This is an Accepted Manuscript, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. This Accepted Manuscript will be replaced by the edited, formatted and paginated article as soon as this is available.

You can find more information about Accepted Manuscripts in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal’s standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this Accepted Manuscript or any consequences arising from the use of any information it contains.
Effect of Apple, Baobab, Red-Chicory, and Pear Extracts on Cellular Energy Expenditure and Morphology of a Caco-2 Cells using Transepithelial Electrical Resistance (TEER) and Scanning Electron Microscopy (SEM)

Enrico Finotti*a, Riccardo Gezzi*b, Fabio Nobili*a, Ivana Garaguso*a and Mendel Friedman*c

aCRAGNUT National Council for Agricultural Research, Research Center for Food and Nutrition, Via Ardeatina, 546, 00178 – Rome, Italy.
bDepartment of Orthodontics, Georg-August-University, Göttingen, Germany
cWestern Regional Research Center, ARS-USDA, Albany, CA 94710, USA

*Corresponding Author: Enrico Finotti, CRA-NUT, Via Ardeatina, 546, 00178 – Rome, Italy. E-mail: enrico.finotti@entecra.it, Fax: +39 06 51494550

Running title: Food Extracts and cellular Energy Expenditure

Keywords: Caco-2 cells, TEER, scanning electron microscopy, food extracts, cellular energy expenditure, physiological equilibrium recovery, mucopolysaccharide production, digestive tract.

Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

Abstract

The present study investigated the effects of four food extracts on the Caco-2 intestinal cell line using a new transepithelial electrical resistance method (TEER) concurrent with electron microscopy (SEM). Caco-2 cells are widely used in transepithelial studies because they can be cultured to create a selective permeable interface similar to that between the intestinal lumen and the basolateral tissue. These cells absorb, secrete, and function as a barrier that limits the passive transepithelial diffusion of hydrophilic solutes from the digestive tract into the circulation. The integrity of this tissue must be maintained when studying cellular physiology. TEER provides useful information on cellular function when a tissue in chemical equilibrium is perturbed by an external compound (such as nutrient, drug, pathogenic microorganism or toxins). In order to restore this equilibrium, the cells need to expend energy that can be calculated through a mathematical TEER value. The change in energy from the equilibrium value depends on the time elapsed and the nature and concentration of the test substance.

The results show that extracts of four commercial foods (with the total phenolic content shown in mg/g gallic acid equivalents) - apples (3.39), baobab (130), red chicory (13.31), and pears (1.15) induced concentration-dependent changes in both the energy and histology (morphology) of the cells as well as the formation of mucopolysaccharide. These changes, reported graphically and mathematically, were altered in the presence of the free radical (oxidant) 2,2'azobis (2-amidinepropane) dihydrochloride (AAPH).

At the highest concentration, measured, the food extract with the highest phenolic content (baobab) completely damage the cells. The new simple in vitro TEER assay offers a novel approach to investigate the influence of nutrients, antinutrients, food toxicants, and drugs on the physiology and morphology of the Caco-2 cells that may predict their behavior in the digestive tract.

Introduction

The intestinal Caco-2 cell line is extensively used for cellular permeability studies of food and xenobiotics (Hidalgo et al., 1989; Berger et al., 2003; Sergent et al., 2005; Boulaba et al., 2013; Rocha et al, 2013; Elhadiy et al., 2014; Rajkovic et al., 2014; Vazquez et al., 2014). Because these cells can be grown on porous plugs, it is possible to utilize them to create an interface (by selective permeable monolayer cells) that simulates both the epithelium of the intestinal mucosa and molecular flow between the lumen and the basolateral tissue (Hidalgo et al., 1989). Caco-2 cells absorb, secrete, and function as a barrier that limits the passive transepithelial diffusion of compounds from the digestive tract into the circulation. Because this barrier is not static (Benson et al., 2013) but is affected by a specific chemical input, it can open and close the tight junction selectively. Thus, the cells are able to control the diffusion from the lumen to the basolateral tissue and vice versa. The capacity of such solutes to cross the lipid membrane is limited and the major route to permeate the monolayer of epithelia is quite specific. The intercellular tight...
junctions operate as a limiting barrier to the movement of the solutes through this specific path (Hubatsch et al., 2007). Because the barrier can modulate adsorption of food-containing microbial toxins and pathogenic bacteria in the tissue and into the circulation, delaying or increasing the adsorption can determine the success of a therapy, it is important to understand the biological mechanism of this barrier in medical treatment (Sköld et al., 2006; Yaheya et al., 2009). The same considerations apply for the bioavailability of nutrients in food (Genser, 2008; McCabe-Sellers et al., 2003; Won et al., 2012).

An important characteristic of Caco-2 cell line is that the compounds added in the apical area (lumen) have to cross the membrane perpendicularly. Epithelial cells are functionally and structurally “polarized” and their basal-lateral extremity differs from the apical one. These properties make this in vitro model very suitable in studies of both morphological and biochemical responses of transepithelial and paracellular transport (Zweibaum et al., 1991; Fonti et al., 1994) under different chemical stimuli (Garaguso et al., 2013; Garaguso et al., 2011; Durazzo et al., 2010; Nobili et al., 2006).

A further property of these cells is that they are structurally similar to human adult colon epithelia (Pinto et al., 1983). Another morphological and structural aspect is that they are also nonhomogeneous: indeed this cell line comprises at least 4 cytotypes (Ferruzza et al., 2002). Caco-2 cells are neoplastic but if they proliferate on plastic discs or on polycarbonate filters, then they act like normal enterocytes, always remaining as a highly polarized (monolayer) without forming clusters typical of neoplasia. Caco-2 cells represent a good model for the study of the damage caused by free radicals and by the protective action of antioxidants (Hidalgo et al., 1989). The principal approach used in the present study to investigate the integrity of tissues grown on a porous support is based on the transepithelial electrical resistance (TEER). This is a non-invasive technique which measures the impedance between the lumen and basolateral tissue. TEER measurements use a constant direct current (DC) applied by two electrodes, one connected with the lumen side and the other one with the basolateral side. By applying Ohm’s law it is possible to measure the related cell resistance (R). If the chemical equilibrium of the tissue is perturbed by an external substance, then the cells tend to restore the equilibrium. For this purpose the cells expend energy by activating a metabolic pathway in response to the chemical injury. This is reflected in the cultured medium and in the resistance value, which is monitored by TEER.

The aim here is to use TEER values to calculate the energy released by the cell to restore its chemical equilibrium. This energy depends on the time elapsed and chemical characteristics and concentration of the tested compound. TEER measurements provide further valuable information, not only when analyzing the conveyance of the compound through lumen and basolateral tissue or when detecting the maximum active dose of a single compound, but also about the cell physiology. The Caco-2 cell line is therefore tested in this study using hydrophilic extracts from different commercial foods such as apples Kalinowska et al., 2014), the African fruit baobab (Lamien-Meda et al., 2008; Mulaudi et al., 2011), red chicory (D’Evoli et al., 2013), and pears (Li et al., 2014). Mathematical analysis of the data, reinforced by scanning electron microscopy (SEM), were used to determine the effect of the food extracts on the energy and histology of the cells.

Materials and Methods

Samples extraction. Commercial fresh samples of apple, baobab gel, red chicory, and pear extracts were used for creating biological triggers for the cell culture. These products were chosen because of their wide-ranging concentration of polyphenols that can interfere with cellular metabolism, especially when they are linked with carbohydrates. Each fresh sample (10 g) was extracted with a water/methanol (50:50 v/v) solution and dried by Rotavapor (Büchi, Flawil, Switzerland) at 50°C and then re-suspended in water. Next, the total free polyphenols were determined by the Folin-Ciocalteu method (Coseteng and Lee 1987). The values obtained range (in mg/g gallic acid equivalents) as follows: pears; 1.15; apples; 3.39; red chicory; 13.31; and baobab, 130. The concentrations of the tested polyphenols were: 10, 50, and 100 µg/ml for apple; 50, 500, 1000, 2500, and 5000 µg/ml for baobab; 5, 10, 15, 20, 50, 100, and 200 µg/ml for red chicory, and 10, 50, and 100 µg/ml for pear. These concentrations, with and without the pro-oxidant AAPH, were added to a monolayer of the Caco-2 cell culture. Fresh oxidation agent (AAPH) solution (6 mg/ml) was used for each sample. TEER values were then measured every 30 min along with a control sample without the extracts or AAPH.

TEER Analysis. TEER values were tested on Caco2 cells in order to measure changes in the tight junction permeability (Lamien-Meda 2008) using a Millicell-ERS (Millipore Corporation, Billerica, MA, USA) at a constant voltage of 0.1 mV. The cells were seeded onto a polycarbonate filter cell culture chamber inserts with the following dimensions: diameter, 6.5 mm; area, 0.33 cm²; pore diameter, 0.4 µm (Costar Corporation Cambridge MA UK), at a density of 1.5x10⁴ cells per filter and placed into a Falcon multwell plate (Costar Transwell; Corning Incorporated, NY, USA). The filter separated the chamber into two parts (apical and basal) which represent the lumen and the basal area of the gastroenteric system, respectively. The TEER was then measured in these two chambers to assess the tight-junction permeability and cell damage. These parameters were adopted to detect the early intestinal barrier function ex vivo damages. Each measurement was performed three times and the results are reported as means and standard deviations.

The human colon adenocarcinoma cell line (Caco-2) was provided by A. Zweibaum (INSERM, Villejuif, Paris, France). The cells were cultivated in vitro in Falcon™ plastic bottles with a surface of 75cm² (Becton, Dickinson and Co., Milan, Italy). The culture medium consisted of Dulbecco (DMEM) containing 25 mM of glucose, and 3.7g/l NaHCO₃ further supplemented with 4 mM of L-glutamine, 10% of fetal horse serum, 1% non-essential amino acids, 10⁻⁵ U/l penicillin and 100 µg/ml of streptomycin. The cells were incubated at 37°C at a 90% humidity in an atmosphere consisting of 95% air and 5% CO₂. The culture medium was replaced three times a week until the attainment of the cell confluence. Each tray cell represented an independent bowel microculture. In this way, with only a few multiwell trays, we were able to obtain a considerable number of cultures, which were monitored daily. Ideal conditions for confluence and differentiation were noted after 14-18 days.

Two fundamental parameters were evaluated: TEER between the apical and basal-lateral compartments (Szczesny, 1978) and the observation of the morphology of the increasing epithelia culture. The latter was observed with the Diavert inverted microscope (Leitz GmbH, Germany).

SEM Analysis. The cellular monolayer was fixed for 12 hours in formaldehyde (10%), glutaraldehyde (2.5%) and then dried with alcohol solution. Next, the sample was dehydrated by Critical Point Drying (Emitech K850, Quorum Technologies, West Sussex, UK). The final dehydration was achieved in CO₂. Finally, the sample was sputtered with gold for 120 sec at 30 mA in a...
modified atmosphere with 2% Argon and analyzed using SEM
(EVO LS10, Carl Zeiss Microscopy GmbH, Jena, Germany).
SEM was used to determine the formation of mucopolysaccharide
by the Caco-2 cells induced by added food extracts and the pro-
oxidant. The Goblet cells, one of the cell types comprising the
Caco-2 cell line, are known to produce mucopolysaccharide
(Dorofeyev et al., 2013; Alvarenga et al., 2014). When the Goblet
cells produce mucopolysaccharide, they are positioned between
the microvilli forming a matrix well visible by SEM, but not by
optical microscopy.

Mathematical treatment and interpretation of the data. The
introduction of a chemical compound induces a modification
of the cellular chemical equilibrium. In order to restore the
equilibrium, the cells expend a certain amount of energy. The
energetic variation might be interpreted in terms of the cellular
reaction to the chemical interference. In order to estimate the
energy variation of the cells, we developed a simple mathematical
model based on the Joule effect, which relates the energy
dissipated by the circuit with the resistance [in this case TEER,
time(t)] and the current flowing through the cells:

\[ P(t) = R(t) \times I(t)^2 \]  

where \( P(t) \) is the calculated power in watts, \( R(t) \) is the
resistance, the time-dependent measured TEER value (in Ohms),
and \( I \) the current (in Amperes), flowing through the circuit that is
kept at a constant voltage of 0.1 mV. The time variation of the
TEER is due to the modification of the chemical composition of
the cellular medium, which is caused by the added compounds,
the consequent chemical response of the cells, and the electric
current. The numerical integration of Eq. (1) over the time
results in:

\[ E = \int_0^t P(t) dt \]  

This equation provides an estimate of the energy released (in
Joules) by the cell due to the passage of an electric current. We
 calculated the energy (\( \Delta E \)) and power (\( \Delta P \)) differences between
the non-equilibrium situation in the cell in the presence of an
added food extract by using equation 3:

\[ \Delta E = E_{\text{food extract or free radical}} - E_{\text{control}}; \quad \Delta P = P_{\text{food extract or free radical}} - P_{\text{control}} \]  

Two different energy situation can occur depending on the sign of
\( \Delta E \):

1) \( \Delta E < 0 \): the energy amount spent by the control is larger than
that of the sample with antioxidant or pro-oxidant. The cells
still try to regain the chemical equilibrium.

2) \( \Delta E > 0 \): the energy amount spent by the control is lower than
that of the sample with antioxidant or pro-oxidant. In this
case, the cells cannot regain equilibrium. If the interference
persists, however, then severe damage to the cells occurs, as
further verified by SEM analysis of the cell morphology.

The outputs, plotted as a function of the different antioxidant or
prooxidant concentrations (see the Results and Discussion
sections) are interpreted as the amount of cell energy that is used
to regain the equilibrium. In this study only the R (Resistance) and the V (Volt) have been
determinate by the instrument. The energy parameters have been
calculated with equations 1-3.

Results

The aim of this work is to present a new approach to identify a
threshold above which the cell cannot regain its physiological
equilibrium after being exposed to an inflammatory stimulus. The
results obtained represent the energy difference between the
control and the sample with added oxidant (\( \Delta E \)) determined by
TEER as function of time. \( \Delta E \) starts from zero then becomes
negative because the cells expend energy to achieve the chemical
equilibrium. By increasing the oxidant concentration, the energy
difference could reach a positive value. This can occur when the
oxidant concentration is so high that it is incompatible with a
complete cellular recovery of equilibrium. We interpret the region
of positive value as a threshold above which the \( \Delta E \) relative to the
concentration of tested compound could severely affect the
cellular equilibrium. Indeed, we identified a concentration
threshold above which the cells cannot recover their equilibrium.

These observations are supported by SEM data of control and
treated cells.

Figure 1 shows the result with the apple, baobab, red chicory and
pear extracts. In the apple samples without added free radical
oxidant (AAPH), all the TEER values (Fig. 1B) are less than the
control value. By contrast, with added oxidant, all TEER values
in Figure 1 are greater than the control. The same figure also
shows the TEER values of baobab extract. The TEER values
without oxidant are greater than the control value, except for the
5000 µg/ml concentration. At all tested concentrations, the TEER
values with oxidant (AAPH) added are greater than the control
value. In red chicory, the TEER values are always less than those
of the control. This trend is more pronounced in the presence of
the oxidant (AAPH). At the end of this figure, we show the TEER
pear values in which the values are quite close to the control
value except for the 100 µg/ml concentration, which illustrates a
negative trend. We repeated the experiment using the free radical
or oxidant (AAPH). The TEER values are similar but with a more
pronounced trend.

Figure 2 illustrates the energy differences values for apple,
baobab, red chicory and pear extracts. In the apple extract, the \( \Delta E \) plot shows all the values to be in the positive field. By contrast,
with added oxidant (AAPH) all energy is negative. It seems that the
presence of the oxidant helps the cell to recover its
equilibrium. Thus, in the baobab extract, the sample without
oxidant shows a negative trend, except for the 5000 µg/ml
concentration. In the samples treated with oxidant (AAPH), the
energy differences are also all negative even at the threshold
concentration near 5000 µg/ml. The plots of \( \Delta E \) support the TEER results (Figure 1). The energy
expenditure is greater and reaches a maximum value of 333
nanojoules (nJ), which is much larger than the maximum value
(12.6 nJ) for the samples without added oxidant. It seems that the
presence of oxidant induces an additional effect, which may be
due to the action of free radicals in the culture medium.

Regarding the observed results with pear extract, it appears that
the changes in energy shown in this Figure 2 reflect the TEER
behaviour (Figure 1), in which all the \( \Delta E \) values are negative
except for 100 µg/ml concentrations. In this trial, this third
concentration (100 µg/ml) appears to be the threshold for the
system. Above this concentration, it is difficult for the cell to
recover the physiological equilibrium. The related \( \Delta E \) value with
oxidant (AAPH) reflects the energy expenditure trend: negative
for the first (10 µg/ml) and second (50 µg/ml) concentrations and
positive for the third one (100 µg/ml).

Figure 3 and 4 illustrate the results of the SEM analyses.
The photos Control Mag 802X and control Mag 12.04 KX represent
the control at the indicated magnifications.
To confirm the apple TEER results (Figure 1), we used SEM to observe changes in the cell morphology. The SEM analysis shows that after introduction of the apple extract, the cell monolayer is in physiological equilibrium; the monolayer reacts to the addition of extract with an increased secretion of mucopolysaccharide located at the level of microvilli. This reaction causes the formation of mucous cords along the edge of the apical wall of the cells (Fig. 3 C). The model with the induced peroxidative trigger shows an even greater secretion of mucopolysaccharide (Fig. 3 F). Cellular morphology, however, is not altered in both models and cell structure is perfectly preserved and functioning.

The SEM analysis of baobab samples shows the differences between the concentration of 2500 µg/ml (near the threshold) with and without oxidant. Samples with oxidant (Fig. 3 F1) show an increased mucopolysaccharide secretion compared with those without oxidant (Fig. 3 C1).

The added baobab fruit extract (5000 µg/ml) with a very high phenolic content induced major cellular damage. At the end of the experimental phase, the monolayer is completely destroyed (Fig. 3 C). The SEM results for the model with the induced peroxidative trigger (AAPH) show an increased secretion of mucopolysaccharides and even some necrotic areas. Overall, however, at this concentration the epithelium culture is preserved intact and is not changed morphologically (Fig. 3 F).

In the red chicory samples, the presence of oxidant (AAPH) induces an additional effect, which can be explained by cellular changes in the culture medium induced by the added free radicals associated with the oxidant. SEM photomicrographs (Fig. 4 C) show that the cell monolayer is in physiological equilibrium. The red chicory extract also induces an increase in the mucopolysaccharide secretion. The flow of mucopolysaccharides, located at the apex of microvilli, results in the formation of mucosa clumps all along the apical wall of the cells (Fig. 4 C). With the peroxidative trigger (AAPH), cell damage is apparent with deep necrotic areas. This extract seems to have a low antioxidant activity in counteracting the peroxidative effect (Fig. 4 F).

The SEM results show that a moderate level of mucopolysaccharide secretion with a normal morphology seems to occur at the concentration of 50 µg/ml (near the threshold) with (Fig. 4 F1) and without oxidant (Fig. 4 C1). The SEM analysis seems to show that the cellular monolayer is in physiological equilibrium. After the introduction of the pear extract (100 µg/ml), the cells react with an increased mucopolysaccharide secretion. Such a secretion, located at the microvilli level, forms a dense glycoalkyl that makes it difficult to observe the apical cells (Fig. 4 C). In the model with the trigger-induced peroxidation, a more pronounced type of secretory mucopolysaccharide accumulation is observed (Fig. 4 F). In both cases, however, the cellular morphology is not modified. The apical structure of the cells is preserved and functional.

Conclusions

Our results show that it is possible to measure the cellular reaction to different chemical stimuli by means of simple electrical measurements. Adding a chemical compound or a food produces an inflammatory stimulus. Consequently, the cell tries to regain the physiological equilibrium. The cells use different mechanisms to inactivate the added prooxidant or food extract that generate chemical stimuli. For instance, cells can overcome adverse effects of added compounds through use of detoxifying enzymes, including intestinal cytochromes P450 (CYPs) (Thummel and Wilkinson, 1998; Lin and Lu, 2001; Paine et al., 2006; Gibbs and Hosen, 2003), esterase (Liederer and Borchardt, 2006), uridine diphosphate glucuronosyl transferase (UGTs) (Peterson et al., 2005; Chang et al., 2007), and sulfonotransferases (SULTs) (Cowgill and Johnston, 2001; Nishimoto et al., 2007; Nishimoto et al., 2005).

As observed in the present study, another cellular defense is to delay the effect of external substances through secretion of mucopolysaccharides. The mucopolysaccharides provide a protective barrier. Yet a third possibility is to avoid adverse effects that involve penetration into the cell by using a permeability barrier with the P-glycoprotein (Huang et al., 2010) and organic anion transport polypeptides (Won et al., 2012; Bailey et al., 2007). The mechanisms of defense influence the composition of the cultural medium and resistance to the electrical current because these need energy to be activated and are strictly related to the concentration and nature chemical compounds in the medium.

The calculated energy expenditure (AE) can be positive or negative, compared to a control that is set equal to zero. When (AE) is negative, the TEER behaviour is always greater than the control, and the cells are not affected by the presence of the tested compound. If the energy expenditure is positive, then the TEER value is always lower than the control and the cells cannot regain their physiological equilibrium. The histological and morphological analysis support these suggestions: the cells are always alive and the morphological differences are related to chemical modifications which can be measured by the observed energy expenditure.

In this study, the tested extracts containing variable amounts of polyphenols. If these are present as glycosylates, then they can penetrate into the cellular membrane and interfere with the enteric oxidative cytochrome P-450 enzymes (CYP-450). If the polyphenols concentration is too high, then the cellular detoxification is more difficult and a degenerative process takes place, as strikingly illustrated above with the data for red chicory.

In our trials we applied extreme stress conditions, because the tested compound remains on the cell surface for a long time. This situation is not usual in a normal gastrointestinal environment; in which there is a regular flow of compounds so that the contact with the cell surface is reduced. We, therefore, believe that this study provides a simple model to detect the maximum compound concentration that can be tolerated by the Caco-2 cell line without irreversible cellular damage. This model could be useful for further studies of cell physiology, using other single compounds at different concentrations or a combinations of nutrients, microbial and plant toxins (Friedman and Rasooly, 2013) or drugs under physiological conditions.

The findings of the present study merit extension to demonstrate the general value of this new bioassay to nutrition and other biomedical sciences. Finally, it would also be of interest to find out whether the described method using Caco-2 cells can replace the more complex method based on monkey kidney Vero cells we used to measure the toxicological activities of native and inactivated toxins, including ricin produced by castor beans (Rasooly et al., 2012), Shiga toxin (Stx2) produced by E. coli bacteria (Rasooly et al., 2014), and Staphylococcus enterotoxin A (SEA) produced by Staphylococcus aureus bacteria (Friedman et al., 2011).

Acknowledgment

We are most grateful to Carol E. Levin for constructive contributions to the creation of the manuscript.
References


Boulalaia M, Mkadmini K, Tsolmon S, Han J, Smaoui A, Alvarenga V, Jr., Pacheco RG, Esposito CC, Buongusto F, Castelo-Branco MT, Madi K, Belmioro CR, Pavo MS, de Souza HS, Schanaider A. (2014) Ascidian (chordate-Gtunicate) and vegetable feeding trial. J Nutr., 137, 890-7.


Ferruza S, Sambuy Y, Onetti-Muda A, Nobili F, Scarnio ML. (2002) Copper toxicity to tight junctions in the human intestinal Caco-2 cell line. In: Massaro EJ, editor. Handbook of Copper Pharmacology and Toxicology. Totowa, New Jersey: Humana Press; p. 397-416.

Pinto M, Robine Leon S, Appay MD. (1983) Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. Biol Cell., 47, 323-30.
Figure 1 Results of TEER Apple, Baobab, Red Chicory and Pear

- **TEER Apple**
  - Graph showing TEER values over time for different conditions.

- **TEER Apple + AAPH**
  - Graph showing TEER values over time for different conditions with AAPH.

- **TEER Baobab**
  - Graph showing TEER values over time for different conditions.

- **TEER Baobab + AAPH**
  - Graph showing TEER values over time for different conditions with AAPH.

- **TEER Red Chicory**
  - Graph showing TEER values over time for different conditions.

- **TEER Red Chicory + AAPH**
  - Graph showing TEER values over time for different conditions with AAPH.

- **TEER Pear**
  - Graph showing TEER values over time for different conditions.

- **TEER Pear + AAPH**
  - Graph showing TEER values over time for different conditions with AAPH.
Figure 2 Results of Energy difference regarding Apple, Baobab, Red Chicory and Pear
Figure 3 Results of SEM analysis regarding Control, Apple and Baobab
Figure 4 Results of SEM analysis regarding Red chicory and Pear

Red Chicory (C)    Red Chicory (F)

Pear (C1)    Pear (C)

Pear (F1)    Pear (F)