HEALTH SCIENCE

Effects of Citric Acid in Lemon Water on the Human Intestinal Microbiota: A Comparison between Pre-Meal Intake of Water and Lemon Water

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ABSTRACT

Objective: **The consumption of citric acid, contained in lemon juice, may effectively improve indigestion and maintain an optimal intestinal environment because it promotes gastric acid function.**

Materials and Methods: **In the present study, we recruited healthy male university students and randomized them into two groups that drank 300 cc of water or lemon water prior to meals for 4 weeks. Stool samples were collected before and after the test period, and the intestinal microbiota was analyzed using next generation sequencing. Bacterial species were identified based on taxonomic classification and binomial nomenclature, and were categorized at the genus level. Bacterial species with a relative abundance rate higher than 0.01% in the water- and lemon water-drinking groups were identified, and changes in the amounts of these bacteria were examined.**

Results and Conclusion: **The results obtained revealed a significant change in the amount of Bilophila, and slight changes in the amounts of Adlercreutzia, Haemophilus, Megamonas, and Roseburia. Therefore, the pre-meal intake of lemon water appeared to promoted peristalsis and digestion, which changed the intestinal microbiota.**

KEY WORDS

lemon water, water, bacterial species, healthy male

INTRODUCTION

Recent studies indicated that the intestinal microbiota plays an important role in the health of the host. An imbalance in the intestinal microbiota results in digestive disorders and abnormal bowel movements, such as diarrhea and constipation, as well as a number of diseases including immunological abnormalities, such as allergy and rheumatoid arthritis, lifestyle diseases, such as diabetes and obesity, and depression[1-3)].

Indigestion due to aging is considered to contribute to the composition and stability of the intestinal microbiota. Elderly individuals were found to develop gastric mucosa atrophy due to *Helicobacter pylori* infection[4)]. Goldschmiedt *et al*. compared gastric acid secretion in middle-aged and young individuals and found decreases as a result of *H. pylori* infection[5)]. Decreases in gastric acid secretion have been shown to reduce resistance to pathogens and the absorption of vitamins and iron[6)]. Reductions in bactericidal effects in the stomach lead to an imbalance in the intestinal environment, which is considered to lower overall resistance to bacteria. Citric acid concentrations differ from those of gastric acid, and citric acid has been shown to suppress the enzymatic activity of urease, which is required for the survival of *H. pylori*[7)]. Since *H. pylori* causes inflammation of the gastric mucosa, citric acid may improve the overall intestinal environment by suppressing urease and subsequently *H. pylori*. Ashida *et al*. demonstrated that the consumption of citric acid increased peristalsis[8)], while Chen *et al*. reported similar findings for water[9)]. Collectively, these findings suggest that citric acid and water improve the gastric environment and promote peristalsis, resulting in overall improvements in the intestinal environment. However, no studies to date have examined the effects of lemon water, which contains citric acid, or compared the effects of water and lemon water.

Therefore, in the present pilot study, we compared the intestinal microbiota of healthy male individuals who consumed water or lemon water to investigate the effects of lemon water on the intestinal microbiota.

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Table 1: Comparison of bacterial species in the intestinal microbiota between water- and lemon water-drinking groups (only those with an abundance > 0.01% are included)

Bacterial species (genus) (%)	Water-drinking group			Lemon water-drinking group			p-value[a]
	pre	post	difference	pre	post	difference	
Eubacterium	0.256 ± 0.341	0.177 ± 0.187	-0.079 ± 0.181	0.312 ± 0.382	0.143 ± 0.168	-0.169 ± 0.308	0.588[b]
Prevotella	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.645 ± 1.443	2.260 ± 4.441	1.615 ± 3.012	0.136
Ruminococcus	3.806 ± 3.770	1.331 ± 0.685	-2.476 ± 3.262	1.532 ± 0.968	1.067 ± 0.874	-0.465 ± 1.413	0.917
Abiotrophia	0.004 ± 0.008	0.001 ± 0.001	-0.003 ± 0.007	0.000 ± 0.000	0.000 ± 0.001	0.000 ± 0.001	0.906
Acidaminococcus	0.550 ± 1.188	0.162 ± 0.235	-0.388 ± 0.995	0.019 ± 0.042	0.018 ± 0.036	-0.001 ± 0.008	1.000
Actinobacillus	0.001 ± 0.003	0.009 ± 0.015	0.008 ± 0.016	0.015 ± 0.033	0.000 ± 0.000	-0.015 ± 0.033	0.290
Actinomyces	0.017 ± 0.019	0.006 ± 0.006	-0.012 ± 0.020	0.012 ± 0.008	0.005 ± 0.006	-0.007 ± 0.012	0.654[b]
Adlercreutzia	0.035 ± 0.068	0.007 ± 0.010	-0.028 ± 0.061	0.000 ± 0.000	0.000 ± 0.001	0.000 ± 0.001	0.095
Aggregatibacter	0.003 ± 0.005	0.006 ± 0.011	0.004 ± 0.013	0.007 ± 0.013	0.000 ± 0.001	-0.007 ± 0.014	0.113
Akkermansia	0.000 ± 0.000	0.016 ± 0.036	0.016 ± 0.036	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.317
Anaerostipes	0.135 ± 0.139	0.062 ± 0.082	-0.073 ± 0.120	0.023 ± 0.027	0.062 ± 0.071	0.039 ± 0.061	0.112[b]
Anaerotruncus	0.004 ± 0.009	0.005 ± 0.008	0.001 ± 0.002	0.001 ± 0.001	0.006 ± 0.007	0.005 ± 0.007	0.577
Atopobium	0.003 ± 0.007	0.004 ± 0.009	0.001 ± 0.012	0.002 ± 0.001	0.001 ± 0.002	0.000 ± 0.002	0.788[b]
Bacillus	0.046 ± 0.103	0.000 ± 0.000	-0.046 ± 0.103	0.000 ± 0.000	0.008 ± 0.017	0.008 ± 0.017	0.180
Bacteroides	30.7 ± 17.3	44.3 ± 7.68	13.6 ± 13.3	33.1 ± 14.5	48.0 ± 12.6	14.9 ± 9.4	0.87[b]
Bifidobacterium	5.216 ± 6.661	1.200 ± 0.477	-4.016 ± 6.404	2.553 ± 3.099	1.302 ± 0.949	-1.252 ± 2.404	0.620
Bilophila	0.119 ± 0.182	0.636 ± 0.880	0.517 ± 0.919	0.366 ± 0.475	0.255 ± 0.344	-0.111 ± 0.133	0.036[c]
Blautia	3.377 ± 0.774	4.535 ± 2.757	1.158 ± 3.315	2.545 ± 1.877	1.945 ± 1.899	-0.600 ± 1.180	0.296[b]
Bulleidia	0.014 ± 0.027	0.004 ± 0.006	-0.010 ± 0.028	0.003 ± 0.002	0.001 ± 0.002	-0.002 ± 0.003	0.759
Butyricimonas	0.233 ± 0.263	0.094 ± 0.116	-0.140 ± 0.315	0.037 ± 0.075	0.047 ± 0.061	0.011 ± 0.044	0.348[b]
Campylobacter	0.001 ± 0.002	0.010 ± 0.012	0.009 ± 0.011	0.003 ± 0.004	0.053 ± 0.073	0.050 ± 0.075	0.675
cc_115	0.004 ± 0.005	0.003 ± 0.004	0.000 ± 0.003	0.005 ± 0.007	0.003 ± 0.007	-0.001 ± 0.005	0.911
Christensenella	0.003 ± 0.006	0.000 ± 0.001	-0.002 ± 0.006	0.002 ± 0.004	0.002 ± 0.003	-0.001 ± 0.002	0.738
Clostridium	0.409 ± 0.274	0.207 ± 0.241	-0.202 ± 0.304	0.037 ± 0.058	0.070 ± 0.071	0.033 ± 0.038	0.159[b]
Collinsella	1.212 ± 1.739	0.361 ± 0.245	-0.851 ± 1.589	0.547 ± 0.486	0.191 ± 0.194	-0.355 ± 0.376	0.834
Coprobacillus	0.016 ± 0.021	0.017 ± 0.019	0.001 ± 0.006	0.063 ± 0.105	0.030 ± 0.041	-0.033 ± 0.066	0.251
Coprococcus	0.707 ± 0.693	0.877 ± 0.896	0.170 ± 0.432	0.169 ± 0.124	0.193 ± 0.162	0.023 ± 0.122	0.5b)
Dialister	0.003 ± 0.003	0.004 ± 0.008	0.001 ± 0.005	0.522 ± 1.165	0.000 ± 0.001	-0.521 ± 1.165	0.754
Dorea	0.728 ± 0.582	0.525 ± 0.413	-0.203 ± 0.368	0.571 ± 0.330	0.201 ± 0.065	-0.370 ± 0.335	0.117
Eggerthella	0.065 ± 0.103	0.015 ± 0.014	-0.050 ± 0.100	0.013 ± 0.012	0.041 ± 0.059	0.028 ± 0.062	0.215
Faecalibacterium	1.275 ± 0.422	0.911 ± 0.492	-0.365 ± 0.620	0.600 ± 0.691	0.506 ± 0.418	-0.095 ± 0.388	0.433[b]
Fusobacterium	0.157 ± 0.321	1.011 ± 2.158	0.854 ± 2.232	6.006 ± 10.266	7.638 ± 7.531	1.633 ± 12.048	0.215
Granulicatella	0.006 ± 0.011	0.005 ± 0.008	-0.001 ± 0.016	0.002 ± 0.003	0.001 ± 0.001	-0.001 ± 0.003	0.941[b]
Haemophilus	0.613 ± 1.330	0.193 ± 0.224	-0.419 ± 1.446	0.761 ± 1.393	0.035 ± 0.067	-0.725 ± 1.414	0.076
Holdemania	0.177 ± 0.382	0.014 ± 0.030	-0.164 ± 0.352	0.019 ± 0.038	0.020 ± 0.033	0.001 ± 0.007	0.281
Klebsiella	0.012 ± 0.021	0.001 ± 0.001	-0.011 ± 0.021	0.012 ± 0.026	0.000 ± 0.000	-0.012 ± 0.025	0.751
Lachnospira	1.601 ± 1.511	1.235 ± 1.242	-0.366 ± 0.898	0.881 ± 1.427	1.729 ± 3.340	0.849 ± 4.022	0.528[b]
Lactobacillus	0.003 ± 0.007	0.000 ± 0.000	-0.003 ± 0.007	0.020 ± 0.045	0.019 ± 0.031	-0.001 ± 0.016	0.316
Lactococcus	0.002 ± 0.002	0.000 ± 0.001	-0.002 ± 0.002	0.013 ± 0.029	0.000 ± 0.001	-0.013 ± 0.029	0.451
Megamonas	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	4.347 ± 9.720	0.470 ± 0.772	-3.877 ± 8.987	0.095
Megasphaera	0.002 ± 0.004	0.008 ± 0.016	0.006 ± 0.011	1.181 ± 1.745	1.492 ± 2.245	0.311 ± 1.657	0.666
Odoribacter	0.235 ± 0.273	0.414 ± 0.443	0.179 ± 0.209	0.060 ± 0.112	0.128 ± 0.205	0.068 ± 0.248	0.466[b]
Oribacterium	0.002 ± 0.002	0.003 ± 0.007	0.002 ± 0.007	0.000 ± 0.001	0.000 ± 0.001	0.000 ± 0.001	0.590
Oscillospira	1.126 ± 0.709	0.673 ± 0.442	-0.454 ± 0.774	0.897 ± 0.872	0.800 ± 0.683	-0.096 ± 1.022	0.551[b]
Parabacteroides	2.299 ± 1.637	2.864 ± 1.070	0.566 ± 2.085	2.649 ± 2.876	4.622 ± 3.808	1.974 ± 4.843	0.567[b]
Paraprevotella	0.001 ± 0.002	0.086 ± 0.191	0.085 ± 0.189	0.125 ± 0.223	0.288 ± 0.366	0.163 ± 0.264	0.435
ph2	0.004 ± 0.007	0.000 ± 0.000	-0.004 ± 0.007	0.000 ± 0.001	0.000 ± 0.000	0.000 ± 0.001	0.368
Phascolarctobacterium	1.354 ± 1.172	1.504 ± 1.289	0.150 ± 0.399	1.068 ± 2.167	0.529 ± 0.783	-0.539 ± 1.394	0.530
Prevotella	0.000 ± 0.001	0.001 ± 0.001	0.001 ± 0.001	7.608 ± 10.935	1.353 ± 2.025	-6.254 ± 8.924	0.117
Providencia	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.029 ± 0.064	0.000 ± 0.000	-0.029 ± 0.064	0.136
Roseburia	0.081 ± 0.111	0.316 ± 0.379	0.235 ± 0.434	2.268 ± 2.268	0.506 ± 0.645	-1.761 ± 1.785	0.065[b]
Rothia	0.002 ± 0.004	0.001 ± 0.001	-0.001 ± 0.004	0.002 ± 0.003	0.000 ± 0.000	-0.002 ± 0.003	0.481[b]
Ruminococcus	0.855 ± 1.623	0.138 ± 0.184	-0.717 ± 1.654	0.162 ± 0.246	0.098 ± 0.076	-0.065 ± 0.206	0.347
Slackia	0.003 ± 0.007	0.001 ± 0.001	-0.003 ± 0.006	0.009 ± 0.020	0.004 ± 0.008	-0.005 ± 0.012	0.700
Streptococcus	0.174 ± 0.225	0.138 ± 0.158	-0.036 ± 0.149	0.163 ± 0.092	0.028 ± 0.021	-0.135 ± 0.091	0.239[b]
Succiniclasticum	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.997 ± 2.229	1.825 ± 4.081	0.828 ± 1.852	0.881
Sutterella	1.343 ± 1.367	3.023 ± 3.734	1.680 ± 3.740	2.057 ± 1.374	3.064 ± 1.200	1.007 ± 1.450	0.717[b]
Turicibacter	0.005 ± 0.006	0.027 ± 0.031	0.022 ± 0.033	0.042 ± 0.060	0.011 ± 0.013	-0.031 ± 0.065	0.144[b]
Veillonella	0.690 ± 1.042	1.220 ± 1.890	0.531 ± 0.879	1.535 ± 2.214	1.074 ± 1.219	-0.461 ± 1.089	0.175

a) The Mann-Whitney U test

b) An unpaired t-test

c) p = 0.144 in the water-drinking group (pre vs post) Wilcoxon's rank sum test, p = 0.134 in the lemon water-drinking group (pre vs post) Paired t-test, p = 0.341 at pre (Water vs Lemon) Mann-Whitney U test, p = 0.393 at post (Water vs Lemon) Unpaired t-test

MATERIALS AND METHODS

Subjects

Ten male students at H University were included in the present study (age: 22.1 ± 1.1 years, height: 173.1 ± 4.6 cm, weight: 63.0 ± 6.5 kg). Subjects were excluded if they 1) were taking warfarin, 2) had a serious medical history, 3) had gastric hyperacidity, 4) had other conditions that were considered to be unsuitable for the present study, or 5) wished to withdraw. The purpose of the present study was explained to all subjects, and written consent was obtained prior to its initiation (Research Ethics Board approval number: 18MH046).

Methods

Study Schedule

Subjects were randomized to a water-drinking group (n = 5, age: 21.6 ± 1.4 years, height: 172.6 ± 5.7 cm, weight: 63.9 ± 7.6 kg) or lemon water-drinking group (n = 5, age: 22.6 ± 1.3 years, height: 173.7 ± 3.9 cm, weight: 62.1 ± 6.0 kg). Subjects were asked to drink 300 cc of water or 10% lemon water (270 cc water + 30 cc lemon juice) every day for 4 weeks. Specifically, they were asked to drink 100 cc of water or lemon water before each meal. Stool samples were collected at home before and after the test period using a stool collection kit. Samples were frozen and transported to the university on ice for analyses.

Measurement and analysis of the intestinal microbiota

Next generation sequencing was performed at Healthcare Systems to identify the bacterial species that constitute the intestinal microbiota. The method is used to characterize the functional properties of the intestinal microbiota, and combines metagenomic, metabolomic, and comprehensive metaproteomic analyses. It allows for the identification of bacterial species that constitute the intestinal microbiota as well as the characterization of host-microbiota interactions based on functional properties, such as the metabolism of carbohydrates and amino acids[10]. We specifically targeted 16S rRNA in this analysis. 16S rRNA exists in all living species other than viruses, and is one of the structural RNAs that constitute ribosomes. It has highly conserved regions as well as variable regions that are prone to mutations. In the present study, we analyzed the metagenome of the V3V4 region of 16S rRNA using the next generation sequencer (MiSeq, Illumina) and Qiime1 software[11]. Bacterial species were identified based on taxonomic classification and binomial nomenclature, and were categorized at the genus level. Bacterial species with a relative abundance rate higher than 0.01% in the water- and lemon water-drinking groups were identified, and the amounts of these bacteria were compared before and after the test period to calculate differences. The Shapiro-Wilk test was performed to test for the normality of data on pre- and post-intake differences in the water- and lemon water-drinking groups. The Student's *t*-test was used when data were normally distributed, and the Mann-Whitney test when data were not normally distributed. $P < 0.05$ was considered to be significant, with 0.05-0.1 indicating a slight difference.

RESULTS

Table 1 summarizes the bacterial species that were identified in the intestinal microbiota. P-values represent differences in the water- and lemon water-drinking groups based on the Student's *t*-test and Mann-Whitney test. A significant change was observed in the amount of Bilophila ($p = 0.036$), and slight changes in the amounts of Adlercreutzia ($p = 0.095$), Haemophilus ($p = 0.076$), Megamonas ($p = 0.095$), and Roseburia ($p = 0.065$).

DISCUSSION

The present results revealed changes in the amounts of Bilophila, Adlercreutzia, Haemophilus, Megamonas, and Roseburia in the water- and lemon water-drinking groups. The composition of the intestinal microbiota is associated with digestion in the stomach, which is affected by peristalsis and gastric acid secretion. Ashida et al. demonstrated that the intake of citric acid increased peristalsis[8], while Chen *et al*. reported similar findings for water[9]. In comparisons with the water-drinking group, peristalsis and digestion were promoted in the lemon-drinking group, which led to changes in the intestinal microbiota.

Adlercreutzia degrades soy isoflavones into equol. Since equol regulates estrogen activity, it is associated with a risk of menopausal osteoporosis and breast cancer[12]. In men, equol has been implicated in the development of osteoporosis and benign prostatic hyperplasia[13,14]. In the present study, a slight increase was noted in Adlercreutzia in the lemon water-drinking group. Since 50% of the Japanese population produces equol[12], the lemon-water-drinking group may have included some equol producers. Due to the small differences observed, further studies on a larger sample size are needed to confirm the results obtained.

Bilophila wadsworthia, which belongs to the genus Bilophila, is one of the bacteria isolated from gangrene, perforated appendicitis, blood, pleural effusion, mandibular osteomyelitis, liver abscess, and synovial fluid[15]. Mathur *et al*. examined the interaction between citric acid and bile acid and demonstrated that it effectively suppressed *Vibrio parahaemolyticus*[16]. Gastric acid damages *V. parahaemolyticus*; however, if this bacterium survives, it may proliferate in the small intestine, and, thus, this activity needs to be inhibited. The present results indicated that citric acid markedly suppressed the proliferative activity of damaged bacteria, which led to an overall reduction in the lemon water-drinking group.

There are many pathogenic bacteria in the genus Haemophilus; however, most do not cause any diseases and only proliferate in immunocompromised individuals[17]. Buchwald *et al*. demonstrated that citric acid in lemon effectively reduced fatigue[18], which has been shown to weaken the immune system[19]. In the present study, we found that the amount of Haemophilus decreased in both groups. This result suggests that lemon water effectively reduced fatigue, thereby improving the immune system and/or preventing bacterial growth, which is consistent with previous findings.

Bacteria in the genus Megamonas are associated with stool consistency[20]. Mahboubi et al. showed that the intake of citric acid in Prunus domestica resulted in stool softening[21]. However, Barreca *et al*. reported that the intake of lemon did not affect bowel movement (frequency and stool consistency)[22]. The present results revealed that the abundance of Megamonas was reduced in the lemon water-drinking group, but not in the water-drinking group. Further studies are needed to clarify whether lemon water effectively attenuates diarrhea and constipation.

Roseburia are butyrate-producing bacteria and their abundance is reduced in patients with diabetes[23]. Butyrate is a short-chain fatty acid involved in the proliferation of epithelial cells and secretion of mucus, and is used as a source of energy for the absorption of water and minerals[24]. It is mostly consumed as a major source of nutrients in the large intestine. The present results revealed a reduction in the abundance of Roseburia in the lemon water-drinking group. This may be attributed to citric acid functioning as a short-chain fatty acid in the intestine because citric acid in lemon water is not absorbed in the stomach.

In the present study, we only analyzed stool samples collected before and after the test period. However, the composition of the intestinal microbiota changes on a daily basis due to diet and lifestyle[25]. Therefore, it is important to collect additional information on the diet and psychological status of subjects using questionnaires. Since the composition of the intestinal microbiota also differs with age[25], future studies also need to be performed on healthy elderly subjects.

CONCLUSION

We herein examined changes in the intestinal microbiota after the consumption of water and lemon water, and detected changes in bacterial species belonging to Adlercreutzia, Haemophilus, Megamonas, Roseburia, and Bilophila. The present results suggest that citric acid in lemon affects digestion, bowel movement, and the immune system. Further studies are needed to clarify the effects of lemon and its relationship with gastric acid secretion.

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