

Original Research Article

Caloric Substitution of Diets with Apple Pomace was Determined to be Safe for Renal and Bone Health Using a Growing Rat Model

Abstract

Aims: to determine the safety of caloric substitution with 10% apple pomace substitution (g/kg) to a healthy or Western diet.

Study design: Growing (age 22-29 days) female Sprague-Dawley rats were randomly assigned (n=8 rats/group) to consume a purified standard rodent diet (AIN-93G), AIN-93G/10% g/kg apple pomace (AIN/AP), Western diet, or Western/10% g/kg apple pomace (Western/AP) diets for 8 weeks.

Results: Histological evaluation showed renal interstitial hypercellularity in rats fed AIN/AP, Western, and Western/AP diets. However, there was no effects on renal expression of oxidative stress and inflammatory genes or serum measures of kidney damage and function among diet groups. Apple pomace is also high in calcium which can affect calcium balance. Dietary calcium consumption was highest ($P < .001$) in rats consuming Western/AP. However, there was no significant differences in calcium absorption and retention among diet groups. Further, there was no evidence of renal calcification. There were also impact on femoral calcium and total mineral content, size, and strength.

Conclusions: Based on the results, apple pomace consumption was safe for renal and bone health, regardless of diet quality and should be considered for repurposing for human consumption.

Keywords: apple pomace, safety, minerals, Western diet, bone, kidney, sustainability

Comment [JFR Rev1]: Can not extrapolate for short animal study to humans.
Change conclusions

26 **1. Introduction**

27 Apple processing generates waste, consisting of skin, stem, seeds, and calyx,
28 collectively known as apple pomace. The environmental pollution and burden of waste disposal
29 costs to apple farmers and producers can be decreased by re-purposing apple pomace as a
30 product for human consumption [1-3]. However, among popular consumed fruits, apples had the
31 highest fructose content [1]. Muir, et al. [4] reported apples to have 10.5 g of fructose/serving
32 compared to 3.2 g/serving for bananas, 6.4 g/serving for blueberries, and 2.5 g/serving for
33 oranges. Further, apple pomace contains 44.7% fructose compared to 5.8-6.0% fructose in
34 whole apple [5]. This is a health concern because fructose overconsumption has been reported
35 to contribute to renal disease and to produce deleterious effects on bone [6,7]. Apple pomace
36 contains a higher mineral content than whole apples, particularly calcium which is required for
37 bone health [1,8]. However, overconsumption of calcium can increase nephrocalcinosis and
38 reduced kidney function [9,10]. In turn, renal dysfunction can lead to bone loss due to mineral
39 imbalance, resulting in increased risk of osteoporosis and other bone-mineral disorders [11].
40 Diets typical of Western countries are characterized by high-fat and high-sucrose.
41 Western diet consumption has been shown to increase the risk of chronic kidney disease by
42 inducing renal steatosis, inflammation, and oxidative stress. Western diet consumption has also
43 been reported to increase risk of kidney stones due to the high sugar content [12,13].
44 Additionally, consuming a Western diet can result in early onset of osteoporosis by promoting
45 mineral balance and inflammation leading to decreased bone mineral density [14,15].
46 Dietary advice suggests replacing calories in the diet with healthier food choices instead of
47 dietary supplementation with a purified isolated nutrient [16].

48 Previously, our laboratory reported caloric substitution of a Western diet with 10% g/kg
49 apple pomace attenuating features of NAFLD [17]. However, the effects of apple pomace on
50 renal and bone was not assessed in this study. To our knowledge no studies have evaluated the
51 safety of apple pomace consumption on renal and bone health. Therefore, the objectives of this

52 study were to determine the safety of apple pomace, due to its high fructose content and
53 increased calcium content, in growing rats consuming a “healthy” and Western diet. Female rats
54 were used due to their increased susceptibility to nephrocalcinosis, and growing rats because
55 kidney disease has been shown to have more severe bone effects in a pediatric population
56 [18,19]. We hypothesize apple pomace will not detriment kidney or bone health in growing
57 female rats consuming “healthy” or Western diets.

58

59 **2. Materials and Methods**

60 **2.1 Diets**

61 Locally sourced apple pomace was provided by Swilled Dog Hard Cider Company
62 (Franklin, WV). Apple pomace was freeze dried in equipment? Nutrient composition analysis of
63 apple pomace was performed by Medallion Laboratories (Minneapolis, MN). Apple pomace
64 contains 32.5% fructose compared to the published average of 5.9% fructose for whole apples.
65 Dietary calcium and phosphorus were determined by inductively coupled plasma mass
66 spectrometry (ICP) (model P400, Perkin Elmer, Shelton, CT). Freeze-dried apple pomace
67 contained 1.47 mg/g calcium and 1.97 mg/g phosphorous (Table 1) compared to respective
68 published values of 0.06 mg/g and 0.11 mg/g in whole apples [17].

69

70 **Table 1.** Composition of locally sourced freeze-dried apple pomace.

Macronutrients (%)	
Protein	3.56
Fat	1.3
Carbohydrates	68.1
Sugars (%)	
Fructose	32.5
Glucose	9.77
Sucrose	13.9
Maltose	<0.1
Lactose	<0.1
Dietary Fiber (%)	
Insoluble Dietary Fiber	22.2
Soluble Dietary Fiber	11.0

Polyphenols (g/kg)	0.029
Minerals (mg/g)	
Total Minerals	15.5
Calcium	1.47
Phosphorous	1.97
Calories (kcal/100 g)	387

71
72 The 'healthy' diet was the standard purified diet American Institute of Nutrition (AIN-93G)
73 for growing rats [20] while a Western diet consisting of 45% fat and 34% sucrose was used to
74 typify the high-fat, high-sugar diet consumed by Western countries [21,22]. AIN-93G and
75 Western diet were calorically substituted with 10% g/kg freeze-dried apple pomace. AIN diets
76 were adjusted to be isocaloric (3.7-3.8 kcal/g) and Western diets were adjusted to be isocaloric
77 (4.7 kcal/g). Table 2 shows diet formulation for macronutrients, sugars, total minerals, calcium,
78 and phosphorous. The complete ingredient composition of experimental diets is provided as a
79 Supplement. Diets were stored at -20°C until fed to animals.

80
81 **Table 2.** Ingredient composition of rodent diets substituted with apple pomace (10% g/kg) fed to
82 growing female rats.

	Diet Groups ¹			
	AIN	AIN/AP	Western	Western/AP
Key Ingredients ¹				
Apple pomace (g/kg)	0.0	100.0	0.0	100.0
Sucrose (g/kg)	100.0	43.9	340.0	283.9
Fructose (g/kg)	50	54.5	170	174.5
Total Minerals (mg/g)	22.1	24.2	26.4	28.0
Calcium (mg/g)	10.4	10.8	12.8	14.6
Phosphorus (mg/g)	7.2	7.5	7.6	7.5
Macronutrients (% kcal)				
Protein	18.8	18.9	14.8	14.8
Fat	17.2	17.3	44.6	44.8
Carbohydrate	63.9	63.7	40.6	40.4
Calories (kcal/g)	3.8	3.7	4.7	4.7

83 ¹ Abbreviations: AIN, the American Institute of Nutrition; AP, apple pomace; TBHQ, tert-
84 butylhydroquinone. ² Insoluble fiber is cellulose. ³ Soluble fiber is mainly pectin [1]. A complete
85 list of ingredients can be found in **Supplemental Table 1**.
86

87 **2.2 Animals**

Comment [JFR Rev2]: Ethical issues

Ethical committee review documents

Any ethical evaluation for the study/approval?

88 Weanling (age 22-29 days) female Sprague-Dawley rats (n=32) were purchased from
89 Harlan-Tekald (Indianapolis, IN). All animal procedures were approved by the Animal Care and
90 Use Committee at West Virginia University and conducted in accordance with the guidelines of
91 the National Research Council for the Care and Use of Laboratory Animals [23]. Rats were
92 individually housed and kept in a room at constant temperature of 21±2°C with a 12 h light/dark
93 cycle throughout the study duration. Following a 7-days acclimation, rats were randomly
94 assigned (n=8 rats/group) to four dietary groups consisting of: 1) AIN-93G, a standard purified
95 rodent diet, 2) AIN-93G with 10% weight (g/kg) substituted with apple pomace (AIN/AP), 3)
96 Western diet (45% fat, 33% sucrose by kcals), or 4) Western diet with 10% of weight (g/kg)
97 substituted with apple pomace (Western/AP). Rats were provided ad libitum access to their
98 assigned diets and deionized distilled water (ddH₂O) throughout the eight weeks study duration.
99 Food intake was measured and assigned diets replaced every other day while ddH₂O was
100 replaced weekly. At the end of the study, rats were fasted overnight then euthanized by carbon
101 dioxide inhalation. The kidney was excised, weighed, and then flash frozen in liquid nitrogen
102 and stored at -80°C until analyzed. Both femurs were removed, cleaned, and stored at -20°C.

103

104 2.3 Kidney histology

105 The left kidney was removed, weighed, flash frozen in liquid nitrogen, and stored at -
106 80°C until analysis. A center sagittal section was cut from each frozen tissue (n=6-8) and stored
107 in 10% neutral buffered formalin for 48 hours (fixation). After fixation, samples underwent a
108 dehydration protocol consisting of 10-15 minutes incubation in increasing ethanol
109 concentrations (50-to-100%) followed by two 20-minute incubations in xylenes. Following xylene
110 incubation, samples were incubated in molten paraffin wax for 20 minutes (infiltration) and
111 embedded into blocks. 5-7µm sections were cut and mounted on charged slides and sections
112 stained with hematoxylin and eosin. Histological evaluation included gross morphological
113 assessment which included the following: glomerular hypercellularity and matrix deposition,

114 interstitial hypercellularity, tubulointerstitial calcification, inflammation, and fibrosis. All slides
115 were analyzed using a Nikon Labophot 2 microscope (Nikon Instruments, New York, NY) at
116 magnification 10X by a trained investigator blinded to the identity of the groups. Images were
117 captured using a LCL-500-LHD digital camera with a PC Method Capture Imaging software
118 (Ludescop, Parkville, MD).

119

120 2.4 Renal RNA isolation and inflammatory gene expression

121 Total RNA was extracted from frozen kidney tissue (50 mg) using the Zymo Research
122 Direct-zol RNA Miniprep Plus Isolation Kit (Irvine, CA, catalog #R2071) according to the
123 manufacturer's instruction for total RNA isolation. Isolated RNA integrity was visualized on a
124 1.5% agarose gel and quantified by spectrophotometry (NanoDrop 100; Thermo Fisher
125 Scientific, Waltham, MA). Following DNase I treatment with TURBO DNA-free kit (Thermo
126 Fisher Scientific), total mRNA was amplified using the Superscript IV First-Strand Synthesis
127 System with oligo dT primers (Thermo Fisher Scientific).

128 Real-time quantitative polymerase chain reaction (RT-qPCR) consisted of 2.5 μ l of
129 SYBR Green Master Mix (Thermo Fisher Scientific), 1 μ L of cDNA (diluted 1:10), 1 μ L of
130 respective forward and reverse primers and 0.5 μ l of deionized distilled water for a total reaction
131 volume of 5 μ l. The reactions were performed in a 7500 ABI Real-Time PCR System (Thermo
132 Fisher Scientific). The thermal profile consisted of 50°C for 2 min, 95°C for 10 min then 40
133 cycles of 95°C for 15 sec and 60°C for 1 min. A melt curve analysis was applied at the end of
134 cycling. Primers that were designed for transcription factors, nuclear factor kappa-light chain
135 enhancer of B cells (NF κ B) and NADPH oxidase 4 (NOX4) and for inflammatory cytokines,
136 tumor necrosis factor-alpha (TNF- α), and interleukin-6 (IL-6) as well as for housekeeping genes,
137 β -actin and glyceraldehyde 2-phosphate dehydrogenase (GAPDH) using the Primer3 program
138 (Howard Hughes Medical Institute) and respective mRNA sequences obtained by NCBI.
139 Forward and reverse primers for genes of interest are listed below:

Gene	NCBI Gene ID	Forward Primer	Reverse Primer
NFκB	81736	5' TTATGGGCAGGAT GGACCTA 3'	5' CCTTTCAGGGCTTT GGTTTA 3'
TNFα	24835	5' CACAAGGCTGCTG AAGATGT 3'	5' GAGGGAAGGAAGG AAGGAAG 3'
IL-6	24498	5' TGGCTAAGGACC AAGACCAT 3'	5' TTGCCGAGTAGAC CTCATAGTG 3'
NOX4	85431	5' CCTCCATCAAGCC AAGATTC 3'	5' CTCCAGCCACACA CAGACTAAC 3'
β-actin	81822	5' TTGCTGACAGGAT GCACAAG 3'	5' CAGTGAGGCCAGG ATAGAGC 3'
GAPDH	24383	5' TCAAGAAGGTGGT GAAGCAG 3'	5' CCTCAGTGTAGCC CAGGATG 3'

140

141

142 2.5 Serum and urinary measures of renal function and health

143 Serum measures of kidney function included: blood urea nitrogen (BUN), creatinine, total
 144 protein, calcium, phosphorous, alanine aminotransferase (ALT). Additionally, serum glucose
 145 and amylase were measured. Values were determined enzymatically using a commercially
 146 available Vet-16 rotor and quantified by a Hemagen Analyst automated spectrophotometer
 147 (Hemagen Diagnostics Inc., Columbia, MD).

148 Serum and urine uric acid was determined by commercially available enzymatic assay (Cayman
 149 Chemical). Briefly, serum and urine samples were aliquoted onto a 96-well plate and incubated
 150 for 15 minutes. Reaction was initiated by adding 15 µl of uricase and horseradish peroxidase
 151 enzyme mixture, and read at an excitation of 535 nm and an emission of 590 nm using a BioTek
 152 Synergy H1 microplate reader (Winooski, VT). Inter-assay coefficient of variation was 32.1% for
 153 both serum and urine.

154

155 2.6 Calcium balance and retention

156 Rats were fasted overnight and euthanized by carbon dioxide inhalation. Blood was
157 collected by aorta puncture. Collected blood was centrifuged at 1,500 g for 10 min at 4°C to
158 obtain serum. Serum samples were stored at -80°C until analyzed. Serum calcium was
159 determined enzymatically using a commercially available Vet-16 rotor and quantified by a
160 Hemagen Analyst automated spectrophotometer.

161 During the initial and final weeks of the feeding study, rats were individually housed in
162 metabolic cages to collect urine and feces for 24 h. Initial and final day urine samples were
163 collected, centrifuged at 1,500 g for 10 min at 4°C, filtered through Whatman no. 1 paper, and
164 then diluted 1:10 in dd H₂O. Initial and final feces were collected and dried for 48 h, then ashed
165 in a muffle furnace (model CP18210, Thermolyne, Dubuque, IA) at 550°C for 24 h. Fecal
166 samples were then acidified in 70% nitric acid, neutralized in ddH₂O, filtered through Whatman
167 no. 1 paper, and further diluted (1:50 v/v) in ddH₂O. Ca content of feces and urine was
168 determined by ICP.

169 Measures of Ca excretion, absorption, and retention were performed according to
170 Maditz, et al. [24]. Briefly, urinary calcium excretion was calculated as urinary Ca
171 concentration/urine volume. Ca apparent absorption was calculated as $[(\text{Ca intake} - \text{fecal Ca excretion}) / (\text{Ca intake})] \times 100$. Calcium retention was calculated as $[(\text{Ca intake} - (\text{fecal Ca excretion} + \text{urinary Ca excretion}))]$.

174

175 2.7 Femur morphometry and mineralization

176 Following CO₂ inhalation, the left and right femur were collected, and then defleshed.
177 After no bilateral differences were determined using a t-test with significance set at $P < .05$ left
178 femurs were used for all analyses. Femoral morphometry measurements of depth, width, and
179 length were determined using a Vernier caliper (Bel-Art Products, Pequannock, NJ, USA).

180 Length was measured from the medial condyle to the greater trochanter. Femurs were weighed
181 using an analytical balance (Mettler Toledo, Columbus, OH, USA).

182 Total bone mineral was determined by ashing in a muffle furnace at 600°C for 24 hours,
183 then weighed. To measure specific minerals, ash was dissolved in 2 mL of 70% nitric acid.
184 Acidified samples were filtered through Whatman no. 1 paper and diluted (1:50 v/v) to volume
185 with ddH₂O and Ca determined using ICP.

186

187 2.8 Femur biomechanical strength

188 Femoral strength indices were assessed using a TA,XT2i Texture Analyzer (Texture
189 Technologies, Scarsdale, NY, USA) fitted with a three point bending apparatus. Femora were
190 placed on supports and force applied to the midshaft marked at a position halfway between the
191 greater trochanter and the distal medical condyle. Bone was broken by lowering a centrally
192 placed blade (1 mm width) at a constant crosshead speed (0.1 mm/s). The load cell was 250 kg.
193 The load-deflection data were collected by a PC interfaced with the TA,XT2i. Sample test
194 distance was set at 10 mm with a signal collection rate of 100 points per second. Peak force,
195 ultimate stiffness, ultimate bending stress and Young's modulus were calculated according to
196 Yuan and Kitts [25].

197

198 2.9 Statistics

199 Results are expressed as mean ± standard error of the mean (SEM). Gene expression
200 was determined as a function of mRNA abundance (A), where $A = 1 / (\text{gene of interest primer}$
201 $\text{efficiency} \times \Delta\text{CT (g.o.i.)} - (\text{average housekeeping primer efficiency} \times \Delta\text{CT (h.k.)})$, where the
202 product of efficiency and average of expression of β -actin was averaged with the product of
203 efficiency and average of expression of GAPDH to determine the overall expression of the two
204 housekeeping gene [17,26,27]. Gene expression data for each treatment group were log-
205 transformed prior to statistical analysis. One-way ANOVA was used to determine differences

206 among dietary groups. Post hoc multiple comparison tests were performed using Tukey's test
 207 with treatment differences considered significant at $P = .05$ and a tendency at $P = .08$. All
 208 statistical analyses were performed using JMP 12.2 statistical software package (SAS Institute,
 209 Cary, NC).

210

211 3. Results and Discussion

212 Rats are susceptible to renal disease and diets high in fructose and high in calcium have
 213 been shown to be detrimental to renal health, and high-fructose diets can detriment bone health
 214 [7,28,29]. In the current study, no differences were observed in body or organ weights (Table 1),
 215 but histological analysis of the kidneys showed no evidence of fibrosis, glomerular
 216 hypercellularity, glomerular matrix deposition, or amyloidosis.

217

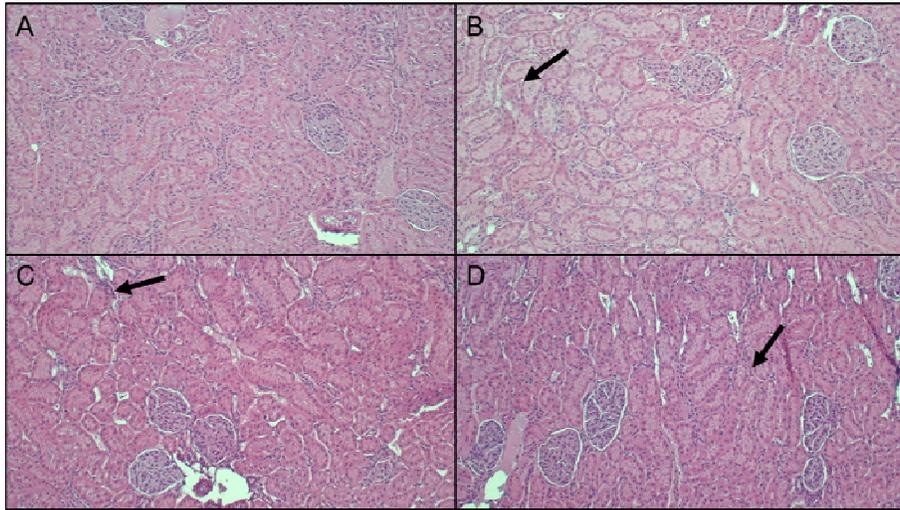
218 **Table 3.** Weekly caloric and macronutrient intake, weekly body weight gain, and kidney and
 219 bone weights of growing female rats consuming different diets substituted with apple pomace
 220 (10% g/kg) for 8 weeks.

Measurements	Treatments ¹				P-Value
	AIN	AIN/AP	Western	Western/AP	
Caloric intake (kcal/week)	368 ± 11 ^b	345 ± 8 ^b	422 ± 9 ^a	430 ± 17 ^a	<.001
Initial bwt (g)	95 ± 3	92 ± 3	95 ± 3	95 ± 3	0.80
Final bwt (g)	216 ± 4	216 ± 8	229 ± 5	234 ± 5	0.08
Average weekly bwt gain (g)	16 ± 3	16 ± 3	18 ± 3	18 ± 3	0.94
Average mineral intake (mg/d)	304.0 ± 9.3 ^b	318.8 ± 7.3 ^b	368.9 ± 7.8 ^a	374.7 ± 15.0 ^a	<.001
Right kidney weight (g)	0.69 ± 0.02	0.68 ± 0.02	0.71 ± 0.02	0.73 ± 0.02	0.28
Left kidney weight (g)	0.69 ± 0.02	0.67 ± 0.02	0.74 ± 0.03	0.74 ± 0.02	0.07
Relative right kidney weight (mg/g)	0.32 ± 0.01	0.31 ± 0.01	0.32 ± 0.01	0.31 ± 0.01	0.86
Relative left kidney weight (mg/g)	0.31 ± 0.01	0.31 ± 0.01	0.31 ± 0.01	0.32 ± 0.00	0.70
Left kidney ash (mg/g)	9.86 ± 0.56	10.07 ± 0.54	9.14 ± 1.09	10.34 ± 0.67	0.71

221 ¹Values expressed as mean ± SEM ($n = 6-8$ rats/group). Different superscript letters a and
 222 b within the same row. Indicate significant difference at $P < .05$ by one-way ANOVA
 223 followed by Tukey's test. Abbreviations: Bwt, body weight; CHO, carbohydrate.

224

225 However, rats consuming Western diet and diets containing apple pomace showed renal
 226 interstitial hypercellularity (Figure 1), suggesting renal inflammation.



227

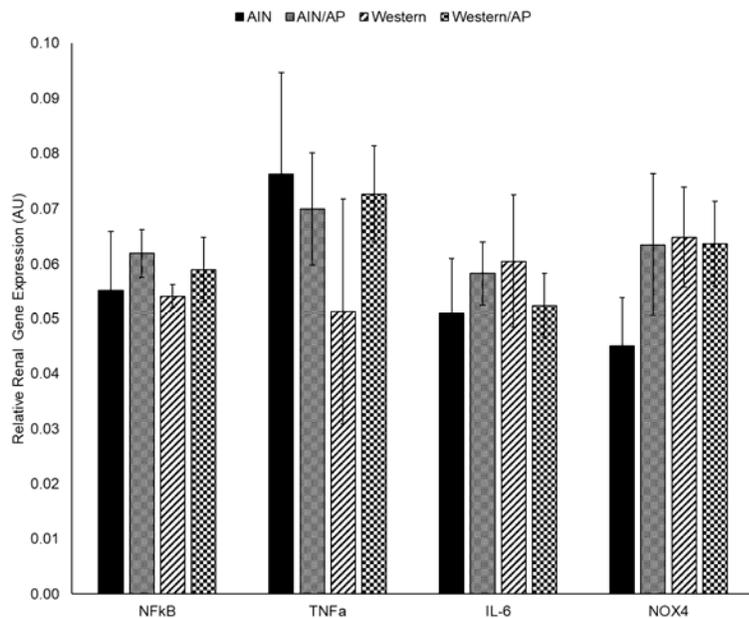
Histological changes	AIN	AIN/AP	Western	Western/AP
Inflammation	0	0	0	0
Fibrosis	0	0	0	0
Glomerular hypercellularity	0	0	0	0
Glomerular matrix deposition	0	0	0	0
Amyloidosis	0	0	0	0
Interstitial Calcification	0	0	0	0
Interstitial hypercellularity	0	2	1	2

228 **Figure 1.** Representative histological staining images of the kidney of growing female rats
 229 consuming (A) AIN, (B) AIN/AP, (C) Western, or (D) Western/AP following 8 weeks of feeding.

230

231 To further investigate, gene expression of inflammatory transcription factor, NFκB and
 232 proinflammatory cytokines, TNF-α and IL-6 as well as NOX4, a highly expressed enzyme
 233 regulating generation of reactive oxygen species, were measured in the kidneys. No significant

234 differences were found in renal expression of any of the genes of interest among diet groups
235 (Figure 2).



236
237 **Figure 2.** Renal expression of genes involved in inflammation and oxidative stress in rats
238 consuming different diets substituted with 10% apple pomace (g/kg). Values expressed as
239 mean \pm SEM (n=5-7 animals/group). Different superscript letters a and b within the same figure
240 indicates significant difference at $P < .05$ by one-way ANOVA followed by Tukey's test.

241
242 Serum creatinine, BUN, ALT, and total protein also showed no significant differences among
243 diet groups, collectively indicating absence of inflammation and oxidative stress (Table 4).

244 Increased fructose consumption and elevated uric acid may play a role in renal
245 inflammation [30-32]. Elevations in uric acid levels have been shown to change the fundamental
246 architecture of renal histology and has been implicated in acute and chronic renal failure [33].

247 The current study results showed no significant difference in serum or urine uric acid among diet
 248 groups (Table 4).

249 **Table 4.** Effect of consumption of different diets substituted with apple pomace (10% g/kg) by
 250 growing female rats on serum and urine measurements of liver function enzymes, and uric acid
 251 following 8 weeks of feeding.

Measurements	Treatments ¹				P-Value
	AIN	AIN/AP	Western	Western/AP	
Serum Creatinine (U/L)	1.46 ± 0.08	1.45 ± 0.11	1.38 ± 0.09	1.43 ± 0.04	0.90
Serum BUN (mg/dl)	17.84 ± 1.59	19.63 ± 1.41	20.25 ± 2.32	16.00 ± 0.94	0.27
Serum ALT (U/L)	107.63 ± 19.59	118.71 ± 43.60	94.5 ± 12.58	133.5 ± 30.59	0.78
Serum Total Protein (g/dl)	3.9 ± 0.25	4.62 ± 0.34	4.08 ± 0.67	4.19 ± 0.34	0.79
Serum Phosphorous (mg/dl)	14.18 ± 0.54	13.46 ± 1.72	15.68 ± 0.53	13.09 ± 1.02	0.35
Serum Ca (mg/dl)	9.56 ± 0.80	11.10 ± 1.09	11.49 ± 0.54	10.51 ± 1.00	0.48
Serum Uric Acid (µM)	7.24 ± 0.31	6.27 ± 1.61	7.19 ± 0.86	7.57 ± 1.25	0.86
Urine Uric Acid (µM)	5.94 ± 2.26	10.35 ± 2.11	10.40 ± 1.12	6.79 ± 1.41	0.23

252 ¹Values expressed as mean ± SEM (n=4-8 animals/group). Different superscript letters a and b
 253 within the same figure indicates significant difference at $P < .05$ by one-way ANOVA followed by
 254 Tukey's test. Abbreviations: ALT, alanine aminotransferase; BUN, blood urea nitrogen.
 255

256 Interstitial hypercellularity was observed in 13-29% of animals, but there were no
 257 significant differences in oxidative stress and inflammatory gene expression or serum and urine
 258 measurements of renal dysfunction and injury were observed among diet groups. These results
 259 indicate renal interstitial hypercellularity was unlikely to be of biological significance. Collectively,
 260 the results indicate the fructose content of apple pomace was not a risk for renal injury and
 261 development of chronic kidney disease in either 'healthy' or Western diet.

262 In our study, Western diets were high in calcium with Western/AP diet having the highest
 263 calcium content (Table 2). Differences in calcium content in diets can have significant effects on
 264 calcium excretion, absorption, and retention [34]. Increased calcium excretion can induce
 265 nephrocalcinosis [35]. Initial urinary and fecal calcium excretion, calcium retention, and calcium
 266 absorption showed no significant differences among diet groups (Table 5). At final week, no
 267 differences were observed in rats urinary calcium excretion among all groups, but an increase
 268 ($P = .04$) in fecal calcium excretion by rats consuming a Western/AP compared to AIN was
 269 observed. This was also likely due to a combination of the high insoluble dietary fiber content in

270 apple pomace possibly binding to calcium and the increased dietary calcium in Western/AP
 271 diets. This also explains the lack of change in apparent calcium absorption among all diet
 272 groups. No differences were observed in calcium retention among all diet groups.

273 **Table 5.** Calcium balance of rats fed different diets substituted with 10% (g/kg) apple pomace.

Calcium Balance	Treatments ¹				P-Value
	AIN	AIN/AP	Western	Western/AP	
Ca Intake (mg/d)	135.6 ± 4.2 ^c	140.1 ± 3.2 ^c	162.4 ± 3.5 ^b	184.9 ± 7.4 ^a	<0.001
Initial					
Urine Ca excretion (mg/dl)	0.16 ± 0.04	0.19 ± 0.04	0.17 ± 0.04	0.18 ± 0.04	0.96
Fecal Ca excretion (mg/d)	25.9 ± 3.6	22.9 ± 3.5	31.3 ± 3.7	34.7 ± 2.7	0.12
Ca retention (mg/d)	89.3 ± 9.4	94.9 ± 5.9	96.4 ± 5.8	109.8 ± 6.2	0.32
Ca absorption (%)	62.5 ± 4.6	68.0 ± 4.7	61.4 ± 4.2	63.3 ± 3.0	0.70
Final					
Urine Ca excretion (mg/ml)	0.15 ± 0.02	0.16 ± 0.04	0.16 ± 0.04	0.10 ± 0.01	0.25
Fecal Ca excretion (mg/d)	60.9 ± 2.9 ^b	79.4 ± 11.6 ^{ab}	81.2 ± 3.9 ^{ab}	99.3 ± 7.1 ^a	0.04
Ca retention (mg/d)	77.7 ± 5.3	66.7 ± 5.3	80.8 ± 5.0	78.9 ± 5.3	0.25
Ca absorption (%)	54.2 ± 4.1	41.8 ± 11.8	49.7 ± 3.2	46.3 ± 5.3	0.65

274 ¹Values expressed as mean ± SEM (n=4-8 animals/group). Different superscript letters a and b
 275 within the same figure indicates significant difference at *P* < .05 by one-way ANOVA followed by
 276 Tukey's test.
 277

278 Further, renal histological evaluation showed no evidence of calcium deposition in any of the
 279 diet groups, further indicating apple pomace consumption to be safe (Figure 2).

280 While Western diet (high fat and high sugar) and fructose consumption have also been
 281 reported to detriment bone health, whole apples have been shown to favorably alter bone
 282 health, through increased bone mineral density, decreased calcium loss, and decreased
 283 inflammation due to antioxidants present in apples [36-39]. Apple pomace has been shown to
 284 contain more calcium than apples [5]. Increasing dietary calcium has been shown to prevent
 285 osteoporosis and to lower the risk of bone fractures [40,41]. Further, children with adequate
 286 calcium consumption had increased bone mineral density [42,43]. The present study showed no
 287 significant differences in femoral calcium content among diet groups. Additionally, there were no
 288 significant differences in femur size or bone strength measurements including: peak force,
 289 ultimate stiffness, ultimate bending stress, and Young's modulus among diet groups (Table 6).

290 **Table 6.** Femoral morphometry and strength measurements of rats fed different diets substituted with 10% (g/kg) apple pomace.

Measurement	Treatments ¹				P-value
	AIN	AIN/AP	Western	Western/AP	
Femur morphometry					
Length (mm)	29.71 ± 0.53	29.09 ± 0.78	30.52 ± 0.56	29.36 ± 0.78	0.09
Medial lateral width (mm)	2.98 ± 0.04	3.12 ± 0.12	3.06 ± 0.08	3.15 ± 0.10	0.13
Depth (mm)	2.78 ± 0.07	2.73 ± 0.12	2.60 ± 0.09	3.06 ± 0.17	0.43
Wet wt (g)	0.77 ± 0.02	0.74 ± .05	0.73 ± 0.03	0.74 ± 0.04	0.89
Dry wt (g)	0.48 ± 0.01	0.46 ± 0.03	0.45 ± 0.02	0.47 ± 0.02	0.77
Femur mineralization					
Ash (mg/g of bone)	407.92 ± 11.42	407.75 ± 9.26	399.66 ± 7.40	396.94 ± 6.46	0.80
Calcium (mg/g of bone)	37.99 ± 0.78	39.09 ± 4.41	40.09 ± 2.26	38.28 ± 2.08	0.75
Femur biomechanical strength					
Peak force (N)	1.74 ± 0.18	1.99 ± 0.25	1.55 ± 0.11	1.23 ± 0.23	0.07
Ultimate stiffness (N/S)	382.03 ± 16.28	399.49 ± 27.07	397.55 ± 38.73	347.15 ± 14.01	0.60
Ultimate bending stress (N/S)	42.32 ± 1.57	38.21 ± 2.19	40.12 ± 3.46	42.19 ± 2.59	0.48
Young's Modulus (N/mm ²)	1604.92 ± 76.18	1484.85 ± 284.92	1549.57 ± 90.13	1275.92 ± 200.17	0.75

291 ¹Values expressed as mean ± SEM (n=6-8 animals/group). Different superscript letters a and b within the same figure indicates
 292 significant difference at $P \leq .05$ by one-way ANOVA followed by Tukey's test.
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298 Another concern is rats consuming Western/AP diets had significantly increased gonadal
299 fat pad weights than rats consuming AIN diets (Table 3). Obesity and diabetes have been
300 reported to be causal factors in diet-induced kidney disease progression [6,44]. In our study,
301 despite higher adiposity in rats fed Western/AP there were no significant differences in fasting
302 serum glucose or amylase among diet groups (data now shown). Our study provides evidence
303 that high fructose and high calcium content of apple pomace was not sufficient to effect renal or
304 bone health in rats, regardless of diet. Studies on apple pomace have reported numerous health
305 benefits including decreases in body weight, as well improvements in serum lipid, insulin,
306 glucose, antioxidant status, and digestion [45-51]. Yet, few studies have evaluated the safety of
307 apple pomace consumption. Devrajan, et al. [52] fed rats unfermented or fermented apple
308 pomace for 2 weeks showed a nonsignificant increase serum BUN, but found no indication of
309 kidney damage [53]. Additionally, histology was not used to evaluate kidney health.

310

311 4. Conclusions

312 Caloric substitution of a healthy or Western diet with 10% apple pomace had no impact on renal
313 or bone health in growing female rodents. Based on our results apple pomace is safe for
314 consumption, despite its high fructose content combined with a high calcium content, regardless
315 of diet quality. The study provides evidence for apple pomace, a “waste” byproduct of apple
316 processing has a favorable nutritional profile and is safe and therefore has potential to be
317 repurposed as a sustainable food source for human consumption.

318

319 Competing Interests

320 Authors have declared that no competing interests exist.

Comment [JFR Rev3]: Not a realistic dosage to extrapolate to human consumption. Just high dosage for the animal study, which can be understood with the objective of verifying innocuousness, but not enough to conclude that this is safe for human consumption.

Comment [JFR Rev4]: Safety profile/Microbiological load? Process effects on bioactives? Presence of undesirable compounds because of the waste generation?

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Comment [JFR Rev5]: Reduce the number of cited references and update the literature review. The majority of the references are more than 10 years old.

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474

475 **Supplemental Material**
 476 **Supplementary Table 1.** Composition of rodent diets substituted with apple pomace
 477 (10% g/kg) fed to growing female rats.

	Diet Groups[*]			
	AIN	AIN/AP	Western	Western/AP
Ingredients (g/kg)[*]				
Apple pomace	0.0	100.0	0.0	100.0
Corn Starch	397.486	392.086	63.36	57.96
Maltodextrin	132.0	132.0	60.0	60.0
Sucrose	100.0	43.9	340.0	283.9
Fructose	50	54.45	170	174.45
Total Dietary Fiber	50.0	50.0	50.0	50.0
Insoluble Fiber †	50.0	39.0	50.0	39.0
Soluble Fiber ‡	0.0	11.0	0.0	11.0
Anhydrous Milkfat	0.0	0.0	210.0	210.0
Soybean Oil	70.0	68.7	20.0	18.7
Casein	200.0	196.0	195.0	191.0
L-Cystine	3.0	3.0	3.0	3.0
Vitamin Mix	10.0	10.0	12.5	12.5
Mineral Mix	35.0	35.0	43.0	43.0
Total Minerals	22.1	24.2	26.4	28.0
Calcium	10.4	10.8	12.8	14.6
Phosphorous	7.2	7.5	7.6	7.5
Choline Bitartrate	2.5	2.5	3.1	3.1
TBHQ, antioxidant	0.014	0.014	0.04	0.04
Polyphenols	0.0015	0.0029	0.0008	0.0032
Macronutrients (% kcal)				
Protein	18.8	18.9	14.8	14.8
Fat	17.2	17.3	44.6	44.8
Carbohydrate	63.9	63.7	40.6	40.4
Calories (kcal/g)	3.8	3.7	4.7	4.7

478 ^v Abbreviations: AIN, the American Institute of Nutrition; AP, apple pomace; TBHQ, tert-
 479 butylhydroquinone. † Insoluble fiber is cellulose. ‡ Soluble fiber is mainly pectin [1].
 480