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The Response of Colonic *Clostridium* Cluster IV to Essential Oil and Aqueous Extract of *Cinnamon Cassia* in Rats

Cong Wang, Shaoting Li, Long Chen, Xichun Peng*, Xiyang Wu

Department of Food Science and Engineering, Jinan University, Guangzhou, China
*Corresponding author: tpxchun@jnu.edu.cn

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Abstract The multiple pharmacological effects of *Cinnamomum cassia* (*C. cassia*) have been reported. The aim of this paper is to test the *in vivo* response of *C. cassia* derived materials on colonic *Clostridium* cluster IV that is supposed to relate the energy metabolism in body. The essential oil and aqueous extract of *C. cassia* were orally administered to 2 tested groups of Sprague-Dawley (SD) rats for 30 days. After 30-days cultivation, the colonic contents of the rats were sampled to investigate the *Clostridium* cluster IV community structure through terminal restriction fragment length polymorphism (T-RFLP) and 16S rRNA gene clone library analysis. The results showed that the total peak areas of colonic *Clostridium* cluster IV, Shannon diversity index and evenness all decreased significantly in 2 tested groups compared with the control group ($P < 0.01$). Jaccard similarity index was under 50% in both tested groups. Putative species corresponding to diet-associated terminal restriction fragments (TRFs), 89, 172 and 177 bp, were identified through gene sequence as *Acetanaerobacterium elongatum*, *Hydrogenoanaerobacterium saccharovorans*, *Anaerotruncus colihominis*, *Ethanoligenens harbinense*, *Clostridium cellulosi*, *Clostridium orbiscindens*, *Ruminococcus bromii* and *Papillibacter cinnamivorans*. In conclusions, both essential oil and aqueous extract of *C. cassia* can alter the bacterial community structure and decline the diversity of colonic *Clostridium* cluster IV in SD rats. Those species of *Clostridium* cluster IV impacted during the experiment may play a role in the energy metabolism of host.

Keywords: *Cinnamomum cassia, essential oil, aqueous extracts, Colon, Clostridium cluster IV*

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1. Introduction

Much concern has been focused on plant-derived bifidus factors towards intestinal bacteria, either promoting or inhibiting the growth of these bacteria. *Cinnamomum cassia* (*C. cassia*) has been extensively used in Chinese cooking not only for its flavor and appetizing effects but also for its preservative and medicinal properties. Its extracts contain several active components such as essential oil (cinnamic aldehyde and cinnamyl aldehyde), tannin, mucus and carbohydrates. They have various biological functions including anti-oxidant [1], antimicrobial [2], anti-inflammation [3], anti-diabetic effects [4], and anti-tumor activity [5].

These published literatures [1,2,3,4,5] mainly concerned about the consequence of cinnamon acting on health status of the host. However many photochemicals interact with colonic microbiota instead of directly absorbed in small intestine [6,7].

There are only two phyla, the *Bacteroidetes* and the *Firmicutes*, are dominant in rodent animal or human colon [8]. Recent investigation on animal and human indicates a reduction of the relative proportion of *Bacteroidetes* to *Firmicutes* in obese individuals; and the *Firmicutes* are responsible for improving the absorbance of energy in gut [9].

The composition of gut microbiota is affected by both endogenous and environmental factors, including antibiotic intake, xenobiotics, etc. [10], among which diet is considered as primary driver for changes in gut bacterial diversity that may modify its functional relationships with the host [11]. We had recently reported the up-regulation of aqueous extracts and essential oil from *C. cassia* on rat *Bacterioidales* [12]. This study aimed at discussing the response of *C. cassia* components (essential oil and aqueous extract) on colonic *Clostridium* clusters IV, dominant in the *Firmicutes*.

2. Experimental

2.1. Preparation of Essential Oil (EOC) and Aqueous Extract (AEC) from *C. cassia*

C. cassia obtained from Er Tiantang Co. (Guangzhou, China) were shattered and meshed with 10 meshes. AEC was prepared as reported by Li, et al [12].

2.2. Animals and Diets

Eight weekS-old healthy male Sprague-Dawley (SD) rats were obtained from the Animal Center of Southern Medical University (Guangzhou, China). The average weight of rats was 200 g ± 20 g. They were cultivated by the procedure described by Li, et al [12]. The animal experiments were approved by the Department of Care and Use of Laboratory Animal in Jinan University (Guangzhou, China).

2.3. Sampling Collection

After 30 days of feeding, 3 rats were randomly selected from each group and etherized after weighed. The colons of all rats were removed aseptically from the abdominal cavity and dissected free from fat and mesentery. Their colonic contents were individually sampled and stored at -20°C for microbiota analysis.

2.4. Total DNA Extraction of Colonic Microbe

The bacterial DNA of each sample was extracted by Stool DNA-out kit (AndyBio, Itasca, IL, USA) according to manufacturer's instructions. The total DNA samples were characterized with 1% agarose gel electrophoresis for integrity and size. The DNA was adjusted to 40 ng/μL and stored at -20°C before used as templates for PCR.

2.5. Amplification of DNA

16S rRNA genes were amplified from each sample using a fluorescent-labeled forward primer, sg-Clep-F (FAM-GCACAAGCAGTGGAGT), and a reverse primer, sg-Clep-R (CTTCCTCCGTTTTGTCAA), both of which were genus-specific for *Clostridium* cluster IV [13]. The PCR consisted of 20 ng template, 0.02 5 U/μL Ex Taq polymerase (Takara Co., Otsu, Japan), 1x Ex Taq buffer (Mg^{2+} plus) (Takara Co., Otsu, Japan), 0.25 mM of each primer (synthesized by Sangon Co., Shanghai, China), and 0.2 mM of each dNTP (Takara Co., Otsu, Japan), and was performed on an Eppendorf Mastercycler^{TM} thermal cycler (Eppendorf Co., Hamburg, Germany) with the following conditions: an initial 8 min pre-denaturation at 94°C, followed by 30 cycles of 45 sec denaturation at 94°C, 30 sec annealing at 55°C, and 1 min extension at 72°C. After the final cycle, there was another 5 min elongating at 72°C to complete the PCR.

PCR products were purified and the purified products were digested with the methods described by Li, et al [12]. The fluorescently labeled fragments were sent to Invitrogen (China) Co. (Shanghai, China) for terminal restriction fragment (TRF) profiles detection.

2.6. T-RFLP Analysis

Fragments differing by less than 1 bp length were clustered [14]. The sizes of TRFs involved were between 30 bp and 600 bp, and only TRFs with relative abundances more than 0.5% were included in further analysis. Putative identities for the most dominant fragments were predicted by the in silico cut sites (*Hha* I digest) on Ribosomal Database Project II (http://rdp.cme.msu.edu) using the T-RFLP analysis program TAP. The analysis of T-RFLP profiles was followed the methods described by Li, et al [12].

2.7. 16S rRNA Genes Clone, Sequencing and Phylogenetic Inference

Total bacterial 16S rRNA genes were first amplified by PCR with the same system and conditions as described above, but with the unlabeled forward primer. The PCR products were purified using the Pearl PCR purification kit (NewProbe, Beijing, China) and attached into pMD19-T vector (Takara Co., Otsu, Japan), reaching up the total amount of 0.1 pmol insert DNA per sample. The transformants of DH5α E. coli competent cells were cultivated on Luria-Bertani (LB) agar plates containing IPTG, X-Gal and Ampicillin Sodium, to get both white and blue colonies. 50 randomly picked white colonies (positive clones) were unidirectional sequenced using dye terminator chemistry and the primer M13 (Sangon, Shanghai, China).

The 16S rRNA gene sequences were further digested in silico to match the specifically sized T-RFLP fragments relevant to C. cassia consumption [15]. The corresponding sequences were assembled into operational taxonomic units (OTUs) with a shreshold of 97% pair-wise identity [16] by DNAMAN version 6.0 and Clustalx version 2.1. A single representative sequence for each OTU was BLASTned on National Center for Biotechnology Information (NCBI) database, and the most similar sequences were included in the construction of phylogenetic tree adopting the neighbor-joining analysis with 1000 replicates p-distance to produce bootstrap values by MEGA version 4.0.

2.8. Statistics

Results are expressed as mean values with their standard deviation (SD). Statistical analyses were conducted with the Statistical Package for Social Science (SPSS for Windows, version 8.0; SPSS Inc., Chicago, IL, USA) to determine if variables differed among treatment groups.

3. Results

3.1. T-RFLP analysis

The total peak areas of *Clostridium* cluster IV in the colon of SD rats were decreased in both groups ($P < 0.01$), with a sharper decline in AEC group than in EOC group compared to control group (Figure 1).

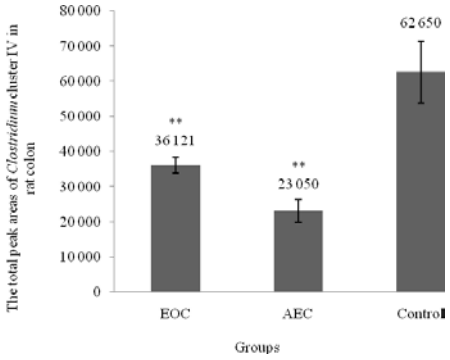

Figure 1. Total peak areas of *Clostridium* cluster IV in the colon of SD rats. ** P < 0.01, compared with the control group.

The total average numbers of terminal restriction fragments (*Hha* I digest) in control group, EOC group and AEC group were 6, 4 and 5, respectively. Shannon diversity index and evenness of EOC group and AEC group experienced a decline compared to that of control group ($P < 0.01$) (Table 1). Furthermore, the difference between EOC and control group was more significant than that between AEC and control group. Jaccard similarity index was under 50% in both groups, that was 32.89% in EOC group and 41.55% in AEC group.

Table 1. Comparison of diversity and evenness indices for the T-RFLP profiles (*Hha* I digest) from the different samples.

Samples	S [a]	H [b]	E [c]
Control group	6	2.132	0.825
EOC	4	1.045**	0.523**
AEC	5	1.478**	0.637**

[a] Ribotype richness; S= total number of brands in profile.

[b] Shannon diversity index; $H = -\sum(p_i)(\log_2 p_i)$, where p_i is the individual peak area; $H_{max} = \log_2 S$.

[c] evenness; $E = H/H_{max}$.

** $P < 0.01$, compared with the control group.

The SIMPER analysis indicated that the differences of T-RFLP profiles (*Hha* I digest) between tested and control groups were driven primarily by variation of 89 and 177 bp in EOC group, while 89 and 172 bp in AEC group (Table 2). Compared to the control group, the relative abundance of 89 bp increased in EOC group ($P < 0.01$), while 177 bp was not detected. On the contrary, the relative abundance of 89 bp decreased in AEC group ($P < 0.05$), while 172 bp increased ($P < 0.01$) (Figure 2).

Table 2. Results of SIMPER analysis of T-RFLP profiles of the samples

Fragments (bp)	MRA (%) [a]		FC (%) [b]	MRA (%) [a]		FC (%) [b]
	Control group	EOC		Control group	AEC	
89	33	77	44	33	9	29
172	27	11	16	27	68	49
177	21	0	21	21	11	12

[a]Relative abundance of each fragment (*Hha* I digest) as a percentage of total fragment abundance. [b]Fragment contribution as a dissimilarity percentage between the two groups. Lists are truncated to contain only those fragments which contribute no less than 5% to the differences between samples.

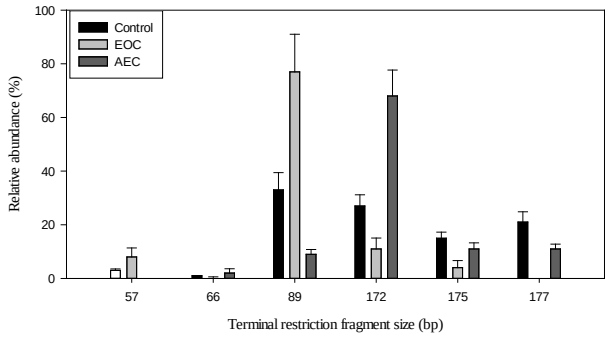

Figure 2. Terminal restriction fragments (*Hha* I digest) and mean relative abundances (%) of colonic *Clostridium* clusters IV in samples. Values are expressed as means ±SD. ** $P < 0.01$, compared with the control group. * $P < 0.05$, compared with the control group.

3.2. Identity of T-RFLP fragments

Comparison of T-RFLP fragments and *in silico* cut sites [17,18] are presented in Table 3 to illustrate the taxonomic identity of 89, 172 and 177 bp. The 89 bp was not detected by the *in silico* cut sites. All the detected fragments were classified into different families as *Ruminococcaceae* and *Clostridiaceae* of *Firmicutes* (Table 4).

Table 3. Comparison of T-RFLP fragments to clone restriction enzyme cut sites predicted from sequence analysis to confirm the identity.

Enriched fragment	Restriction enzyme	TRFs	Sequence data
1		89	- [a]
2	*Hha* I	172	174/175
3		177	178/179

[a]-: Not found.

3.3. Phylogeny

The 50 clones in EOC, AEC and control bacterial library were divided into 11, 17 and 24 OTUs, respectively. Comparison between the relative abundance of EOC group, AEC group and control group illustrated that 3 TRFs were significantly associated with tested diets. By the in silico digestion of sequenced clone genes, we found out the clones which represented TRFs linked to EOC and AEC (Table 5).

Putative species of Clone 0-42, 0-49, 0-86 and 10-18 were not detected in EOC group, so was Clone 0-95 in AEC group. On the other hand, putative species of Clone 01-10, 01-13, 01-21 and 01-48 did not exist in control group as far as we learnt. These diet-associated species were identified as *Acetanaerobacterium elongatum*, *Hydrogenoanaerobacterium saccharovorans*, *Anaerotruncus colihominis*, *Ethanoligenens harbinense*, *Clostridium cellulosi*, *Clostridium orbiscindens*, *Ruminococcus bromii* and *Papillibacter cinnamivorans*. Phylogenetic tree was constituted of the clone sequences influenced by EOC and AEC, and reference sequences from GenBank (Figure 3).

4. Discussion

Hypothesis based on the *in vitro* and *in vivo* researches suggest that change in the microbiota ratio may influence food components ingestion [19-26], when the commensal microbiota regulates the processing and absorption of dietary carbohydrates and complex lipids, which altogether leads to more fat storage and weight gain in the host [8]. Thus manipulation of the relative proportion of *Bacteroidetes* to *Firmicutes*, *Bacteroidetes* or to cut down *Firmicutes*, could be an option to treat with metabolic disorders of energy.

Generally speaking, the gut bacterial community of healthy adult individuals is relatively stable despite minor fluctuation over a short period of time [27]. Short-term dietary shifts, however, are reported to tip this balance [28,29,30,31,32,33]. Based on the result of T-RFLP analysis in our study, there was a shift of bacterial community structure and a drop in the diversity of colonic *Clostridium* clusters IV of SD rats given by gavage with EOC and AEC. Additionally, Jaccard similarity index indicated a low level of similarity between bacterial communities of control and tested samples. We speculated the shifts may derive from the ingestion of phytochemicals in EOC and AEC. On one hand, the phytochemicals contained in plant extracts have either inhibitory or promoting effects on different species of bacteria [8]. On

the other hand, the colonic bacterial transformation also altered the bioactivities of some phytochemicals in *C. cassia* back [34]. Some components of phytochemicals cannot be absorbed entirely in the upper intestine entirely after intake. Instead, they are passed to colon and subjected to microbial metabolism by colonic microbiota [35]. Unabsorbed flavonoids of AEC, for instance, will arrive at the large intestine where they are further metabolized by gut microbiota into simpler molecules such as phenolic acids and valerolactones [36], concluding in a reorganization of new metabolites. These dietary phenolics and their metabolites are believed to show antimicrobial or bacteriostatic capabilities [34].

Table 4. Putative phylogenetic affiliation of each enriched fragment (*Hha* I digest) as determined with the Ribosomal Database Project analysis tool "classifier".

Fragment (bp)	Phylum	Class	Order	Family	Genus
174	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	*Acetanaerobacterium*
175	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	*Hydrogenoanaerobacterium*
175	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	*Anaerotruncus*
178	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Clostridium*
178	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Clostridium*
178	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	*Ethanoligenens*
178	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	*Papillibacter*
179	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Clostridium*
179	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	*Ruminococcus*

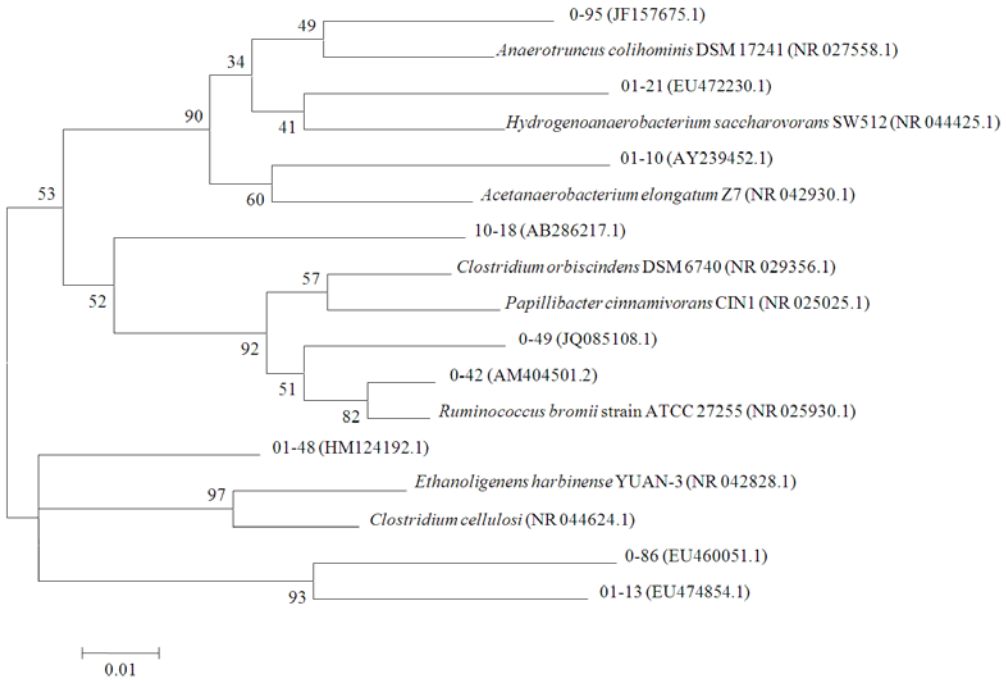

Figure 3. Phylogenetic tree of representative *Clostridium* cluster IV 16S rRNA gene sequences that were significantly associated with the two types of tested diets and reference sequences from GenBank. Data in parentheses are GenBank accession numbers. Numbers at the nodes demonstrate the levels of bootstrap support depending on neighbor-joining analysis of with 1000 replicates *p*-distance.

Table 5. T-RFLP fragments that were significantly associated with the two types of tested diets.

Fragments (bp)	Associated diet	Clone name	Closest relatives	Similarity to closest relatives [a]
89	EOC, AEC	- [b]	-	-
172	AEC	01-10	*Acetanaerobacterium elongatum*	93%
		01-21	*Hydrogenoanaerobacterium saccharovorans*	94%
		0-95	*Anaerotruncus colihominis*	95%
177	EOC	01-13	*Ethanoligenens harbinense*	90%
		01-48	*Clostridium cellulosi*	93%
		0-42	*Ruminococcus bromii*	98%
		0-49	*Clostridium orbiscindens*	95%
		0-86	*Clostridium orbiscindens*	90%
		10-18	*Papillibacter cinnamivorans*	93%

[a]Percentages of sequence similarity between the cloned 16S rRNA gene sequences and those of their closest relatives using BLAST. [b]-: Not found.

As our results presented, TRFs significantly associated with tested diets were 89 and 177 bp in EOC group ($P < 0.01$), while 89 bp ($P < 0.05$) and 172 bp ($P < 0.01$) in AEC group. To identify putative species impacted by EOC and AEC intake, a molecular fingerprinting approach based on 16S rRNA gene was adopted to describe the responses of colonic *Clostridium* cluster IV community structure [14,37]. 172 bp TRFs were identified as *Acetanaerobacterium elongatum*, *Hydrogenoanaerobacterium saccharovorans* and *Anaerotruncus colihominis*. 177 bp TRFs were identified as *Ethanoligenens harbinense*, *Clostridium cellulosi*, *Clostridium orbiscindens*,

Ruminococcus bromii and *Papillibacter cinnamivorans*. The above species of *Clostridium* clusters IV are considered to probably associate with metabolism of the ingredients of EOC and AEC, that is, EOC inhibits the growth of *Clostridium orbiscindens*, *Ruminococcus bromii* and *Papillibacter cinnamivorans*, while promotes the growth of *Ethanoligenens harbinense* and *Clostridium cellulosi*, and AEC inhibits the growth of *Anaerotruncus colihominis*, while promotes the growth of *Acetanaerobacterium elongatum* and *Hydrogenoanaerobacterium saccharovorans*. As for our explore of *C. cassia* pharmacological mechanism, those species may have catalytic and hydrolytic action on components processing of EOC and AEC, and thus serve as the intermedium for components of EOC and AEC to affect host at the same time. An increase in Firmicutes has been found to be accompanied with body obesity [38]. Based on the hypothesis of previous research state the hypotheses for better understanding [39], we reasonably anticipate *C. cassia* derived materials to modulate the intestinal microbiota, as well as the energy metabolism of host as a result.

In conclusion, this paper reported the response of rat colonic *Clostridium* clusters IV on AEC and EOC. It was found that both essential oil and aqueous extract of *C. cassia* can alter the bacterial community structure and decline the diversity of colonic *Clostridium* cluster IV in SD rats. Furthermore, the impacted species of *Clostridium* clusters IV have been identified. Those down-regulated species may promote the energy ingestion of host and the others may inhibit the energy ingestion of host.

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