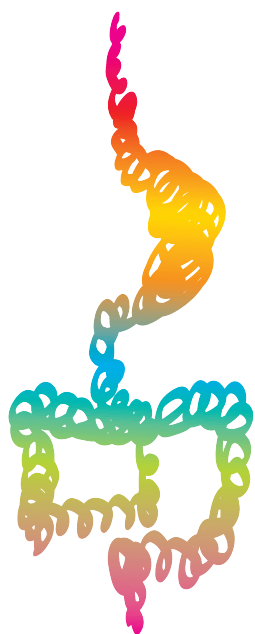


# The Intestinal Microbiota and Gut Health:

*Contribution of the Diet, Bacterial Metabolites,  
Host Interactions and Impact on Health and Disease*

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**Effect of different apple varieties on human gut microbiota composition using an *in vitro* colonic model.** By A. Koutsos<sup>1,2</sup>, M. Lima<sup>1</sup>, F. Fava<sup>1</sup>, L. Conterno<sup>1</sup>, Julie A. Lovegrove<sup>2</sup>, R. Viola<sup>1</sup> and K. Tuohy<sup>1</sup>, <sup>1</sup>*Department of Food Quality and Nutrition, Fondazione Edmund Mach, IASMA Research and Innovation Centre, Via E. Mach 1, 38010 S. Michele (TN), Italy and* <sup>2</sup>*Hugh Sinclair Unit of Human Nutrition and Institute of Cardiovascular and Metabolic Research, University of Reading, Reading, RG6 6AP, United Kingdom*

## Introduction

Apples are among the most frequently consumed fruits and a good source of both polyphenols and soluble fibre. A major proportion of apple polyphenols escape digestion in the stomach and small intestine, and together with apple non-digestible polysaccharides reach the colon and may serve as substrates for bacterial fermentation (1). Pectin, the main soluble fibre of apples, is almost completely fermented *in vitro* by bacteria resident in the colon and thus, could change the composition of the human gut microbiota (2). In contrast, it has been suggested that apple polyphenols may inhibit bacterial growth, but their impact on the gut microbiota has not been fully investigated (3). Animal studies suggest a synergistic interaction between apple polyphenols and pectin which could increase their biological activity and beneficial effects compared to the individual compounds (4). Most of the previous studies have focused on the effects of specific apple components, with only a limited number exploring in detail how whole apples from different varieties may potentially modulate the human gut microbiota. The aim of the current study was to assess the effect of four commercial apple varieties - Gold Rush, Renetta, Golden Delicious and Pink Lady - on human gut microbiota composition, compared to a readily fermented fiber (inulin) and a fiber poorly fermented by the human gut microbiota (cellulose), by using *in vitro* batch culture models.

## Material and methods

*In vitro digestion and fermentation.* A protocol was followed according to Mandalari *et al.* (2008) (5) to simulate the *in vitro* gastric and duodenal digestion of the four commercial apples, inulin (positive control) and cellulose (negative control). Following the *in vitro* digestion, fermentation was conducted in triplicate using three healthy faecal donors. Donors, two male and one female, were in good health and aged between 30 and 50, had not received antibiotic treatment for at least 3 months, had not knowingly consumed pre- or probiotic supplements prior to experiment, and had no history of bowel disorders. Water-jacket vessels (200 ml) were pH and temperature controlled (pH 5.5-6.0, 37°C) throughout the experiment and anaerobic conditions were maintained to simulate the conditions in the colon. Vessels were dosed with 2 g of each freeze dried apple variety, inulin and cellulose and inoculated with 10% w/v fresh human faeces to a final concentration of 1%. A 5 ml sample from each vessel was taken immediately for analysis (T0), similarly samples were taken at 5 (T5), 10 (T10), and 24 (T24) hours.

*Bacterial enumeration.* *Bifidobacterium spp* enumeration, for all the time points in fermentation vessels, was performed by real time polymerase chain reaction (RT-PCR) with DNA intercalating dye chemistry using the following 16S-targeted primers: Primer Forward:

TCG CGT C(C/T)G GTG TGA AAG Primer Reverse: CCA CAT CCA GC(A/G) TCC AC (6). Total bacteria population was enumerated by RT-PCR with TaqMan chemistry using 16S-targeted primers: F\_Bact 1369: CGG TGA ATA CGT TCC CGG (forward) and R\_Prok 1492 TAC GGC TAC CTT GTT ACG ACTT (reverse) with TaqMan probe: P\_TM1389F: [6FAM]-CTT GTA CAC ACC GCC CGT C [TAM] and Takara Master Mix (7). A DNA target standard curve was generated using the PCR product from DNA extract of a pure culture of *Bifidobacterium animalis BB12* and RT-PCR carried out using a Rotor Gene (Corbett Life Science).

*Statistical Analysis.* A paired t-test was used to determine differences between the baseline (T0) and the different time points (T5, T10 and T24). The effect of the different treatments (apple varieties and controls) on the bacterial population was determined by univariate analysis of variance. When statistical differences were found ( $P < 0.05$ ), data were further tested by the Tukey post hoc test.

## Results

Concerning differences between the baseline and the three time points, Bifidobacteria significantly increased as early as 5 h in the cases of Gold Rush and Golden Delicious ( $P=0.013$  and  $P=0.010$ , respectively) (Table 1). Furthermore, Bifidobacteria population increased significantly at 10 h and 24 h after a treatment with Gold Rush ( $P=0.007$  and  $P=0.013$ , respectively), Renetta ( $P=0.008$  and  $P=0.013$ , respectively) and Pink Lady ( $P=0.015$  and  $P=0.038$ , respectively) (Table 1); in the case of Golden Delicious, there was an increase after 10 h ( $P=0.001$ ), while a trend was observed after 24h ( $P=0.063$ ) (Table 1). Bifidobacteria levels did not differ between T0 and T24 for cellulose and blank (only faecal inoculum), whereas after treatment with inulin there was an increase, but this was not statistically significant. There were no significant changes in total bacteria populations for any of the fermentations, with the exception of a significant increase observed at 5h and 10h ( $P=0.022$  and  $P=0.000$ , respectively) for Golden Delicious treatment.

The effect of the different treatments (apple varieties and controls) on the bacterial population at T24 is also shown in Table 1. Treatment with the four apple varieties resulted in higher numbers of bifidobacteria compared with cellulose ( $P < 0.05$ ) and blank ( $P < 0.05$ ). Compared with inulin, only treatment with Renetta significantly increased bifidobacteria population ( $P=0.024$ ), whereas a trend was observed for Golden Delicious and Pink Lady ( $P=0.061$  and  $P=0.085$ , respectively).

## Discussion

The results from the current study suggest that commercial apple varieties - Gold Rush, Renetta, Golden Delicious and Pink Lady - may beneficially modulate the human gut microbiota by increasing bifidobacteria population *in vitro*. Pectin and polyphenols, which are the most important apple components, could be responsible for these effects. Pectin is degraded by many intestinal bacteria including Bacteroides, Eubacteria, Clostridia, Bifidobacteria (8), however, selectivity towards bifidobacteria has been shown only for pectic oligosaccharides (8). On the other hand, data measuring the impact of apple polyphenols on the gut microbiota are scarce. In an *in vitro* batch culture study, Bazzocco *et al.* (2008) (3) found that apple proanthocyanidins, the major fraction of apple polyphenols, resulted in a

reduction of short chain fatty acids (SCFA) production, indicating an inhibition of saccharolytic fermentation. However, when apple polyphenols and pectin are combined, they may exert a synergistic effect, which could increase their biological effects in the gut, compared with the individual compounds (4). Aprikian *et al.* (2003) (4) reported that caecal pH in rats was beneficially decreased only after a combined diet of apple pectin (5%) and high polyphenol freeze dried apple (10%) compared with the individual diets. Nevertheless, the specific gut microbiota composition was not determined. Sembries *et al.* (2006) (9) reported that a 4-week consumption of an apple juice, rich in polyphenols and fibre, increased the population of *Lactobacillus* and *Bifidobacterium* in rats. The fairly stable total bacteria numbers in our study, together with the significant increase of bifidobacteria, might indicate a potential modulation between beneficial and harmful bacteria.

Regarding human trials, Shinohara *et al.* (2010), observed that bifidobacteria population was significantly increased after the daily consumption of 2 apples for 2 weeks by 8 healthy subjects (10). However, in a more recent human study of 23 subjects, gut microbiota composition was not significantly affected after the daily consumption of whole apples, apple pomace, clear and cloudy apple juice for 4 weeks (11).

According to our study, Renetta variety resulted in significantly higher bifidobacteria numbers compared to inulin (P=0.024) at 24h. In a study of Vrhovsek *et al.* (2004) (12) where 8 of the most widely cultivated varieties in western Europe were tested, Renetta had the highest polyphenol content (211.9 mg/100g) which is much higher than the average polyphenol content (110.2 mg/100g) of all the apple varieties tested (12).

<b>Bifidobacteria</b>							
	<b>Treatment</b>						
<b>Time Point</b>	<b>Blank</b>	<b>Inulin</b>	<b>Cellulose</b>	<b>Gold Rush</b>	<b>Renetta</b>	<b>Golden Delicious</b>	<b>Pink Lady</b>
<b>T0</b>	7.33 ±0.34	7.43±0.18	7.36±0.21	7.51±0.15	7.39±0.23	7.55±0.19	7.39±0.27
<b>T5</b>	7.56±0.30	7.80±0.43	7.66±0.39	8.05±0.42*	8.29±0.78	8.52±0.11*	8.23±0.25
<b>T10</b>	7.71±0.49	7.74±0.61	7.77±0.57	8.37±0.17*	8.38±0.18*	8.65±0.23*	8.34±0.08*
<b>T24</b>	7.42±0.04	7.84±0.50	7.56±0.10	8.49±0.13*#°	8.62±0.03*#§°	8.51±0.33#°	8.47±0.12*#°
<b>Total bacteria</b>							
	<b>Treatment</b>						
<b>Time Point</b>	<b>Blank</b>	<b>Inulin</b>	<b>Cellulose</b>	<b>Gold Rush</b>	<b>Renetta</b>	<b>Golden Delicious</b>	<b>Pink Lady</b>
<b>T0</b>	9.03±0.15	9.00±0.30	9.12±0.21	8.99±0.12	8.82±0.19	9.00±0.05	8.96±0.05
<b>T5</b>	9.16±0.16	9.20±0.21	9.21±0.17	8.91±0.49	8.99±0.63	9.30±0.04*	9.08±0.13
<b>T10</b>	9.12±0.03	9.08±0.27	9.16±0.04	9.19±0.33	9.17±0.54	9.39±0.04*	9.27±0.20
<b>T24</b>	8.94±0.12	9.19±0.23	8.96±0.17	9.01±0.10	9.10±0.23	9.17±0.38	9.21±0.24

**Table 1.** Bacterial populations ( $\log_{10}$  cells/ml batch culture fluid) determined by RT-PCR in anaerobic, stirred, pH (5.5-6.0) and temperature (37°C) controlled faecal batch cultures at 0, 5, 10 and 24 h using inulin as a positive control, cellulose as negative control and blank vessel (only faeces) as a baseline in order to compare the microbiota-modulating abilities of 4 different apple varieties (n=3 healthy adults). (\*) Mean value was significantly different from that at 0 h: P<0.05. (#) Mean value was significantly different from Blank at 24h: P<0.05. (§) Mean value was significantly different from inulin at 24h: P<0.05. (°) Mean value was significantly different from cellulose at 24h: P<0.05.

Our preliminary results have shown that apples may beneficially modulate the human gut microbiota composition by increasing the levels of bifidobacteria *in vitro*. Renetta was

associated with the highest bifidogenic effect. However, the potential balance between beneficial and harmful bacteria is critical for our health and thus, the determination of other bacteria groups or genera in our study is necessary. Ongoing work includes fuller characterisation of the gut microbiota and the end products of their metabolism of apples, short chain fatty acids and polyphenolic catabolites. Finally, a randomized controlled human intervention study is required to determine the effects of the Renetta variety on gut microbiota *in vivo* and potential benefits to other health outcomes.

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