

## RESEARCH ARTICLE

## Butyric Acid Bacteria Culture Solution Improves Hyperglycemia in Alloxan-Induced Diabetes Mellitus Rats

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### Abstract

Butyric acid bacteria (BAB) are the primary intestinal flora present in all mammalian digestive tracts. Prior studies have found the association between decreased intestinal BAB population and development of diabetes, and BAB was suggested as a new treatment of type 2 diabetes. However, few studies have examined the effect of BAB on type 1 diabetes (DM1), which is frequently diagnosed in pet animals. Therefore, the aim of this study was to examine the therapeutic effects of BAB culture solution in the DM1 model. Thirty female rats were included for induction of DM1 by alloxan (200 mg/kg, IP). After one week, DM1 was developed in 13 rats (blood glucose level >300 mg/dL) which were then treated with BAB culture solution at a dose rate of 300 µL/kg/day for two weeks. The result revealed a reduction in blood glucose level (P<0.05) and improvement of polydipsia and polyuria in six diabetic rats; meanwhile, 7 rats did not respond to the treatment. The blood pressure showed no change. In conclusion, the administration of BAB culture solution alleviates symptoms of DM1 by improving glycemic control in the model. The effectiveness of BAB as an alternative or supportive therapy for the treatment of DM1 needs further studies on pet animals.

**Keywords:** Alloxan, Butyric acid bacteria, Hyperglycemia, Rat diabetes model

## Bütirik Asit Bakteri Kültür Solüsyonu Alloksan İle Diabetes Mellitus Oluşturulmuş Ratlarda Hiperglisemi İyileştirir

### Öz

Bütirik asit bakterileri (BAB), tüm memeli sindirim sistemlerinde bulunan birincil bağırsak florası etkenleridir. Önceki çalışmalar, azalan bağırsak BAB popülasyonu ile diyabet gelişimi arasındaki ilişkiyi saptamışlar ve BAB, tip 2 diyabetin yeni bir tedavisi olarak önerilmiştir. Bununla birlikte, BAB'nin, evcil hayvanlarda sıklıkla teşhis edilen tip 1 diyabet (DM1) üzerine etkisini inceleyen az sayıda çalışma mevcuttur. Bu nedenle, bu çalışmada, DM1 modelinde BAB kültürü solüsyonunun terapötik etkilerinin incelenmesi amaçlanmıştır. Çalışmada, alloksan (200 mg/kg, IP) ile DM1 oluşturulması için otuz dişi rat kullanılmıştır. Alloksan uygulamasından bir hafta sonra 13 ratta DM1 oluşturulmuş (kan şekeri seviyesi >300 mg/dL) ve sonraki iki hafta boyunca bu ratlar 300 µL/kg/gün dozu ile BAB kültür solüsyonu ile tedavi edilmiştir. Tedavi sonrası, diyabetik 6 ratta kan glukoz seviyesinde bir azalma (P<0.05) ve polidipsi ve poliüri tablolarında iyileşme saptanırken, 7 rat tedaviye yanıt vermemiştir. Kan basıncında bir değişiklik görülmemiştir. Sonuç olarak, BAB kültür solüsyonunun uygulanması, modeldeki glisemik kontrolü iyileştirerek DM1 semptomlarını hafifletmektedir. DM1 tedavisinde alternatif veya destekleyici bir terapi olarak BAB'nin etkinliği, evcil hayvanlarda yapılacak daha fazla çalışmaya ihtiyaç duymaktadır.

**Anahtar sözcükler:** Alloxan, Butirik asit bakterileri, Hiperglisemi, Rat diyabet modeli

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## INTRODUCTION

Populations of butyrate-producing bacteria, such as *Clostridium butyricum*, a gram-positive spore bacillus, are recognized as important intestinal bacteria which possess great health benefits [1,2]. Because of the spore forming characteristic of *C. butyricum*, it can be utilized when administered orally for therapeutic applications as it can pass through stomach to the intestine without degradation by the gastric acid [3]. Various studies have demonstrated the treatment benefits of BAB. In this regard, *C. butyricum* administration normalizes intestinal flora [4] and inhibits the activity of some pathogenic bacteria [5,6]. In humans, commercialized products containing BAB are available as nutritional supplements, which tout probiotic effects [7]. In the dairy industry, butyrate-producing bacteria are administered to livestock to improve intestinal healing and enhance appetite [8,9]. In broiler chickens, butyrate products are known to improve levels of hepatic enzymes and uric acid [10].

Recently, BAB culture solution, unlike the existing *C. butyricum* products, containing various contents such as BABs, produced-butyric acid and BAB culturing environment, have been developed which is expected to have the potential to normalize the intestinal environment after pancreatic disorder. The BAB fermentation was effective in suppressing the growth of pathogenic microorganisms, such as *E. coli*, *S. aureus* (ATCC25923), *C. difficile* (ITO) and *C. sporogenes* (GAI95048) [5,6]. After culturing, vitamins (B2, B6, B12 and C) and amino acids (Alanine, Valine,  $\beta$ -Alanine and  $\gamma$ -Aminobutyric acid) were produced with BAB fermentation (non published data). Administration of BAB culture solution was also effective in the treatment of atopic dermatitis and was suggested to decrease mast cell population and mitigation of allergic symptom in mice models (non published data).

Diabetes mellitus (DM) is a common ailment in small animal veterinary medicine, and the only available treatment modality is glycemic management with insulin. Home monitoring of DM, particularly measuring blood glucose, is often difficult for small patients such as hamsters and ferrets, so dietary treatments play a major role in management of diabetic complications. Recently, intestinal flora, including butyric-producing bacteria, was reported to improve lifestyle diseases such as metabolic disorders and obesity in human patients [4,11-13]. A moderate degree of gut bacterial disorder has been reported in type 2 DM, in which the usefulness of butyrate-producing bacteria in metabolism is decreased concomitantly with increasing number of opportunistic pathogens [11,12]. In a study of obese mice, *C. butyricum* inhibited progression of metabolic syndrome to type 2 DM and further improved insulin resistance by reducing cytokine production characteristic of diabetes onset [14]. Furthermore, *C. butyricum* alleviated hyperglycemia in type 2 DM mice [15]. Notably, previous

studies identified the BAB therapeutic approach as a new treatment modality for type 2 DM through in depth studies of the anti-inflammatory and antioxidant effects of *C. butyricum* and its associated butyric acid [15]. Most of the studies of BAB for DM used spontaneous animal model and were focused on suppressing the onset of hyperglycemia [1]. Until now, no prior study highlighting the efficacy of BAB culture solution in alleviating the hyperglycemia in type 1 DM, an irreversible pancreatic disorder, in a small animal clinical veterinary setting has been reported. Therefore, the aim of the present study is to evaluate whether oral administration of BAB culture solution controls hyperglycemic state in alloxan (ALX)-induced DM1 rat model.

## MATERIAL AND METHODS

### Ethical Statement

This study was conducted with pre-approval from the ethical committee to use experimental animals in Tokyo University of Agriculture and Technology (approval number: 29-74).

### Animals

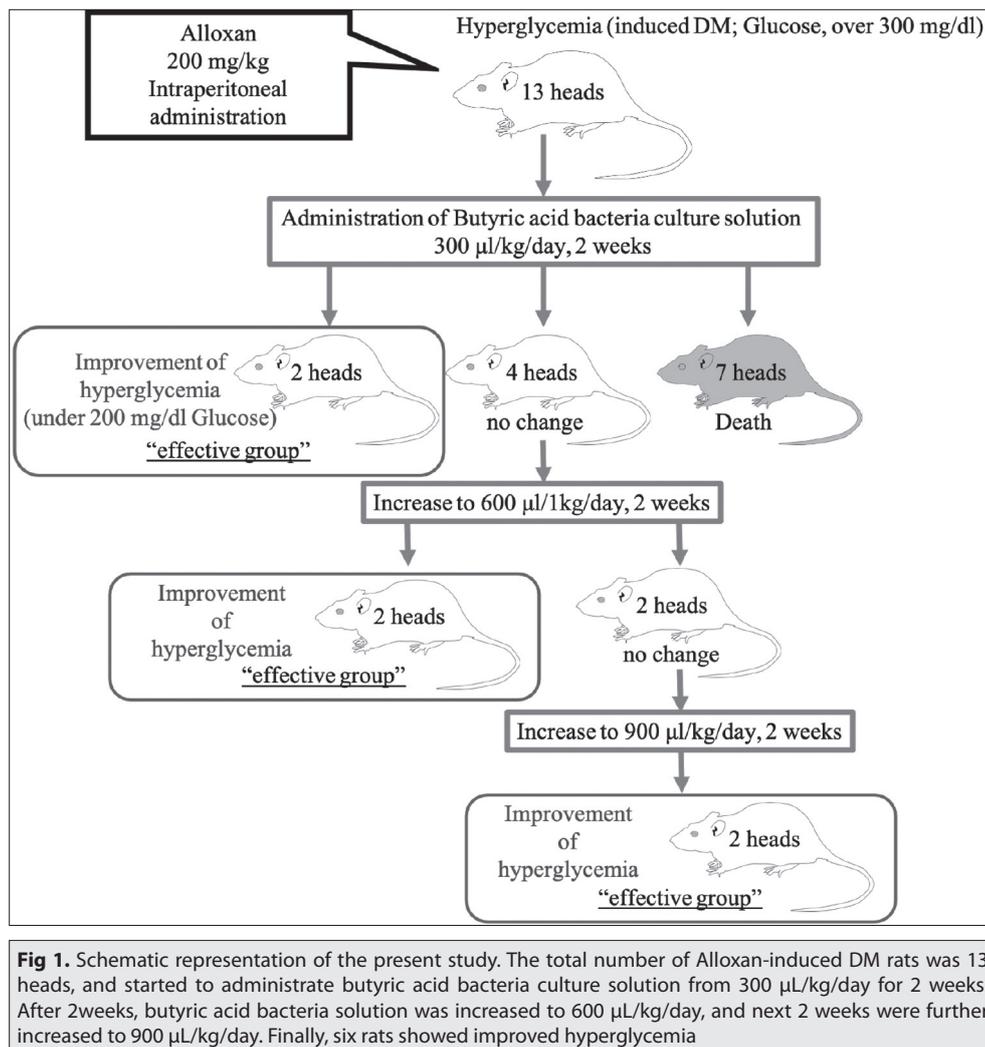
Three-months-old, 30-Sprague-Dawley female rats (CHARLES RIVER LABORATORIES JAPAN, INC., Japan) were bred in-house. The average body weight of 30 rats was 379.31 g. All rats were cared under the same management conditions. Animals were single-housed, identified based on cage number, and maintained under *ad libitum* feeding and a 12 h light/dark cycle. The used diet was supplied by a commercial company (Oriental yeast CO., LTD., Tokyo, Japan) which provides energy and protein levels of 359 kcal/100g and 23.1 g/100g, respectively.

### Induction of DM and Administration

Figure 1 shows the schema of the present study. DM was induced by alloxan injection (ALX; Alloxan Monohydrate, Tokyo Chemical Industry CO., LTD., Japan; 200 mg/kg IP, dissolved in saline) [16]. One week after injection, rats exceeding blood glucose level of 300 mg/dL were used as diabetic rats for subsequent experiments [17] and were started with the administration of BAB culture solution.

### Administration of Butyric Acid Bacteria Culture Solution for Treatment of DM1

Butyric acid bacteria culture solution (ACE BIO PRODUCT CO., Japan) was administered by mixing with the drinking water for an estimated dosage of 300  $\mu$ L/kg/day for two weeks. When this dosage did not significantly affect the blood glucose level, the dosage was increased to double and then to triple the initial dose for the preceding two weeks. The dosage of BAB was considered effective when blood glucose level became <200 mg/dL and the experiment was terminated.



### Blood Sampling and Blood Glucose Monitoring

Blood samples were collected prior to DM induction and every week thereafter. Conscious rats were placed in a holding tool for blood pressure measurement (Rat holder M/L, Muromachi Kikai CO., Ltd., Japan). While the animals were restrained, a small amount of blood was sampled from the tail vein using a 1 mL syringe (TOP Corporation, Japan) and 30G needle (Becton, Dickinson and Company, Japan). For measurement of blood glucose level, blood was dropped onto the blood glucose monitoring system (Glucose PILOT, technicon internal Inc., Japan).

### Blood Pressure Measurement

Blood pressure measurement was conducted before and after DM induction and at the end of the experiment. Rats were placed in the holding tool for blood pressure measurement, and measurement was performed on the tail using a rodent blood pressure measurement device (BP MONITOR FOR RATS & MICE Model MK-2000, Muromachi Kikai CO., Ltd., Japan). After the pulse pressure waveform stabilized, data acquisition was conducted five times for each animal. Systolic, diastolic and mean blood pressure were recorded.

### Statistical Analysis

Sample size was determined based on the outcomes and calculation performed with the G\*Power 3.1.9.2 software (University Kiel, Germany, 1992-2014)<sup>[18]</sup>, assuming a moderate effect of BAB culture solution on DM1 rat model according to Cohen<sup>[19]</sup> with 0.37 effect size. The data were categorized as before induction of DM (negative control, NC), after induction of DM1 (ALX-DM), and after administration of BAB culture solution (BAB). Data were expressed as mean  $\pm$  standard deviation through one-way ANOVA analysis and a  $P < 0.05$  was considered statistically significant. Each data figure was prepared using software for statistical analysis (GraphPad Prism, version 5.0a, GraphPad Software, USA).

## RESULTS

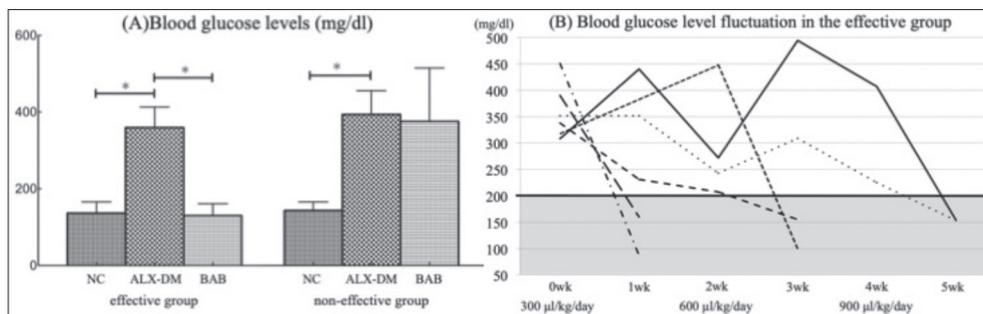
### Induction of Type 1 DM

A week after the ALX injection, 13 rats out of 30 (43.3%) developed DM1 as indicated by elevation of the blood glucose (BG) level. After ALX injection, the BG was significantly increased from the basal level ( $P < 0.05$ ). These

**Table 1.** Fluctuation of blood glucose levels of individual animals in the effective group

Parameter	NC	ALX-DM	BAB
Blood glucose level (mg/dL)	140	318	100
	82	338	126
	149	451	87
	152	390	160
	166	352	153
	131	308	154
<b>Average</b>	136.7	359.5*	130.0**

Before alloxan-injection is expressed as NC (negative control), and ALX-DM indicates one week after alloxan-injection. BAB means post administration of BAB culture solution. Significant differences are indicated by \* (vs. NC,  $P < 0.05$ ) and \*\* (vs. ALX-DM,  $P < 0.05$ )



**Fig 2.** Changes (A) and fluctuation (B) in blood glucose level. Blood glucose level was significantly increased to over 300 mg/dL after ALX injection (ALX-DM). This significant increase of blood glucose level suggested that induction of diabetes mellitus was successful. Blood glucose level after administration of butyric acid bacteria culture solution was significantly decreased (BAB)

rats were ultimately included in the analysis as type 1 DM model.

### BAB Alleviates Hyperglycemia in Type 1 DM

The BAB culture solution was administered daily at an initial dose of 300 µL/kg/day followed by duplication of doses in non-responsive rats. Table 1 shows fluctuations in blood glucose level in the effective group. Among thirteen DM1 rats, only six rats showed significant decrease in blood glucose level after administration of BAB culture solution and were grouped as the “effective group” (Fig. 2-A). However, the effective dose, which is the dose required to reduce the BG to the non-diabetic level, was not the same among the effective group. On the other hand, seven rats did not respond to BAB administration and died. These rats were classified as the “non-effective group”.

Notably, in the present study, we used increasing dose of BAB depending on the response of the blood glucose level. These doses were equally suppressing DM1 (2 rats for each dose). In this regard, low dose, 300 µL/kg/day, decreased blood glucose level in two rats, moderate dose (600 µL/kg/day) decreased blood glucose level in two additional rats, and the high dose (900 µL/kg/day) was effective in another two rats (Fig. 2-B).

Before induction of the DM, the blood glucose level was not significantly different between the two groups (Fig. 3).

### Blood Pressure Measurement

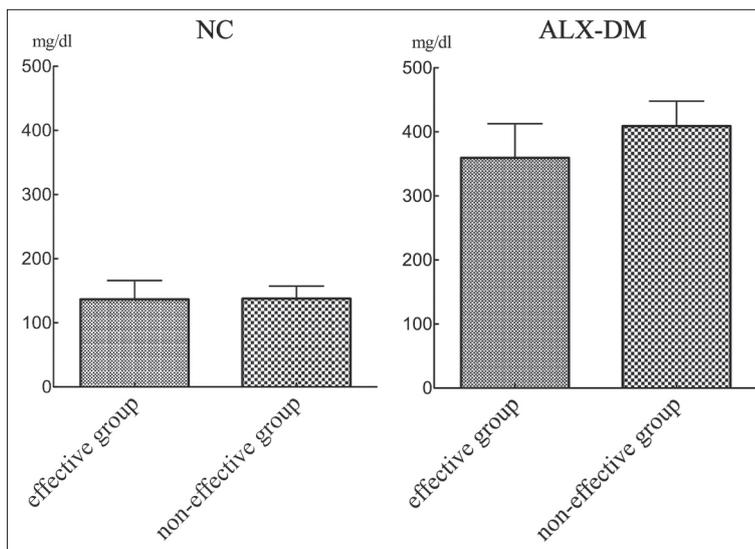
Blood pressure measurements before and after ALX injection are shown in Table 2. The data revealed no significant difference between groups at the basal time and after development of type 1 DM ( $P > 0.05$ ). Additionally, significant differences were not observed between effective and non-effective groups after administration of BAB culture solution.

### General Physical Conditions

In the effective group, concomitantly with reduction in blood glucose level, polyuria, as indicated by the cage environment, was improved. Macroscopically the pigmentation of the abdominal hair was reduced. However, urine excretion was not directly quantified. The average body weight of the effective group was 369.5 g.

## DISCUSSION

The new trend for treatment of diseases recommends usage of probiotics and prebiotics on large scale as a supportive or replacement therapy, particularly metabolic ones. Natural products are organic, safe, and characterized by multi-pathway mechanisms to combat diseases. Recently, BAB has been discussed as a potential therapeutic target for treatment of metabolic disorders including DM [4,13-15]. The



**Fig 3.** Severity of diabetic mellitus in each group. NC, negative control (before alloxan injection); ALX-DM, alloxan induced diabetes mellitus. The effective group (decreased blood glucose level with butyric acid bacteria culture solution treatment) and the non-effective group had no significant differences in blood glucose level before and after injection of alloxan

**Table 2.** Blood pressure before and after alloxan injection

Groups	Systolic (mmHg)		Mean (mmHg)		Diastolic (mmHg)	
	Pre ALX	Post ALX	Pre ALX	Post ALX	Pre ALX	Post ALX
Effective	94.0±15.3	101.2±22.9	63.8±9.8	70.3±10.0	48.7±11.8	52.7±8.2
Non-effective	102.0±6.3	93.9±12.2	66.4±8.3	64.8±11.9	48.5±11.8	50.3±13.9

Results are expressed as average ± SD. No significant differences were detected

current study investigated the hypoglycemic efficiency of BAB culture solution in DM1 model.

Alloxan induces irreversible destruction of pancreatic islet  $\beta$  cells [20]. It was used in the present study to induce DM1, which resulted in elevation of blood glucose [21]. At least 50% of diabetic dogs have DM1, and epidemiological factors closely match those of the latent autoimmune diabetes of adult form of human DM1 [22]. In the present study, the results suggest that BAB culture solution is effective against DM1. Interestingly, 46% (6 rats) of the enclosed rats showed euglycemia after oral administration of BAB culture solution. After alloxan administration, the blood glucose level which exceeded 300 mg/dL was definitely decreased to non-diabetic level after BAB administration, which may be attributed to the ability of BAB to attenuate pancreatic inflammation [4]. BAB, especially *C. butyricum*, was reported to decrease pancreatic damage through reducing inflammation cytokine level [15]. From the present study, this BAB culture solution was suggested to relieve pancreatic inflammation. On the other hand, the remaining rats (54%) did not respond to BAB administration, and resulted in hyperglycemia. This difference in outcomes may be due in part to differences in the extent of pancreatic disorder; however, the present study did not perform histopathology and was unable to confirm the level of pancreatic disorder. Because there was no statistical difference in the blood glucose level before BAB culture solution administration between these two groups, blood glucose alone may not be sufficient

for evaluation of DM severity. In the present study, the dosage of BAB culture solution was sequentially increased until an effective dose for glycemic control was reached. This effect was observed immediately after the increase in BAB culture solution dosage. Accordingly, it can be said that the effectiveness of BAB against type 1 DM is likely to be dose-dependent. In aquatic animals, *C. butyricum* was shown to exert a dose-dependent effect on the immune system as probiotics, and have been administered up to  $1.0 \times 10^{12}$  CFU/kg [23]. In the present study, BAB culture solution contained  $1.0 \times 10^5$  CFU/mL of *C. butyricum*, and administration dose was decided from previous mice experiment conducted by the manufacturer of BAB culture solution. Moreover, *C. butyricum* has no known contraindications and considered as a potential complementary therapy for effective control of DM [14]. Accordingly, administration of BAB culture solution in “non-effect” rats of this study failed to acclimatize the hyperglycemia state even at final dose, because the dose of administration may not be appropriate for the alloxan-induced DM model.

In human medicine, DM is a well-known risk factor for cardiovascular disease [24] including cardiomyopathy and vascular endothelial disorders leading to hypertension that sometimes exacerbate type 1 and type 2 DM, and these interrelationships have been widely investigated [25-29]. In a previous study, anti-hyperglycemic compounds were effective for cardiovascular disorders associated with DM [30]. In the present study, the effect of BAB culture solution

on cardiac complications associated with diabetes was evaluated by measuring blood pressure. However, BAB culture solution did not significantly affect blood pressure. Because this diabetic model was acute<sup>[31]</sup>, the evaluation may have been conducted prior to development of cardiovascular complications. In a previous study, alloxan-DM rats were donated for the cardiovascular examination 8 weeks after induction<sup>[32]</sup>. Therefore, longer term observation will be necessary to fully assess the therapeutic effects of BAB culture solution on the cardiovascular system in DM.

In small-sized animals such as hamsters, hedgehogs, and birds, therapeutic modalities for diabetes are focused on preventing death and severe complications and improving hygiene control<sup>[33]</sup>, rather than on curative therapy. Osmotic diuresis secondary to hyperglycemia worsens cage conditions in DM animals due to polydipsia and polyuria<sup>[34]</sup>. In the present study, the cage environment was notably improved after administration of BAB culture solution. We speculate that this change was due to improvement in polydipsia and polyuria, a common clinical sign of DM. This finding suggested that BAB administration increases the hygienic condition of the cage, which in turn may prevent secondary infections or complications in DM. Although quantitative assessment was not conducted, decreased consumption of drinking water and reduction of hair pigmentation due to polyuria were noticeable. Improving the living environment will significantly improve the quality of life for DM animals that receive BAB culture solution. The results of the present study indicated that BAB culture solution for dietary control of DM in small animals is convenient and safe for small animal caretakers and had multiple beneficial outcomes, including glycemic control, improved quality of life, and decreased water consumption and subsequently improved polyuria. Although the mechanism of action for BAB culture solution is not fully understood, some previous studies suggested that this modality improves insulin resistance.

This is a preliminary study which highlights the utility of BAB culture solution in DM1. However, further studies addressing the dose response manner, histopathological and molecular pathways are warranted.

In conclusion, BAB culture solution is an important potential complementary therapeutic for type 1 DM, and the utility of this modality is particularly notable for small animal veterinary medicine, including exotic animals.

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#### CONFLICT OF INTEREST

The authors declare no conflicts of interest.

#### AUTHOR CONTRIBUTIONS

K. Shimada planned the present study, conducted this experiment, and wrote this manuscript. S. Hara and S. Goya conducted and supported this experiment. A. S. Mandour, P. Kitpipatkun and A. Uemura supported to write this manuscript. L. Hamabe corrected English of this manuscript. J. Takizawa researched about butyric acid products. R. Tanaka supported and supervised the present study.

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