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Degradation of chondroitin sulfate by the gut microbiota of Chinese individuals

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ABSTRACT

Oral preparations of chondroitin sulfate (CS) have long been used as anti-osteoarthritis (anti-OA) drugs. However, little is known about the degradation of CS by human gut microbiota. In the present study, degradation profiles of CSA (the main constituent of CS drugs) by the human gut microbiota from six healthy subjects were investigated. Each individual's microbiota had differing degradation activities, but ΔUA-GalNAc4S was the end product in all cases. To elucidate the mechanisms underlying this phenomenon, different CSA-degrading bacteria were isolated from each individual's microbiota and tested for CSA degradation. In addition to *Bacteroides thetaiotaomicron* J1, *Bacteroides thetaiotaomicron* 82 and *Bacteroides ovatus* E3, a new CSA-degrading bacterium, *Clostridium hathewayi* R4, was isolated and characterized. Interestingly, at least two different CSA-degrading species were identified from each individual's gut microbiota. Predictably, these functional bacteria also had differing degradation rates, but still generated the same end product, ΔUA-GalNAc4S. In addition, the human fecal isolates produced different degradation profiles for CSC, CSD, and CSE, suggesting that CS could be readily metabolized to varying extents by diverse microbial consortiums, which may help to explain the poor bioavailability and unequal efficacy of CS among individuals in OA treatment.

1. Introduction

Osteoarthritis (OA) is the most common form of arthritis and is a major source of pain, disability, and socioeconomic costs worldwide [1]. In 2010, the global OA prevalence was estimated to be 3.8% [2] and, among those over 60 years old, 10% of males and 18% of females are affected [3]. Chondroitin Sulfate (CS), whether used as a dietary supplement or as a proprietary drug (Condrosulf®, CSA, CSC *ca.* 3:2 ratio), has long been championed as a potential disease modifier for OA [4–7]. However, in clinical trials, a high dosage (1,200 mg/d) is required to achieve the desired therapeutic effects [8]. This is due to the poor absorption of CS (a high-molecular-weight glycosaminoglycan) by the intestine after oral administration [8,9] and the bioavailability of CS is estimated to be 0–13% [4,9,10]. Therefore, as no intestinal pancreatic or brush border enzymes have been discovered that metabolize CS, the removal and degradation of this drug must be performed by gut microbiota [11]. However, there is currently little information available regarding the degradation profiles of CS by human gut microbiota. In addition, as drug bioavailability can be directly linked to therapeutic effects, the cause of poor CS bioavailability needs to be investigated, rather than simply ascribing the effects to the high molecular weight and charge density of CS [9].

The gut microbiota is composed of complex consortia that encompass trillions of microorganisms and has been observed to play a central role in regulating multiple physiological processes [12]. Often called the "forgotten organ," the gut microbiota has also been documented to actively participate in drug metabolism [13,14] and multiple biotransformations of clinical drugs performed by intestinal bacteria—including reduction, hydrolysis, dehydroxylation, acetylation, deacetylation and deconjugation—have all been reported [15]. CS degradation by *Bacteroides* strains isolated from human gut microbiota have been studied by Salyers et al. [16] and Ahn et al. [17]. who found that *Bacteroides thetaiotaomicron*, *Bacteroides ovatus*, *Bacteroides fragilis* and *Bacteroides stercoris* were all capable of utilizing CS during *in vitro*

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Table 1
Structural comparison between CSA, CSC, CSD, and CSE.

Types of CS	Repeating disaccharide unit	Mw	Sulfate content
CSA	→4GlcAβ(1 → 3) GalNAc4Sβ1→	24 kDa	17.22%
CSC	→4GlcAβ(1 → 3) GalNAc6Sβ1→	130 kDa	17.71%
CSD	→4GlcA2Sβ(1 → 3) GalNAc6Sβ1→	40 kDa	26.12%
CSE	→4GlcAβ(1 → 3) GalNAc4S6Sβ1→	190 kDa	27.06%

fermentation. However, no direct comparisons have been made regarding the CS-degrading capability between the gut microbiota from discrete individuals. In addition, even though metagenomic studies have confirmed the CS degradation capabilities of *B. thetaiotaomicron* and *B. ovatus* [18], little is known about other species of gut anaerobes that can degrade CS.

In the present study, CS degradation profiles by human gut microbiota were assessed and compared. CSA is used clinically worldwide [5,19,20], establishing it as an ideal representative in investigating CS degradation. In sum, CSA was observed to be readily degraded by human gut microbiota, but CSA-degrading capabilities varied strongly between individuals. Moreover, in addition to *Bacteroides*, a new CS-degrading bacterium, *Clostridium hathewayi* R4, was also isolated and identified from human feces. Together, this study provides new evidence of CS degradation by human gut microbiota, which could help in explaining the poor bioavailability and efficacy of CS in clinical trials.

2. Materials and methods

2.1. Origin of fecal samples

Six healthy adult volunteers (aged 24–47 years old) who resided in Hangzhou, China were recruited for the current study. All individuals had not received any antibiotics, pre- or probiotics treatment for at least one year prior to sample collection. All volunteers provided well-informed, written consent and all studies were supported by the Ethics Committee of Ocean University of China.

Fresh fecal samples were collected anaerobically and six grams of each sample were homogenized with 0.1 M anaerobic phosphate-buffered saline (PBS, pH 7.0) to prepare 20% (wt/vol) slurries in the stomacher bag. Large fecal residues were removed by passing the slurries through a 0.4 mm sieve and the slurries were then transported quickly to an AW 500 anaerobic chamber (Electrotek Ltd., Shipley, U.K.) for batch fermentation.

2.2. Batch fermentation of CSA

Batch fermentations were conducted under strict anaerobic conditions using a previously-described protocol [21,22]. CSA, purchased from Sangon Biotech Co. Ltd. (Shanghai, China), was added as the sole carbon source to growth medium VI. The molecular weight (Mw) and sulfate content of CSA are listed in Table 1. The Mw was determined by high performance liquid chromatography (HPLC) equipped with multi-angle laser light scattering system [23]. The sulfate content was determined by a turbidimetric method [24]. Growth medium VI contained the following (g/L): starch, 8.0, tryptone, 3.0; peptone, 3.0; yeast extract, 4.5; bile salts No. 3, 0.4; L-cysteine hydrochloride, 0.8; haemin, 0.05; NaCl, 4.5; KCl, 2.5; $MgCl_2{\cdot}6H_2O$, 0.45; $CaCl_2{\cdot}6H_2O$, 0.2; KH_2PO_4, 0.4; Tween 80, 1 mL and 2 mL of trace elements solution [21]. The medium was adjusted to pH 6.5 and CSA was added to a final concentration of 8 g/L before autoclaving. 45 mL of VI growth medium was inoculated with 5 mL of human fecal suspensions and then incubated at 37 °C in an anaerobic chamber. 3 mL samples of fermentation broth were removed regularly at 0 h, 24 h, 48 h, and 72 h for carbohydrate degradation analysis.

2.3. Thin layer chromatography (TLC)

The samples collected during fermentation were first centrifuged for 10 min at 8,000 × *g* at 4 °C to remove bacteria and other insoluble substances. Then, samples were analyzed by TLC to detect degradation end products [25]. Briefly, 0.2 μL of each sample was loaded onto a pre-coated silica gel-60 TLC aluminum plate (Merck, Darmstadt, Germany). The samples were resolved using solvent system containing formic acid/n-butanol/water (6:4:1, v/v/v). The resulting plate was completely immersed in orcinol reagent and then visualized by heating for 3 min at 120 °C.

2.4. Total carbohydrate analysis

The total carbohydrate concentration in the samples was determined using a previously described phenol-sulfuric acid method [25,26]. Analysis was performed in parallel and in triplicate. Results were expressed as the mean amount of carbohydrate remaining during fermentation, relative to the amount detected at 0 h.

2.5. Isolation of CSA-degrading bacteria

Two grams of fresh fecal samples were homogenized in stomacher bags with 0.1 M anaerobic phosphate-buffered saline (PBS, pH 7.0) to produce 20% (wt/vol) slurries. Then 5 mL of slurry was inoculated into 45 mL of VI growth medium containing 5 g/L of CSA. After incubation at 37 °C for 24 h in an anaerobic chamber, fermentation samples were spread onto CSA agar plates (basic growth medium VI plus 8 g CSA and 15 g agar) using a 10-fold dilution method. ~470 colonies were randomly picked from dilution plates and re-inoculated into CSA growth medium. CSA degradation was assessed using TLC analysis of the supernatant. Positive colonies were further segregated by repeating the 10-fold dilution method.

2.6. Characterization of CSA-degrading bacteria

The fecal isolates that were responsible for CSA degradation were further classified by 16S rRNA gene sequencing. In brief, genomic DNA was extracted using a Bacterial DNA Kit-200 (OMEGA Biotech Shanghai Co. Ltd., Shanghai, China) following the manufacturer's instructions. Then a pair of universal primers 27F (5′-CAGAGTTTGATCCTGGCT-3′) and 1492R (5′-AGGAGGTGATCCAGCCGCA-3′) were used to specifically amplify the 16S rRNA gene by PCR [27]. DNA sequencing was conducted by TSINGKE Biological Technology Co. Ltd. (Beijing, China). DNA sequences were aligned with the BLAST server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) of the National Centre for Biotechnology Information.

2.7. Degradation of CSA, CSC, CSD, and CSE by human fecal isolates

CSC was purchased from B&BIO Co. Ltd. (Qingdao, China). CSD and CSE were purchased from Rushan Wantongming Co. Ltd. (Weihai, China). CSA, CSC, CSD and CSE are different members from the CS family which have the same disaccharide composition and linkage mode but differ in sulfate position and sulfate content. The Mw and sulfate content of CSC, CSD and CSE were determined as previously described [23,24], and are listed in Table 1. The basic growth medium VI was supplemented with 8 g/L of CSA, CSC, CSD, and CSE and used for batch fermentation. Human fecal isolates that were capable of degrading CSA were inoculated into the growth media and incubated at 37 °C under anaerobiosis for 72 h. Meanwhile, *Bacteroides xylanisolvens* G25, a xylan-degrading bacterium, and *Bacteroides uniformis* L8, an AO-degrading bacterium, were also