Investigation of Butyrate-producing Bacteria and Genes from Fecal Samples of Thai Volunteers

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บทคัดย่อ

สารบิวทีเรท (Butyrate) ในลำไส้เกิดขึ้นจากการหมักอาหารจำพวกคาร์โบไฮเดรต โดยแบคทีเรียซึ่งอาศัยอยู่ในลำไส้ บิวทีเรทเป็นแหล่งพลังงานที่สำคัญสำหรับเซลล์เยื่อบุผิวลำไส้ นอกจากนี้ยังมีบทบาทสำคัญในการรักษาสมดุลของเซลล์เยื่อบุผิวลำไส้ ได้แก่ ควบคุมการเพิ่มปริมาณ การเปลี่ยนสภาพและการตายของเซลล์เยื่อบุผิวลำไส้ ปริมาณบิวทีเรทที่ผลิตได้ขึ้นอยู่กับหลายปัจจัยเช่น ชนิดของอาหารที่รับประทาน ปริมาณของแบคทีเรียกลุ่มที่ผลิตบิวทีเรท การศึกษาก่อนหน้านี้พบว่า มีแบคทีเรียที่ผลิตบิวทีเรทสองกลุ่มคือ แบคทีเรียคลอสติเดียลกลุ่มที่สี่ (CIV) และแบคทีเรียคลอสติเดียลกลุ่มสิบสี่เอ (CXIVa) วัตถุประสงค์ของการศึกษาครั้งนี้คือ หาปริมาณของแบคทีเรีย *Faecalibacterium prausnitzii* (ตัวแทนของ CIV) และปริมาณของ *Clostridium coccoides-Eubacterium rectale* (ตัวแทนของ CXIVa) และ วัดปริมาณของยีนที่ควบคุมการผลิตบิวทีเรทซึ่งได้แก่ยีน *butyryl-CoA: acetate CoA-transferase* (*but*) และยีน *butyrate kinase* (*buk*) ในตัวอย่างอุจจาระของอาสาสมัครจำนวน 14 คน ด้วยวิธี quantitative PCR ในการศึกษานี้แบ่งตัวอย่างอุจจาระของอาสาสมัครออกเป็นสองกลุ่มตามดัชนีมวลกาย กลุ่มแรกมีดัชนีมวลกายปกติ (BMI≤๒๓) จำนวน 6 คน และกลุ่มที่สองมีดัชนีมวลกายเกิน (BMI> 23) จำนวน 8 คน ผลการศึกษาพบว่า สัดส่วนปริมาณแบคทีเรีย *Faecalibacterium prausnitzii* ในอุจาระของอาสาสมัครที่มีดัชนีมวลกายเกินปกติมีแนวโน้มสัดส่วนแบคทีเรียชนิดดังกล่าวสูงกว่าในอุจจาระของอาสาสมัครที่มีดัชนีมวลกายปกติ อย่างไรก็ตามสัดส่วนแบคทีเรียกลุ่ม *Clostridium coccoides-Eubacterium rectale* ไม่แตกต่างกันระหว่างสองกลุ่ม จากการประมวลทางสถิติเพื่อหาความสัมพันธ์ระหว่างสัดส่วนของแบคทีเรีย ยีนและค่าดัชนีมวลกาย พบว่าสัดส่วนของแบคทีเรีย *Faecalibacterium prausnitzii* มีความสัมพันธ์ทางบวกอย่างมีนัยสำคัญกับสัดส่วนของยีน *but* ($r = 0.752$, $p < 0.05$) และค่าดัชนีมวลกายมีความสัมพันธ์เชิงบวกกับสัดส่วนของยีน *buk* ($r = 0.812$, $p < 0.01$)

คำสำคัญ: แบคทีเรียที่ผลิตบิวทีเรท ยีนที่ควบคุมการสร้างบิวทีเรท อุจจาระ อาสาสมัครไทย

Abstract

Butyrate, produced by intestinal anaerobic bacteria via dietary carbohydrate fermentation, is an important energy source for gut epithelial cells. The butyrate also plays a crucial role in homeostasis of intestinal epithelial cells including cellular proliferation, differentiation, and apoptosis. Butyrate production depends on the type of diet and availability of butyrate-producing bacteria. Bacteria of *Clostridial* cluster IV (CIV) and cluster XIVa (CXIVa) are major butyrate producers. This study investigated: i) the availability of *Faecalibacterium prausnitzii* (a representative of CIV) and *Clostridium coccoides–Eubacterium rectale* group

(a representative of CXIVa), and ii) the presence of the butyrate-producing genes, *butyryl-CoA: acetate CoA-transferase* (*but*) and *butyrate kinase* (*buk*), in fecal samples of butyrate-producing volunteers using quantitative PCR. The samples were obtained from six normal (BMI$\leq$23) and eight overweight (BMI$>$23) Thai volunteers. The results showed the trend toward higher levels of *Faecalibacterium prausnitzii* found in the overweight volunteers as compared to the normal-weight volunteers. However, the proportion of *Clostridium coccoides–Eubacterium rectale* was not different between the two groups. Interestingly, the availability of *Faecalibacterium prausnitzii* showed significant positive correlation to the presence of *but* (r=0.752, p<0.05) and the BMI was positively correlated to the presence of *buk* (r=0.812, p<0.01).

Keywords: Butyrate-producing bacteria: Butyrate-producing genes: Fecal sample: Thai volunteers

Introduction

Butyrate plays a crucial role as a major source of energy to gut epithelial cells. Moreover, it also regulates gene expression, inflammation, and apoptosis of the host cells[1]. Butyrate is produced by colonic bacteria that belong to un-cultureable gram-positive anaerobic *Firmicutes*. Even though they are phylogenically distinct they share the same features of energy metabolism. The main butyrate producers in human colon are *Clostridial* cluster XIVa (CXIVa) and cluster IV (CIV). Examples of the CXIVa members are *Roseburia hominis*, *R. intestinalis*, *R. faecis*, *R. inulinivorans*, *Eubacterium rectal*, *E. ventriosum*, *E. ramulus*, *E. hallii*, *Butyrivibrio fibrisolvens*, *Coprococcu scatus*, *C. eutactus*, *C. comes,* and *Anaerostipe scaccae*. Members of the CIV are *Faecalibacterium prausnitzii* (hereafter referred to as *F. prausnitzii*), *Anaerotruncus colihominis,* and *Subdoligranulum variabile*[2]. Butyrate is a reduced end-product of alternative pathways in response to carbon source availability and to balance the obtained ATP and redox state. Butyrate-producing gene clusters are organized in the same genomic arrangement co-regulated in response to available carbon sources. The core butyrate biosynthesis pathway is the sequential formation of butyryl-CoA from acetyl-CoA which is the response of six functional genes. Then there are two final routes for the butyrate production. The conventional route harbored in most of the butyrate producers is finished by the conversion of butyryl-CoA and acetate to butyrate and acetyl-CoA respectively, by *butyryl-CoA:acetate CoA-transferase* (*but*). The *but* genes are highly conserved among the butyrate producers which is applicable for using degenerated primers for quantification strategy[3]. The other route is the turning of the butyryl-CoA to butyryl-phosphate and then to butyrate by *phosphotransbutyrylase* (*ptb*) and *butyrate kinase* (*buk*) respectively. The *buk* route is mainly found in species such as*C. eutactus* and related species. It has been shown that the availability of the butyrate producers restrictively depended on the polysaccharide constituent in the diet intake and on the metabolic cross-feeding environment. In addition, many butyrate producers can also produce lactate, formate, hydrogen, and carbon dioxide as by-products. Importantly, these by-products can affect the gut environment, especially pH change, and promote co-domination of other gut bacteria [4]. Louis and Flint[2] reported that *C. coccoides–E. rectale* and *F. prausnitzii* can be used to represent *clostridial* cluster XIVa and cluster IV respectively. To test the availability of the butyrate producers (*c. coccoides–E. rectale* and *F.*

prausnitzii) and their correlation to the butyrate-producing genes, quantitative PCR technique targeting the phylogenic conserved 16S ribosomal RNA and the key genes in butyrate production routes (*but* and *buk*) was used to quantify the bacterial and gene proportion from fecal samples of healthy volunteers in this study.

Materials and Methods

Volunteers

Fourteen volunteers, seven male and seven female, were recruited. The ages of volunteers were between 25 and 35 years. Basic information was collected including weight (w, kg) and height (h, m) for calculation of the body mass index (BMI) by w/h^2 (kg/m^2). According to their BMI ranges, they were classified into two groups, Normal ($BMI \leq 23$, n=6) and Overweight ($BMI > 23$, n=8). This study was approved by Mae FahLuang University human ethics committee (license number REH57027).

DNA extraction from fecal samples and quantitative PCR (qPCR)

Stool samples of 400 mg were processed to isolate total genomic DNAs using innuPREP Stool DNA Kit (Analytikjena, Biometra). Quantitative PCR (qPCR) technique was used for relative quantification of bacterial proportion using sets of group/species-specific primers (Table 1) targeting 16S rRNA and *but*/*buk* genes related to all bacteria (universal primer). The qPCR reaction was analyzed by qPCR under CFX96 PCR thermocycle (BIO-RAD). Each reaction was done in triplicate in a total volume of 10 µl by SensiFAST^{TM} SYBR No-ROX Kit (BIOLINE, USA) containing the final concentration of 1X Reagent mix, 100-200 nM each of forward and reverse primers, and 4 ng of genomic DNA template in 96-well PCR white plate.

Table 1 Set of primers targeting bacterial 16S rRNA and butyrate-producing genes

Targets	Sequences of primers (5' to 3')	Product size (bp)	Ta (°C)
F. prausnitzii (representative of CIV)[a]	F:GGAGGAAGAAGGTCTTCGG [7] R:AATTCCGCCTACCTCTGCACT [8]	247	63
C. coccoides– E. rectale group (representative of CXIVa)[b]	F:CGGTACCTGACTAAGAAGC [9] R:AGTTTYATTCTTGCGAACG [9]	429	60
Butyryl-CoA:acetate CoA-transferase (but)	F:GCIGAICATTTCACITGGAAYWSITGGCAYATG [3] R:CCTGCCTTTGGAATRTCIACRAANGC [3]	530	60
Butyrate kinase (buk)	F:TGCTGTWGTTGGWAGAGGYGGA [10] R:GCAACIGCYTTTTGATTTAATGCATGG [10]	279	56
All bacteria (Universal-200)	F:ACTCCTACGGGAGGCAGCAG [11] R:ATTACCGCGGCTGCTGG [11]	~170-200	63

[a]The primers had low specificity to *A. colihominis* and *S. variabile* by bioinformatics analysis

[b]The primers had low specificity to *E. ventriosum* and *B. fibrisolvens* by bioinformatics analysis

A cyclic condition was performed with the following temperature profile: a polymerase activation cycle at 95°C for 2 minutes, 40 cycles of denaturation at 95°C for 5 seconds, annealing at the indicated Ta (Table 1) for 10 seconds, and extension at 72°C for 20 seconds. The Ta for each pair of primers was optimized for the best sensitivity and specificity. The specificity of the PCR product was analyzed from the melting-curve analysis after denaturation at 95°C for 10 seconds and slow heating the mixture from 65°C to 95°C with the plate read every 0.5°C for 5 seconds. The amplification efficiency of the target (E_{Target}) and universal ($E_{Universal}$) primers was determined form Ct value of 3 points of 10-fold dilutions of a pooled sample and the slope was from the linear regression fit. The mean of the Ct value was used for calculation of amplification efficiency (E) of each set of primer by Equation 1[5].The proportion (relative quantification) of the interested specific taxa to the total population was calculated by the Ct value of the targeted product to all bacteria (Universal-200) product by Equation 2[6].

$$E = 10^{\left(\frac{-1}{slope}\right)} \quad (1)$$

$$\text{Proportion}\ (\%) = \frac{(E_{Universal})^{Ct_{Universal}}}{(E_{Target})^{Ct_{Target}}} \times 100 \quad (2)$$

Statistical analysis

Statistical analysis was performed by the use of SPSS software version 21 (purchased order: 10-58878). Correlation analysis was calculated using Pearson's correlation analysis. Mann-Whitney U-Test was used for the determination of the bacterial proportion distribution between groups.

Result and Discussion

In this study, qPCR analysis of 16S rRNA genes belonging to the butyrate-producing *F. prausnitzii* and *C. coccoides–E. rectale* group revealed that they accounted up to 5% and 13%respectively among the volunteers, whereas the functional butyrate-producing *but* and *buk* were all less than 1% of the population. As diet has a major impact on physical appearance (BMI), the BMI value ranged and correlated with the proportions of the producers and the genes. It was found that BMI was not correlated to the availability of the *F. prausnitzii* and *C. coccoides–E. rectale* group. In contrast, higher BMI was strongly correlated with the presence of *buk* (r=0.812, $p<0.05$) (Table 2) but not *but*, implying that detection at the functional genes level might give more precious details than the broad 16S rRNA analysis. As some of the phylotypes in the clusters are not butyrate producers, it was possible to over-estimate the butyrate producers by the 16S rRNA analysis. However, the *but* had a positive association with the presence of *F. prausnitzii* (r=0.752, $p<0.05$), but not the *C. coccoides–E. rectale* group, indicating that the *F. prausnitzii* mayhave been the major butyrate producer among the observed population. After grouping the volunteers into 2 sets based on BMI range (Normal and Overweight), little differences in the bacterial proportions were observed (Figure 1). Even the presence of *F. prausnitzii* between the two groups was not significantly different (p=0.147) but the range of distribution among the Overweight was wider than the Normal, indicating the unstable composition of *F. prausnitzii* among the overweight group. In contrasted to *F. prausnitzii*, the phylogenically distinct *C. coccoides–E. rectale* group was more distributed among the Normal, but

not the Overweight, implying that the group of *C. coccoides* and *E. rectal* group among the normal range BMI mayhave been more available than the higher BMI range. Moreover, distributions of the *but* and *buk* as well as the accumulative value of the *but* and *buk* were higher and wider in the Overweight that were correlated with the distribution of *F. prausnitzii* in the Overweight group. From the results, it seemed likely that the major butyrate producers might belong to *F. prausnitzii* with the minor group as the *C. coccoides–E. rectale* group among the observed population.

Table 2 Correlation of BMI, butyrate producers, and genes

Parameters	BMI	*F. prausnitzii*	*C. coccoides–E. rectale*	*but*	*buk*	*but*&*buk*
BMI	1					
F. prausnitzii	-0.167	1				
C. coccoides–E. rectale	-0.191	0.210	1			
but	0.031	* 0.752	0.428	1		
buk	** 0.812	-0.466	-0.140	-0.229	1	
but &*buk*	* 0.554	0.395	0.305	** 0.777	0.434	1

* Significant (p) value is lower than 0.05. ** Significant (p) value is lower than 0.01. Absence of the indicated p value means not significant ($p>0.05$).

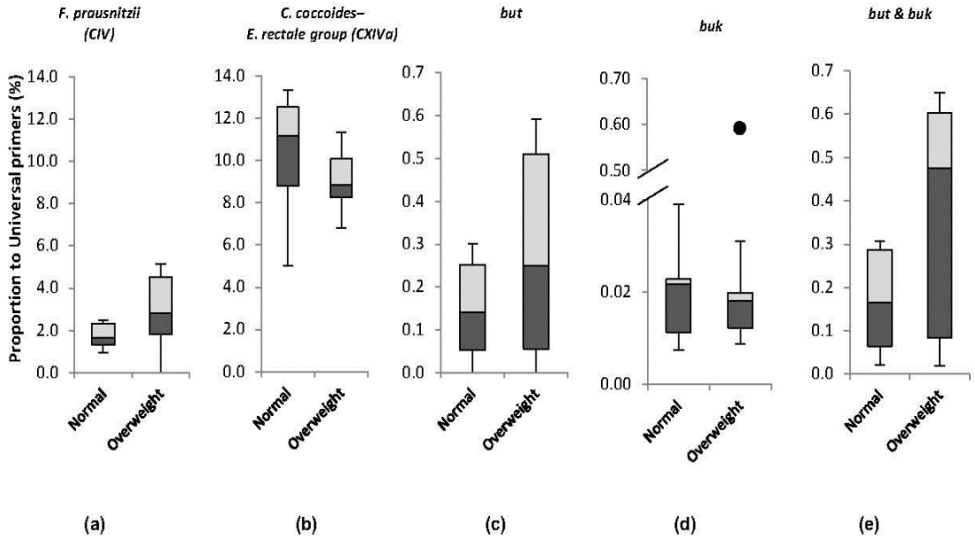

Figure 1 Box-and-whisker plot distribution of butyrate producers and genes: *F. prausnitzii* (a), *C. coccoides– E. rectale* group (b), *Butyryl-CoA:acetate CoA-transferase (but)* (c), *Butyrate kinase (buk)* (d), and accumulative values of the *but* and *buk* (e)

Conclusions

The study demonstrated butyrate-producing bacteria were not found to be different among the two groups. In addition, *but* gene was also not different between the two groups, but it was found that *buk* gene was significantly higher in the overweight group. This is the first pilot study of the butyrate producers and the genes in Thai population. To better understand the roles of butyrate producers and genes in association to other physiological conditions, such as obesity, type II diabetes, and colorectal diseases, a larger population using these established detection systems should be studied in the future.

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