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Effect of Irradiated Natto on Thrombus Mice Model and Intestinal Microbiota

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Abstract Natto is a traditional fermented functional food to adjust intestinal function and exert fibrinolytic effect. Additionally, natto is one of the few fermented foods that can be eaten directly. Microbial contamination is the biggest challenge for both the fermentation process and the end products. So that the microbial safety of natto is particularly crucial. Radiation is a sterilization technique, widely used in food processing. Irradiation is a viable safety measure. However, the question of whether irradiation preserves natto's intrinsic functionality in animal experiments still lies in a research vacuum. We compare the effects of feeding irradiated and non-irradiated natto on blood parameters and coagulation in mice using a thrombosis model. Thrombus dissolution was observed through carotid artery sections. We found that, whether feeding irradiated or non-irradiated natto, for 20 days the thrombosis was resolved , with blood parameters and coagulation indices returning to normal. But the recovery was more pronounced in the irradiated group. Specifically, the irradiated group achieved normalization of Gran# and Lymph% at 10 days, while the control group required 20 days. Gut microbiota analysis revealed that, after 10-20 days, the irradiated group saw Bacteroidota increase from 36% to 46% and Bacillota decrease from 42% to 35%. In the control group, Bacteroidota decreased from 30% to 23%, Bacillota increased from 15% to 52%, and Chlamydiota decreased from 23% to 0.4%. The irradiated group's gut microbiota showed greater stability than the control group with significantly lower abundances of TMAO-related genera such as Lachnospirales, Erysipelotrichia, and Odoribacter, which may contribute to enhanced thrombus resolution.

Keywords: *irradiation, thrombus mice model, blood parameters, coagulation indicators, intestinal microbiota*

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1. Introduction

Natto, a traditional fermented soybean product with a centuries-long history in East Asian cuisine, is valued for its distinctive nutritional and functional properties [1]. Unlike many fermented foods that mainly improve flavour or shelf-life, natto is renowned for its health benefits, particularly in cardiovascular health [2]. These benefits are largely attributed to nattokinase, a fibrinolytic enzyme unique to natto. This enzyme has been shown to regulate thrombosis effectively: it breaks down fibrin, the main component of blood clots, and may help treat conditions like deep vein thrombosis and ischemic stroke [3,4]. Its ability to dissolve fibrin without causing excessive bleeding makes it a key focus in functional food research for preventing cardiovascular disease.

However, natto production faces a challenge due to its main ingredient-soybeans. Rich in starch, protein, and lipids, soybeans provide an ideal environment for microbial growth [5]. While fermentation relies on beneficial strains like *Bacillus subtilis* natto to produce functional components such as nattokinase, it also encourages the growth of undesirable microbes, including pathogens (e.g., *Salmonella spp*.) and spoilage organisms. This microbial diversity in bean-fermented products not only raises food safety concerns but also affects product consistency-variations in microbial composition can lead to unstable nattokinase activity and reduced nutritional quality, limiting natto's potential as a functional food for clinical or preventive use.

To address this microbial challenge, the food industry has adopted various sterilisation techniques, with irradiation emerging as the most prevalent and effective method for fermented soybean products [6]. Beyond its core role in inactivating pathogenic and spoilage microorganisms, irradiation offers additional advantages that align with natto processing goals. Research shows controlled irradiation (e.g., using ^{60}Co gamma rays) can positively influence the nutritional composition of fermented foods: it reduces anti-nutritional factors (e.g., trypsin inhibitors and phytic acid) that impair nutrient absorption while preserving essential amino acids and polyunsaturated fatty acids-key nutrients contributing to natto's health benefits [7,8]. Notably, recent preclinical

studies associate irradiated fermented foods with improved outcomes in animal models of chronic diseases. For example, irradiated natto mitigated gut microbiome dysbiosis in insulin-deficient type 2 diabetic rats, suggesting a potential synergistic interaction between irradiation and natto's bioactive components in modulating host physiology [9]. Despite these promising findings, critical gaps remain in understanding how irradiation influences.

Natto's efficacy against thrombosis-one of its most well-recognized health benefits. Thrombosis models are widely used to evaluate the functional potential of foods and supplements, as they replicate the pathological conditions of abnormal blood clot formation [10,11]. However, existing studies have focused either on nattokinase's direct fibrinolytic activity in vitro or on natto's general effects in healthy or diabetic models, with limited attention to two key questions: 1) Does irradiation enhance or preserve natto's ability to promote thrombus degradation in a thrombosis-specific animal model? 2) How does irradiation modulate the gut microbiota of thrombosis-model animals, and could these microbial changes contribute to improved thrombotic outcomes-particularly given the growing evidence that gut dysbiosis is closely linked to cardiovascular disease development via pathways such as trimethylamine-N-oxide (TMAO) Production? Currently, no studies have explicitly clarified the impact of irradiation on natto's anti-thrombotic function in thrombosis models, nor have the associations between gut microbiota and thrombosis been verified in natto-related research.

To address these gaps, this study systematically evaluated irradiated natto's effects on thrombus resolution and intestinal microbiota in a thrombotic mouse model. We hypothesized that irradiation would ensure natto's microbial safety while enhancing its therapeutic potential against thrombosis-either by preserving nattokinase activity or modulating gut microbiota toward coagulation homeostasis. We compared irradiated versus unirradiated natto feed on: 1)Hematological and coagulation parameters (e.g., neutrophil-lymphocyte ratio, prothrombin time), reflecting systemic coagulation status; 2) Histological changes in the common carotid artery (a primary thrombus site) to assess thrombus size and vascular damage; 3) Gut microbial composition (via metagenomic analysis) to identify microbiota-coagulation associations. These findings offer critical insights into optimizing natto processing for cardiovascular health and advance understanding of fermented food processing, gut microbiota, and coagulation function.

2. Materials and Methods

Animal feed preparation: We weighed out 50 g of commercial natto powder (containing nattokinase activity ≥40000 IU/g, purchased from Taizhou Youlikang Biotechnology Ltd Co., batch number: YLK20250318), then mixed it thoroughly with the premixed feed (purchased from Jiangsu Xietong Pharmaceutical Bioengineering Co., Ltd.) before granulating the mixture using a standard granulator (Model: LGJ-15, Shanghai Lituo Machinery Equipment Co., Ltd.). Analysis of the resulting animal feed showed the following nutritional composition: crude protein 20.26% (determined by Kjeldahl method), crude ash 8.1% (determined by muffle furnace combustion method), crude fat 5.1% (determined by Soxhlet extraction method), crude fiber 5.0% (determined by acid-base digestion method), total phosphorus 0.85% (determined by molybdenum blue colorimetry), calcium 1.71% (determined by EDTA titration), and moisture 9.1% (determined by direct drying method). For feed sterilization, we subjected the granulated feed to ^{60}Co high-energy irradiation (irradiation source: Jiangsu Yuxiang Radiation Technology Co., Ltd.; dose rate: 1 kGy/h; total dose: 3 kGy; irradiation temperature: ≤ 25°C) at an intensity of 3 kGy for 3 hours; the control group feed remained unirradiated.

Animal Experiments: This study was approved by the Animal Ethics Committee of Yancheng Institute of Technology (Approval No.: YCIT-AEC-2024-072) and strictly followed the "Guidelines for the Ethical Review of Laboratory Animal Welfare" and international laboratory animal ethical standards. Six-week-old male C57BL/6 mice (SPF grade) , purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd., certificate number: SCXK (Beijing) 2023-0009) were randomly assigned to three groups, with 12 mice per group and 12 mice housed per cage (cage size: 40cm×30cm×25cm, stainless steel material). The feeding environment was controlled at a temperature of 22 ± 2°C, humidity of 55 ± 5%, and a 12h light/12h dark cycle. Sterile corn cob bedding was used and replaced twice a week; sterile deionized water was provided ad libitum. Mice were acclimatized for 7 days to confirm good health before the experiment began. The thrombosis model was established using the method described by Ja-Young Jang et al. [12], with minor adjustments to fit our experimental conditions. Mice were intraperitoneally injected with carrageenan (10 mg/kg body weight, dissolved in normal saline) to induce thrombosis. The irradiated group received irradiated feed, while the CK group was provided with non-irradiated feed. All animals had ad libitum access to feed. On days 10 and 20 post-intervention, three mice from each group were euthanized by carbon dioxide inhalation (painless and compliant with ethical standards) for sample collection. Blood samples were obtained for complete blood count and coagulation analyses. Intestinal contents and vascular tissue specimens were collected and stored at -20°C for subsequent analyses.

Detection method: Routine blood tests: Whole blood was analyzed using a fully automated hematology analyzer (EXZ6000, Zhongyuan Huiji Biotechnology Ltd., Co.) within 2 hours of collection. The detection indicators included white blood cell count (WBC), lymphocyte count (Lymph#), neutrophil-lymphocyte ratio (NLR), monocyte count (Mon#), neutrophilic granulocyte count (Gran#), and other 16 routine blood parameters. Coagulation indicators: Following the reaction of whole blood with the kit (purchased from Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions, measurements were obtained using a fully automated coagulation analyzer (YX2000, Shenzhen Chuanshi Biomed Ltd., Co.). Common carotid artery staining: Blood vessels were dehydrated using a graded ethanol series (75%, 80%, 85%, 90%, 95% and 100%), then treated with xylene for 30 minutes, and embedded in paraffin. After

fixation, 5 μm sections were prepared , mounted on glass slides, baked, and subjected to hematoxylin and eosin (HE) staining. The methodology followed that described by Wang [13]. Gut microbial metagenomic analysis:100 mg samples were used for DNA extraction with a DNA extraction kit (Fastpure Microbiome DNA Isolation Kit, Vazyme, China) following the manufacturer's protocol. DNA concentration was measured using a NanoDrop 2000c spectrophotometer (Thermo Scientific, USA). The quality and quantity of the DNA extract were evaluated with gel electrophoresis and a Quantus fluorometer (Promega, Madison, WI, USA). DNA sequencing libraries and subsequent Illumina HiSeq sequencing services were provided by Novogene (Beijing, China).

Statistical analysis: All data were analyzed using SPSS 26.0 statistical software. Measurement data were expressed as mean. Differences between multiple groups were compared using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. $P < 0.05$ was considered statistically significant, and different letters (a, b, c) in tables indicate significant differences between groups.

Table 1. Routine blood indexes of thrombosis model mice

Routine blood indexes	CK group		Irradiated group		Reference values for healthy mice
	10 d	20 d	10 d	20 d	
WBC	4.95^{c}	3.64^{a}	4.00^{b}	4.04^{b}	0.8 - 6.8
Lymph#	2.28^{b}	1.88^{a}	2.98^{c}	2.92^{c}	0.7 - 5.7
NLR	2.25^{c}	1.94^{b}	1.34^{a}	1.38^{a}	-
Mon#	0.18^{c}	0.12^{ab}	0.10^{a}	0.10^{a}	0.0 - 0.3
Gran#	2.50^{c}	1.64^{b}	1.83^{b}	1.24^{a}	0.1 - 1.8
Lymph%	46.48^{a}	51.63^{ab}	58.68^{b}	67.06^{c}	55.8 - 90.6
Mon%	3.80^{b}	3.84^{b}	3.75^{b}	2.96^{a}	1.8 - 6.0
Gran %	49.73^{c}	44.53^{b}	42.15^{b}	31.13^{a}	8.6 - 38.9
RBC	8.12^{b}	7.07^{a}	8.06^{b}	7.33^{a}	6.36 - 9.42
HGB	107.25^{b}	91.69^{a}	118.75^{c}	100.56^{b}	110 - 143
HCT	36.10^{c}	31.75^{a}	36.55^{c}	33.44^{b}	34.6 - 44.6
MCV	45.08^{a}	45.17^{a}	45.10^{a}	45.55^{a}	48.2 - 58.3
MCH	13.63^{a}	13.01^{a}	13.73^{a}	13.33^{a}	15.8 - 19.0
MCHC	303.50^{b}	289.63^{a}	304.75^{b}	293.69^{a}	302 - 353
RDW	14.15^{a}	14.49^{a}	14.53^{a}	14.77^{a}	13.0 - 17.0
PLT	879.75^{a}	1134.94^{c}	882.00^{a}	964.50^{b}	450 - 1590
MPV	4.90^{ab}	4.79^{a}	5.03^{b}	4.97^{ab}	3.8 - 6.0

Abbreviated meaning: WBC = white blood cell count; Lymph# = lymphocyte count; NLR = neutrophil-lymphocyteratio; Mon# = monocyte count; Gran# = neutrophilicgranulocyte count; Lymph% = lymphocyte percent; Mon% = monocyte percent; Gran % = neutrophilicgranulocyte percent; RBC = red blood cell; HGB = hemoglobin; HCT = hematocrit; MCV = mean corpuscular volume; MCH = mean corpsular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; RAD = red blood cell distribution width; PLT = platelet; MPV = mean platelet volume.

Abbreviated meaning: PT = Prothrombin Time; APTT = Activated partial thromboplastin time; TT = Thrombin time; FIB = Fibrinogen.

Table 2. Coagulation of four items of thrombosis model mice

Coagulation of four items	CK group		Irradiated group		Reference values from healthy mice
	10 d	20 d	10 d	20 d	
PT (s)	9.50^{b}	9.96^{c}	9.35^{a}	9.94^{c}	9.74^{bc}
AP(s)	18.70^{b}	17.31^{a}	18.88^{b}	19.78^{c}	17.93^{ab}
TT (s)	21.00^{c}	21.92^{c}	19.98^{b}	19.20^{a}	20.39^{b}
FIB (g/L)	2.98^{b}	2.38^{a}	2.86^{b}	2.67^{ab}	2.67^{ab}

3. Results and Discussion

In 16 types of routine blood tests, there were 5 non-conformities in CK-10d, while both CK-20 and CK had 4 non-conformities. Furthermore, the differences in all non-conformities were minimal compared to the normal values. In CK-10d, Gran# and Lymph% levels were observed to be below normal, but these two indicators returned to normal after 20 days of administration. In the irradiated group at 10d, these two indicators had already normalized. Additionally, in the measurements of HCT and MCHC, the markers for mice administered for 20 days were lower than those at 10 days. Compared with healthy mice, the outliers in the CK group exhibited prolonged TT. In the irradiated group, the PT value decreased at 10 days and the APTT value increased at 20 days. Futhermore, no significant differences were observed among all groups or in other assays from healthy mice.

The blood vessel wall structure in the CK-10 group was indistinct, with sparse elastic lamina fractures, minor vascular wall necrosis (red arrow), and limited calcification foci in the wall and lumen (gray arrow). The intima showed marked hyperplasia with sporadic lymphocyte infiltration (blue arrow). A thrombus (yellow arrow) composed of erythrocytes, leukocytes, and insoluble fibrin occluded the lumen. Intimal smooth muscle arrangement was disorganized (black arrow), while adventitial connective tissue displayed mild proliferation with occasional lymphocytic infiltrates. In the CK-20 group, the vessel wall structure was also indistinct, with extensive necrosis (red arrow). Calcifications were more prevalent in the wall and lumen (gray arrow), and sporadic lymphocyte infiltration was noted in the adventitia and surrounding areas (blue arrow).

In the 10-day irradiated group, the blood vessel wall structure was indistinct. Extensive necrosis occurred in the vascular wall (red arrow), with calcifications mainly in the vessel wall and lumen (gray arrow). A thrombus (yellow arrow) of leukocytes and insoluble fibrin obstructed the vascular lumen. Macrophage presence increased (green arrows). The vascular adventitia and surrounding connective tissue showed mild proliferation, with significant lymphocyte infiltration (blue arrows) and brownish-yellow pigmentation (orange arrows).

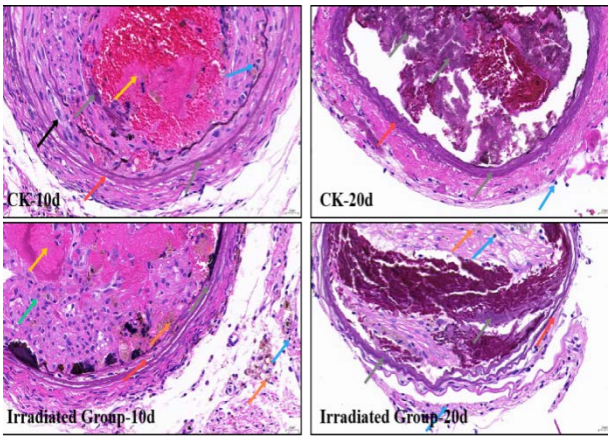
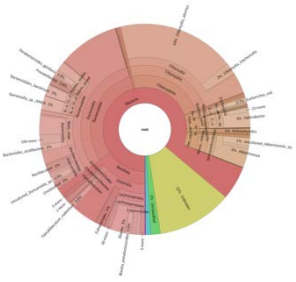


Figure 1. HE staining of common carotid arteries in thrombosis model mice

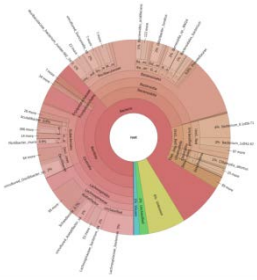
In the 20-day irradiated group, the blood vessel wall structure remained indistinct. Extensive necrosis persisted in the vascular wall (red arrow), while calcification foci were rare in the vessel wall and lumen (gray arrow). Connective tissue proliferation in the lumen was minimal, and lymphocyte infiltration (blue arrow) was sparse. Brownish-yellow pigmentation (orange arrow) was seldom observed, and lymphocyte infiltration in the adventitia and surrounding vasculature was also uncommon.

These results indicate that both irradiated and non-irradiated natto can promote thrombus dissolution in thrombosis model mice after 20 days of feeding, but the irradiated group has a more significant effect on repairing vascular wall damage, which is consistent with the changes in blood routine and coagulation indicators.

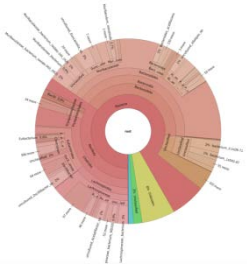


(a) Diversity of intestinal microbiota in CK-10d

(b) Diversity of intestinal microbiota in CK-20d



(c) Diversity of intestinal microbiota in Irradiated group-10d



(d) Diversity of intestinal microbiota in Irradiated group-20d

Figure 2. Diversity of intestinal microbiota of thrombosis model mice

A complete blood count can be used to simply determine the condition of the blood. Neutrophil count, neutrophil-lymphocyte ratio (NLR), erythrocyte sedimentation, and so on can be used to describe the impact of blood clots [14]. In the case of an overall normal blood count, we can find that NLR, which is an important association with thrombosis, is significantly different under different treatments. Wherher CK-20d or irradiated group, both are lower than CK-10d. The blood routine indicators of the model mice were basically in a normal state. Coagulation of four items are the most commonly used items for diagnosing thrombosis [15]. Evidence suggests that APTT, PT, and other indicators can be used to assess the effect of *Bacillus subtilis* K-54 on blood clots [16]. In the PT and FIB indicators, the trend of performance is consistent in both the CK group and the irradiation group. From 10d to 20d, PT rises and FIB decreases. However, there was no significant change compared to healthy mice. The control group and the irradiation group had obvious differences in the four coagulation items, and the APTT and TT indexes were significantly different. In the control group, from 10 to 20 days, the APTT content decreased, but there was no significant difference with the healthy mice, while the TT was significantly higher than that of the healthy mice. In the irradiated group, from 10 to 20 days, APTT increased significantly, which was significantly higher than that of healthy mice, while TT was significantly lower than that of healthy mice. Clinically, a high APTT value means a low fibrinogen content and a decrease in the precursor material for the formation of new blood clots. A high TT value is more common with an increase in fibrinogen degradation products in the blood. From this point of view, there were still thrombotic symptoms at CK-20d, while the irradiated group had fully recovered.

From Figure 1, blood vessel sections, obvious thrombus can still be seen in mice with CK-10d and irradiated -10d, while no thrombus can be observed in the 20d group. We demonstrated the effects of the CK group and the irradiated group on thrombosis model mice through results at multiple levels.

Figure 2(a) displayed the Krona map of microbiota composition in CK-10d samples showing 30% Bacteroidota, 23% Chlamydiota, 13% unknown/unclassified taxa, 15% Bacillota, 5% Verrucomicrobiota, 3% Campylobacterota, 2% Pseudomonadota, and 0.8% viruses. After 20 days, the relative abundance of Bacillota increase to 52%, while Bacteroidota decreased to 23%, Verrucomicrobiota decreased to 3%, 8% unknown/unclassified taxa, and viruses represented 0.7%. In Figure 2(c), Bacillota and Bacteroidota dominate at 38% and 32%, respectively, with the unclassified/unknown portion accounting for 15%. As feeding duration increased the differences in intestinal microbiota composition at the phylum level between the 10-day and 20-day irradiated groups diminished. Figure 2(d) illustrated that Bacillota and Bacteroidota remain predominant at 35% and 42%, respectively,while the unclassified/unknown portion comprised 14%.

In fermented foods, the formation of numerous microorganisms during the fermentation process is unavoidable. The primary function of irradiation is sterilization. In the irradiated group, the intestinal microbiota of mice exhibited minimal changes between 10

and 20 days. This was primarily evidenced by an increase in Bacteroidota from 36% to 46%, and a decrease in Bacillota from 42% to 35%. Further analysis revealed a reduction in Odoribacter from 7% to 0.6%, Barnesiella from 5% to 0.4%, and an increase in Alistipes from 4% to 8%, as well as Muribaculaceae from 37% to 49% (as a percentage of Bacteroidota). In the control group, the change from 10 to 20 days was subtantial. Bacteroidota decreased from 30% to 23%, Bacillota increased from 15% to 52%, and Chlamydiota declined from 23% to 0.4%. Within Bacteroidota, Muribaculaceae primarily increased from 20% to 46%, while Barnesiella decreased from 7% to 0.6% (expressed as a percentage of Bacteroidota). In Bacillota, Lachnospirales rose from 41% to 52%, Eubacteriales increased from 9% to 30%, Erysipelotrichia declined from 12% to 2%, and Bacilli decreased from 3% to 1% (expressed as a percentage of Bacillota).

Natto is a food fermented by *Bacillus subtilis*, we examined the *Bacillus* content in the intestinal microbiota. In CK-10d, *Bacillus* accounted for only 0.005%, whereas in CK-20d and the irradiation groups, the content reached 0.02%-0.03%. In the intestinal microbiota, *Muribaculaceae* showed a clear upward trend, with abundances of 6%, 12%, 12% and 21% of the total in CK-10d, CK-20d, Irradiated-10d and Irradiated-20d, respectively. *Muribaculaceae* is a class of intestinal probiotics that has a certain effect on growth performance and immunity [17,18]. In the treatment of blood deficiency syndrome, the abundance of Muribaculaceae increased with an increase in the dosage of the traditional Chinese medicine Danggui Wuji granules [19]. *Alistipes* is a microorganism associated with Trimethylamine-N-Oxide (TMAO) content but does not directly reflect its relationship with thrombosis [20]. *Lachnospirales* and *Erysipelotrichia* are also associated microorganisms associated with TMAO production [21]. In other studies, *Odoribacter* and *Lachnospiraceae* have also been reported to be negatively correlated with TMAO [22]. Evidence suggests that gut microbes induce thrombus production primarily by producing TMAO [23].

Muribaculaceae is a class of intestinal probiotics that has a certain effect on growth performance and immunity [17,18]. In the treatment of blood deficiency syndrome, the abundance of Muribaculaceae increased with an increase in the dosage of the traditional Chinese medicine Danggui Wuji granules [19], which may be related to the improvement of blood circulation. Alistipes is a microorganism associated with TMAO content but does not directly reflect its relationship with thrombosis [20]. Lachnospirales and Erysipelotrichia are also associated with TMAO production [21], and Odoribacter and Lachnospiraceae have been reported to be negatively correlated with TMAO [22]. Evidence suggests that gut microbes induce thrombus production primarily by producing TMAO [23]. In this study, the irradiated group had significantly lower abundances of Lachnospirales, Erysipelotrichia, and Odoribacter (TMAO-related genera) than the CK group, which may reduce TMAO production and thereby promote thrombus resolution. Additionally, the stability of the gut microbiota in the irradiated group may create a favorable intestinal environment for improving coagulation function.

However, the chemical method used in this thrombosis model is modeled, so the TMAO associated microorganisms of the intestinal microbiota can only be used to describe whether the changes in the intestinal microbiota are not conducive to the elimination of the thrombus. In the experimental group, due to the function of irradiation sterilization, the microorganisms in the feed were controlled, and the changes in the gut microbial population were much smaller than those in the control group. In addition, *Bacillus subtilis*, a fermentation bacterium used in natto foods that eliminates blood clots, does not multiply in large numbers in the intestines of mice. In both the control and irradiated groups, Bacillus subtilis was almost undetectable.

4. Conclusions

In the thrombus mice model, the inclusion of natto in the diet effectively restored pathological indicators. Irradiated food accelerated the recovery of thrombus model mice as evidenced by earlier normalization of blood routine indicators (Gran# and Lymph% at 10d), more significant improvement in coagulation function (increased APTT and decreased TT at 20d), and better repair of vascular wall damage. Analysis of intestinal microbial diversity revealed that mice fed irradiated food exhibited greater stability in their intestinal microbiota, particularly with a significantly lower abundance of Lachnospirales, Erysipelotrichia, and Odoribacter associated with TMAO, which were abundant in the control group. This suggests that the regulation of gut microbiota may be one of the mechanisms by which irradiated natto enhances anti-thrombotic effects. Moreover, Bacillus subtilis associated with natto fermentation, did not colonize extensively in the mouse intestine. These findings provide experimental evidence for the application of irradiated natto as a functional food for cardiovascular health.

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