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Modulation of Gut Microbiota in *Salmonella* Typhimurium-infected Sprague Dawley Rats by Treatment with *Pichia kudriavzevii* 2P10 in Combination with Prebiotic Mannan-Oligosaccharide

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Abstract

Gut microbiota is significantly influenced by diet, including probiotics and prebiotics, and can be disrupted through infection with the foodborne pathogenic bacteria *Salmonella* Typhimurium. The effects of dietary probiotics yeast on modulating gut microbiota have not been widely explored. Therefore, this study aimed to investigate the effects of administering *Pichia kudriavzevii* 2P10 (PRO), mannan-oligosaccharide (MOS), and their combination (PMOS) on male Sprague Dawley (SD) rats, followed by injecting *S.* Typhimurium (ST) ATCC 14028 to induce non-typhoidal infection. The composition of rats' cecum microbiota was analyzed through next-generation sequencing analysis, and histological changes were observed using Hematoxylin-Eosin (HE) staining. The results showed that rats treated with *P. kudriavzevii* 2P10 and infected with *S.* Typhimurium (PRO.ST) had gut microbiota community structure closest to CONTROL, without the occurrence of dysbiosis. Firmicutes levels increased after the administration of *P. kudriavzevii* 2P10, MOS, and PMOS. Moreover, *P. kudriavzevii* 2P10 treatment increased Bacteroidetes, followed by a decrease in Firmicutes/Bacteroidetes ratio in gut microbiota. This phenomenon affected the production of short-chain fatty acids (SCFAs) propionate to impair ST. In conclusion, this study suggested that all treatments could modulate gut microbiota to prevent dysbiosis severity and damage to the histological structure of the cecum after ST infection, with *P. kudriavzevii* 2P10 showing the optimal efficacy.

Keywords: gut microbiota, *Pichia kudrivazevii*, probiotics yeast, *Salmonella* Typhimurium, prebiotics, mannan-oligosaccharide

1. Introduction

A stable and balanced gut microbiota is essential in health maintenance, providing various advantages, such as increasing the immune system against pathogens (Hasan and Yang, 2019; Ji *et al.*, 2020). The diversity and abundance of microbiota in the digestive tract are significantly influenced by diet, health status, lifestyle, microbial infection, age, genetics, and use of drugs or antibiotics (Rinninella *et al.*, 2019). Among the infections that can cause dysbiosis is a non-typhoidal *Salmonella* (NTS), namely *Salmonella enterica* serovar Typhimurium or *Salmonella* Typhimurium (ST), a food poisoning bacterium responsible for diarrheal infections, particularly in children (Kirk *et al.*, 2017). Among bacterial pathogens, ST is the primary cause of foodborne diseases that lead to hospitalizations and deaths globally. This infection causes acute inflammatory diarrhea capable of progressing to invasive systemic disease in susceptible patients (Anderson and Kendall, 2017).

Several studies showed that a healthy diet was mainly practiced by consuming probiotics and prebiotics to modulate gut microbiota, avoiding digestive tract infections (Hasan and Yang, 2019; Ji *et al.*, 2020). Furthermore, consuming probiotics has a good effect on the digestive tract in inhibiting the growth of pathogenic bacteria and has many healthy effects, such as increasing blood protein content (Adriani *et al.*, 2021). These probiotics are live microorganisms capable of conferring health benefits on the host when administered in sufficient amounts (Hill *et al.*, 2014). Compared to bacteria-based, there are probiotics yeast such as *Saccharomyces cerevisiae* var. *boulardii* (*S. boulardii)*, which have been explored for their effectiveness in treating various gastrointestinal disorders. This yeast plays a significant role in maintaining normal gut microbiota and inhibiting the pathogenicity of diarrheal. Numerous studies reported

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S. boulardii as a biotherapeutic agent due to its antibacterial, antioxidant, anti-inflammatory, antiviral, anticarcinogenic, and immunomodulatory properties (Czerucka, Piche and Rampal, 2007; Abid *et al.*, 2022). Among the most often used genera of probiotic yeasts in Indonesia are *Saccharomyces, Pichia*, and *Candida*, which are widely applied in poultry and human health (Astuti *et al.*, 2023). A previous study isolated *P. kudriavzevii* 2P10, a probiotics yeast from the cocoa fermentation process, which showed antioxidant abilities and co-aggregates with ST (Wulan *et al.*, 2021).

Prebiotics are substrates selectively used by microbial hosts that are capable of conferring a significant health benefit (Gibson *et al.*, 2017). Mannan-oligosaccharide (MOS) is among prebiotics that is widely used to improve and maintain the structure of gut microbiota, facilitating the body's resistance to pathogens (Wang *et al.*, 2018). Furthermore, MOS prebiotics are used to treat pathogenic bacterial infections that have type 1 fimbriae, such as ST. The mannose structure in MOS constituent can impede the attachment of type 1 fimbriae to the intestinal epithelial wall, preventing ST infection (Zeiner, Dwyer and Clegg, 2012).

Based on the background above, this study aimed to evaluate the potential of yeast *P. kudriavzevii* 2P10 as probiotics and MOS prebiotics, with their combination in modulating gut microbiota *in vivo*. ST-infected-Sprague Dawley (SD) rats were used in in vivo study to elaborate on the potential of *P. kudriavzevii* 2P10 and MOS in interfering with gut microbiota, potentially lowering ST infection.

2. Material and Methods

2.1. Culture and Cultivation

In this study, *P. kudriavzevii* 2P10 was used as yeast probiotics, which had been characterized in previous investigations (Wulan *et al.*, 2021). Initially, a microbial growth curve was created to determine the harvest period for probiotic yeast and the pathogenic bacteria ST. *P. kudriavzevii* 2P10 was grown for 48 hours at 28 °C in a liquid yeast extract peptone dextrose (YPD) medium. Subsequently, *P. kudriavzevii* 2P10 cells were counted every 3 hours based on the total yeast count method on YPD agar media and incubated for 48 hours at 28°C (Rahmadhani *et al*., 2022). For the growth curve, ST was grown in a Mueller-Hinton (MH) liquid medium for 48 hours at 37 °C, while ST cells were counted every 2 hours based on the total plate count (TPC) method on *Salmonella Shigella* Agar (SSA) medium incubated for 24 hours at 37 °C. *Salmonella* will form transparent colonies with a black dot in the middle

For cultivation, *P. kudriavzevii* 2P10 was cultured in YPD liquid medium for 15 hours at 28 ºC aerobically, while ST ATCC 14028 was cultured with Mueller-Hinton (MH) liquid medium for 18 hours at 37 ºC aerobically. Each culture was harvested separately by washing and resuspending in PBS twice through centrifugation at 4000 rpm for 15 minutes, followed by dissolution in PBS to obtain a cell number of 10^8 CFU/mL.

2.2. Animals and Treatments

The methods used in this study were authorized by the Animal Ethics Committee of the Faculty of Veterinary Medicine, Bogor Agricultural University, Indonesia, No. 46/KEH/SKE/XI/2021. The experiment was carried out using male SD (10 weeks old) rats purchased from iRATco Veterinary Laboratory Services, Bogor, Indonesia. A total of 2 rats were placed in cage in a temperature-controlled room, with the experimental design shown in Figure 1. Before treatment, rats were acclimatized for 2 weeks and given Ciprofloxacin (10 mg/kg) treatment to eradicate ST in gut. The treatments conducted included CONTROL, *P. kudriavzevii* 2P10 (PRO), PMOS, as well as MOS without and with ST ATCC14028. The diet was supplemented with 5% MOS (Bio-Mos, Alltech, USA) and 1 mL 10^8 CFU/ mL *P. kudriavzevii* 2P10 for 15 days. Subsequently, rats were infected by 1×1 mL 10^8 CFU/ mL ST ATCC 14028 and allowed 3 days for an infection response, with water provided ad libitum.

The group without ST (CONTROL, PRO, PMOS, and MOS) infection was sacrificed on the 16th day, while those with ST (CONTROL.ST, PRO.ST, PMOS.ST, and MOS.ST) infection was carried out the 20th day (3 days after infection) by euthanasia ketamine-xylazine. From the contents of the cecum, bacterial DNA was extracted and used for 16S rRNA gene amplicon sequencing.

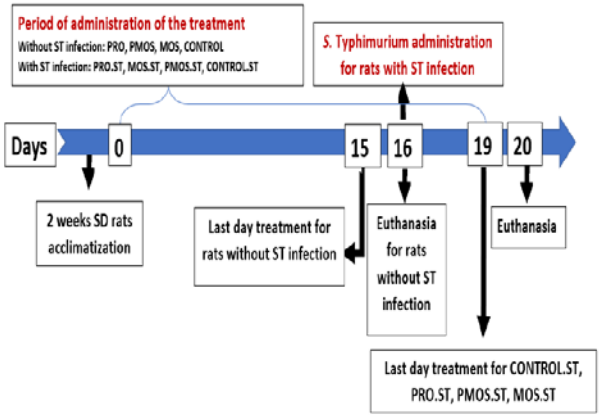


Figure 1. Chronological experimental design showing the treatments in the study. PRO: 1 mL (10^8 CFU/mL) of probiotics yeast *P. kudriavzevii* 2P10, MOS: mannan-oligosaccharide 5% in fed, PMOS: the combination of PRO and MOS, ST: Infection of 1 × 1 mL *S.* Typhimurium 10^8 CFU/mL.

2.3. Bacterial DNA extraction and 16S rRNA gene amplicon sequencing

The total genome DNA from cecum content samples was extracted using CTAB/SDS method (Sambrook and Russell, 2001). Subsequently, PCR reactions were conducted using Phusion® High-Fidelity PCR Master Mix (New England Biolabs), with the genomic DNA as a template. Amplification of the hypervariable region V3-V4 of bacterial 16S rRNA gene was performed using specific primers 341F 5′-TTTCTGTTGGTGCTGATATTGCCCTACGGGNGGCWGCAG-3′ and 806R 5′-ACTTGCCTGTCGCTCTATCTTCGGACTACHVGGGTWTCTAAT-3′ (Takahashi *et al.*, 2014; Matsuo *et al.*, 2021), where the underlined sequence served as an index or adapter.

PCR products quantification and qualification were performed by mixing the same volume of 1X loading buffer (contained SYB green) and operating electrophoresis on 2% agarose gel for detection. The mixture was purified with a Qiagen Gel Extraction Kit

(Qiagen, Germany). NEBNext® Ultra DNA Library Pre Kit for Illumina was generated for the sequencing library, which was assessed using the Agilent Bioanalyzer 2100 instrument and the Qubit@ 2.0 Fluorometer (Thermo Scientific). Subsequently, the library was sequenced using an Illumina NovaSeq platform, producing 250 bp paired-end reads.

2.4. Data analysis

The sequencing results were subjected to paired-end reads assembly and quality control, which included data splitting, sequence assembly, filtration, and chimera removal. Pairing-end reads were trimmed by removing the primer sequence and barcode, followed by sample distribution based on distinct barcodes for data splitting. In sequence assembly, paired-end reads were collected using FLASH V1.2.7, and the splicing sequences were called raw tags. Data filtration was carried out by quality filtering the raw tags, with specific settings applied to obtain high-quality clean tags following QIIME (V1.7.0) quality-controlled method (Caporaso *et al.* 2010). UCHIME Algorithm was used to determine chimera sequences by comparing the tags with the reference database (Gold database) to remove chimeras and obtain effective tags.

Sequence analysis was carried out using UPARSE software, where sequences with 97% or more similarity were assigned to the same OTU. Additional annotations were checked on representative sequences from each OTU. For each representative sequence, the Green Gene Database (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi) (DeSantis *et al.*, 2006) was used based on the RDP 3 classifier 2.2 algorithm to annotate taxonomic information.

Alpha diversity was applied to assess the complexity of sample species diversity using six indices, including observed species, Chao1, Shannon, Simpson, and ACE. QIIME (Version 1.7.0) was used for calculation, while R (Version 2.15.3) was applied to visualize all indices. Meanwhile, beta diversity analysis was used to evaluate the variations in species complexity in the sample using QIIME software (version 1.7.0) to calculate unweighted UNIFRAC beta diversity.

2.5. Histological Analysis of the Cecum

Histological evaluation of the cecum was performed by fixing the fragments of 10% formalin for 24 hours, followed by dehydration and embedding in paraffin. Specifically, the 5-µm paraffin cecum sections were prepared and stained with hematoxylin as well as eosin. Histological micrographs were captured using an OptiLab Advance viewer and an Olympus CX23 Microscope device (Tokyo, JAPAN).

3. Results

3.1. P. kudriavzevii 2P10, Salmonella Typhimurium ATCC 14028, and Mannan-Oligosaccharide

Figure 2 shows the morphology of *Pichia kudriavzevii* 2P10, ST ATCC 14028, and MOS used in this study. The probiotic yeast *P. kudriavzevii* 2P10 forms round, white colonies on YPD agar media, with microscope observation showing oval cells and reproduces through budding. The pathogenic bacteria used to infect rats in this study was ST ATCC 14028, which appears black in SSA media, as shown in Figure 2C. The prebiotic used is the commercial prebiotic Bio-Mos Alltech, which has a dark brown color in powder form, as presented in Figure 2D.

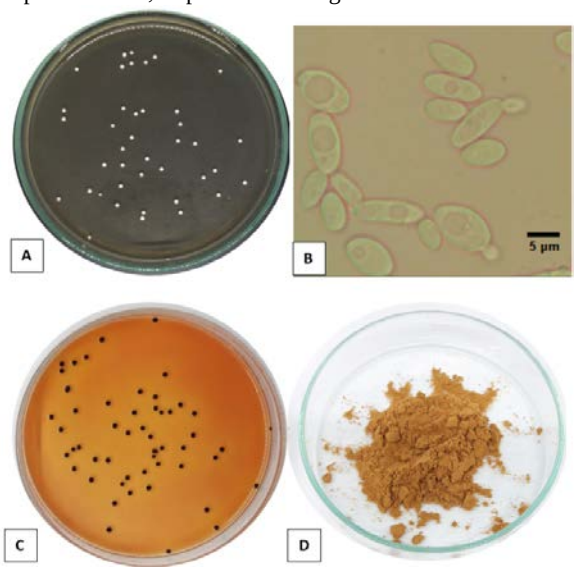
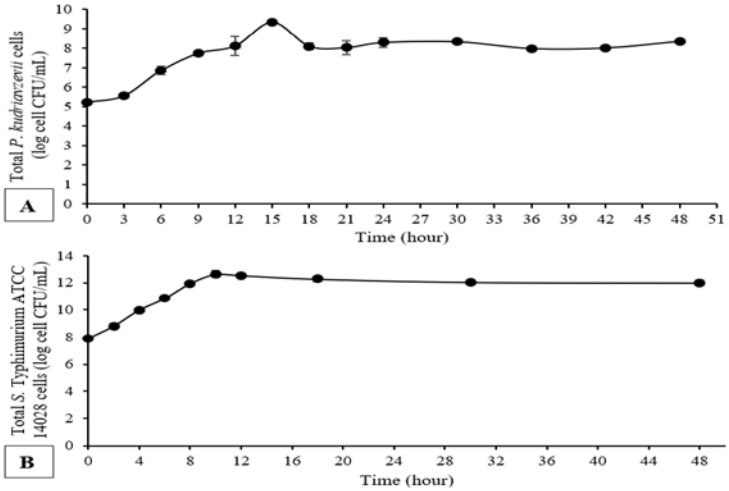


Figure 2. The morphology of: (A) Pure colony of probiotic yeast *Pichia kudriavzevii* 2P10 on yeast extract, peptone, and dextrose (YPD) agar medium at 28°C for 24 hours of incubation, (B) Probiotic yeast *Pichia kudriavzevii* 2P10 oval cells shape, (C) Black colony of *Salmonella* Tyhipimurium 2P10 *Salmonella Shigella Agar* (SSA) agar medium at 37°C for 24 hours of incubation, (D) Mannan-Oligosaccharide (Bio-Mos Alltech, USA)

3.2. The Growth Curve of P. kudriavzevii 2P10 and Salmonella Typhimurium ATCC 14028

Figure 3 shows the growth curve of both probiotics and prebiotics, with *P. kudriavzevii* 2P10 having a maximum cell number (N_{max}) at the 15th hour, reaching 9.33 log cell CFU/mL. Meanwhile, ST has a maximum cell number (N_{max}) at the 10th hour with a cell number of 12.62 log cell CFU/mL and tends to be stable at the 48th hour. The maximum growth rate (μ_{max}) of *P. kudriavzevii* 2P10 is 0.43 hour^{-1}, while μ_{max} value for ST is 0.43 hour^{-1}.



Microbes	N_{max} (log cell CFU/mL)	μ_{max} (hour^{-1})
P. kudriavzevii 2P10	9,33±0,0165	0,43±0,0746
S. Typhimurium ATCC 14028	12,62±0,2370	0,58±0,1039

Figure 3. Growth curve of (A) Probiotic yeast *Pichia kudriavzevii* 2P10 in liquid YPD medium at 28°C for 48 hours of incubation, (B) ST ATCC 14028 in liquid Mueller-Hinton medium at 37°C for 48 hours of incubation.

3.3. Gut Microbiota Analysis

3.3.1. Number of OTU in Rats' Gut Microbiota

The sequencing of 16S rRNA gene region V3-V4 in rats' cecum content across all treatments produced an average total tags of 102,245 and OTU of 1638. Moreover, OTU is a collection of V3-V4 16S rRNA sequences with more than 97% similarity. Based on the results, the most abundant OTU was found in the treatment with probiotic yeast (PRO) and PMOS, as shown in Table 1. The number of OTU in ST ATCC 14028-infected samples was decreased compared to those without infection in all treatments of PRO and MOS.

Table 1. Number of observed OTU (97% similarity) and alpha diversity indices of bacterial microbiota in cecum rats with and without **ST** infection

Sample name	Observed species (OTU)	Shannon	Simpson	Chao1	ACE
CONTROL	1657	6.89	0.957	1756	1799.331
CONTROL.ST	1630	6.91	0.965	1767	1855.420
PRO	1686	7.60	0.984	1789	1727.083
PRO.ST	1638	7.40	0.983	1656	1689.173
PMOS	1686	6.98	0.967	1822	1793.863
PMOS.ST	1642	7.16	0.975	1783	1661.841
MOS	1614	7.10	0.978	1710	1787.539
MOS.ST	1549	7.15	0.973	1642	1785.156

Note: CONTROL, control without ST infection (negative control); CONTROL.ST, control positive with ST infection; PRO, 1 mL of probiotics yeast *P. kudriavzevii* 2P10 10^8 CFU/mL; PRO.ST, 1 mL of probiotics yeast *P. kudriavzevii* 2P10 10^8 CFU/mL with ST infection; MOS, mannan-oligosaccharide 5% in fed; MOS.ST, mannan-oligosaccharide 5% in fed with ST infection; PMOS, the combination of PRO and MOS; PMOS.ST, the combination of PRO and MOS with ST infection; ST infection of 1 mL *Salmonella* Typhimurium ATCC 14028

3.3.2. Alpha Diversity of Rats' Gut Microbiota

The alpha diversity index of rats subjected to all treatments had higher Shannon and Simpson indices compared to those without treatment. The highest observed OTU (1686) was in rats fed with PRO without ST infection. The results were also confirmed by the highest Shannon and Simpson diversity index, with values of 7.60 and 0.984, as shown in Table 1. However, in the Chao1 diversity index, the highest was PMOS (1822), followed by PRO (1789) due to the influence of ST infection.

3.3.3. Beta Diversity Index in Rats' Gut Microbiota

Beta diversity was examined using principle component analysis (PCoA) graph and UPGMA cluster tree. Principle component analysis (PCoA) graph showed clustering patterns among microbiota community structures across treatments. Based on Figure 4, gut bacterial community structure of PRO.ST was similar to CONTROL on the same plot. Based on the results, rats fed with *P. kudriavzevii* 2P10 and infected with ST had normal gut microbiota without dysbiosis. However, ST infection in MOS-supplemented rats showed a similar community structure to infected CONTROL.ST. This showed that the administration of MOS without *P. kudriavzevii* 2P10 did not prevent dysbiosis. PRO and PMOS.ST treatments have similar gut microbiota community structures, while PMOS and MOS produced identical results.

Clusterization of the cecum bacterial communities based on the UPGMA cluster tree divided into three clusters. The first cluster was rat administrated with PRO, MOS, and PRO.ST, and the second cluster was rat administrated with PMOS, MOS.ST, CONTROL.ST, and PMOS.ST, and the last is control with no infection cluster (Figure 5).

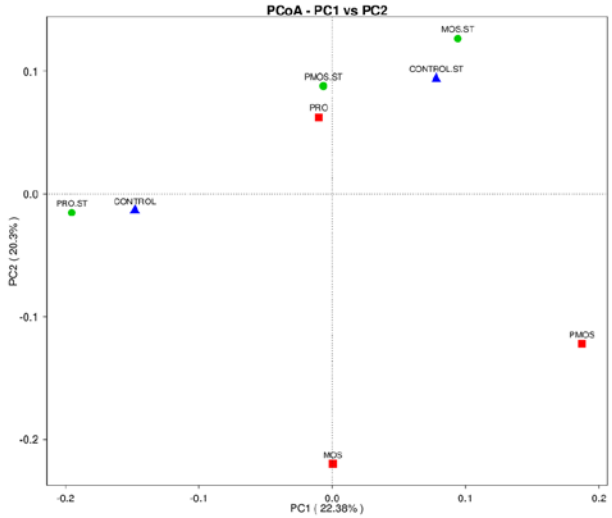


Figure 4. Principle component analysis (PCoA) based on unweighted UNIFRAC of gut microbial community of male SD rats in all treatments. Treatments: PRO (1 mL of *Pichia kudriavzevii* 2P10 10^8 CFU/mL), MOS (5% of Mannan-oligosaccharide), PMOS (PRO and MOS combination), ST (*Salmonella* Typhimurium infection 1×10^8 CFU/mL

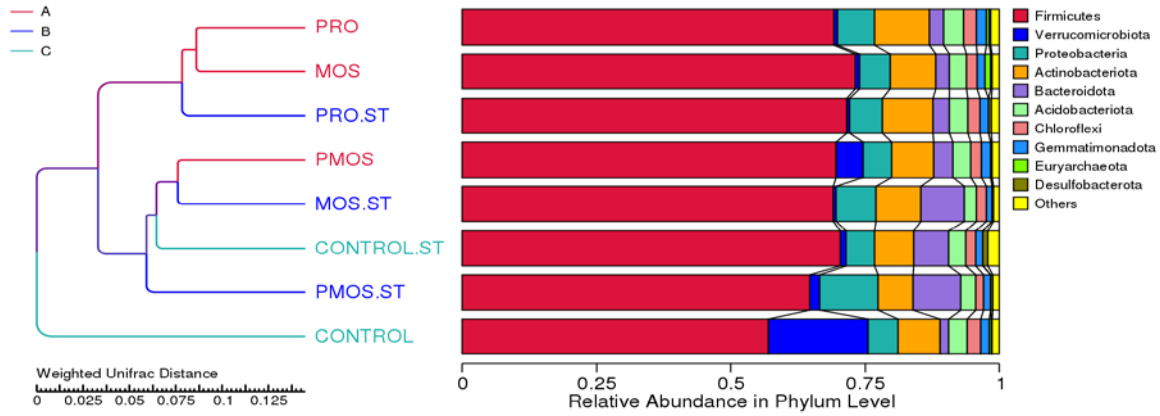


Figure 5. Clusters of bacterial communities in the caecum of rats treated with PRO (1 mL of *Pichia kudriavzevii* 2P10 10^8 CFU/mL), MOS (5% of Mannan-oligosaccharide), PMOS (PRO and MOS combination), ST (*Salmonella* Typhimurium infection 1×10^8 CFU/mL

3.3.4. Profile of Bacterial Gut Community at Filum Level

Firmicutes, Verrucomicrobiota, Proteobacteria, Actinobacteriota, Bacteriodota, Acidobacteriota, Chloroflexi, Gemmatimonadota, Euryarchaeota, and Desulfobacterota, formed the composition of the top 10 phyla in all treatments. According to Figure 6, Firmicutes dominated the phyla of bacterial microbiota with more than 65% relative abundance, compared to CONTROL at 57%. The highest relative abundance of Firmicutes was found in the MOS treatment (73.19%).

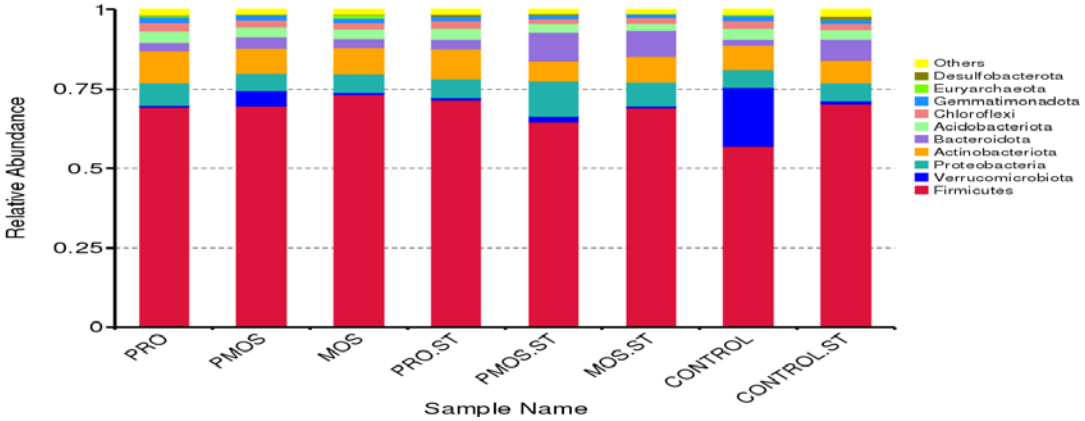


Figure 6. Profile of gut bacterial community at phylum levels following probiotic yeast and prebiotic treatments in Salmonella Typhimurium-infected and non-infected SD rats. The figure presents the relative abundance of OTU with more than 97% similarity. Treatments: PRO (Pichia kudriavzevii 2P10), MOS (Mannan-oligosaccharide), PMOS (PRO and MOS combination), ST (Salmonella Typhimurium infection).

3.3.5. Relative Abundance of Bacterial Gut Community at Family Level

Another phylum that is influential in gut microbiota is Bacteroidota (synonym: Bacteriodetes). Based on the results, all treatments without ST infection had a lower Firmicutes/Bacteroidetes ratio in the cecum compared to CONTROL, as shown in Table 2. Specifically, *P. kudriavzevii* 2P10, MOS, and PMOS showed potential to reduce the Firmicutes/Bacteroidetes ratio in gut microbiota. This showed that treatment with ST infection produced a lower Firmicutes/Bacteroidetes ratio than those ST.

Table 2. The relative abundance of cecum microbiota at the family level following treatment of *Pichia kudriavzevii* 2P10 (PRO), prebiotic mannan-oligosaccharide (MOS), probiotic yeast + prebiotic mannan-oligosaccharide (PMOS) in both ST ATCC 14028-infected and non-infected SD rats.

Relative abundance (%)	CONTROL	CONTROL.ST	PRO	PRO.ST	PMOS	PMOS.ST	MOS	MOS.ST
Firmicutes	0.488	0.633	0.590	0.623	0.613	0.556	0.645	0.617
Lachnospiraceae	0.213	0.234	0.365	0.296	0.242	0.165	0.353	0.227
Lactobacillaceae	0.100	0.315	0.080	0.161	0.238	0.264	0.076	0.231
Erysipelotrichaceae	0.023	0.016	0.047	0.054	0.074	0.049	0.083	0.086
Oscillospiraceae	0.072	0.018	0.029	0.038	0.027	0.034	0.032	0.018
Peptostreptococcaceae	0.046	0.040	0.057	0.048	0.026	0.033	0.053	0.028
Clostridiaceae	0.034	0.010	0.012	0.026	0.006	0.011	0.048	0.027
Verrucomicrobiota								
Akkermansiaceae	0.178	0.004	0.002	0.002	0.043	0.013	0.002	0.002
Bacteroidota	0.009	0.047	0.016	0.021	0.027	0.062	0.018	0.048
Muribaculaceae	0.008	0.004	0.011	0.020	0.025	0.046	0.015	0.037
Prevotellaceae	0.001	0.043	0.005	0.001	0.002	0.016	0.003	0.011
Proteobacteria								
Succinivibrionaceae	0.001	0.001	0.004	0.001	0.001	0.058	0.001	0.001
Others	0.324	0.315	0.390	0.354	0.317	0.320	0.335	0.334
F/B ratio*	36.37	10.81	26.84	23.57	19.33	7.22	28.32	8.55

Note: CONTROL, control without ST infection (negative control); CONTROL.ST, control positive with ST infection; PRO, 1 mL of probiotics yeast *P. kudriavzevii* 2P10 10^8 CFU/mL; PRO.ST, 1 mL of probiotics yeast *P. kudriavzevii* 2P10 10^8 CFU/mL with ST infection; MOS, mannan-oligosaccharide 5% in fed; MOS.ST, mannan-oligosaccharide 5% in fed with ST infection; PMOS, the combination of PRO and MOS; PMOS.ST, the combination of PRO and MOS with ST infection; ST. infection of 1 mL *Salmonella* Typhimurium ATCC 14028

*The Firmicutes/Bacteroidetes ratio

All treatments of PRO and MOS had a higher relative abundance of Firmicutes family members compared to CONTROL, as shown in Table 2. Lachnospiraceae had the highest abundance as observed in PRO (0.365) and MOS (0.353) among the top 10 families, followed by Lactobacillaceae. However, ST infection reduced the relative abundance of Lachnospiraceae in the treatment of PRO.ST, MOS.ST, and PMOS.T. The family with the second highest relative abundance was Lactobacillaceae, which was highest in CONTROL.ST (0.315), followed by PMOS (0.238), PMOS.ST (0.264), and MOS.ST (0.231). The reduction in the abundance of Lactobacillaceae could be attributed to treatment with ST infection. Regarding Clostridiaceae, the highest relative abundance was found in CONTROL (0.034) and MOS (0.042) in addition to Akkermansiaeceae. Moreover, Provotellaceae in CONTROL had the lowest value compared to other treatments.

In phylum Bacteriodota, the relative abundance in all treatments was higher than CONTROL, as shown in Table 2. Based on the results, ST infection also increased the abundance of Bacteriodota, with the highest being in observed PMOS.ST (0.062) and MOS.ST (0.048). In this study, the highest abundance of family members of phylum Bacteriodota were Muribaculaceae and Prevotellaceae.

3.4. Histopathology of Rats' Cecum

The confirmation of gut microbiota results was achieved through histological examination of rats' cecum. The histology results showed that the control-treated cecum, which was given standard feed and infected with ST ATCC 14028 (CONTROL.ST), had more severe cecal epithelial damage, as presented in Figure 7. This damage manifested as epithelial desquamation (ed) and a more tenuous structure of the villous epithelium of the cecum. Specifically, the histology of the cecum appeared normal in CONTROL treatment without ST infection, PRO, MOS, and PMOS.

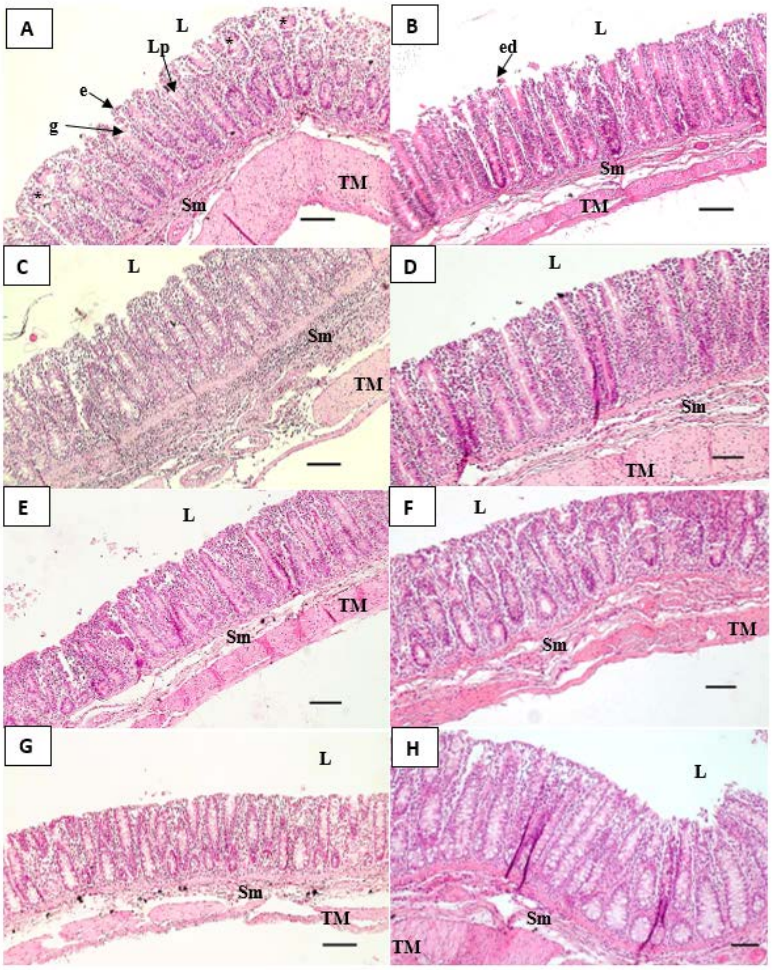


Figure 7. Photomicrographs of rat's cecum histology in all treatments with Hematoxylin-Eosin (HE) staining: A) CONTROL, B) epithelial desquamation in treatment CONTROL.ST= control with ST infection, C) PRO= Probiotic yeast *P. kudriavzevii* with no ST infection, D) PRO.ST= Probiotic yeast *P. kudriavzevii* with ST infection, E) MOS= 5% of prebiotic MOS with no ST infection, F)MOS.ST= 5% of prebiotic MOS with ST infection, G) PMOS= Combination of probiotics yeast and prebiotics with no ST infection, H) Combination of probiotics yeast and prebiotics with ST infection (PMOS.ST). After 4th days ST ATCC 14028 10^8 CFU/mL infection. L= cecal lumen; e= epithelial, ed= epithelial desquamation, g= goblet cell*; Lp= lamina propria; Sm= Submucosa; TM= Tunica Muscularis. Scale bar= 50 µm.

4. Discussion

Pichia sp. is a yeast from Ascomycota division isolated from fermented foods (Astuti *et al.*, 2023). As shown in Figure 2, *P. kudriavzevii* 2P10 used in this study is a yeast or single-cell fungi that reproduces through budding. This yeast is native to Indonesia from the cocoa fermentation process and has been characterized as a probiotic (Wulan *et al.*, 2021; Astuti *et al.*, 2023), due to the ability to survive at pH 3, 0.5% bile salts tolerance, withstand temperatures of 37°C and 41°C. Other characteristics include the non-hemolytic ability to auto-aggregate and co-aggregate with ST ATCC 14028, acting as an antioxidant (Wulan *et al.*, 2021). Generally, probiotic yeast at a dose of 10^8 CFU/mL is considered capable of modulating gut microbiota (Roobab *et al.*, 2020). A dose of ST ATCC 14028 1×10^8 CFU/mL through oral gavage is also effective in causing changes in beta diversity in broiler chickens consistently (Martins *et al.*, 2010; Sheets *et al.*, 2022). This infection is supported by the high growth rate in ST at μ_{max} 0.58 ± 0.1039 $hour^{-1}$ on the Mueller-Hinton medium (Figure 3).

ST is a major foodborne pathogen that causes diarrheal infections globally, particularly in children (Kirk *et al.*, 2017). Generally, rats are often used as biomedical models to explore the correlation between gut microbiota and gastrointestinal (GI) disease. Gut microbiota consists of bacteria, yeast, and viruses, with cecum of rats serving as the largest and most active fermentative digestive chamber. Specifically, cecum has the highest microbial community compared to the stomach and small intestine (Li *et al.*, 2017).

The top 10 gut microbiota in cecum of rats were Firmicutes, Verrucomicrobiota, Proteobacteria, Actinobacteriota, Bacteriodota, Acidobacteriota, Chloroflexi, and Gemmatimonadota, as shown in Figure 6. Firmicutes were found to be the most dominating phylum, with a relative abundance of over 65% (Li *et al.*, 2017; Sivixay *et al.*, 2021). Rat fed with *P. kudriavzevii* 2P10, MOS, and PMOS increased the abundance of Firmicutes, with MOS showing the best efficiency. Previous studies showed that the administration of a high-cholesterol diet with 1% MOS for 14 weeks increased the abundance of *Bacteroides ovatus*, a genus of Firmicutes known as mannan fermenter (Hoving *et al.*, 2018). Additionally, MOS reduced the colonization of *S.* enteritidis,

Salmonella, and *Escherichia coli* (Ghasemian and Jahanian, 2016; Azad *et al.*, 2020), serving as prebiotic commonly used in poultry to promote gut health and combat *Salmonella* infection (Micciche *et al.*, 2018). The administration of mannan-oligosaccharides has a dominant growth effect on firmicutes. The Firmicutes phylum plays a crucial role in maintaining health, and members of the Firmicutes phylum belong to commensal butyrate-producing bacteria (Lindstad *et al.,* 2021). Previous studies also reported that the most dominant phylum Firmicutes was found after the turkey was given Mannan-oligosaccharides (Corrigan *et al.*, 2012).

In this study, microbial diversity analysis was carried out by analyzing alpha and beta diversity to determine the influence of various treatments (Asriatno *et al.*, 2023). Alpha diversity indices, including the Simpson and Shannon indices, increased after administration of all treatments, with the highest value observed in *P. kudriavzevii* 2P10. This suggested that the diversity of gut microbiota could be affected by the administration of *P. kudriavzevii* 2P10, MOS, and PMOS. Significant improvements in gut microbiota richness are associated with positive health status, while decreases in species diversity are related to disease symptoms (Manor *et al.*, 2020; Bao *et al.*, 2022)**.** Based on the results, ST infection across all treatments caused a significant decrease in the Simpson and Shannon indices. Furthermore, this infection led to Salmonellosis, which caused a reduction in the diversity of gut microbiota. Several studies showed that Salmonellosis could lead to inflammatory bowel disease (IBD) and inflammation-induced dysbiosis (Bratburd *et al.*, 2018; Gillis *et al.*, 2018; Rinninella *et al.*, 2019; Bao *et al.*, 2022).

Based on beta-diversity analysis, the bacterial community cluster in rat cecum, PRO, MOS, and PRO.ST are in the same cluster, which is different from CONTROL.ST (Figure 3). The presence of PRO, MOS, and PRO.ST in the same cluster indicates the similarity of the bacterial community structure. ST infection in rats treated with probiotic yeast *Pichia kudriavzevii* 2P10 does not affect the structure of the cecum microbiota, so dysbiosis does not occur. Other studies have reported that probiotic yeast *Pichia kudriavzevii* Y33 has antibacterial properties against various pathogenic bacteria such as *Salmonella typhi, Escherichia coli, Shigella, Pseudomonas aeruginosa, Bacillus cereus, Staphylococcus aureus, Aeromonas hydrophila,* and *Listeria monocytogenes* (Lata *et al*., 2022). The mechanism of action of the probiotic yeast *S. boulardii* is by inhibiting the abundance of pathogenic bacteria, thereby increasing microbial diversity and restoring microbiota dysbiosis in mice (Gao *et al*., 2023).

Firmicutes and Bacteroidota (synonym Bacteroidetes) are the two main species that determine the homeostasis of gut microbiota, thereby serving as marker of health status. Bacteria from these species are the most common, representing 90% of gut microbiota (Rinninella *et al.*, 2019; Stojanov *et al.*, 2020). Phylum Firmicutes includes Gram-positive bacteria with 'firm' or rigid or semi-rigid cell walls, predominantly originating from the genera *Bacillus, Clostridium, Enterococcus, Lactobacillus,* and *Ruminococcus* (Seong *et al.*, 2018; Stojanov *et al.*, 2020). In PRO, PRO.ST, PMOS, PMOS.ST had a lower relative abundance of Firmicutes compared to CONTROL.ST. This is in line with previous yeast probiotic studies, that *Saccharomyces boluardii* also increased the relative abundance of Bacteroidetes and decreased Firmicutes and Proteobacteria. This condition is associated with improved metabolic health and reduced inflammation (Yu *et al.,* 2017).

In Bacteroidota, the relative abundance across all treatments was higher compared to CONTROL, as shown in Table 2. Specifically, ST infection increased the abundance of Bacteriodota in all treatments, with the highest being observed in PMOS.ST (0.062) and MOS.ST (0.048). Bacteroidetes is the largest phylum of Gram-negative bacteria that inhabit the digestive tract, consisting of approximately 7000 species of predominantly Gram-negative bacteria from the genera *Bacteroides, Alistipes, Parabacteroide*s, and *Prevotella*. Specific roles have been attributed to several Bacteroidetes genera play specific roles in the development of immune dysregulation, systemic diseases such as metabolic syndrome, and neurological disorders (Gibiino *et al.*, 2018). This study found two families in phylum Bacteriodota, namely Muribaculaceae and Prevotellaceae.

PRO, MOS, and PMOS treatments increased the abundance of phylum Bacteroidota compared to CONTROL after ST infection, with the highest observed PMOS.ST and MOS.ST. Previous studies reported that *Bacteroides* spp., in rats, mediated colonization resistance to ST by producing propionic short-chain fatty acids (SCFAs). Propionate directly inhibited the growth of pathogens in vitro by disrupting intracellular pH homeostasis, and increased intestinal propionate levels chemically to protect rats from ST infection (Jacobson *et al.*, 2018).

The results showed that the administration of *P. kudriavzevii* 2P10, MOS, and PMOS, including ST infection reduced Firmicutes/Bacteroidetes ratio in the cecum microbiota of rats compared to CONTROL. Moreover, this study is the first to report the effect of *P. kudriavzevii* 2P10 on gut microbiota in male SD rats. A previous investigation focusing on probiotic yeast *S. boulardii* in supernatant form reduced Firmicutes/Bacteroidetes ratio in 6-week-old male C57BL/6J rats (Gu *et al.*, 2022). *S. boulardii* also significantly improved the relative abundance of Bacteroidetes but decreased Firmicutes and Proteobacteria in adult BALB/c rats (Yu *et al.*, 2017). Furthermore, the administration in type 2 diabetic and obese rats (db/db) was associated with a significant increase in Bacteroidetes by approximately 37% and a 30% decrease in Firmicutes (Everard *et al.*, 2014).

The treatment with ST infection had a lower Firmicutes/Bacteroidetes ratio than those without infection due to a significant increase in the abundance of Bacteroidetes. This showed that a decrease in Firmicutes/Bacteroidetes ratio served as a marker of ST infection. Proteobacteria contribute to dysbiosis (Shin, Whon and Bae, 2015), showing a significant correlation between a decrease in Firmicutes and general microbial diversity in inflammatory bowel disease (IBD) (Morgan *et al.*, 2012).

At the family level, Lachnospiraceae and Lactobacillaceae were dominant in all treatments. Although the abundance of Lachospiraceae increased after the administration of *P. kudriavzevii* 2P10, MOS, and

PMOS, there was a significant decrease on the fourth day due to the effect of ST infection on rats. The family Lachnospiraceae is a phylogenetically and morphologically heterogeneous taxon of the class Clostridia, phylum Firmicutes. All members are anaerobic, fermentative, and chemoorganotrophic, some with hydrolyzing solid activities and fermenting carbohydrates (Stackebrandt, 2014). Furthermore, gut microbiota in the human cecum is formed by two anaerobic bacteria families, Lachnospiraceae and Ruminococcaceae (Lee *et al.*, 2018).

Carbohydrate metabolism by gut microbiota is an essential mechanism that provides the host with nutrition and energy. Butyrate and short-chain fatty acids (SCFAs) could be produced through the hydrolysis of starch and other sugars by Firmicutes families Lachnospiraceae, Lactobacillaceae, and Ruminococcaceae. Although Lachnospiraceae members are among the top producers of SCFAs, several taxa have been related to various intra- and extraintestinal diseases (Biddle *et al.*, 2013; Vacca *et al.*, 2020). Treatment of *P. kudriavzevii* 2P10, MOS, and PMOS reduced the abundance of Lactobacillaceae, which were not commonly found in the cecum as their main habitat is the ileum (Lee *et al.*, 2018).

The histological evaluation of the cecum structure confirmed dysbiosis in the gut microbiota. The results showed that the control cecum, which was fed standard food and infected with ST ATCC 14028 (CONTROL.ST), had more epithelial desquamation (ed) and tenuous cecum villous epithelial structure. The report of Setyawardani *et al.*, (2017) also showed that ST ATCC 14028 caused epithelial detachment in the ileum 10 days after infection. *Salmonella* Enteritidis P125109 infection caused mild erosion of the cecum mucosal epithelium in control treatments without probiotic administration. However, when probiotic *Lactobacillus rhamnosus* EM1107 was administered, there was no cecum epithelium erosion (Rolim *et al.*, 2021). In this study, control treatment without ST infection, PRO, MOS, and PMOS, cecum histology appeared normal. According to Gu *et al.*, (2022), there were no apparent pathological lesions in rats' colon tissue in a group of irritable bowel syndrome (IBS) model rats given the probiotic yeast *Saccharomyces boulardii* supernatant.

5. Conclusion

In conclusion, this study showed that ST infection could disrupt gut microbial community in SD rats. However, the treatments of *P. kudriavzevii* 2P10 and MOS modulated gut microbiota, potentially preventing the severity of dysbiosis after ST infection, which reduced Firmicutes/Bacteroidetes ratio. *P. kudriavzevii* 2P10 treatment in SD rats infected with ST provided Firmicutes/Bacteroidetes ratio similar to controls and other treatments without infection. The administration of *P. kudriavzevii* 2P10 also prevented damage to the histological structure of the cecum. The results showed that probiotic yeast *Pichia kudriavzevii* 2P10 treatment optimally modulated gut microbiota compared to MOS and PMOS.

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Authors' contributions

R.W. contributed to the experimental design, study work, and writing of the manuscript preparation. R.I.A. contributed to the experimental design and revision of the manuscript before submission. Y.R. and S.E. contributed to the experimental design. A.M. contributed to the study plan and was responsible for the overall study and funding.

Declaration of Competing Interest

The authors declare no competing personal or financial interests regarding the publication of this study.

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