

EFFECT OF *BACILLUS SUBTILIS* CGMCC 32826 ON THROMBUS MOUSE MODEL AND INTESTINAL MICROBIOTA

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Abstract

Bacillus subtilis is an unusual microorganism with the widest range of applications. As a common microorganism in traditional fermented foods, *Bacillus subtilis* has a unique function of regulating human health. In this study, *Bacillus subtilis* CGMCC 32826 was fed to thrombosis model mice to observe its ability to degrade thrombosis and its effect on the diversity of intestinal *Bacillus*. We observed that after the thrombosis model mice were fed with normal feed in the CK group (normal diet-fed thrombosis model mice group), the number of *Bacillus* in their intestines increased sharply (with extremely high diversity on the 20th day). It was known through routine blood tests, four coagulation indicators, and common carotid artery HE staining that the thrombosis mice had a certain self-healing ability. Mice fed with *Bacillus subtilis* CGMCC 32826 showed diversity of *Bacillus* in their intestines on the 10th day. By the 20th day, it could be determined from the routine blood tests, four coagulation indicators, and common carotid artery HE staining that the thrombosis mice had fully recovered their health.

Key words: *Bacillus subtilis*, thrombosis model mice, intestinal microbiota

Introduction. *Bacillus subtilis* is a safe bacterium used as food probiotic [1]. The most typical, *Bacillus subtilis* has been proved to improve intestinal microbiota, health, disease resistance, and inflammatory response [2,3]. The functional food natto is fermented by *Bacillus subtilis* using soy as a substrate [4].

Natto contains a fibrinolysis enzyme (called nattokinase) produced by *Bacillus subtilis*, which can ameliorate the effect of in vivo endogenous thrombolysis [5,6]. Animal thrombosis models are used to assess the effect of thrombosis

treatment drugs or food, such as atherogenic rat (*Rattus norvegicus*) model and the carrageenan-induced rat model [7,8].

Bacillus subtilis does not act solely on blood clots through nattokinase. It is also an intestinal probiotic that can help the body recover health through intestinal action.

In this study, we employed a thrombus mouse model and gut microbial metagenomic analysis techniques to evaluate the effect of *Bacillus subtilis* CGMCC 32826 on thrombotic animals.

Materials and methods. Medium. The flask culture medium contained (w/w): glucose 10 g/L, yeast powder 30 g/L, Na₂HPO₄ 2 g/L, NaH₂PO₄ 1 g/L. Secondary fermentation liquid contained (w/w): peptone 20 g/L, glucose 10 g/L, Na₂HPO₄ 1.42 g/L, KH₂PO₄ 0.24 g/L, MgSO₄ 0.30 g/L, CaCl₂ 0.1 g/L. Fermentation culture medium contained (w/w): red bean powder 50 g/L, buckwheat flour 10 g/L, glucose 20 g/L, Na₂HPO₄ 4 g/L, KH₂PO₄ 0.3 g/L, MgSO₄ 0.5 g/L, CaCl₂ 0.1 g/L, NaCl 5 g/L.

Preparation for *Bacillus subtilis* powder. The strain was purchased from the China General Microbiological Culture Collection Center (Collection number CGMCC No. 32826). *Bacillus subtilis* 32826 was grown at 37 °C in a 250 mL flask containing 150 mL of culture medium at 240 r/min for 16 h. Then, the bacterial solution was transferred into a 20 L fermentation tank as seed liquid. The volume of liquid in the fermentation tank was 12 L of secondary fermentation liquid, and the inoculation rate was 1%. The cultivation conditions were 37 °C, mixing speed was 400 r/min, and pH was controlled between 6.8–7.2. After 8 h of strain cultivation, all the secondary fermentation liquid was transferred to a 1-ton fermentation tank to conduct the final fermentation. The cultivation conditions were the same as above, but the fermentation time was 24 h. After fermentation was completed, the spraying process began. For every one volume of fermentation liquid, 10% maltodextrin and 2% skim milk powder were added. The feed temperature of the spray machine was 150 °C, and the discharge temperature was 80 °C. The *Bacillus subtilis* powder was collected and stored in a cool warehouse.

Animal feed preparation. Fifty grams of *Bacillus subtilis* powder were mixed with premixed feed, and then granulation was performed. The nutrition of the animal feed was tested as crude protein 20.26%, crude ash 8.1%, crude fat 5.1%, crude fibre 5.0%, total phosphorus 0.85%, calcium 1.71%, and moisture 9.1%. The CK animal was fed feed composed of the same raw material but without adding *Bacillus subtilis* powder.

Animal experiments. Six-week-old male C57BL/6 mice (SPF level) were divided into three groups. Each group consisted of 12 mice, housed four per cage. The thrombosis model referenced the method in the paper by JANG et al. [9]. The *Bacillus* group was fed animal feed with *Bacillus subtilis* powder. The CK group was fed CK animal feed. The feeding method was free intake. We chose to take three mice from each group on the 10th and 20th days for blood collection.

The blood was used to perform routine blood and coagulation tests. Intestinal contents and vascular samples were also collected and stored in a -20°C freezer for follow-up testing.

Detection method. *Routine blood tests:* Whole blood was tested using a fully automated hematology analyzer (EXZ6000, Zhongyuan huiji Biotechnology Ltd., Co.).

Coagulation indicators: After the whole blood was reacted with the kit, it was read with a fully automatic coagulation analyzer (YX2000, Shenzhen Chuanshi Biomed Ltd., Co.).

Common carotid artery staining: The blood vessels were first dehydrated by gradient ethanol (75%-80%-85%-90%-95%-100%) solution, then treated with xylene for 30 min, and embedded with paraffin. After fixation, they were cut into $5\text{ }\mu\text{m}$ wax pieces and transferred them to glass slides, baked and subjected to HE staining (Hematoxylin-eosin staining). The method used the paper of HUANG et al. [10].

Gut microbial metagenomic analysis: One hundred milligrams samples were used for DNA extraction by a DNA extraction kit (Fastpure Microbiome DNA Isolation Kit, Vazyme, China) according to the manufacturer's protocol. The DNA concentration was measured using a NanoDrop 2000c spectrophotometer (Thermo Scientific, United States). The quality and quantity of the DNA extract were evaluated with gel electrophoresis and a Quantus fluorometer (Promega, Madison, WI, USA). The DNA sequencing libraries and subsequent Illumina HiSeq sequencing services were provided by Novogene (Beijing, China).

Results and discussion. *Routine blood indexes of thrombosis model mice.* From the perspective of the blood routine on the 10th day and 20th day, the thrombosis model mice are relatively healthy as can be seen in Table 1. The exception parameters are gran#, gran%, HGB, and MCH. On the 20th day mice in the CK group showed gran returning to normal. HGB and MCH remained below normal. These phenomena also occurred in the Bacillus group.

In comparison, the blood routine indicators of the thrombosis model mice were relatively mild, and no extreme indicators were observed. Whether it was the CK group or the Bacillus group, after a relatively long period of feeding, the abnormal items in the blood routine of the mice were eliminated. Of course, the effect of the Bacillus group was better.

Coagulation of four items of thrombosis model mice. It can be seen from Table 2 that the CK of the model mice shows a trend of low PT, high APTT, TT, and FIB. In the CK group from the 10th day to the 20th day, PT increased (insignificantly), FIB decreased (insignificantly), and APTT and TT decreased (significantly). Among the four indicators of CK-20d, except for PT, which is still lower than that of healthy mice, the other three are gradually transitioning to healthy mice. The trend presented by the Bacillus group-10d is consistent with that of CK-10, but the values are all better than those of the CK group. By

T a b l e 1
Routine blood indexes of thrombosis model mice

Routine blood indexes	CK		Bacillus Group		Reference values for healthy mice
	10d	20d	10d	20d	
WBC	3.55 ^a	6.06 ^c	4.95 ^b	6.6 ^c	0.8–6.8
Lymph#	1.55 ^a	4.09 ^c	2.28 ^b	4.81 ^c	0.7–5.7
Mon#	0.13 ^a	0.18 ^b	0.18 ^b	0.15 ^a	0.0–0.3
Gran#	1.88 ^a	1.80 ^a	2.50 ^b	1.64 ^a	0.1–1.8
Lymph%	40.4 ^a	62.21 ^b	46.48 ^a	65.69 ^b	55.8–90.6
Mon%	3.50 ^{ab}	3.10 ^a	3.80 ^b	3.23 ^a	1.8–6.0
Gran%	56.08 ^b	34.69 ^a	49.73 ^b	31.08 ^a	8.6–38.9
RBC	7.66 ^a	7.61 ^a	8.12 ^b	7.73 ^a	6.36–9.42
HGB	107.25 ^{ab}	103.37 ^a	107.25 ^{ab}	103.58 ^a	110–143
HCT	34.83 ^a	34.84 ^a	36.10 ^{ab}	35.37 ^{ab}	34.6–44.6
MCV	45.65 ^{ab}	45.90 ^{ab}	45.08 ^a	45.75 ^{ab}	48.2–58.3
MCH	13.85 ^{bc}	13.49 ^a	13.63 ^{ab}	13.27 ^a	15.8–19.0
MCHC	305.00 ^{ab}	295.00 ^a	303.50 ^{ab}	291.50 ^a	302–353
RDW	14.8 ^a	15.33 ^{bc}	14.15 ^a	17.00 ^d	13.0–17.0
PLT	912.30 ^a	1207.90 ^b	879.75 ^a	1359.50 ^{bc}	450–1590
MPV	4.85 ^{ab}	4.77 ^{ab}	4.9 ^b	4.59 ^a	3.8–6.0

Abbreviations: WBC – white blood cell count; Lymph# – lymphocyte count; Mon# – monocyte count; Gran# – neutrophilic granulocyte count; Lymph% – lymphocyte percentage; Mon% – monocyte percentage; Gran% – neutrophilic granulocyte percentage; RBC – red blood cell; HGB – hemoglobin; HCT – hematocrit; MCV – mean corpuscular volume; MCH – mean corpuscular hemoglobin; MCHC – mean corpuscular hemoglobin concentration; RAD – red blood cell distribution width; PLT – platelet; MPV – mean platelet volume; CK means the thrombosis model mice fed normal diet; Bacillus Group means the thrombosis model mice fed the feed with *Bacillus subtilis* CGMCC 32826.

T a b l e 2
Coagulation of four items of thrombosis model mice

Coagulation of four items	CK		Bacillus Group		Reference values from healthy mice
	10d	20d	10d	20d	
PT (s)	9.25 ^a	9.35 ^{ab}	9.50 ^b	9.98 ^c	9.74 ^c
APTT (s)	18.73 ^c	17.36 ^a	18.70 ^c	17.16 ^a	17.93 ^b
TT (s)	21.48 ^c	20.10 ^{ab}	21.00 ^c	19.78 ^a	20.39 ^{ab}
FIB (g/L)	2.97 ^c	2.74 ^{bc}	2.98 ^c	2.34 ^a	2.67 ^{abc}

Abbreviations: PT – Prothrombin time; APTT – Activated partial thromboplastin time; TT – Thrombin time; FIB – Fibrinogen; CK means the thrombosis model mice fed normal diet; Bacillus Group means the thrombosis model mice fed the feed with *Bacillus subtilis* CGMCC 32826.

the 20th day of the Bacillus group, all indicators returned to normal, specifically manifested as high PT, low APTT, TT, and FIB.

The four coagulation items are the simplest methods used to determine whether there is a blood clot. The coagulation indicators of the mice in the Bacillus Group-20d have fully recovered to health. The coagulation indicators of CK-20d also improved compared with those of 10d. This indicates that mice have a certain ability to degrade blood clots. Taking *Bacillus subtilis* can significantly accelerate the speed of self-healing.

HE staining of common carotid arteries in thrombosis model mice.

Figure 1 presents the HE staining of common carotid arteries (40×). On the 10th day, thrombi could be seen in the vascular lumen of the CK group (Yellow arrows), and the arrangement of smooth muscle within the membrane was disordered (Black arrows). Moderate hyperplasia of the outer vascular membrane and surrounding connective tissue was observed, accompanied by a considerable infiltration of lymphocytes (Blue arrows). In the Bacillus subtilis experimental group, there was small-scale necrosis of the vascular wall (Red arrow), rare calcification foci in the vascular wall and vascular lumen (Gray arrow), severe hyperplasia of the vascular intima, occasional lymphocyte infiltration (Blue arrow), thrombosis in the vascular lumen (Yellow arrow), and disordered arrangement of smooth muscle in the vascular media (Black arrow).

On the 20th day, in the control group, there was extensive necrosis of the vascular wall (Red arrow), and calcification foci were rare in the vascular wall and vascular lumen (Gray arrows). Thrombi can be seen in the vascular lumen (Yellow arrows). Connective tissue hyperplasia can be observed in the vascular lumen, and a small number of lymphocytes can be seen (Blue arrows). Moderate hyperplasia of the outer vascular membrane and the surrounding connective tissue was accompanied by a considerable infiltration of lymphocytes and granulocytes (Green arrows), and rare brownish-yellow pigment deposition (Orange arrows). The vascular wall structure of mice in the Bacillus group was unclear, with extensive necrosis of the vascular wall (Red arrow). Calcification foci were frequently seen in the vascular wall and vascular lumen (Gray arrow), and lymphocyte infiltration was occasionally observed in the outer membrane of the blood vessel and its surroundings (Blue arrow).

It can be clearly observed in Fig. 1 that the thrombus in the blood vessels of CK-20d and Bacillus Group-10d is smaller than that of CK-10d, while no thrombus can be observed in Bacillus Group-20d. This is consistent with the results of blood routine and coagulation indicators, indicating that taking *Bacillus subtilis* can significantly accelerate the rate of self-healing.

Diversity of Bacillus in intestinal microbiota of thrombosis model mice. In Figure 2, we used pie charts to show the composition of Bacillus in different groups.

In CK-10d, *Bacillus* accounts for 0.0005% of the total Bacteria, 0.08% of Bacilli, and 5% of the total Bacillaceae. Among *Bacillus*, the ones with the largest quantities were *Bacillus sp. SA1-12* (46%) and *Bacillus cereus* (42%). In

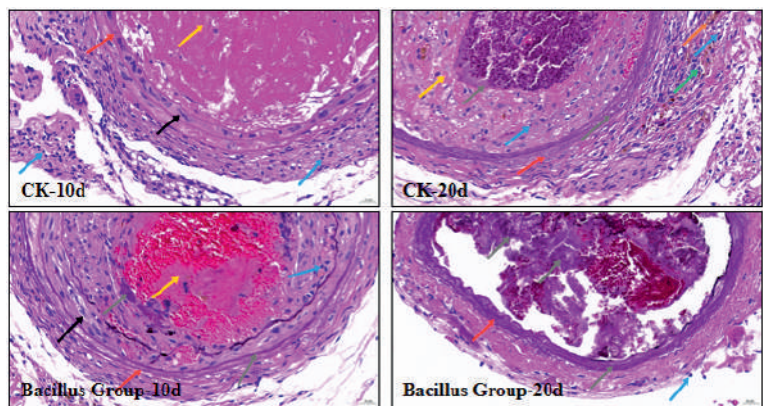


Fig. 1. HE staining of common carotid arteries in thrombosis model mice

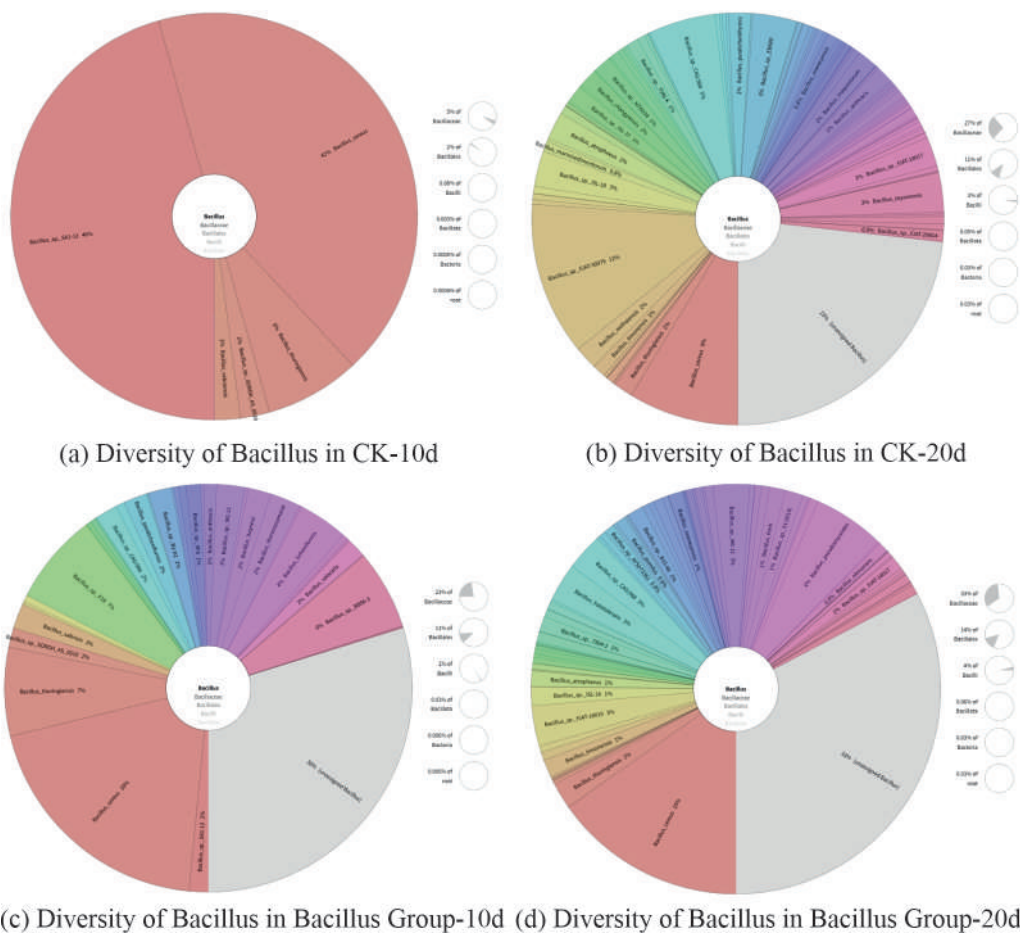


Fig. 2. Diversity of *Bacillus* in intestinal microbiota of thrombosis model mice

CK-20d, *Bacillus* accounts for 0.03% of the total Bacteria, 3% of Bacilli, and 27% of the total Bacillaceae. Among *Bacillus*, the ones with the largest quantities were *unassigned Bacillus* (23%), *Bacillus sp. FJAT-50079* (12%), and *Bacillus cereus* (9%).

In *Bacillus* group-10d, *Bacillus* accounts for 0.006% of the total Bacteria, 1% of Bacilli, and 23% of the total Bacillaceae. Among *Bacillus*, the ones with the largest quantities were *unassigned Bacillus* (30%), *Bacillus cereus* (20%), *Bacillus thuringiensis* (7%), and *Bacillus sp. F19* (7%). In *Bacillus* group-20d, *Bacillus* accounts for 0.03% of the total Bacteria, 4% of Bacilli, and 33% of the total Bacillaceae. Among *Bacillus*, the ones with the largest quantities were *unassigned Bacillus* (33%), *Bacillus cereus* (15%), *Bacillus thuringiensis* (7%), and *Bacillus pseudomycoloides* (7%).

We observed that the diversity and species of intestinal bacillus in CK-20d significantly increased, which might be related to the self-degradation of thrombosis in the model mice to some extent. Taking *Bacillus subtilis* CGMCC 32826 can significantly increase the diversity of *Bacillus*. In many studies, it is not only *Bacillus subtilis* that has plasmin ability, but also *Bacillus cereus*, *Bacillus amyloliquefaciens*, and *Bacillus thuringiensis* [11–13]. There are also some species of *Bacillus cereus* that can cause thrombosis [14]. In this experiment, as the thrombus in the model mice decreased, the number of *Bacillus cereus* also decreased.

Conclusions. The results from blood routine tests, coagulation indicators and HE staining of carotid artery sections indicate that thrombotic model mice exhibit a degree of spontaneous recovery upon resuming a normal diet. Dietary supplementation with *Bacillus subtilis* CGMCC 32826 was found to accelerate this recovery process. After 20 days of continuous administration of *Bacillus subtilis* enriched food, the thrombotic mice demonstrated complete restoration of health, with all measured parameters (blood routine, coagulation indicators, and carotid artery HE staining return to normal level). In contrast, the CK group exhibited persistent carotid artery thrombi and subnormal PT values. Analysis of intestinal microbiota composition revealed that the *Bacillus subtilis* supplemented food significantly enhanced early-stage (first 10 days) bacillus diversity formation in the intestinal tract of thrombotic model mice. These findings suggest that dietary *Bacillus subtilis* CGMCC 32826 supplementation may facilitate recovery in thrombotic patients.

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