Wound Medicine 26 (2019) 100169

Contents lists available at ScienceDirect

Wound Medicine

journal homepage: www.elsevier.com/locate/wndm

Original research article

In vitro investigation of antibacterial activity against fecal bacteria infecting wounds

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ARTICLE INFO

Keywords:
Fecal bacteria
Wound infection
Antibacterial activity
16S ribosomal DNA

ABSTRACT

Background: Fecal bacterial wound infection is common in both gastrointestinal surgery and decubitus ulcers. Various approaches have been developed to suppress bacterial growth. Antibacterial activity shown by the conventional assays using a few representative strains may not necessarily be similar to *in vitro* activity at infection sites.

Purpose: The purpose of the present study was to develop a simple and easy *in vitro* assay system to investigate antibacterial activity against fecal bacteria and to evaluate its validity.

Methods: The cultures contained Hank's balanced salt solution containing 10% fetal calf serum as a culture medium and were incubated at 37 °C with constant stirring in room air. Fecal bacterial growth was evaluated by several methods, including optical density at 600 nm, analysis of bacterial DNAs, real time-polymerase chain reaction, and terminal restriction fragment length polymorphism. Four methods were investigated for inhibiting fecal bacterial growth.

Results: Facultative anaerobes, including *E. coli*, *Enterococcus faecaliss,* and *Lactobacillus* species, increased after culture in contrast to the decrease in obligate anaerobes, *Clostridium coccoides*, *Clostridium leptum*, *Bactroides fragilis*, *Prevotella,* and *Bifidobacterium* species. The addition of 5 mM EDTA, activated carbons, maintenance of the medium pH at 4.4, and the combination of the above inhibited the increase in optical density.

Conclusion: This *in vitro* assay is easy to use, partly simulates fecal bacterial growth *in vivo*, and can be used to measure fecal bacterial growth inhibition.

1. Introduction

1.1. Roles of fecal bacteria in wound infections

Fecal bacteria, together with bacterial skin commensals, are the major source of bacteria in wound infections associated with gastrointestinal surgery. These infections include parastomal infection due to detachment of the intestinal wall from the skin, incisional wound infection close to the intestinal stoma, and perianal abscess. In addition, bedsores (decubitus/pressure ulcers), which develop on skin covering the bony areas of the hips and tail bones, have a high risk for fecal bacterial infection.

The wound infections listed above are due to daily attack from billions of fecal bacteria coming from the inevitable stool excretion. Repeated exposure to a large amount of bacteria and consequent infection cause a delay and/or failure of wound healing, septic complications, and death [1]. Therefore, local control of fecal bacterial infections is a research target with the goal of reducing bacterial loads and bacterial growth *in situ*.

1.2. Limitation of conventional assays to investigate antibacterial activity against fecal bacteria

Various approaches have been developed to suppress bacterial growth in infected wounds. Those include the use of silver because of its antibacterial activity [2,3], local application of antibiotics [4], and nanoparticle-based therapies against wound biofilm infection [5]. The detection of the presence or absence of antibacterial activity has been one of the essential goals in any novel approach.

Although several methods for evaluating antimicrobial activities

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https://doi.org/10.1016/j.wndm.2019.100169
Received 12 March 2019; Accepted 22 July 2019
Available online 24 July 2019
2213-9095/

have been established *in vitro* [6], those methods are growth inhibition assays using a few representative strains and media with or without agar specifically designed for bacterial culture. Those established assays are generally simple and reliable. However, those assay conditions obviously do not reflect the bacterial growth conditions *in vivo* in infected wounds. Furthermore, new methods that display growth inhibition of fecal bacteria, which are a mixture of many bacterial strains, may also not be fully representative of *in vivo* infected wounds. Complete physiological *in vitro* simulation of fecal infection sites is not possible. However, we defined several essential factors in fecal bacterial wound infections that could be important in mimicking the fecal bacterial wound environment. Such factors include the presence of fecal obligate and facultative anaerobes, culture under either aerobic or microaerobic conditions, interstitial fluids containing a certain amount of serum components, host immune and mesenchymal cells and derivatives from those cells, degraded tissue components, a small amount of digestive enzymes, bile, mucins, and food debris present in the stool.

The purpose of the present study was to develop a simple, easy, and more physiological *in vitro* assay system to investigate antibacterial activity against fecal bacteria and to evaluate its validity.

2. Materials and methods

2.1. Stool samples and preparation of fecal bacterial solutions

We obtained stool samples from 7 healthy volunteers (5 men and 2 women whose mean age was 30.1 years) under informed consent. The volunteers were free from any medications except one man was taking daily anti-hypertension drugs. The study was approved by the Ethics Committee of Tohoku University, Graduate School of Medicine.

Fecal samples were combined with 1 ml/100 mg of 0.1 M phosphate buffered saline (PBS) immediately after defecation, then mixed well with 2.5 mm glass beads (BIOSPEC PRODUCTS Inc., Bartlesville, USA) by vortexing at 2000 rpm for 10 min using a cell crusher (CD-100E, EYELA Co., Tokyo, Japan). After the centrifugation at 1000 rpm for 5 min, the supernatants were collected as bacterial solutions for further experiments.

2.2. Preliminary culture of fecal bacteria

Five μl of bacterial solutions from feces were added to 4 ml Hank's balanced salt solution (HBSS) (Invitrogen Japan, Tokyo, Japan) with or without 10% fetal calf serum (FCS) (Sanko Junyaku Co., Ltd., Tokyo, Japan). Two types of HBSS (with and without calcium and magnesium) were also used for the cultures. The bacterial solutions were incubated at 37 °C with constant stirring at 200 rpm for 24 h. After mixing 200μl of the indicated solutions, the cultures were transferred into the wells of a 96-well plate in duplicate, and then optical density (OD) at 600 nm was measured using an iMark microplate reader (Bio-Rad Laboratories Inc., Tokyo, Japan). Changes in OD were calculated after correction for the background OD before culture.

Alternatively, bacterial solutions from two fecal samples were diluted at 1:10,000 fold, and 100 μl of the diluted solutions were plated on HBSS agar plates containing 1.5% Bacto-Agar (Becton and Dickinson Japan Co., Tokyo, Japan) with or without 10% FCS. The plates were incubated for 24 h at 37 °C under aerobic conditions, then photographs were taken. HBSS agar plates were prepared by combining autoclaved 400 ml H_2O containing 1.85% (w/v) agar with 50 ml of pre-warmed 10X HBSS, containing either 50 ml FCS or water. Bacteria were picked from the colonies, smeared onto glass slides, Gram-stained, and then photographed using a BZ 8100 microscope (KEYENCE Japan, Osaka, Japan).

2.3. Analysis of the bacterial composition

It is important to be able to identify and quantify the bacteria at the species level in a simulation system for fecal bacterial wound infection.

2.3.1. Extraction of the bacterial DNA

Fecal bacterial solutions were added to 4 ml HBSS with 10% FCS and incubated at 37 °C for 3 days with constant stirring. After 3 days, 2 ml of the culture media were centrifuged at 20,000 *g* for 3 min, and the resulting pellet was dissolved in 100 μl of Tris-EDTA buffer (10 mM Tris − HCl, 1 mM EDTA). Five hundred μl of GES buffer containing 60% (w/v) guanidine thiocyanate (Wako Pure Chemical, Osaka, Japan), 0.1 M EDTA (Dojindo Laboratories, Kumamoto, Japan), and 0.2% Sarcosyl (Sigma, St. Louis, USA) were added and the cultures were maintained at room temperature for 10 min, and then mixed well with 0.5 mm glass beads (BIOSPEC PRODUCTS Inc.) using a cell crusher at 2500 rpm for 1 min. After the addition of 250 μl of 7.5 M ammonium acetate, the solution was mixed and left on ice for 10 min. Then, after the addition of 1 ml phenol-chloroform-isoamylalcohol (Nippon gene Co., Tokyo, Japan), the solution was mixed well and centrifuged at 13,000 *g* for 3 min. The resulting upper layer solution was carefully transferred to a new tube, mixed with 1 ml chloroform by turning the tube up-side down 50 times, and centrifuged. The upper layer was transferred to a new tube. This procedure was repeated one more time, and the DNA was precipitated with a half volume of isopropanol. The mixture was centrifuged at 20,000 *g* for 10 min, and the resulting pellet was washed with 70% ethanol twice, air-dried, and re-suspended in 100 μl of nuclease-free water (Takara Bio Inc., Ohtsu, Japan). Fecal bacterial DNAs were also extracted directly from bacterial solutions as day 0 samples. The DNA concentration was estimated from absorbance at 260 nm.

2.3.2. Real-time polymerase chain reaction (PCR) for quantification of bacteria

Duplicate samples of 10 ng bacterial DNA were used for 16S rRNA and *tuf* gene quantification with either SYBR Green Realtime PCR Master Mix (TOYOBO Co., Ltd., Osaka, Japan) or EagleTaq Master Mix with ROX (Roche Diagnostics Co., Tokyo, Japan) for *Lactobacillus* species) and an ABI 7500 Real-time PCR system (Applied Biosystems Japan, Tokyo, Japan) as previously described [7]. The amplification program consisted of 1 cycle at 95 °C for 1 min, 45 cycles at 95 °C for 15 s, 57 °C for 15 s, and 72 °C for 1 min. When *Lactobacillus* species were measured, the amplification profile was 1 cycle at 95 °C for 10 min, 45 cycles at 95 °C for 15 s, 60 °C for 1 min, and 72 °C for 1 s. The sequences of the primers used in this study are listed in Table 1. The copy number per microgram of bacterial DNA was calculated relative to plasmid DNA controls, and the median and percentile values in each group were evaluated.

2.3.3. Terminal restriction fragment length polymorphism (T-RFLP) analysis

T-RFLP was performed to allow the finger printing of the bacterial community by analyzing the number of dominant bacteria. Ten ng of bacterial DNA from 7 paired samples before and after culture with HBSS solutions containing 10% FCS were applied to a Phusion Bacterial profiling kit (FINZZYMES, Espoo, Finland) according to the manufacturer's protocol. Briefly, 10 μl reaction mixtures were added to the PCR reaction. PCR generated 16S rRNA amplified products labelled 5′- and 3′-ends with a different fluorescent color, respectively. Resulting products were digested with the endonuclease solution (MT1), then denatured with formamide, combined with 0.5 μl GeneScan 1200 LIZ Size Standard (Applied Biosystems), and analyzed with an ABI PRISM 3100 genetic analyzer (Applied Biosystems) in GeneScan mode. We counted the number of peaks larger than that of the 400 bp marker in GeneScan 1200 LIZ Size Standard.

2.3.4. Test for survival of anaerobic bacteria

When we cultured fecal bacteria in HBSS containing 10% FCS with constant stirring in room air, it was important to investigate whether

Table 1
Primer and probe sets used in this study.

Target bacterial group	Target gene	Primer sequence	Amplicon size (bp)	Reference
All eubacteria	**16SrRNA**	TCCTACGGGAGGCAGCAGT GGACTACCAGGGTATCTATCCTGTT	**466**	[15]
Esherichia coli	**16SrRNA**	GACCTCGGTTTAGTTCACAGA CACACGCTGACGCTGACCA	**585**	[16]
***Enterococcus* species**	**16SrRNA**	CCCTTATTGTTAGTTGCCATCATT ACTCGTTGTACTTCCCATTGT	**144**	[17]
Enterococcus faecalis	**16SrRNA**	AACCTACCCATCAGAGGG GACGTTCAGTTACTAACG	**358**	[18]
***Streptococcus* group**	***Tuf***	GAAGAATTGCTTGAATTGGTTGAA GGACGGTAGTTGTTGAAGAATGG	**560**	[19]
***Staphylococcus* species**	**16SrRNA**	ACGGTCTTGCTGTCACTTATA TACACATATGTTCTTCCCTAATAA	**257**	[20]
***Lactobacillus* species**	***Tuf***	TACATYCCAACHCCAGAACG AAGCAACAGTACCACGACCA	**71**	[21]
	Probe	FAM- AAGCAACAGTACCACGACCA-TAMRA		
***Clostridium coccoides* group**	**16SrRNA**	AAATGACGGTACCTGACTAA CTTTGAGTTTCATTCTTGCGAA	**440**	[22]
***Clostridium leptum* subgroup**	**16SrRNA**	GCACAAGCAGTGGAGT CTTCCTCCGTTTTGTCAA	**239**	[22]
***Bacteroides fragilis* group**	**16SrRNA**	ATAGCCTTTCGAAAGRAAGAT CCAGTATCAACTGCAATTTTA	**495**	[22]
***Atopobium* cluster**	**16SrRNA**	GGGTTGAGAGACCGACC CGGRGCTTCTTCTGCAGG	**190**	[22]
Prevotella	**16SrRNA**	CACRGTAAACGATGGATGCC GGTCGGGTTGCAGACC	**513**	[22]
Bifidobacterium	**16SrRNA**	CTCCTGGAAACGGGTGG GGTGTTCTTCCCGATATCTACA	**550**	[22]
***Desulfovibrio* species**	**16SrRNA**	CCGTAGATATCTGGAGGAACATCA ACATCTAGCATCCATCGTTTACAGC	**135**	[23]

anaerobic bacteria survived or eventually died. After 9 days of culture, 20 µl of the culture solution were spread on the selection plates for *Bacteroides* (Bacteroides Agar "Nissui", Nissui Pharmaceutical Co., Tokyo, Japan) and cultured in an anaerobic box with AnaeroPack-Anaero (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan) for 24 h at 37 °C.

Bacteria picked from colonies were suspended in 200 µl of TE buffer in microfuge tubes and subjected to 10 min of boiling. Immediately after boiling, the microfuge tubes were centrifuged for 5 min at 15,000 rpm at room temperature.

Two µl of the supernatants was combined with 3.125 units Taq polymerase (*Takara Taq* Hot Start Version, Takara Bio Inc.), 1X LA PCR buffer II supplied by the manufacturer, 0.2 mM dNTP, and the primer set specific for *B. fragilis* 16S rDNA (Table 1). The reaction mixtures were incubated in a thermal cycler (G-Storm 482, Gene Technologies Ltd., Essex, England). The amplification profile consisted of an initial denaturation at 94 °C for 2 min, then 35 cycles of denaturation at 94 °C for 30 s, annealing at 59 °C for 30 s, and extension at 72 °C for 30 s with final extension at 72 °C for 5 min. The resulting products were electrophoresed on a 1% agarose gel, visualized by ethidium bromide staining, and photographed.

2.4. Validation of the culture method for inhibition of fecal bacterial growth

Inhibition of bacterial growth is essential for the local management of infected wounds. We evaluated 4 compounds/conditions that were added at the start of the cultures to validate the use of the cultures to test for inhibition of fecal bacterial growth. These inhibitors included 5 mM EDTA, 200 mg dry weight activated carbons (Wako Pure Chemical), 10 mM acetic acid buffer to maintain pH at 4.4, or all of the above. Activated carbons were washed twice with distilled water, autoclaved, saturated with HBSS at 4 °C overnight, then 200 mg dry weight of charcoal were added to the cultures. After culture with constant stirring at 37 °C in room air, the ODs at 600 nm were measured, and changes in the ODs were calculated.

2.5. Statistical analysis

Data are presented as median and percentile values within each group. Kruskal-Wallis rank test was used to determine if there was a significant correlation among the groups. Mann–Whitney *U* test was used to compare two groups, with significance at $P < 0.05$.

3. Results

3.1. Fecal bacteria grew in HBSS containing 10% FCS

The ODs minimally increased in HBSS without FCS. When HBSS containing 10% FCS was used, the ODs dramatically increased, indicating that fecal bacteria grew well in this solution (Fig. 1). Comparable OD values were observed in cultures with 20% FCS (data not shown). The addition of calcium and magnesium to the HBSS did not change the OD patterns.

Better bacterial growth in 10% FCS-containing HBSS was confirmed in agar plates (Fig. 2). Colonies more than 1 mm in size were very limited in the HBSS agar plate. In contrast, several types of colonies with more than 1 mm diameter were detected on the agar plates with HBSS containing 10% FCS, and a yellowish color change was observed, suggesting pH changes from neutral (pink) to acidic (yellow) values. Gram staining demonstrated that those colonies included gram-negative cocci, gram-positive cocci, gram-negative coccobacilli, and gram-negative bacilli (Fig. 3).

3.2. Molecular analysis of the bacterial composition after culture with HBSS containing 10% FCS

The bacterial composition was compared before and after 3 days of culture. As expected, most facultative anaerobes, including *E. coli*, *Enterococcus faecalis,* and *Lactobacillus* species, significantly increased (Fig. 4). In contrast, obligate anaerobes, including *Clostridium coccoides*, *Clostridium leptum*, *Bactroides fragilis*, *Prevotella,* and *Bifidobacterium* species, decreased.

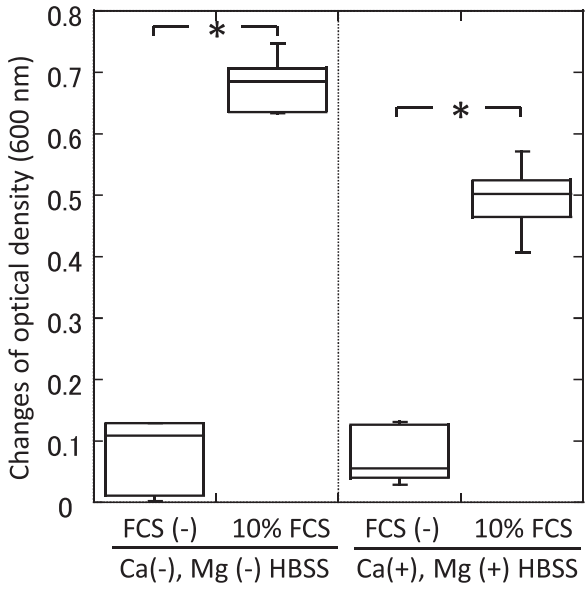

Fig. 1. Preliminary culture of fecal bacteria with or without FCS. Fecal bacteria grew well in the presence of 10% FCS regardless of the presence or absence of calcium and magnesium. The data are presented as a box plot from 5 independent experiments. FCS: Fetal calf serum, HBSS: Hank's balanced salt solution. Asterisks indicate statistical significance ($p < 0.05$).

Using T-RFLP analyses, the number of peaks from each display was counted. The number of 5′- and 3′-peaks decreased after 3 days of culture with statistical significance observed for the 5′-peaks (Fig. 5)

We investigated whether an obligate bacteria, *B. fragiilis*, was still viable and capable of growth after 9 days of culture. Thousands of colonies were observed on the Bacteroides Agar plate after 24 h, and we confirmed that 7 out of 8 colonies were *B. fragilis* by detection of *B. fragilis*-specific 16S rDNA (Fig. 6).

3.3. Validation of the culture protocol for testing inhibition of fecal bacterial growth

All treatments resulted in a decreased OD, suggesting the inhibition of fecal bacterial growth as compared to that of the controls (Fig. 7). In the treatments with EDTA, pH at 4.4, and the activated carbons, the decreased OD changes were the most remarkable when 5 mM EDTA was added into the culture solution.

4. Discussion

The normal phases of wound healing involve inflammation, proliferation/regeneration, and remodeling, and generally proceed without difficulty in normal physiological conditions. Feces contains a large amount of bacteria, often reaching 10^{11}/gram [8]. Since bacteria and their products (*e.g.*, endotoxins, metalloproteinases) each impair the healing process, bacterial contamination of wounds should be minimized. Although systemic antibiotic therapies are powerful and constitute the gold standard to prevent disease progression, *in situ* inhibition of bacterial growth has increasing significance because of the emergence and expansion of antibiotic-resistant bacteria. A method that evaluates antibacterial activity *in vitro* is essential to develop better ways to detect such bacteria in wounds and to control infected wounds.

4.1. Fecal bacteria grow in HBSS containing 10% FCS

All wounds exposed to feces should be considered contaminated without exception. Once fecal bacteria colonize a wound, the bacteria must obtain nutrients from the infection sites to replicate themselves [9]. We speculated that both the components in the medium and the aerobic or anaerobic conditions were critical for the survival and growth of fecal bacteria. Bacterial culture media which are nutrient-rich and designed for rapid growth of bacteria are very different from the nutrient conditions of infected wounds.

We selected HBSS containing 10% FCS as a culture medium. HBSS is composed of ion formulations similar to those of tissue interstitial fluid,

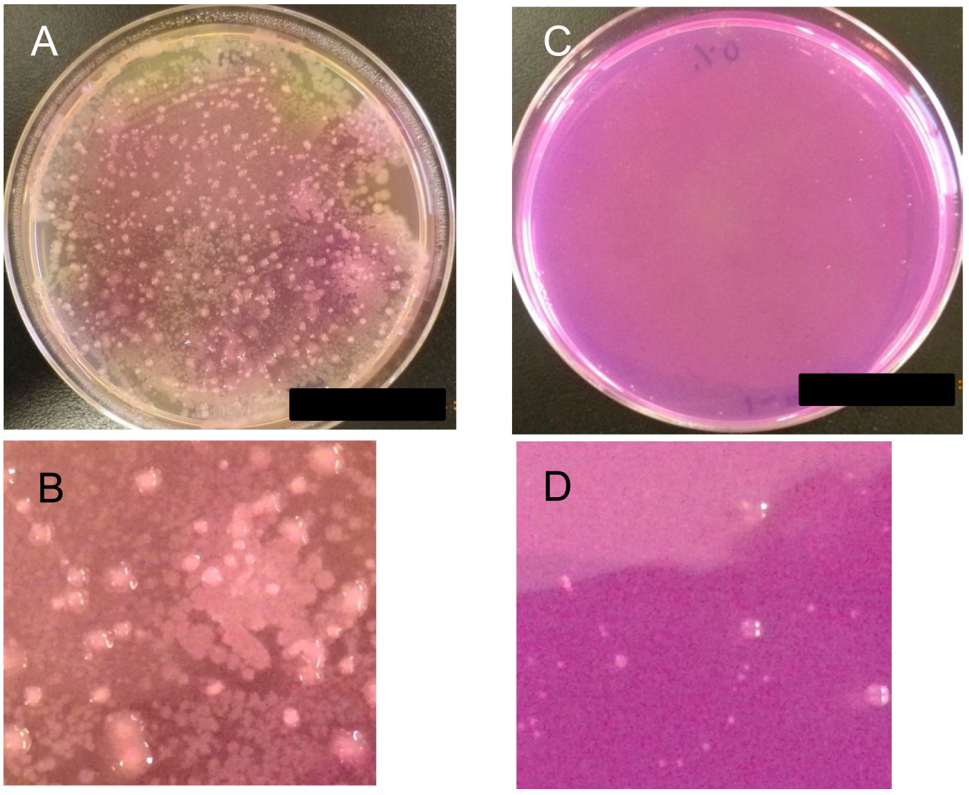

Fig. 2. Bacterial growth on the HBSS agar plates with (A, B) and without (C, D) 10% FCS under aerobic conditions. B and D are magnified pictures. Note that the color of the medium in A and B is yellowish in contrast to the pink color in C and D, suggesting a decrease in the pH in the plate with 10% FCS. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

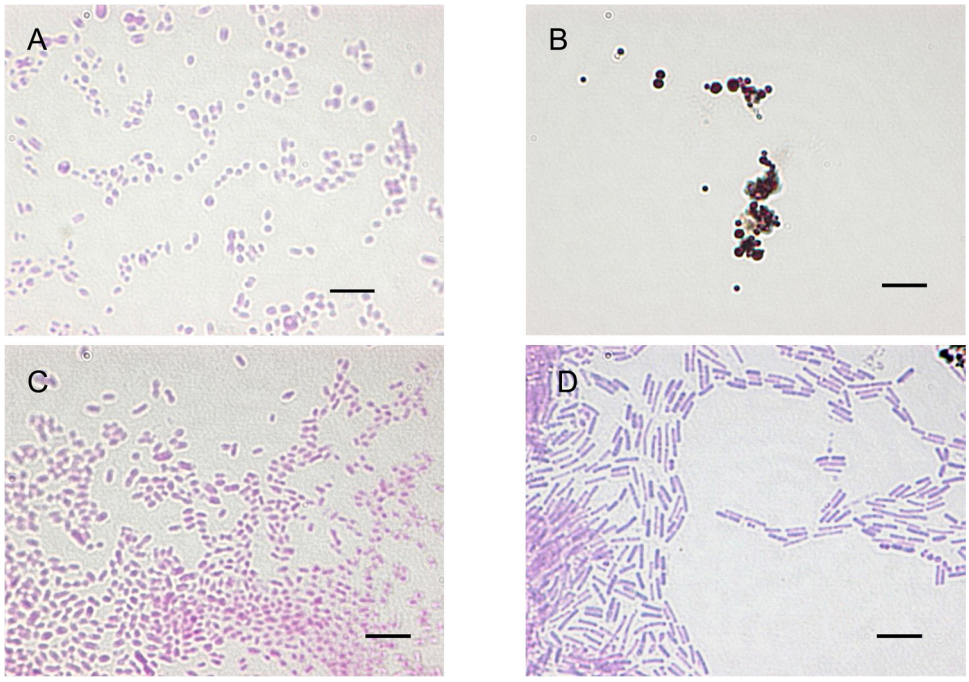

Fig. 3. Gram staining of the bacteria growing on the HBSS agar plate with 10% FCS. A. Gram-negative cocci, B. Gram-positive cocci, C. Gram-negative coccobacilli, D. Gram-negative bacilli. Bars indicate 10 μm.

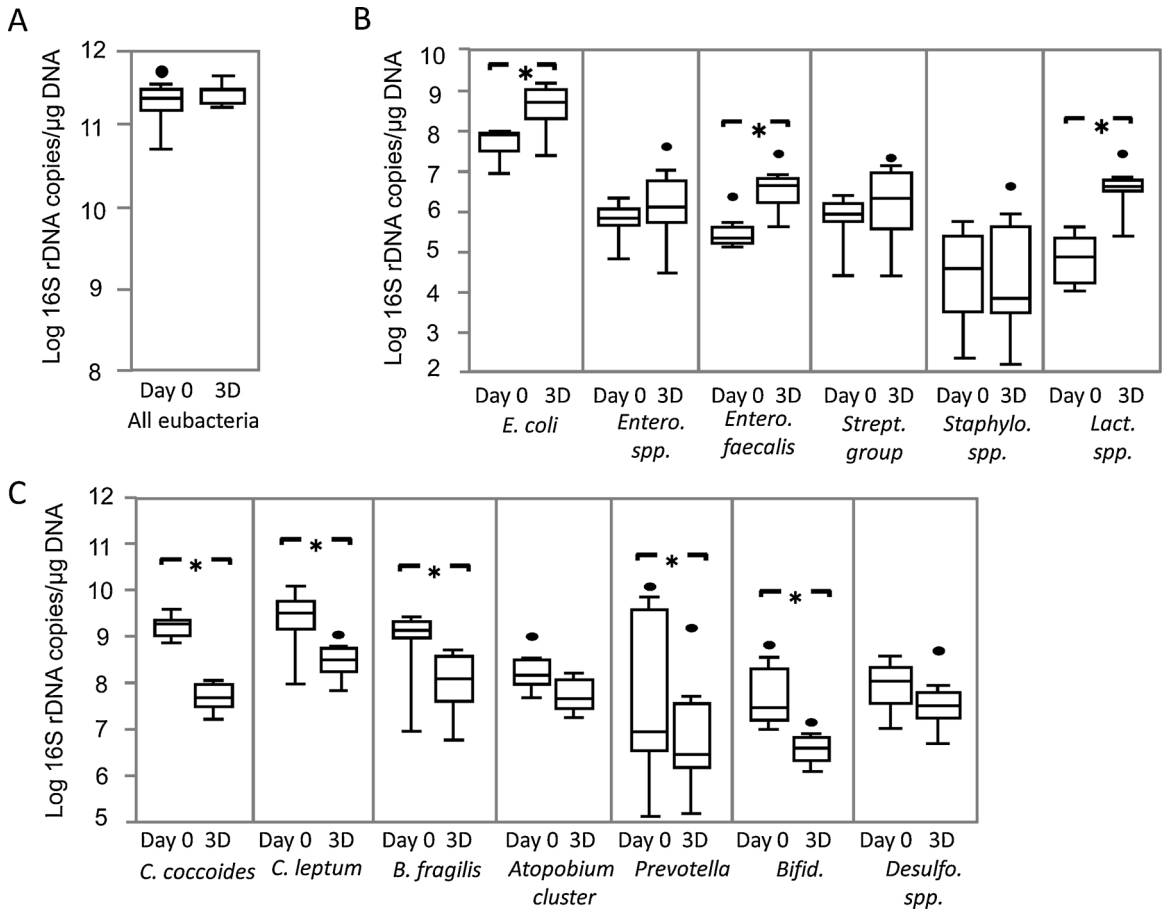

Fig. 4. Quantification of bacteria after 3 days in culture in HBSS containing 10% FCS. The data from each group are presented as a box plot (n = 7). A. Eubacteria. B. Facultative anaerobes. C. Obligate anaerobes. Entero.: *Enterococcus*, Strept.: *Streptococcus*, Staphylo.: *Stahylococcus*, Lact.: *Lactobacillus*, Desulfo.: *Desulfovibrio*. 0D: Before starting culture, 3D: After 3 days of culture.

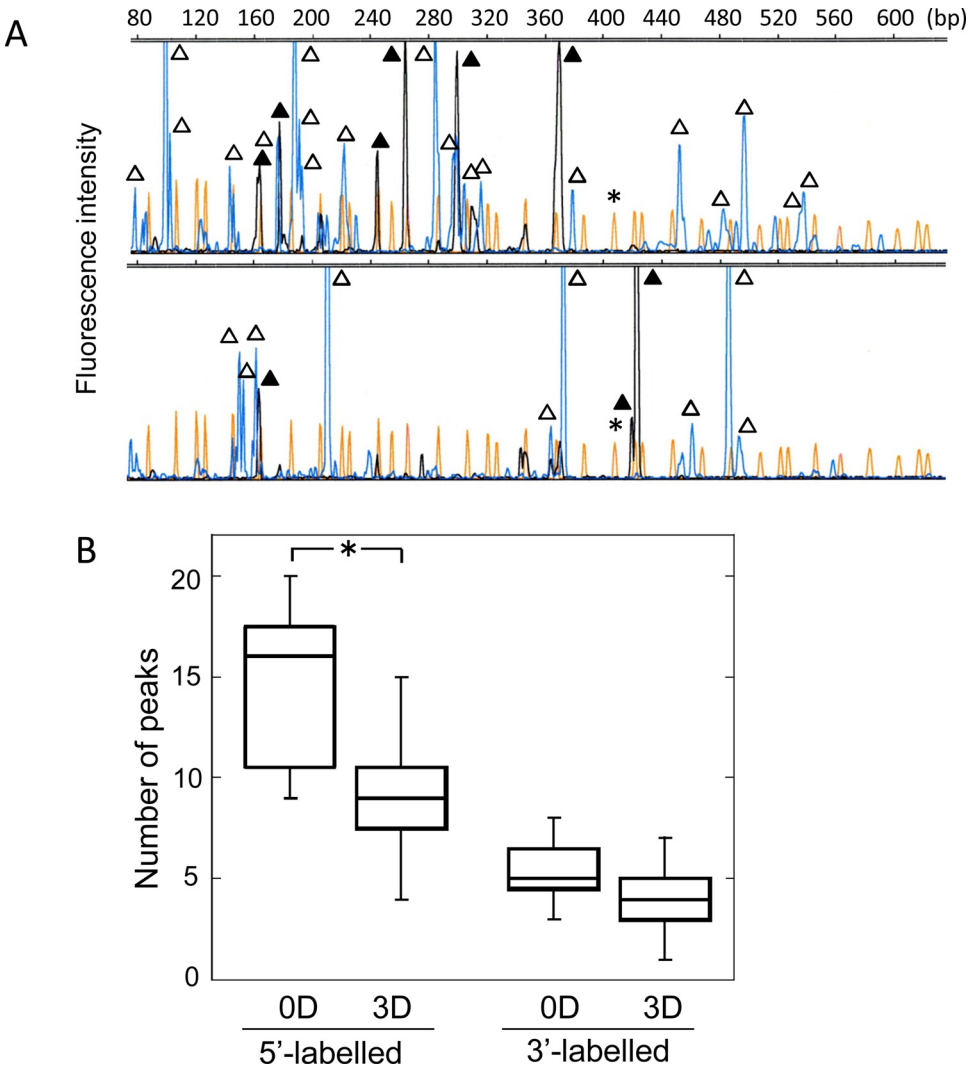

Fig. 5. A. T-RFLP patterns of DNA before (*upper panel*) and after 3 days (*lower panel*) of culture with HBSS containing 10% FCS. The horizontal axis indicates the relative length of DNA fragments (bp). The vertical axis indicates the relative fluorescence intensity. Blue (marked with open triangles) and black peaks (marked with closed triangles) indicate 5′-labeled or 3′-labeded fragments, respectively. Yellow peaks indicate molecular weight markers, GeneScan 1200 LIZ Size Standard. Asterisks indicate the 400 bp marker fragments, which are the standard of peak magnitude. B. Number of peaks before and after 3 days of culture in HBSS containing 10% FCS. An asterisk indicates statistical significance ($p < 0.05$). The data from each group are presented as a box plot ($n = 7$).0D: Before starting culture, 3D: Culture for 3 days (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

HBSS is widely used for cell isolation from a variety of tissues. FCS was added as a serum component because it probably leaks from blood capillaries at wound sites. Fibroblasts, neutrophils, macrophages, and lymphocytes are cellular components found in the microenvironment of infected wounds. A co-culture system of fecal bacteria with those cells is feasible because those cells are viable in HBSS with 10% FCS for several hours.

More than two-thirds of the fecal bacteria so far have been found to be difficult to cultivate. Therefore, it is not surprising that only a part of fecal bacteria, in particular, facultative anaerobes could grow in HBSS containing 10% FCS. All obligate anaerobes examined decreased but were measurable after 3 days of culture, indicating that obligate anaerobes might be alive but not able to proliferate. Alternatively, those bacteria died and their DNAs were not degraded. We found that *B. fragilis* could grow even after 9 days of culture when the bacteria in the resultant culture solution was incubated under anaerobic conditions. This result supports the former idea that the bacteria are alive but not proliferating and indicates that oxygenation of the media was not necessarily complete by the constant stirring at 200 rpm. The oxygenation in infected wounds appears to be variable and partly insufficient due to the degree of exposure to room air and to the blood supply to tissues.

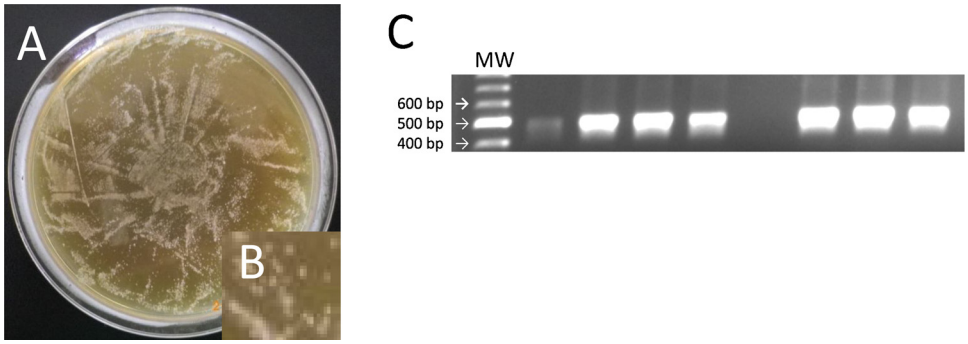

Fig. 6. A. Bacterial growth on the plate, Bacteroides Agar "Nissui". Fecal bacteria were cultured in HBSS containing 10% FCS with constant stirring for 9 days in room air. Then, 20 μl of cultured solution were seeded on the plates and incubated overnight under anaerobic conditions. B. Magnified picture. C. 16SrRNA gene specific for *B. fragilis* was detected by PCR in 7 out of 8 colonies from the plate, Bacteroides Agar "Nissui". MW: Molecular weight.

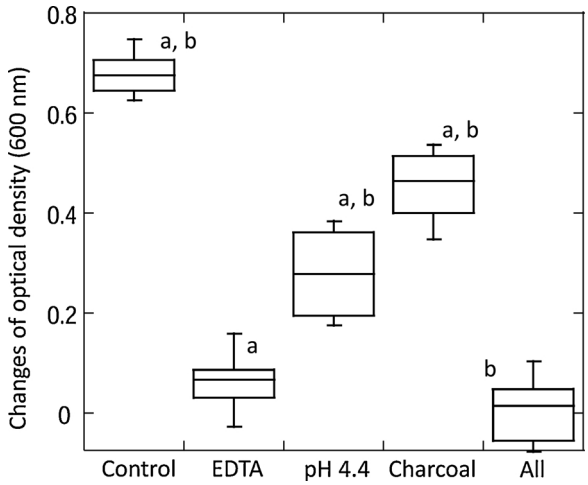

Fig. 7. Inhibition of bacterial growth by either EDTA, charcoal, or low pH. Control: Fecal bacteria was cultured in HBSS containing 10% FBSS for 24 h. EDTA: EDTA was added to the solution at a concentration of 5 mM. pH 4.4: The solution pH was maintained at 4.4 by 10 mM acetic acid buffer. Charcoal: Twenty mg (dry weight) charcoal was added to the solution. All: Fecal bacteria were cultured in HBSS containing 10% FCS, 5 mM EDTA, and 20 mg charcoal at pH 4.4. The data are presented as a box plot from 5 independent experiments.The letter a or b indicates significant difference ($p < 0.05$) from each other, respectively.

4.2. Bacterial identification and quantification

In the present study, we utilized molecular approaches to both identify and quantify bacteria before and after culture because the conventional culture or biochemical methods are time-consuming and laborious. Recent PCR-based molecular approaches, such as 16S ribosomal DNA amplification with group- or species-specific primers, T-RFLP analysis, and pyrosequencing of 16S rDNA amplicons, are rapid and sufficiently sensitive [10].

4.3. Application and limitation of the present culture method to develop a novel inhibition assay for detecting fecal bacteria in wounds

The present *in vitro* culture differs from the microenvironment of fecal bacterial-infected wounds. However, this approach has several advantages over other assays to investigate possible antibacterial activities against fecal bacteria. The conventional methods usually examine the presence or absence of antibacterial activity against a representative strain. In contrast, the present culture method evaluates all of the fecal bacteria. Because the bacterial growth rate depends on the genetic background, the physiological history of the bacteria, and on the nutrients in the growth medium [11], researchers must consider that the medium composition, level of oxygen, and bacteria-bacteria interactions significantly influence bacterial growth. Our method fulfills some but not all of these *in vivo* factors.

We evaluated four conditions of fecal bacterial growth inhibition, including the addition of 5 mM EDTA, activated carbons into the culture solutions, maintenance of medium pH at 4.4, or all of the above together. Iron is required for the growth by most bacteria [12]. Other trace minerals are also required for the activation and synthesis of bacterial enzymes, and deficiency leads to the impairment of bacterial growth. EDTA sequesters metal ions in aqueous solution and has been predicted to reduce bacterial growth [13] Activated carbons adhere to bacteria and decrease their viability to some extent [14]. The effect of medium pH changes during the culture period varies due to both the growth rate and type of bacteria. The maintenance of pH at 4.4 inhibits the growth of neutrophils and alkaliphiles. We successfully used this culture method to observe the inhibition of fecal bacterial growth. Further studies are needed to continue with the development of new dressing materials that reduce or prevent bacterial growth.

5. Conclusion

Fecal bacterial wound infection is common in gastrointestinal surgery and decubitus ulcers. We propose a new more physiological *in vitro* culture method to investigate possible antibacterial activities against fecal bacteria. The method involves HBSS containing 10% FCS, liquid culture under aerobic conditions, and molecular detection of bacteria. This method is useful for the screening of antibacterial agents and may lead to the development of a new dressing material and better management of fecal bacterial wound infection.

Ethical statement

The present study was approved by the Ethics Committee of Tohoku University, Graduate School of Medicine (2013-1-556).

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Tohoku University

Funding source

This work was supported by a research grant from the Japanese Society of Stoma and Continence Rehabilitation (to K.F.).

Declaration of Competing Interest

All authors declare no conflict of interest.

Acknowledgements

Some of the data were generated using the facilities at the Biomedical Research Core of Tohoku University Graduate School of Medicine. This work was supported by a research grant from the Japanese Society of Stoma and Continence Rehabilitation (to K.F.).

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