The Journal of Nutrition
Nutrient Physiology, Metabolism, and Nutrient-Nutrient Interactions

Dietary Flavonoids from Modified Apple Reduce Inflammation Markers and Modulate Gut Microbiota in Mice[1–3]

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Abstract

Apples are rich in polyphenols, which provide antioxidant properties, mediation of cellular processes such as inflammation, and modulation of gut microbiota. In this study we compared genetically engineered apples with increased flavonoids [myeloblastis transcription factor 10 (*MYB10*)] with nontransformed apples from the same genotype, ''Royal Gala'' (RG), and a control diet with no apple. Compared with the RG diet, the *MYB10* diet contained elevated concentrations of the flavonoid subclasses anthocyanins, flavanol monomers (epicatechin) and oligomers (procyanidin B2), and flavonols (quercetin glycosides), but other plant secondary metabolites were largely unaltered. We used these apples to investigate the effects of dietary flavonoids on inflammation and gut microbiota in 2 mouse feeding trials. In trial 1, male mice were fed a control diet or diets supplemented with 20% *MYB10* apple flesh and peel (MYB-FP) or RG apple flesh and peel (RG-FP) for 7 d. In trial 2, male mice were fed MYB-FP or RG-FP diets or diets supplemented with 20% *MYB10* apple flesh or RG apple flesh for 7 or 21 d. In trial 1, the transcription levels of inflammation-linked genes in mice showed decreases of >2-fold for interleukin-2 receptor (*Il2rb*), chemokine receptor 2 (*Ccr2*), chemokine ligand 10 (*Cxcl10),* and chemokine receptor 10 (*Ccr10*) at 7 d for the MYB-FP diet compared with the RG-FP diet ($P < 0.05$). In trial 2, the inflammation marker prostaglandin E_2 (PGE_2) in the plasma of mice fed the MYB-FP diet at 21 d was reduced by 10-fold ($P < 0.01$) compared with the RG-FP diet. In colonic microbiota, the number of total bacteria for mice fed the MYB-FP diet was 6% higher than for mice fed the control diet at 21 d ($P = 0.01$). In summary, high-flavonoid apple was associated with decreases in some inflammation markers and changes in gut microbiota when fed to healthy mice. J. Nutr. 144: 146–154, 2014.

Introduction

The consumption of plant-derived polyphenols has been associated with a reduced incidence of coronary heart disease, age-related degeneration, and some cancers (1). Evidence from in vitro, animal, and human studies suggests that these bioactive benefits can be attributed to antioxidant and anti-inflammatory properties of polyphenols and by their effect on the gut microbiota (2–6). Oxidative stress is implicated in the pathogenesis of many diseases, and the direct antioxidant activity of polyphenols on free radicals and reactive oxygen species is well known (7,8), whereas the indirect antioxidant effects are due to modulation of endogenous cell signaling pathways (9,10).

Polyphenols can also influence immune response, especially in the intestine. This response involves regulatory mediators, such as cytokines, or cellular events that modulate inflammation (11,12). Many studies have shown that dietary polyphenols can modulate intestinal inflammation and reduce the incidence or severity of chronic disorders such as inflammatory bowel disease (2). The bioavailability and biologic activity of polyphenols also depends on interaction with the microbiota and the complex role bacteria play in mediating bioactivity of these compounds (2,4). Many polyphenols undergo conversion by gut bacteria with further metabolism occurring in the body postabsorption to the extent that plasma and tissues are not exposed to the original forms (13). Metabolic transformations of compounds such as quercetin can have a profound effect on their antioxidant and anti-inflammatory properties (14). In addition, the degree of antioxidant and anti-inflammatory effects of polyphenols can vary according to the food matrix and on the interaction of different subclasses of polyphenols within that matrix (15).

[1] Supported by the Ministry for Science and Innovation (contract COX0207), New Zealand.
[2] Author disclosures: R. V. Espley, C. A. Butts, W. A. Laing, S. Martell, H. Smith, T. K. McGhie, J. Zhang, G. Paturi, D. Hedderley, A. Bovy, H. J. Schouten, J. Putterill, A. C. Allan, and R. P. Hellens, no conflicts of interest.
[3] Supplemental Table 1 and Supplemental Figure 1 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.
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Manuscript received July 31, 2013. Initial review completed September 11, 2013. Revision accepted November 18, 2013.
First published online December 18, 2013; doi:10.3945/jn.113.182659.

Although many in vitro cell-based studies have been undertaken on the effects of phytochemicals, there is an increasing focus on studies that show these effects in vivo. For example, long-term feeding of rats with maize rich in anthocyanin increased cardioprotection in a model of induced heart failure (16), and feeding cancer-susceptible mice genetically engineered tomatoes with a high anthocyanin content significantly reduced the incidence of tumorigenesis and prolonged the animals' life span (17).

It is known that a diet rich in fruit and vegetables has nutritional and health benefits (18,19), and for apple these beneficial effects have been well documented (20,21). Apple and apple products are one of the major dietary sources of polyphenolics (22). The 5 major classes of polyphenols in apple are as follows: phenolic acids; anthocyanins; flavan-3-ols, such as catechin; dihydrochalcones, such as phloridzin; and flavonols, such as quercetin (23). The in vitro antioxidant properties of flavonoids (flavonols and anthocyanins in apple) have also been described (24) and are mainly concentrated in the peel. A recent review on the potential health benefits of apple consumption concluded that there is compelling evidence that apple consumption is linked to a reduced risk of various chronic diseases (25). However, the mechanisms behind these benefits are still not fully understood. Furthermore, these health benefits may depend on the presence or interaction of different classes of bioactives because a number of studies suggest that apple as a whole food, rather than its individual components, may be more effective in disease prevention.

In this study we aimed to determine the effects of high-flavonoid apple consumption on inflammation and on gut microbiota. We used a genetically engineered apple variety modified by overexpression of the apple myeloblastis transcription factor 10 (*MYB10*)[8], which regulates flavonoid biosynthesis, particularly anthocyanin. Using the transgenic and nontransgenic apples of the same genotype in a mouse feeding trial, we were able to isolate the dietary effect of flavonoids while other phytochemicals present in the apple food matrix remained largely unaltered. To evaluate the effects on inflammation and bacterial growth, we measured differences between inflammation-related gene expression and markers and bacterial populations in mice fed diets containing the 2 apple genotypes.

Materials and Methods

Fruit tissue

Apple fruit used to supplement diets were either the readily available variety "Royal Gala" (RG) or "Royal Gala" transformed with the apple MYB transcription factor, *MYB10*, fused to a constitutive promoter, Cauliflower mosaic virus 35S (*MYB10* apple) (26). As in other plant species, this MYB transcription factor regulates biosynthetic genes in the anthocyanin-specific part of the flavonoid pathway (27). Fruit were harvested at a similar maturity (according to internal ethylene values and starch index) and either processed intact (without core) or separated into peel and the outer cortex (fruit flesh excluding the core and peel). Samples were immediately frozen in liquid nitrogen, freeze-dried, finely ground, and stored under vacuum in aluminum foil bags at −20°C until use.

Antioxidant activity

Total antioxidant activity of the apple flesh and peel samples was measured by using the Trolox equivalent antioxidant capacity (TEAC) assay (28). Vitamin C in the apple was extracted and measured as previously described (29).

Mouse feeding trials

The experimental procedures for these studies were approved by the AgResearch Grasslands Animal Ethics Committee (Palmerston North, New Zealand) according to the Animal Welfare Act of 1999.

Trial 1. Thirty Swiss male mice, ~30 g live weight, 6–7 wk of age, bred in the Food Evaluation Unit, Plant and Food Research, Palmerston North, New Zealand, were kept under standard conditions at room temperature (22 ± 1°C), a humidity of 60 ± 5%, an air exchange of 12 room volumes/h, and with a 12-h light-dark cycle. Weanling male mice (21 d old) were housed in family groups and fed commercial pelleted diet (Prolab RMH 1800; PMI Nutrition International) until 6 wk of age, after which they were transferred to individual metabolism cages and fed AIN-76A diet ad libitum for 4 d. These cages are designed with open wire mesh floors to allow the quantitative collection of spilled diet and waste matter (urine and feces). The cage structure thus makes coprophagy unlikely but not impossible. On day 5, the mice were randomly allocated to an experimental dietary treatment (n = 10) and fed 1 of the experimental diets: control, RG apple flesh and peel (RG-FP), or *MYB10* apple flesh and peel (MYB-FP) (**Supplemental Table 1**). The control diet was diet AIN-76A, and the freeze-dried apple (flesh and peel) was included (20%) at the expense of sucrose. Sugars (11%) comprise the major component of the dry matter (14.6%) in apple (30). Experimental diets were offered at 5 g/mouse per day and were fed for 7 d. The General Health Score, a visual assessment of healthy appearance (31), for each mouse was noted daily, and the mice were weighed and food intake recorded. Urine and feces output was collected each day and stored at −80°C.

Trial 2. After the next season's harvest of *MYB10* apples, we undertook a second mouse feeding trial to confirm the first study and to extend the feeding period to 21 d. The mice were kept under the same conditions as for trial 1. The dietary treatments were fed for 7 and 21 d and consisted of a control, RG flesh (RG-F), *MYB10* flesh (MYB-F), RG flesh and peel (RG-FP), and *MYB10* flesh and peel (MYB-FP) (Supplemental Table 1). The apple treatments included the comparison between apple flesh only and flesh and peel for both apple genotypes. The experimental conditions were as for trial 1 with the following changes. The mice were staggered on to the experiment over several days to allow for the tissue sampling at the end of the study. The diets were offered at 6 g/mouse per day and fed for 7 or 21 d. Urine and feces output was collected and weighed each day during the first 7-d period and stored at −80°C, and were collected each day and stored in 7-d aliquots for the remaining 14 d of the study. At the end of the 2 trials, the mice were killed by CO_2 asphyxiation, and blood was immediately withdrawn via cardiac puncture for the preparation of plasma for cytokine analysis. The colon contents were gently removed, snap-frozen in liquid nitrogen, and then stored at −80°C for real-time PCR quantification of microbiota analysis. The jejunum tissues were excised, thoroughly rinsed with PBS, and stored at −80°C for gene expression analysis.

Mouse diet analysis

Approximately 3 g of diet was mixed with 10 mL solvent (ethanol: water:formic acid, 80:20:1) and homogenized at 300 g for 4 min. After overnight storage at 1°C, the samples were centrifuged and diluted with methanol before analysis by liquid chromatography–quadruple time-of-flight–high resolution mass spectrometry. The LC-MS system used has previously been described, together with the source parameters and calibration method (32).

Mouse gene expression analysis

RNA was extracted from the flash-frozen jejunum sections of the gut from the mice in trial 1 and treated with DNase (Qiagen). The jejunum

[8] Abbreviations used: *Ccr2*, chemokine (C-C motif) receptor 2; *Ccr10*, chemokine (C-C motif) receptor 10; *Cox-2*, cyclooxygenase-2; *Cxcl10*, chemokine ligand 10; *Cxcl15*, chemokine (C-X-C motif) ligand 15; DW, dry weight; gDNA, genomic DNA; *Hprt1*, hypoxanthine guanine phosphoribosyl transferase 1; *Hprt1*, hypoxanthine guanine phosphoribosyl transferase 1; *Hsp90ab1*, heat shock protein 90α (cytosolic), class B member 1; *Il11*, interleukin-11; *Il2rb*, interleukin-2 receptor, β chain; *Il6*, interleukin-6; LTB_4, leukotriene B_4; *MYB10*, myeloblastis transcription factor 10; MYB-F, *MYB10* apple fruit flesh; MYB-FP, *MYB10* apple flesh and peel; *Nfkb*, nuclear factor kappa-light-chain-enhancer of activated B cells; RG, Malus × domestica "Royal Gala" apple; RG-F, "Royal Gala" apple fruit flesh; RG-FP, "Royal Gala" apple flesh and peel; TEAC, trolox equivalent antioxidant capacity.

has been previously reported to be the major site of anthocyanin uptake (33). cDNA was synthesized (SABioscience) and relative gene expression assayed by using RT^2 qPCR SYBR Green MasterMix (SABioscence) on Mouse Inflammatory Cytokines and Receptors RT^2 Profiler PCR arrays (SABioscence) with the Roche LightCycler 480 (Roche). qPCR conditions and subsequent analyses were carried out according to the manufacturer's recommended protocol (SABioscience). Of the 5 mouse housekeeping genes tested, 2 were used for the final analysis: hypoxanthine guanine phosphoribosyl transferase 1 (*Hprt1*) and heat shock protein 90α (cytosolic), class B member 1 (*Hsp90ab1*), which showed a fold difference of between 1.06 and −1.06 across all replicates. Each plate included control wells for genomic DNA (gDNA) contamination and RT-PCR efficiencies. One mouse replicate for each treatment was excluded due to poor RT-PCR efficiency or gDNA contamination, leaving a sample set of 9 mice for each treatment. Melt curve analysis confirmed the amplification of single products.

Cytokine analysis

Plasma concentrations of cytokines were determined by flow cytometry according to the manufacturer's instructions (Cayman Chemical Company). Mouse plasma samples were diluted 4-fold with Luminex Assay Buffer. A 100-μL diluted plasma sample, 50-μL phycoerythrin tracers, and 50-μL Luminex beads were added to the 96-well plate. Each plate contains 2 maximum binding wells (B0) and an 8-point standard curve run in duplicate. The plate was covered by a foil plate cover and incubated for 4 h at room temperature on an orbital shaker. The plate was read on a flow cytometer, and the median fluorescent intensity for each well was obtained. An 8-point standard curve was obtained by plotting % Standard/B for standard versus cytokine concentration by using linear (y) and log (x) axes and fitting the data to a 4-parameter logistic equation. The concentration of cytokines in samples was determined from their standard curve by calculating % Sample/B for each sample with the correction of dilution factors.

Quantification of colonic microbiota

The colonic microbiota was quantified by real-time PCR as described previously by Paturi et al. (34). gDNA was extracted from the overnight cultures of *Bacteroides fragilis* NZRM 964, *Bifidobacterium adolescentis* American Type Culture Collection 15703, *Lactobacillus reuteri* DPC 16, and *Escherichia coli* American Type Culture Collection 35150 according to the QIAamp DNA mini kit protocol (Qiagen). The oligonucleotides used to construct standard curves and to quantify the *Bacteroides-Prevotella-Porphyromonas* group, *Bifidobacterium* spp., *Lactobacillus* spp., and total bacteria were as previously described (34).

Statistical analysis

Data are presented as means ± SEs. Statistical analysis was performed by Minitab version 15 (Minitab) or Genstat version 14 (VSN International). For trial 1, the weights, food intakes, urine and fecal output, and fiber intake data were all analyzed with 1-factor ANOVA, with the factor being diet. For trial 2, the urine and fecal output, weight gain, food and fiber intakes, cytokine data, and the bacterial populations were analyzed by using 2-factor ANOVA, and the factors were diet and 2 time periods (7 d, 21 d). The urine and fecal output, weight gain, food and fiber intakes after 21 d were compared by using 1-factor ANOVA, with the factor being diet. The weekly weight gain and fecal and urine output data from the 21-d group were analyzed by using a split-plot-in-time ANOVA, with diet as the main factor and week as the subplot factor. The post hoc analysis was carried out by a least significant difference test ($P < 0.05$). Residuals were inspected for all analyses, and, if necessary, data were log-transformed to stabilize variance. Statistical analysis for antioxidant capacity and vitamin C concentration was performed by 1-factor ANOVA. Statistical analysis of gene expression data was performed by using the software provided and according to the manufacturer's instructions (SABiosciences), where the relative gene expression level ($2^{-\Delta Ct}$) was calculated and normalized to the housekeeping genes by using the following equation: $\Delta Ct = C_t$ (GOI) – average [C_t (HKG)], where Ct was the crossing threshold, GOI was each gene of interest, and HKG were the housekeeping genes.

Results

Flavonoid content in transgenic apples. Transgenic RG apple showed an increase in polyphenols in the flavonoid subclasses anthocyanins, flavanol monomers (epicatechin), flavanol oligomers (procyanidin B2), and flavonols (quercetin glycosides). Anthocyanin-derived pigmentation was visible in virtually all parts of the tree and fruit (**Supplemental Fig. 1**). Fruit tissue from RG apple and transgenic *MYB10* apple was used in the mouse diets, and the diets were analyzed for polyphenol content (**Table 1**). The major anthocyanin, cyanidin galactoside, was undetectable in the RG-F diet and ranged from 29 μg/g in the RG-FP–supplemented diet to 379 μg/g in the MYB-FP diet, a 14-fold difference. Similarly, procyanidin B2 ranged from 68 to 232 μg/g and epicatechin ranged from 66 to 352 μg/g. Some classes of flavonoids were reduced in the MYB-FP diet, including quercetin-arabinoside and phlorizidin. Cyanidin and quercetin were not detected in the RG-F diet. None of the dietary polyphenols we tested were detected in the control diet (no apple).

Antioxidant capacity. The antioxidant capacity of RG-FP [63 ± 14 mmol Trolox/kg dry weight (DW)] was less than that of MYB-FP (271 ± 17 mmol Trolox/kg DW) ($P < 0.01$). The antioxidant capacity of RG-F (34.5 ± 20 mmol Trolox/kg DW) was less than that of MYB-F (94 ± 5 mmol Trolox/kg DW) ($P < 0.01$). This was despite the observation that vitamin C was reduced by 54% and 7% in MYB-F and MYB-FP, respectively ($P < 0.05$) compared with RG, suggesting that the increased flavonoid content more than compensated for the low ascorbate content.

Mouse feeding trials. The mice remained healthy throughout both feeding trials, with a daily General Health Score of 5 recorded at all times. There was a small loss of weight for the mice in trial 1, but this was not significantly different between dietary treatments and was likely due to the restricted quantity of food offered (5 g/d) (**Table 2**). There were also no significant differences in food intake and urine output for the *MYB10* or RG diets compared with the control diet in trial 1. Fiber intake ($P < 0.01$) and fecal output ($P < 0.01$) were significantly different between the dietary treatments (Table 2). Mice fed

TABLE 1 Polyphenol concentrations in the mouse diets supplemented with RG and *MYB10* apple fruit tissue (trials 1 and 2)[1]

	Diet			
Compound	RG-F	RG-FP	MYB-F	MYB-FP
			$\mu g/g$	
Cyanidin galactoside	ND	29 ± 0. 9	197 ± 7.1	397 ± 34.1
Catechin	22 ± 0.4	22 ± 0.9	31 ± 1.3	37 ± 1.5
Epicatechin	66 ± 4.6	96 ± 0.9	261 ± 13.6	352 ± 26.9
Phlorizidin	6 ± 0.4	14 ± 0.4	8 ± 0.7	9 ± 0.9
Phlorizidin-xyloside	16 ± 2.1	46 ± 0.3	14 ± 1.4	25 ± 3.9
Procyanidin B2	68 ± 4.2	91 ± 0.5	173 ± 5.7	238 ± 16.3
Quercetin-arabinoside	ND	14 ± 0.1	1 ± 0.9	11 ± 1.6
Quercetin-galactoside	ND	9 ± 0.2	6 ± 0.2	21 ± 2.4

[1] Liquid chromatography-MS values represent the means of 3 technical replicates ± SEs. Polyphenols listed in this table were not detected in the control diet. *MYB10*, myeloblastosis transcription factor 10; MYB-F, *MYB10* apple fruit flesh; MYB-FP, *MYB10* apple flesh and peel; ND, not detected (limit of detection 1 μg/g); RG, ''Royal Gala''; RG-F, ''Royal Gala'' apple fruit flesh; RG-FP, ''Royal Gala'' flesh and peel.

TABLE 2 Food and fiber intake and output of urine, feces, and fecal dry matter of mice fed control, RG-FP, or MYB-FP diets for 1 wk (trial 1)[1]

	Food intake	Total fiber intake	Weight gain	Urine output	Fecal output	Fecal dry matter	Fecal dry matter output
	g/7 d	*g/7 d*	*g/7 d*	*g/7 d*	*g/7 d*	*%*	*g/7 d*
Control	26.6 ± 2.8	1.52 ± 0.2[a]	−1.6 ± 1.3	13.2 ± 15.8	2.55 ± 0.4[a]	87.7 ± 4.4[c]	2.23 ± 0.2[a]
RG-FP	25.9 ± 1.9	2.23 ± 0.2[b]	−2.2 ± 1.1	11.4 ± 6.0	3.50 ± 0.5[b]	82.1 ± 4.4[b]	2.86 ± 0.3[b]
MYB-FP	26.7 ± 2.1	2.62 ± 0.2[c]	−2.3 ± 1.1	8.9 ± 4.5	5.79 ± 0.9[c]	71.6 ± 5.4[a]	4.12 ± 0.5[c]
P value (2 df)	0.68	<0.001	0.30	0.64	<0.001	<0.001	<0.001

[1] Values are means ± SDs, $n = 10$. Labeled means in a column without a common letter differ, $P < 0.05$. *MYB10*, myeloblastosis transcription factor 10; MYB-FP, *MYB10* apple flesh and peel; RG-FP, ''Royal Gala'' flesh and peel.

the MYB-FP diet produced significantly more feces (as fecal matter or on a dry matter basis) than mice on the RG-FP diet, which, in turn, produced significantly greater fecal output than mice fed the control diet (Table 2).

In trial 2, the weight gain for the mice fed the control diet was significantly higher ($P = 0.01$) than for mice fed the RG-FP and MYB-FP diets (**Table 3**). There was no effect of dietary treatment on food intake, but there was a significant ($P < 0.01$) effect of diet on fiber intake over 21 d (Table 3). When the time period was included as a factor in the ANOVA, there was a significant effect of diet ($P = 0.02$) and time ($P = 0.01$) for weight gain (data not shown). Weight gains were significantly lower for mice fed RG-FP and M-FP diets compared with the control diet ($P < 0.01$), and for all treatments weight gain decreased over time as the mice neared their mature adult body weight. Food intake was not significantly different between diets, but there was a significant effect of time ($P < 0.01$) in which food intake was lower in the final week of the study compared with the first week (data not shown). Fiber intake was significantly ($P < 0.01$) lower for the control diet as for trial 1, and there was a significant reduction in food intake and weight gain with time ($P < 0.01$). There were no significant effects for the interaction (diet × time) for weight gain, food intake, or fiber intake.

Analysis of fecal output over the 2 time periods (7 d and 21 d) confirmed the findings of trial 1 (**Table 4**) in which the fecal output from mice fed the MYB-FP diet was significantly greater ($P < 0.01$) than that for mice fed the MYB-F, RG-F, and RG-FP diets, which, in turn, was significantly greater than for mice fed the control diet. Fecal dry matter was significantly ($P < 0.01$) higher for mice fed the control diet than for mice fed the apple-supplemented diets. There was a significant effect of time for fecal output ($P = 0.02$) and fecal dry matter output ($P < 0.01$), reflecting the slowing of growth and food intake with time. After taking into account the differences in fiber intake between diets by calculating the ratios of fecal output to fiber intake (**Table 5**), there were still significant effects of diet, with mice fed the control diet giving the least output and mice fed the *MYB10* diets giving greater output than mice fed the RG diets ($P < 0.01$). Similarly, over time, there was a reduction in outputs for mice fed the RG-F, RG-FP, MYB-F, and MYB-FP diets at the 15–21-d period compared with the 1–7-d period ($P < 0.01$).

Inflammatory pathway gene expression. Of the 83 mouse inflammatory-related genes tested, the expression of 4 genes was reduced for mice fed the MYB-FP diet when compared with the RG-FP diet in trial 1. The volcano plot (**Fig. 1**) shows the $\log_2$ of the fold change in gene expression between the samples compared with its *P* value from the *t* test. In particular, interleukin-11 (*Il11*) showed a 3-fold decrease in expression ($P < 0.01$), whereas there were >2-fold decreases for chemokine (C-C motif) receptor 2 (*Ccr2*) ($P < 0.01$), chemokine (C-C motif) receptor 10 (*Ccr10*) ($P < 0.05$), and interleukin-2 receptor, β chain (*Il2rb*) ($P < 0.05$) in mice fed the MYB-FP diet compared with the RG-FP diet.

Circulating plasma cytokines. Circulating cytokine protein concentrations in the plasma of mice in trial 2 were measured at 7 and 21 d, and we found significant decreases in the immune mediator leukotriene B_4 (LTB_4) between mice fed the control and MYB-FP diets ($P < 0.01$) (**Fig. 2**). For PGE_2, there were also significant differences between diets, with a reduction in PGE_2 concentrations in mice fed *MYB10* diets over 21 d compared with RG-F, RG-FP, and control diets ($P < 0.01$). For PGE_2 we observed a time effect, with significant differences in concentrations between 7 and 21 d for the control, RG-F, and MYB-FP diets ($P = 0.02$). This was not the case for LTB_4. We also found a reduction in the concentration of TNF-α for mice fed the apple diets (RG and *MYB10*) compared with the control diet ($P < 0.01$). There was an increase in concentrations at 21 d compared with 7 d for mice fed control and RG-FP diets ($P < 0.01$).

Quantification of colonic microbiota. There were significant effects of diet ($P < 0.01$) and time ($P < 0.01$) on total bacteria (**Fig. 3**). Total bacteria were significantly lower for mice fed the control diet compared with mice fed RG-F and MYB-FP diets. Total bacteria were significantly higher after 7 d of feeding than after 21 d for mice fed the control diet. The numbers of *Lactobacillus* spp. relative to total bacteria in colon were significantly higher ($P < 0.01$, diet; $P < 0.01$, day; $P < 0.01$, diet × day) for the control diet on day 21 compared with all the other dietary treatments. For the *Bacteroides-Prevotella-Porphyromonas* group, there was a significant effect of diet ($P = 0.03$) and day ($P = 0.05$) where bacteria counts in mice fed the MYB-FP diet were higher than those in mice fed the RG-F

TABLE 3 Weight gain and food and fiber intake for mice fed control, RG-FP, RG-F, MYB-FP, and MYB-F diets for 3 wk (trial 2)[1]

	Weight gain	Food intake	Fiber intake
Control, *g/21 d*	5.0 ± 1.1[c]	87 ± 3.2	5.4 ± 0.2[a]
RG-FP, *g/21 d*	3.6 ± 1.4[a,b]	85 ± 5.5	7.2 ± 0.5[c]
RG-F, *g/21 d*	4.5 ± 1.5[b,c]	88 ± 5.6	6.9 ± 0.4[b]
MYB-FP, *g/21 d*	3.4 ± 1.7[a]	87 ± 4.8	7.6 ± 0.4[d]
MYB-F, *g/21 d*	4.2 ± 1.7[a,b,c]	89 ± 6.0	6.9 ± 0.5[b]
P value (4 df)	0.009	0.13	<0.001

[1] Values are means ± SDs, $n = 10$. Labeled means in a column without a common letter differ, $P < 0.05$. *MYB10*, myeloblastosis transcription factor 10; MYB-F, *MYB10* apple fruit flesh; MYB-FP, *MYB10* apple flesh and peel; RG-F, ''Royal Gala'' apple fruit flesh; RG-FP, ''Royal Gala'' flesh and peel.

TABLE 4 Fecal output, fecal dry matter, and fecal dry matter output for mice fed control, RG-FP, RG-F, MYB-FP, and MYB-F diets for 1 (1–7 d) and 3 (15–21 d) wk (trial 2)[1]

	Fecal output		Fecal dry matter		Fecal dry matter output	
	1–7 d	15–21 d	1–7 d	15–21 d	1–7 d	15–21 d
	g/7 d		*%*		*g/7 d*	
Control	2.85 ± 0.3[a]	2.71 ± 0.2[a]	81.3 ± 4.1[c]	82.4 ± 3.5[b]	2.31 ± 0.1[a]	2.23 ± 0.1[a]
RG-FP	4.52 ± 0.6[b,c]	4.11 ± 0.6[b]	70.6 ± 6.1[a,b]	72.9 ± 4.7[a]	3.17 ± 0.2[b]	2.99 ± 0.4[b]
RG-F	4.06 ± 0.4[b]	3.95 ± 0.5[b]	74.9 ± 5.5[b]	71.0 ± 7.1[a]	3.02 ± 0.2[b]	2.77 ± 0.1[b]
MYB-FP	5.27 ± 0.8[d]	4.98 ± 1.0[c]	69.2 ± 4.7[a,b]	67.7 ± 7.0[a]	3.63 ± 0.5[c]	3.31 ± 0.4[c]
MYB-F	4.61 ± 0.6[c]	4.16 ± 0.6[b]	68.3 ± 7.9[a]	68.9 ± 6.9[a]	3.11 ± 0.2[b]	2.84 ± 0.2[b]
P value						
Diet (4 df)	<0.001		<0.001		<0.001	
Time (1 df)	0.022		0.84		<0.001	
Diet × time (4 df)	0.86		0.48		0.70	

[1] Values are means ± SDs, $n = 10$. Labeled means in a column without a common letter differ, $P < 0.05$. *MYB10*, myeloblastosis transcription factor 10; MYB-F, *MYB10* apple fruit flesh; MYB-FP, *MYB10* apple flesh and peel; RG-F, ''Royal Gala'' apple fruit flesh; RG-FP, ''Royal Gala'' flesh and peel; 1–7 d, group killed at 7 d; 15–21 d, group killed at 21 d.

diet. *Bifidobacterium* spp. were significantly higher in mice fed RG-F and RG-FP diets compared with all other experimental diets ($P < 0.01$, diet; $P < 0.01$, day; $P < 0.01$, diet × day).

Discussion

Because the beneficial dietary components from fruit may act in a synergistic manner (35) and may be partly dependent on the food matrix, it is especially important to consider the effect on health of using whole fruit in in vivo studies. The apple fruit used in this study were of the same genotype (RG) and genotypically differed only in the transgenic overexpression of the MYB transcription factor *MYB10*. The transgenic fruit were tested for both primary and secondary metabolites; the major differences were in the polyphenolics, whereas smaller differences in sugars, carotenoid amounts, and vitamin C were observed (36). This allowed us to test the dietary effect of 1 group of potential bioactive compounds while other phytochemicals and the overall food matrix remained essentially unchanged. The precise nature of the flavonoid elevation is due to the transcriptional regulation of *MYB10*.

Studies in humans have shown that apple can increase antioxidant concentrations in plasma (37) and serum (38) and reduce DNA damage and oxidative stress levels (39,40). The antioxidant capacity of apple has been shown to vary by as much as 2-fold according to genotype (41). Here we showed that the antioxidant capacity of RG and *MYB10* apples as measured by the TEAC assay increased by as much as 4-fold in the peel of *MYB10* apples. However, it is not clear from the TEAC assay as to which bioactives are associated with this antioxidant capacity. A more in-depth analysis of the antioxidant capacity of these high-flavonoid fruit would be required to confirm the bioactive source. Previous studies in other plant species have shown that anthocyanins and ascorbate tend to correlate positively (42). However, vitamin C amounts are relatively low in RG fruit (43) and are unlikely to affect antioxidant capacity.

In our preliminary mouse feeding trial we showed that transcription levels of 4 inflammatory genes, including 2 chemokine receptors (*Ccr2* and *Ccr10*), an interleukin (*Il11*), and an interleukin receptor (*Il2rb*), were significantly altered in the mouse jejunum when comparing mice fed a MYB-FP diet with those fed an RG-FP diet. Chemokines are involved in many pathologic and physiologic processes, including inflammation, tumorigenesis, and

TABLE 5 Ratios of mouse fecal output, fecal dry matter output, and fiber intake for mice fed control, RG-FP, RG-F, MYB-FP, and MYB-F diets for 1 (1–7 d) and 3 (15–21 d) wk (trial 2)[1]

	Fecal output:total fiber intake		Fecal dry matter output:total fiber intake	
	1–7 d	15–21 d	1–7 d	15–21 d
	g/7 d		*g/7 d*	
Control	1.56 ± 0.1[a]	1.56 ± 0.1[a]	1.26 ± 0.1[a]	1.28 ± 0.1[a]
RG-FP	2.25 ± 0.3[c]	1.80 ± 0.2[a,b,c]*	1.58 ± 0.1[b,c]	1.32 ± 0.2[a]*
RG-F	1.96 ± 0.2[b]	1.73 ± 0.2[a,b]	1.46 ± 0.1[b]	1.20 ± 0.2[a]*
MYB-FP	2.48 ± 0.2[c]	2.01 ± 0.3[c]*	1.71 ± 0.1[c]	1.34 ± 0.1[a]*
MYB-F	2.28 ± 0.3[c]	1.87 ± 0.2[b,c]*	1.54 ± 0.1[b]	1.28 ± 0.1[a]*
P value				
LSD (90 df)	0.27		0.16	
Diet (4 df)	<0.001		<0.001	
Time (1 df)	<0.001		<0.001	
Diet × time (4 df)	0.09		0.009	

[1] Values are means ± SDs, $n = 10$. Labeled means in a column without a common letter differ, $P < 0.05$. *Different from day 7, $P < 0.05$. LSD, least significant difference test; *MYB10*, myeloblastosis transcription factor 10; MYB-F, *MYB10* apple fruit flesh; MYB-FP, *MYB10* apple flesh and peel; RG-F, ''Royal Gala'' apple fruit flesh; RG-FP, ''Royal Gala'' flesh and peel; 1–7 d, group killed at 7 d; 15–21 d, group killed at 21 d.

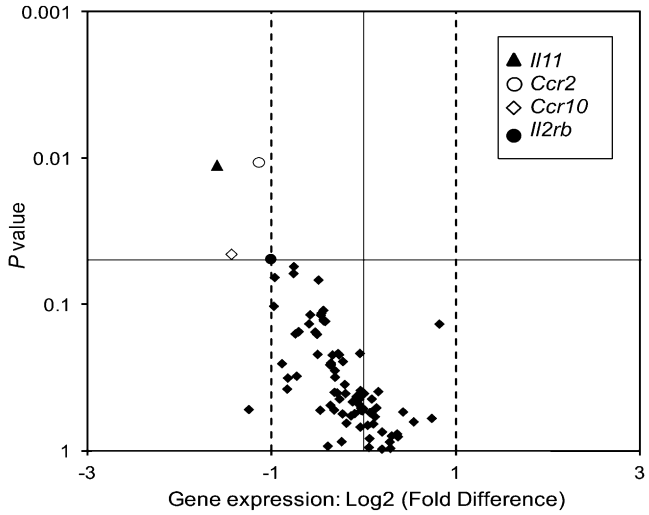

FIGURE 1 Fold changes in inflammatory pathway gene expression in jejunum tissue of mice fed the MYB-FP diet compared with those fed the RG-FP diet (trial 1). The data show a threshold for fold difference of 2 (vertical dotted lines) ($P < 0.05$). *Ccr2*, chemokine (C-C motif) receptor 2; *Ccr10*, chemokine (C-C motif) receptor 10; *Il11*, interleukin-11; *Il2rb*, interleukin-2 receptor, b chain; *MYB10*, myeloblastosis transcription factor 10; MYB-FP, *MYB10* flesh and peel; RG-FP, ''Royal Gala'' flesh and peel.

immune system development (44). Previous studies suggest that other markers of the inflammation pathway, such as nuclear factor kappa-light-chain-enhancer of activated B cells (*Nfκb*) and Toll-like receptors, may be modulated by phytochemicals and the downstream inflammation genes such as tumor necrosis factor-α (*Tnf-α*), interleukin-6 *(Il6*), or chemokine (C-X-C motif) ligand 15 (*Cxcl15*) would be likely targets for change (2); however, significant changes in expression for these genes were not observed in our study. Our results concur with previous studies using anthocyanin extracts in which the expression of cyclooxygenase-2 (*Cox-2*), an enzyme linked with PGE_2 synthesis, is downregulated (45).

We also examined the dietary effects on the concentrations of the protein markers for immune modulation in plasma and observed significant decreases in LTB_4, PGE_2, and TNF-α. There are previous rodent studies that showed the inhibitory effect of polyphenols on induced intestinal inflammation models. This included a reduction in LTB_4 concentrations due to inhibition by various flavonoid subclasses, such as quercetin, flavones, and flavanone derivatives (2). A recent study of induced oxidative stress and inflammation in intestinal Caco-2/15 cells showed a strong reduction in markers of both oxidative stress and inflammation, including PGE_2 and TNF-α, when cells were preincubated with apple peel extracts (46). The observed decrease in plasma TNF-α concentrations in our research was not matched by the gene expression data, but this may be due to the tissue type selected for RNA isolation (jejunum).

Results from the bacterial assay indicated that there were changes in both the overall population and in the numbers of individual species. There were significant changes in the overall bacterial population, with mice fed any of the apple-supplemented diets (RG-F, RG-FP, MYB-F, and MYB-FP) showing greater bacterial numbers than mice fed the control diet. Changes in specific bacterial groups were varied, with a significant increase in *Lactobacillus* spp. at 21 d in mice fed the control diet compared with all other diets, but a significant increase in *Bifidobacterium* spp. in mice fed the RG-F and RG-FP diets compared with mice

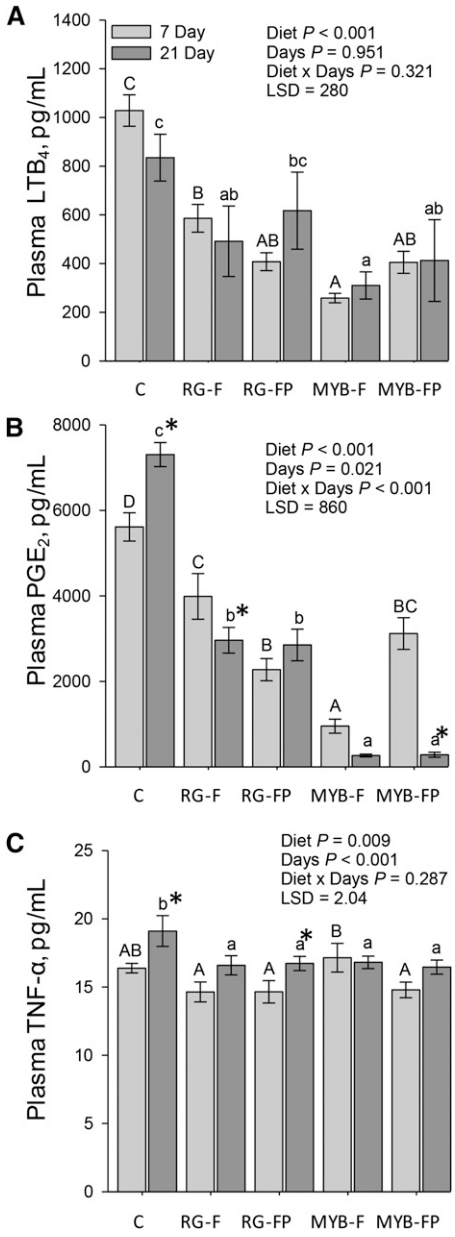

FIGURE 2 Concentrations of protein markers for immune modulation in mouse plasma collected at 7 or 21 d (trial 2). (*A*) LTB_4, (*B*) PGE_2, and (*C*) TNF-α. Labeled means at a time without a common letter differ, $P < 0.05$ (capital letters for 7 d, lowercase letters for 21 d). *Different from day 7, $P < 0.05$. Bars represent means ± SEMs, n = 10/group. C, control; LSD, least significant difference test; LTB_4, leukotriene B_4; MYB10, myeloblastosis transcription factor 10; MYB-F, *MYB10* apple fruit flesh; MYB-FP, *MYB10* apple fruit flesh and peel; RG-F, ''Royal Gala'' apple fruit flesh; RG-FP, ''Royal Gala'' apple fruit flesh and peel.

fed control, MYB-F, or MYB-FP diets. A more comprehensive study to assess a greater number of bacterial groups would help determine the effect of high-flavonoid diets on the microbiota. It has been shown in animal models that symbiotic bacteria, such as members of the *Bacteroides-Prevotella-Porphyromonas* group, can mediate immune response and protect against inflammation (47). There is much evidence that the composition of the human intestinal microbiota has an influence on health and the incidence of disease and that gut health is largely determined by the complex interaction between host and gastrointestinal microbiota (48).

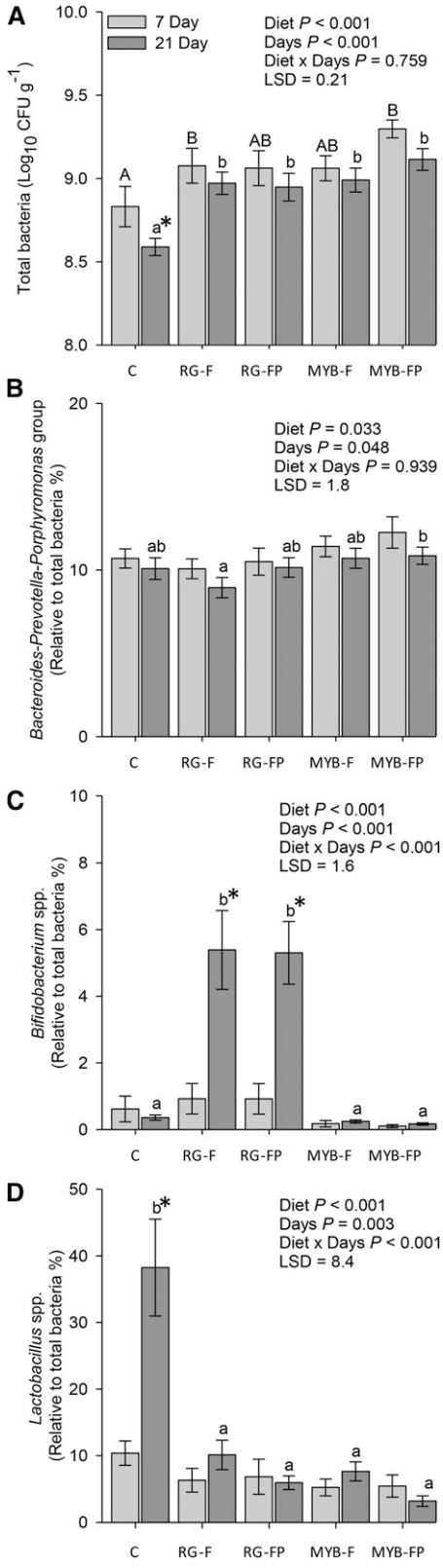

FIGURE 3 Genomic DNA of bacteria in the colonic contents of mice collected at 7 or 21 d (trial 2). (*A*) Total bacteria, (*B*) *Bacteroides-Prevotella-Porphyromonas* group, (*C*) *Bifidobacterium* spp., and (*D*) *Lactobacillus* spp. The total bacteria (*A*) mean values are $\log_{10}$ CFU/g of colonic content, whereas microbial populations in panels *B*, *C*, and *D* are expressed relative to total bacteria (%). Labeled means at a time without a common letter differ, $P < 0.05$ (capital letters for 7 d, lowercase letters for 21 d). *Different from day 7, $P < 0.05$. Bars represent means ± SEMs, $n = 10$/group. C, control; LSD, least significant difference test; MYB10, myeloblastosis transcription factor 10; MYB-F, *MYB10* apple fruit flesh; MYB-FP, *MYB10* apple fruit flesh and peel; RG-F, ''Royal Gala'' apple fruit flesh; RG-FP, ''Royal Gala'' apple fruit flesh and peel.

Whereas the diversity and complexity of both microbial flora and its interaction with the host cannot be underestimated (49), our results further suggest that a diet high in apple polyphenols can alter microbial populations and thus affect inflammation and general gastrointestinal health.

An additional observation from the mouse studies was that, whereas most of the physiologic variables we measured remained largely unaltered by the dietary treatments, there was a significant increase in fecal output. Fecal movement and output is associated with many aspects of the diet, particularly fiber (50). Dietary fiber intake differed across the treatments due to differences between the transgenic and control apples and because of differences in food intake (Table 2). We found that apple consumption had an effect on fecal output, and although fiber is the most likely cause of this there is evidence that a high-polyphenol diet can also increase fecal output more than that expected from increased fiber intake (51). A study in rats using anthocyanin-rich extracts, which are unlikely to have significantly altered fiber content, also showed an increase in total fecal output (52). In humans, a proposed explanation for fecal bulking is that high-antioxidant, polyphenol-rich foods can slow bacterial growth in the gut and so reduce fiber fermentation (53). The synergistic activity of polyphenols and fiber is an interesting target for further studies. Alternatively, the observed change in bacterial populations may contribute to the increased fecal output. Previous studies have shown a relation between a high-antioxidant diet and fecal output, which may be partly due to high amounts of polyphenols in the diet acting in a prebiotic manner, changing the bacterial populations (54).

In summary, this targeted approach to engineering fruit provides opportunities to test the relation between different classes of phytochemicals and the gut microbiota to understand the mechanisms behind the proposed health benefits. The findings here further demonstrate that apple consumption affects aspects of inflammatory pathways and the gut microbiota. Future studies might include human or human gut model confirmation of changes in inflammation marker concentrations and a more detailed analysis of modulation of particular groups of gut microbial populations.

Acknowledgments

We thank Tim Holmes for photography. R.V.E. conducted the research, wrote the manuscript, and has responsibility for the final content; S.M., H.S., A.B., T.K.M., J.Z., and G.P. conducted the research; D.H. performed statistical analysis; C.A.B. and W.A.L. planned the research, analyzed the data, and edited the manuscript; and J.P., H.J.S., A.C.A., and R.P.H. planned and designed the research. All authors read and approved the final manuscript.

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