Identification of commensal bacterial metabolites that enhance the integrity of the gastrointestinal barrier

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ABSTRACT

The objective of this research was to identify metabolites produced by commensal bacteria that enhance the integrity of the gastrointestinal barrier (GIB). The integrity of the GIB is mainly determined by the complexity of interactions between tight junctions of opposing epithelial cells of the GI wall. This was measured using an *in vitro* bioassay, consisting of a monolayer of intestinal epithelial cells grown on a membrane. The trans-epithelial electrical resistance (TEER) across this monolayer was measured to determine the strength of the tight junctions. Preliminary results showed that a commercial probiotic product caused an increase in TEER across non-differentiated and differentiated monolayers by 178 ± 61% and 40 ± 9%, respectively. The maximum increases in TEER obtained with conditioned media fractions (media fractions containing components secreted by the probiotic product) was between 23 ± 6% and 43 ± 4%. The conditioned media fraction containing components less than 3 kDa accounted for the total activity of the whole conditioned media. Metabolite profiling of active conditioned media compared to inactive conditioned media using fast-LCMSMS identified numerous metabolites that may be responsible for this positive effect. Future work will isolate the candidate metabolites and screen them for GIB enhancing activity both *in vitro* and *in vivo*.

Keywords: metabolomics; gastrointestinal barrier; tight junctions; commensal bacteria; probiotics.

INTRODUCTION

The gastrointestinal barrier (GIB) is the largest interface between an animal and its external environment. It acts as a "biological bouncer" that protects the host from the entry of bacteria and hence it is critical in maintaining health and wellness. The GIB is comprised of physical (Balda & Matter, 1998; Schneeberger & Lynch, 2004), chemical (Deplancke & Gaskins, 2001), immunological (Nagler-Anderson, 2001; Rescigno *et al.*, 2001; Rescigno *et al.*, 2001) and microbiologic barriers (Sartor, 2004). The physical barrier is a single layer of epithelial cells that form tight junctions between adjacent cells to exclude bacteria and larger particles. The integrity of the GIB is defined by the tightness of these junctions. In animals, impaired GIB integrity is often associated with acute or chronic inflammation and diarrhoea, which leads to, at best, impaired growth and reduced productivity.

It is well accepted that bacteria present in the intestinal lumen can affect GIB integrity. For example, pathogens such as *Salmonella* reduce GIB integrity (Tafazoli *et al.*, 2003); whereas, probiotic (beneficial bacteria) preparations can maintain GIB integrity during pathogenic infection (Otte & Podolsky, 2004). The mechanisms by which probiotics mediate this effect is poorly understood but there is evidence to suggest that metabolites secreted by the probiotic bacteria may be involved (Madsen *et al.*, 2001; Menard *et al.*, 2004; Otte & Podolsky, 2004).

The hypothesis of this research is that metabolites secreted by commensal bacteria are able to reduce the drop in GIB integrity during infection and maintain GIB integrity during wellness. Our objective was to identify such metabolites using an *in vitro* bioassay which enabled us to easily and sensitively measure GIB integrity enhancement and metabolic profiling to identify candidate metabolites in the active solution(s). Because there is no clear evidence about the structure of the possible active compound(s), a metabolomics approach was required. This is an alternative to the traditional method of progressively purifying fractions and testing them for activity, which is time-consuming, labour intensive and tedious.

MATERIALS AND METHODS

Treatment preparation

To prepare the probiotic solution for testing in the *in vitro* bioassay, an active bacterium from a commercial probiotic product was suspended in sterile water and inoculated into MRS broth (de Man, Rogosa and Sharpe Broth, Difco,

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Sparks, USA). The culture was incubated aerobically at 37° C overnight. The bacterial cells were collected by centrifugation (12000 rpm for 5 minutes in a microcentrifuge) and suspended in M199 (Medium 199 with HEPES modification, Sigma-Aldrich, St Louis, USA) with 1% non-essential amino acids (MEM non–essential amino acids 100x solution, Sigma-Aldrich, St Louis, USA) to the required optical density at 600 nm because turbidity of the solution at this wavelength is proportional to the bacteria cell concentration. This is subsequently referred to as the probiotic solution.

For the preparation of the conditioned media, the probiotic solution (OD_{600nm} 0.9) was incubated aerobically at 37° C overnight. The bacterial cells were removed by centrifugation as described above and the supernatant was filter-sterilised (Millex GP 0.22 µm syringe driven filter, Millipore, Cork, Ireland). This is subsequently referred to as the conditioned medium. The conditioned media was fractionated by passing it through centrifugal filters with molecular weight cut-offs of 30, 10 and 3 kDa (Centricon Filters YM-30, 10 and 3, Millipore, Bedford, USA).

***In vitro* bioassay**

The *in vitro* bioassay was used to test the effect of the treatments on GIB integrity. Caco-2 cells (a human intestinal epithelial adenocarcinoma cell line, ATTC HTB-37, Manassas, USA) were grown on semi-permeable membranes to mimic the GIB. The trans-epithelial electrical resistance (TEER) across the monolayer of epithelial cells was measured to determine its integrity. TEER is a correlates to the strength of the tight junctions between the epithelial cells of the monolayer.

Caco-2 cell stock cultures were grown in T75 flasks in M199 with 10% foetal bovine serum (GIBCO, Invitrogen Corporation, Auckland, NZ), 1% non-essential amino acids and 1% penicillin-streptomycin (10000 units penicillin G sodium salt and 10000 µg streptomycin sulphate in 0.85% saline, GIBCO, Invitrogen Corporation, Auckland, NZ) at 37° C in 5% CO_2. The medium was replaced every 3-4 days and the cells were subcultured weekly at a ratio of 1:3.

For the *in vitro* bioassay, the Caco-2 cells were seeded onto 14 mm collagen membrane inserts (Cellagen™ Discs CD-24, MP Biomedicals, Ohio, USA) at a density of 10^5 cells/insert. Each insert was placed in a well in a 12-well plate with 1 mL of medium in the bottom and 250 µL medium in the top of the insert. The medium was replaced every 2-3 days. For the majority of the experiments the Caco-2 monolayers were grown for 5 days until confluency, except for the experiment comparing differentiated monolayers which were grown for 18 days.

The monolayers were prepared the day before the TEER assay by removing the medium, washing three times with PBS and adding M199 with 1% non-essential amino acids (without foetal bovine serum and penicillin-streptomycin), to ensure growth of the probiotic solution. Before the initial resistance readings were taken on the day of the experiment, the medium was removed from the top of the Caco-2 membrane inserts and replaced with the treatment solutions (e.g. probiotic solution vs. control solution or conditioned medium vs control solution). For the controls the spent media was replaced with fresh medium (M199 and 1% non-essential amino acids). Each treatment was performed in quadruplicate.

To measure the resistance across the monolayers, each insert was lifted into an electrode chamber with electrodes at the top and bottom (ENDOHM-12 tissue culture chamber, World Precision Instruments, Florida, USA) using sterile tweezers and the resistance was measured using a voltohmmeter (EVOM Epithelial Tissue Voltohmmeter, World Precision Instruments, Florida, USA). For the experiments using live bacteria, the resistance was measured every 2 hours for 14 hours and then after 24 hours. For the experiments using conditioned media the resistance was measured every 1.5 hours for 4.5 hours.

The TEER was calculated from the resistance using the formula: TEER (Ω cm^2) = resistance (Ω) x membrane area (cm^2), where the membrane area was 1.54 cm^2. The change in TEER for each insert was calculated using the following formula: change in TEER (%) = TEER (Ω cm^2) / initial TEER ($\Omega.cm^2$) - 100 (%). The mean change in TEER was plotted against time, with the error bars showing the standard error of the mean. A Student's t-Test was used to compare treatments. Statistical differences between treatments were declared at a probability less than 0.05 whilst a probability below 0.1 but above 0.05 was considered to represent a trend.

Identification of candidate metabolites

Six replicates of active conditioned media, inactive conditioned media and control media (untreated) with a molecular weight cut off of 3 kDa were prepared as described above. The samples were lyophilised, dissolved in 2 ml H_2O and centrifuged at 13000 rpm. The supernatant was transferred to an HPLC vial. The samples were kept at 5°C in an autosampler, which injected 20 µl. This was eluted through a Strata-X on-line cartridge (Phenomenex; 20 x 2 mm, 25 µm) at a flow rate of 300 µl/min using a HPLC system

(Thermo Finnigan Surveyor, Waltham, USA). The solvent gradient started with 100% H_2O for 1.5 minutes and then increased to 100% MeCN with 0.1% formic acid over 3 minutes followed by a 2 step column wash (First wash: 100 % MeCN with 0.1% formic acid for 1.5 minutes at 300 µl/min; Second wash: 100% H_2O for 1.5 minutes 600 µl/min) after which the column was reconditioned again for 1.5 minutes with 100% H_2O at 300 µl/min. The total run-time was 10 minutes.

The first 0.5 minutes were diverted to waste and for the other 9.5 minutes mass spectra were determined (mass range 150-1000 m/z) with a linear ion trap mass spectrometer (Thermo LTQ, Waltham, USA) using electrospray ionisation in positive mode. The spray voltage was 5.0 kV and the capillary temperature was 275°C. The flow rates of sheath gas, auxiliary gas, and sweep gas were set to 20, 5, and 0 (arbitrary units), respectively. Spectra were collected in data dependent mode, isolating (± 1 mass unit (Da)) the most intense ion in the MS1 spectrum and subsequently fragmenting with 35% relative collision energy, followed by the isolation and fragmentation of the most intense ion in the MS2 with the same parameters. A dynamic exclusion list was used, in which ions appeared for 30 seconds after repeated fragmentation. In addition, UV spectra were obtained using a Thermo surveyor PDA detector.

An average mass spectrum was taken across the whole chromatogram and exported into MS Office Excel 2003. The masses were rounded to their nominal mass and aligned. The intensity of each nominal mass to charge ratio (*m/z*) was normalised against the total intensity (sum of all intensities). The normalised data was then exported to a statistical software package (MINITAB® Release 14.20) and analysed by principle component analysis.

RESULTS

Effect of the treatments on TEER

The effects of a probiotic solution of the TEER of confluent non-differentiated monlayers and differentiated monolayers were compared. Differentiated monolayers are a better model of a health GIB than confluent non-differentitated monolayers because the initial TEER is high. The probiotic solution induced a greater increase in TEER across confluent non–differentiated monolayers compared to differentiated monolayers as shown in Figure 1. After fourteen hours, the mean increases in TEER for non-differentiated and differentiated monolayers were 178 ± 61% and 40 ± 9%, respectively.

FIGURE 1: Change in trans-epithelial electrical resistance (TEER) across confluent non-differentiated (5 day old) and differentiated (18 day old) Caco-2 monolayers over time in the presence of a probiotic bacteria. The change in TEER is the percentage change compared to the initial TEER for each inserts. The values plotted are the means for four membrane inserts and the error bars show the SEM. For the differentiation monolayers, the change in TEER due to the probiotic bacteria was significantly different ($P < 0.05$) from the controls from 6 hours onwards; whereas, for the confluent non-differentiated monolayers, the change in TEER due to the probiotic bacteria was significantly different ($P < 0.05$) from the controls at 2 and 4 hours and showed a trend ($0.05 < P < 0.1$) from 8 hours onwards.

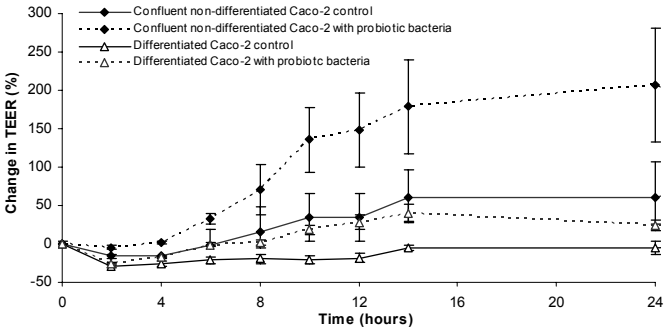


FIGURE 2: Change in trans-epithelial electrical resistance (TEER) across confluent Caco-2 monolayers over time in the presence of probiotic bacteria conditioned media with different molecular weight cut-offs. The change in TEER is the percentage change compared to the initial TEER for each inserts. The values plotted are the means for four membrane inserts and the error bars show the SEM. The changes in TEER due to the treatments were all significantly different ($P < 0.05$) from the controls from 1.5 hours onwards.

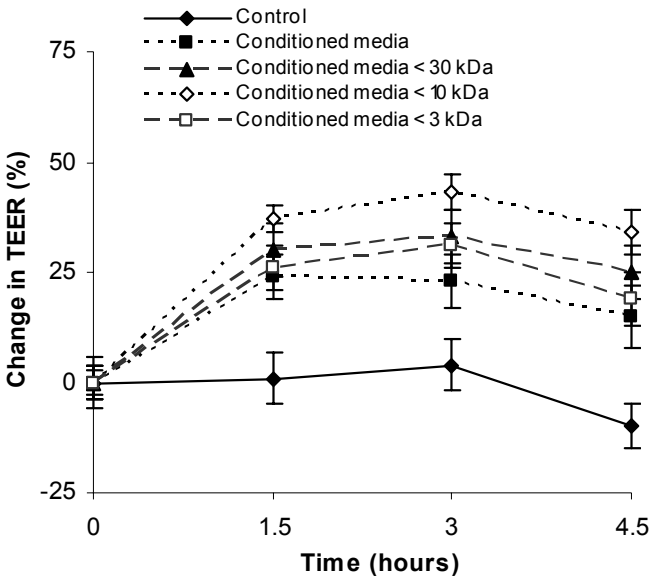
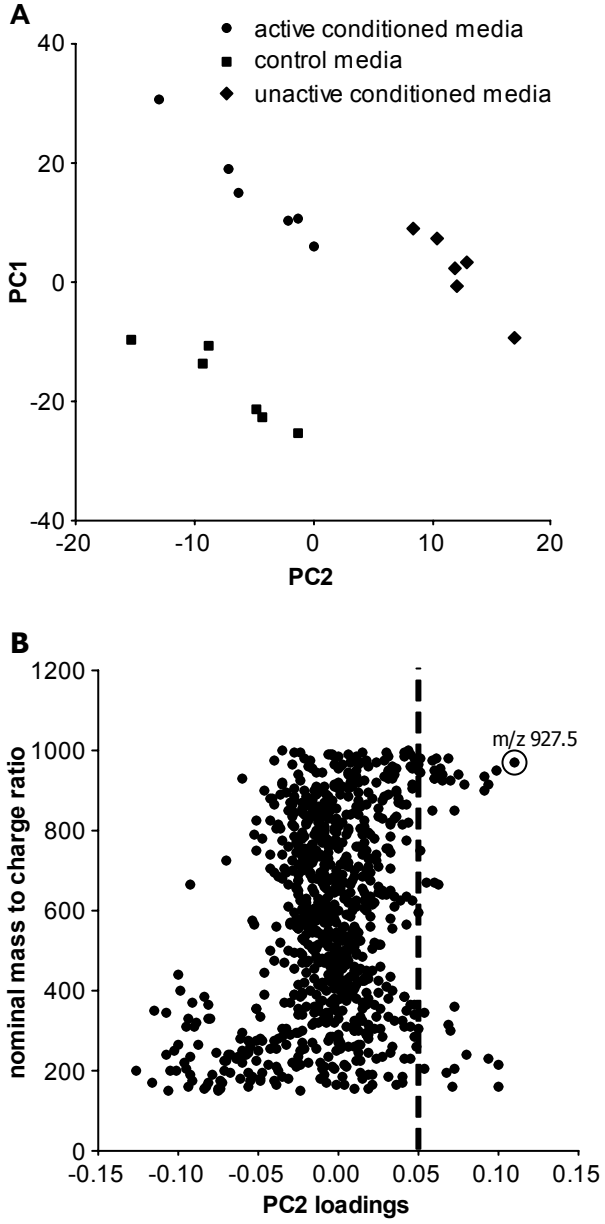


FIGURE 3: Principle component 1 (PC1) versus PC2 for active conditioned media, control media and inactive conditioned media all with molecular weight cut-offs of 3 kDa (A) and contribution of each nominal mass to charge ratio to the separation of PC2.



In comparison to the probiotic solution, the conditioned media and conditioned media fractions caused a more rapid increase in TEER, which was sustained for the 4.5 hours of the experiment. The maximum increases in TEER obtained with the conditioned media fractions were between 23 ± 6% and 43 ± 4% after 3 hours as shown in Figure 2.

Identification of candidate metabolites

The metabolic profile of six replicates of active conditioned media, inactive conditioned media and control media (untreated) with a molecular weight cut off of 3 kDa were compared. Samples from the different treatment groups were clearly resolved by principle component analysis (based on correlation) of the average aligned metabolic profiling data. The graph of the first two principle components (PC1 vs PC2) for the active conditioned media, inactive conditioned media and control media, with molecular weight cut-offs of 3 kDa, is given in Figure 3A. In this experiment we only collected mass spectral information between 150-1000 *m/z*. Many larger metabolites (oligopeptides, oligosaccharides) with masses above 1000 Da often carry multiple charges and can be detected by this method. Each of the treatment groups cluster together which shows that the preparation of the samples was repeatable. The active conditioned media are clearly separated from the inactive and control media groups. The PC1 separated the conditioned media from the control media and the PC2 separated the active from the inactive conditioned media.

The graph of the contribution of each nominal *m/z* to the separation of PC2 is given in Figure 3B. Components with a PC2 loading greater than 0.05, are present in greater amounts in the active compared to the inactive conditioned media; therefore, they may be responsible for the difference in activity between the active and inactive samples. These are referred to as candidate metabolites. The candidate metabolite with the highest PC2 loading (furthest right in Figure 3B) has a *m/z* of 927.5

DISCUSSION

Both the probiotic solution and the conditioned media induced an increase in TEER across the Caco-2 monolayers, which indicates that they enhanced the integrity of the tight junctions. For the bacterial solution the TEER increased slowly over time, with the maximum TEER being reached at least 12 hours after addition. In contrast, the TEER increase due to the conditioned media was apparent at the first measurement 1.5 hours after addition. This may be attributed to the concentration of the active metabolite(s) increasing with time as the bacteria grow during the bioassay, but in the case of the conditioned media the metabolite may be present at a high enough concentration at the start of the TEER experiment to have an immediate effect.

An active bacterial component from the probiotic product induced a larger response in confluent non-differentiated compared to differentiated Caco-2 monolayers: however, the variation between inserts was also much greater for the non-differentiated monolayers. This may be attributed to the differentiated monolayers having

more stable tight junctions compared to the non-differentiated monolayers which are still forming tight junctions.

The activity of the conditioned media after passing through a molecular weight cut-off filter of 3 kDa accounted for the total activity of the whole conditioned media. This is in agreement with previous results which indicated that the active metabolites produced by *Streptococcus thermophilus* and *Bifidobacterium breve* were also less than 3 kDa in size (Menard *et al.*, 2004). In contrast, the active metabolite produced by the probiotic mixture VSL#3 was reported to be greater than 50 kDa (Otte & Podolsky, 2004). In the published studies, the effect of the VSL#3 conditioned media was decreased by heat-treatment, proteases and changes in pH, which indicates that the active component was a protein In contrast, the activity of the *Streptococcus thermophilus* and *Bifidobacterium breve* conditioned media were resistant to digestive enzymes.

The next step in this research will be to obtain enriched fractions of candidate metabolites by HPLC, beginning with the component with the highest PC2 loading, and to then test them in the *in vitro* bioassay. When these fractions show a significant increase in TEER, the responsible metabolite will be isolated and its structure elucidated.

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