, Boston, MA, USA.

Bacterial strains

Three bacterial strains were obtained from Alimentary Health Ltd., Kinsale, Co. Cork, and the Department of Microbiology, University College, Cork, Ireland. *Lactobacillus salivarius* (UCC 118) and *Escherichia coli* non-pathogenic culture (*E. coli*; as control strain) were both received at a concentration of 10^9 cfu/ml. *Bifidobacterium infantis* (UCC 35624) was received at a concentration of 10^8 cfu/ml. Bacteria used in calcium transport experiments were seeded at a density of 10^7 cfu/ml onto Caco-2 cell monolayers that had been grown on permeable membrane supports (24 mm diameter, 0.4 μm pore size, Costar, Cambridge, MA, USA) for 16 days.

Conditions of cell culture

The human colon adenocarcinoma cell line, Caco-2, was purchased from the European Collection of Animal Cell Cultures (Salisbury Wiltshire, UK). Cells were routinely grown in 75 cm^2 plastic culture flasks (Costar, Cambridge, MA, USA) in Dulbecco's modified Eagle's medium supplemented with non-essential amino acids (10 ml/l) and FBS (100 ml/l). Caco-2 cells were maintained at 37˚C

in a CO_2-air (5:95, v/v) atmosphere. Cells were seeded at $3\times10^4/cm^2$ and passaged when reaching 90% confluence. Cell culture media was changed on alternate days for 16 days.

Transepithelial electrical resistance
For all transport experiments, the transepithelial electrical resistance (TEER; a measure of the integrity of polarized epithelial cell monolayers) was assessed before the experiment by a Millicell® ERS meter (Millipore Corp., Bedford, MA, USA) connected to a pair of thin side-by-side electrodes as described by Tanaka *et al.* (1995). TEER readings were taken at 37º C. A TEER value ≥800 $\Omega.cm^2$ was used as an indicator that the epithelial layer was intact and ready to use for calcium transport studies.

Cell treatments
For calcium transport experiments, cells grown in the Transwell® (Costar) inserts for 16 d were exposed apically to PBS only (for control) or to 10^7 cfu/ml of one of the three microbe solutions (*E. coli*, UCC 118 or UCC 35624). The bacterial strain was added after the complete culture medium had been added to the Caco-2 cells. The Caco-2 monolayers were treated for 1 h with the bacterial suspension followed by a coincubation with 50 µl of a gentamycin solution (0.2 mg/ml) for 5 h or 23 h at 37°C in a CO_2-air (5:95 v/v) atmosphere. The gentamycin treatment was required to prevent excessive bacterial proliferation which has been shown to adversely affect Caco-2 cells and their monolayer integrity (L. O'Mahony, unpublished). TEER measurements were taken immediately before treatment with test compounds and 24 hours after treatment.

Calcium transport and uptake studies
The method used for determining calcium transport across the Caco-2 membrane in the present study is a modification of the methods of Giuliano and Wood (1991) and Fleet and Wood (1994). Transepithelial transport of calcium was studied with Caco-2 cells grown on permeable membrane supports for 16 d, by which time cells are fully differentiated. On the day of an experiment, after exposure of the cells to *E. coli*, UCC 118 or UCC 35624 for 1 h, gentamycin solution (0.2 mg/ml) was added for a further 5 h or 23 h. At time zero, ^{45}Ca (1 µl/ml) (with an activity of 148 kBq/ml) was added to the apical chamber of the Transwell® inserts (Costar). Following the addition of ^{45}Ca to the Caco-2 cell monolayers, the plates were covered and incubated at 37°C in a shaking water bath (set to 48 oscillations/min) for 60 min. At 30 and 60 min, aliquots (100 µl) of the basolateral buffer were taken for determination of ^{45}Ca content. Samples of the basolateral buffer were placed in scintillation vials and 5 ml of liquid scintillation cocktail (biodegradable counting scintillant; Amersham International Plc., Amersham, Bucks., UK) was added to each vial. Radioactive counts were measured on a Beckman LS 6500 multipurpose liquid scintillation counter (Beckman Instruments Inc, Fullerton, CA, USA). An equal volume of fresh basolateral buffer was added back to the lower chamber following each sampling point. To evaluate uptake of ^{45}Ca by the Caco-2 cell monolayers at the end of the 60 minutes, the membranes were removed, gently washed with PBS and placed in the scintillation vials. Five ml of liquid scintillation cocktail (biodegradable counting scintillant; Amersham International Plc., Amersham, Bucks., UK) was added to each vial. The amount of ^{45}Ca appearing in the basolateral buffer was expressed as a percentage of the total ^{45}Ca applied to the upper chamber. This represented total transepithelial ^{45}Ca transport (i.e. by both the paracellular and transcellular transport routes) and was expressed as both %/h and nmol transported/min per well. Uptake of calcium by cell monolayers was expressed as nmol/well. In all studies, at least three wells were examined per treatment and experiments were repeated three times. Therefore, there was at least an n = 9 wells per treatment.

Statistical methods
Data for all variables were normally distributed and allowed for parametric tests of significance. Results are presented as mean values with their standard errors. Treatment effects were compared by one-way analysis of variance (ANOVA), with variation attributed to bacterial strains (Snedecor and Cochran, 1967). To follow up the ANOVAs, all pairs of means were compared by Tukey-Kramer's multiple comparisons test. A P-value <0.05 was considered as significant, while a P-value in the range 0.05–1.0 was considered reflective of a trend.

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