Different Intestinal Microbiota Composition Inhibits the *Lactobacillus* Growth in Rheumatoid Arthritis Patients in Malang, Indonesia

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Abstract

Rheumatoid arthritis (RA) is an autoimmune disease that can cause progressive damage to the joints of patients. The number of patients is expected to increase, along with the exact cause of this disease remains unknown. However, there are several risk factors associated with RA, including dysbiosis. The purpose of this study was to characterize the composition of intestinal microbiota in the RA and control groups through fecal analysis and reveal the association of microbiota composition with RA disease in Indonesia, especially Malang. Fecal samples were obtained from RA patients and controls. Fecal analysis was carried out through several stages, namely the calculation of total bacterial colonies, isolation and characterization of anaerobic bacteria, calculation of the Simpson diversity index, and DNA isolation. Analysis of bacterial composition profiles in fecal was carried out using 6 specific primer sets through PCR analysis. The results of the 16S rRNA PCR analysis showed different microbiota compositions between RA patients and controls. The number of *Enterococcus* bacterial group was lower in the control patients than the RA group, whereas the *Lactobacillus* bacteria decreased in RA patients. In addition, our study found that the existence of bacterial isolate 11 changed the composition of microbiota in RA patients, and the DNA band only appeared in Universal primers. The diversity of bacterial species can provide symbiotic and pathogenetic effects in RA patients.

Keywords: Dysbiosis, intestinal microbiota, PCR, rheumatoid arthritis.

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease in humans, which is characterized by metabolic disorders and damage to several joints [1]. This disease has received serious attention in Indonesia since the prevalence increased from 6.12% in 2013 [2] to 11.9% in 2018 [3]. The exact cause of RA disease is still unknown hitherto. This phenomenon is caused by RA including in group of multifactor disease. Therefore, the incidence of RA is expected to increase continously.

Previous studies have reported that several factors increase the risk of RA, including age, sex, ethnicity, genetics, and environment [4]. One factor that has been a concern of researchers is the relationship between the emergence of this disease with abnormalities of microbiota (dysbiosis) in the intestinal tract. Dysbiosis may be involved with RA pathogenicity [5]. The condition of dysbiosis in the human digestive tract causes changes in the lining of the intestinal epithelial cells leading to inflammation [6].

Inflammation can trigger the synovial tissue of the joints to form pannus tissue. Pannus tissue will invade bone resulting in damage to the cartilage in the joints [7]. Synovial tissue inflammation can be initiated by several factors, namely immunological and microbial disorders, including oral bacterial DNA in the mouth, increased expression of the peptidylarginine deiminase (PAD) enzyme, unmethylated CpG oligonucleotide, and lipopolysaccharide in bacterial membranes [8].

The human digestive tract contains an abundant and diverse microbial community. Bacterial abundance in the digestive tract is estimated to reach 10^{11} to 10^{12} per-milliliter [9]. In normal condition, the dominant intestinal microbiota originates from the phyla *Firmicutes, Bacteroidetes, Actinobacteria,* and *Verrucomicrobia* [10]. Besides being involved in the digestion and absorption of food, bacteria are also known to be involved in the modulation of the immune system [11-13]. Previous studies have reported changes in the composition of microbiota in the intestine with the immune system lead to autoimmune diseases, which is rheumatoid arthritis [14].

The intestinal microbiota composition of RA patients and controls showed a difference, wherein RA patients, there was a reduction of certain bacteria in the *Bifidobacterium* and *Bacteroides* family [15]. Other studies showed the increase of *Lactobacillus salivarius* species in RA [16]. However, the correlation of the

intestinal microbiota composition in affecting the development of RA in humans is still unclear due to various factors of the disease. Therefore, this study was conducted to characterize the composition of intestinal microbiota in RA and control (Non-RA) patients in Indonesia, especially Malang city, through fecal bacteria analysis based on 16S rRNA using PCR. Fecal bacterial analysis can describe the microbial community in the intestine that initiates the emergence of disease in humans because it is related to the fact that fecal is the final product of the digestive process that occurs in the intestine [17]. This research is expected to reveal new things about the composition of microbiota in the intestines of RA sufferers in Indonesia.

MATERIAL AND METHOD
Ethics Clearance

This study was approved with the code of ethics (ethical clearance) No. 051/EC/KEPK-FKIK/2019 by the Health Research Ethics Commission (KEPK) Faculty of Medicine and Health Sciences UIN Maulana Malik Ibrahim Malang.

Sampling

Fecal samples were obtained from the hospital with a doctor's intermediary. Patients were recruited based on the sampling method after obtaining an explanation and agreement through the signing of informed consent. Fecal samples were divided into two groups, namely the control (non-RA) and RA group, which consists of three patients as replicates in each. Recruitment of patients as control (Non-RA) and RA group was based on medical records from the hospital with several inclusion criteria of not taking antibiotics, not consuming yogurt during the last four weeks before sampling, and having Indonesian citizenship based on an identity card. Fecal sampling was done by giving patients a sterile set of sampling equipment. Furthermore, fecal samples were stored at 4°C for further analysis [18].

Culture and Determination of Aerobic Mesophilic Bacteria

The 0.5 grams of fecal samples from each patient were collected and then put into NaCl 0.9% using the serial dilution technique. Dilution was carried out to the desired concentration. The suspension was cultured in plate count agar (PCA) media and then aerobically incubated at 37°C for 48 hours. The aerobic bacterial colonies were counted using a colony counter. The calculation was established in triplicate for each control (Non-RA) and the RA group.

Isolation and Characterization of Anaerobic Bacteria

Anaerobic bacterial isolation was done using the serial dilution technique with peptone yeast glucose (PYG) agar. A diluted sample solution of 0.1 mL was put into a sterile petri dish using the pour plate technique and then incubated in the anaerobic jar at room temperature for 48 hours. Anaerobic bacterial colonies were counted and observed for the morphological characteristics. They were purified using the four-way streak plate technique until the pure culture was obtained.

Simpson index calculation

The diversity index used in this study was the Simpson index. The total number of colonies and the number of different species' colonies found in each sample were calculated. The data obtained were analyzed using the Id formula [19]. The Simpson diversity index ranged from 0 to 1. Id values could be calculated using the following formula [20]:

$$Id = \frac{\Sigma\, Ni(Ni-1)}{N(N-1)}$$

Description:
Id = Diversity index
Ni = Number of specific individuals / species found
N = Total number of individuals found

The representation of the index value is as follows:
0.00 - 0.3 = Low dominance, high diversity
0.31 - 0.6 = Moderate dominance, moderate diversity
0.61 - 1.0 = High dominance, low diversity

Bacterial DNA isolation

Anaerobic bacteria were grown in 3 mL of Luria Bertani (LB) broth media for 16 hours at 37°C. Bacterial culture in LB media was centrifuged at 10,000 rpm, 25°C for 5 minutes. The pellets obtained were added with buffer lysis (25 mM EDTA pH 8, 10 mM Tris-HCl pH 8, 100 mM NaCl, 1% SDS, 5 mg.mL^{-1} proteinase-K, and distilled water) and then incubated at 37°C for an hour. Furthermore, the isolation steps of anaerobic bacterial DNA in fecal samples followed the previous method with several modifications [21]. The obtained DNA was stored at -20°C until used.

Bacterial DNA amplification

The PCR technique was based on the previous method with several modifications [21]. The PCR was performed using a thermal cycler with the PCR program set up as follows: hot start at 95°C for 3 minutes; 31 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 1 minute; and post

extension at 72°C for 5 minutes. Primers used for amplifying the DNA of digestive tract bacteria were the primary set of Universal, *Bacteroides, Clostridium, Bifidobacterium, Enterococcus,* and *Lactobacillus* (Table 1) [22]. The amplification results were detected using 1.5% agarose gel and visualized using the UVP Biodoc-IT Imaging System.

Table 1. Primers used in bacterial DNA amplification

Primer	Primer sequences (5'-3')	Ref
Uni-F	TACGGGAGGCAGCAG	23, 24
Uni-R	ATTAACCGCGGCTGCTGG	
g-Bifid F	CTCCTGGAAACGGGTGG	23
g-Bifid R-	GGTGTTCTTCCCGATATCTACA	
Ent.1017F	CCTTTGACCACTCTAGAG	23
Ent.1263R-	CTTAGCCTCGCGACT	
Lac1-F	AGCAGTAGGGAATCTTCCA	23
Lac2-R	ATTYCACCGCTACACATG	
Bact_F	TCAGTTGTGAAAGTTTGCG	23
Bact_R-	GTRTATCGCMAACAGCGA	
Ccoc_F	TGACGGTACCTGACTAA	24
Ccoc_R-	CTTTGAGTTTYATTCTTGCGAA	

Source: Scientific jounnal [22]

Statistical data analysis

Data were analyzed through descriptive statistics using Microsoft Excel and one-way ANOVA using SPSS 16.0 for windows. Statistical tests performed normality and homogeneity tests with a significance level of 5% ($p < 0.05$).

RESULT AND DISCUSSION

The Amount of Aerobic Mesophilic Microbiota in the Fecal Samples of Control and RA Patients

The aerobic mesophilic count (AMC) performed in three control groups and three RA patients showed differences. The calculation results on Control C1, C2, and C3 were $0.84 \times 10^8 \pm 0.34$, $43800 \times 10^8 \pm 4.79$, and $2.58 \times 10^8 \pm 0.10$, respectively (Table 2). This value indicated that the amount of AMC of C2 was higher than that of C1 and C3. AMC values of RA patients RA1, RA2 and RA3 were $550 \times 10^8 \pm 0.23$, $8.13 \times 10^8 \pm 0.36$ and $0.37 \times 10^8 \pm 0.04$, respectively.

Table 2. Aerobic mesophilic count of bacterial colonies in control and RA patients

No	Sample	Total bacteria (x 10^8 CFU.mL^{-1})	
		P < 0.01	P < 0.05
1	C1	0.84 ± 0.34^{ab}	0.84 ± 0.34^{a}
2	C2	43800 ± 4.79^{abc}	43800 ± 4.79^{abcd}
3	C3	2.58 ± 0.10^{ab}	2.58 ± 0.10^{b}
4	RA1	550 ± 0.23^{abc}	550 ± 0.23^{c}
5	RA2	8.13 ± 0.36^{cd}	8.13 ± 0.36^{d}
6	RA3	0.37 ± 0.04^{bc}	0.37 ± 0.04^{a}

Notes: Values indicate the mean ± standard deviation. Values with the same notation in the column do not differ significantly ($p < 0.01$ and $p < 0.05$).

The RA1 had a higher AMC value than that of RA2 and RA3. The AMC value of C1 was significantly lower than RA2 ($p < 0.01$). There were no significant differences between C2, C3, RA1, and RA3. However, the AMC values of C1, C3, RA1, RA2, and RA3 showed significant differences with a significance level of 5% ($p < 0.05$) (Table 2).

Morphological Characters and Diversity of Microbiota

The most abundant type of microbes found in the human digestive tract was anaerobic bacteria. Previous data reported that the total number of bacteria in each individual's fecal sample consisted of aerobic and anaerobic bacteria. The results of anaerobic bacterial isolation showed the presence of bacterial colonies with 30 different morphological characters. Morphological characters observed included shape, configuration, elevation, optical characteristics, texture, and color. A total of 30 isolates were screened representing each group until eight selected isolates were obtained. The selection was based on characters, including the bacterial isolates found in the control group and RA, found only in the control group, and found only in the RA group (Fig. 1, Table 3).

Our study reported differences in bacterial composition in RA and control patients. The data were used to analyze the diversity index using the Simpson index, so that bacterial dominance could be investigated in each group.

The microbial diversity index in RA and control patients showed variations. The Simpson index values of C1, C2, and C3 were 0.79, 0.68, and 0.73, respectively. However, the Simpson index values of RA1, RA2, and RA3 were 0.63, 0.71, and 0.65, respectively (Table 4). The higher Simpson index value indicated the dominating bacterial group, so it could be inferred that several species of bacteria predominated in each RA and control patients.

Our study observed that isolate 1 dominated in C3, RA1, and RA2. Isolate 10 dominated in C2, RA1, and RA2, while isolate 9 dominated C1. Of the three isolates found in C3, isolate 16 was the least. This indicated that there was a competition between each isolate in C3, which caused the different numbers of each isolate. Differences in the abundance of isolates found in the control and RA group could also represent dysbiosis in the RA group (Table 4).
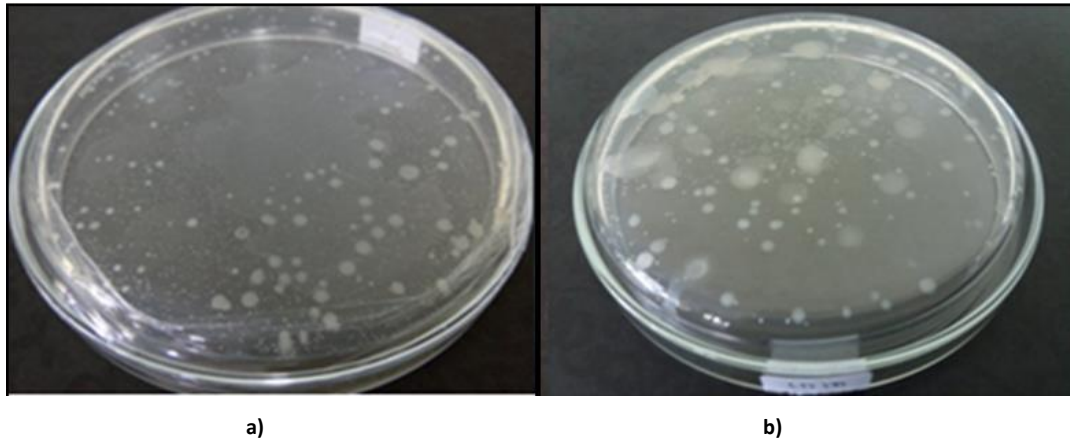
Figure 1. Morphology of anaerobic bacterial colonies found in fecal samples. a) Control, b) RA patient

Table 3. Morphological characterization of anaerobic bacterial colonies in fecal samples of control and RA patients

Isolate	Shape	Configuration	Elevation	Optical Characteristics	Texture	Color
3*	Oval	Entire	Flat	Clear	Smooth	Clear & White
7*	Round	Entire	Flat	Clear	Smooth	Clear & White
16*	Round	Entire	Pulvinate	Oblique Steep	Smooth	White Milk
18**	Round	Entire	Flat	In diameter	Smooth	Clear, Cloudy & White
1**	Round	Entire	Flat	Clear	Smooth	Clear & White
9**	Round	Entire	Flat	Small	Smooth	White Milk
10**	Round	Entire	Pulvinate	Oblique	Smooth	White Milk
11***	Irregular	Entire	Flat	Oblique	Rough	Clear & White

Description: C = control; RA= RA patient; *= only found in control; **= found in control and RA; ***= only found in RA.

Table 4. Data interpretation on the level of bacterial dominance using the Simpson index in each fecal sample

Isolate	C1	C2	C3	RA1	RA2	RA3
Isolate 1	5	12	124	248	52	27
Isolate 3	1	0	0	0	0	0
Isolate 7	17	0	0	0	0	0
Isolate 9	31	3	0	0	7	0
Isolate 10	2	19	0	207	56	17
Isolate 11	0	0	0	2	4	5
Isolate 16	0	0	23	0	0	0
Isolate 18	0	0	62	3	0	0
Id Simpson	0.79	0.68	0.73	0.63	0.71	0.65

Description: C = control; RA= RA patient

16S rRNA gene analysis

The 16S rRNA gene analysis showed that all selected isolates were successfully amplified at 200bp using universal primers (Fig. 2). Isolate 1, isolate 9, and isolate 18 were identified as *Enterococcus* group with a band size of 300bp. Isolate 3, isolate 7, and isolate 16 were *Lactobacillus* with a band size of 400bp (Fig. 2). However, isolate 10 and isolate 11 were not identified in all markers used in this study.

DISCUSSION

This study showed a significant difference in the amount of aerobic mesophilic number of microbes in the RA and control groups. The amount of aerobic mesophilic number of microbes in the RA group was higher significantly than in the control group ($P<0.01$). Among the 3 control groups, C2 was the sample that had the highest number of microbes. It can be influenced by various factors, such as age, diet, and the disease being suffered [9]. In addition, these factors can cause dysbiosis [25]. This condition results in an imbalance amount of microbes in the digestive tract [26]. Previous studies reported a link between food protein consumption and intestinal microbiota composition based on 16S rRNA gene sequencing analysis [27]. Subjects who consumed beef food showed a reduction in the number of groups of *Bifidobacterium*, *Bacteroides*, and *Clostridium*

bacteria compared with subjects who consumed meatless food [28]. It is known that consumption of peas can increase the group of *Bifidobacterium* and *Lactobacillus* bacteria, while consumption of whey protein can reduce pathogenic *Bacteroides fragilis* and *Clostridium perfringens* [29-30]. This causes the majority of researchers to reveal that protein consumption is positively correlated with microbial diversity [30-32]. We assume the types of food and nutrients consumed are some of the factors that cause the diversity of microbiota composition in the intestine to change, so that it can lead to the emergence of RA disease.

Previous research revealed that the condition of dysbiosis can trigger RA disease through mucosal immune responses induced by collagen-induced arthritis (CIA) [33]. The intestinal microbiota composition correlates with the overall increase in RA symptoms in CIA mice [34]. The susceptibility and severity of arthritis in some rodents kept in a germ-free environment has shown to be reduced compared to rodents induced by the CIA [35]. The part that plays an important role in the interaction between the host and the external environment is the surface of the mucosa, one of which is found in the digestive tract [36]. The mucous layer is continuously exposed to microorganisms that are both commensal and pathogenic [37].

The mucosal immune response is initiated through the introduction of bacterial antigens by mucosa-associated lymphoid tissue (MALT) [38]. MALT follicles containing various immunocompetent cells (T cells, B cells, and APCs) subsequently initiate an immune response [39]. In the RA condition, the barrier which becomes the defense site in the intestine is damaged [40]. This causes bacterial antigen to penetrate the mucosa. The incoming antigen can be eliminated by regulator T cells with specific functions [41].
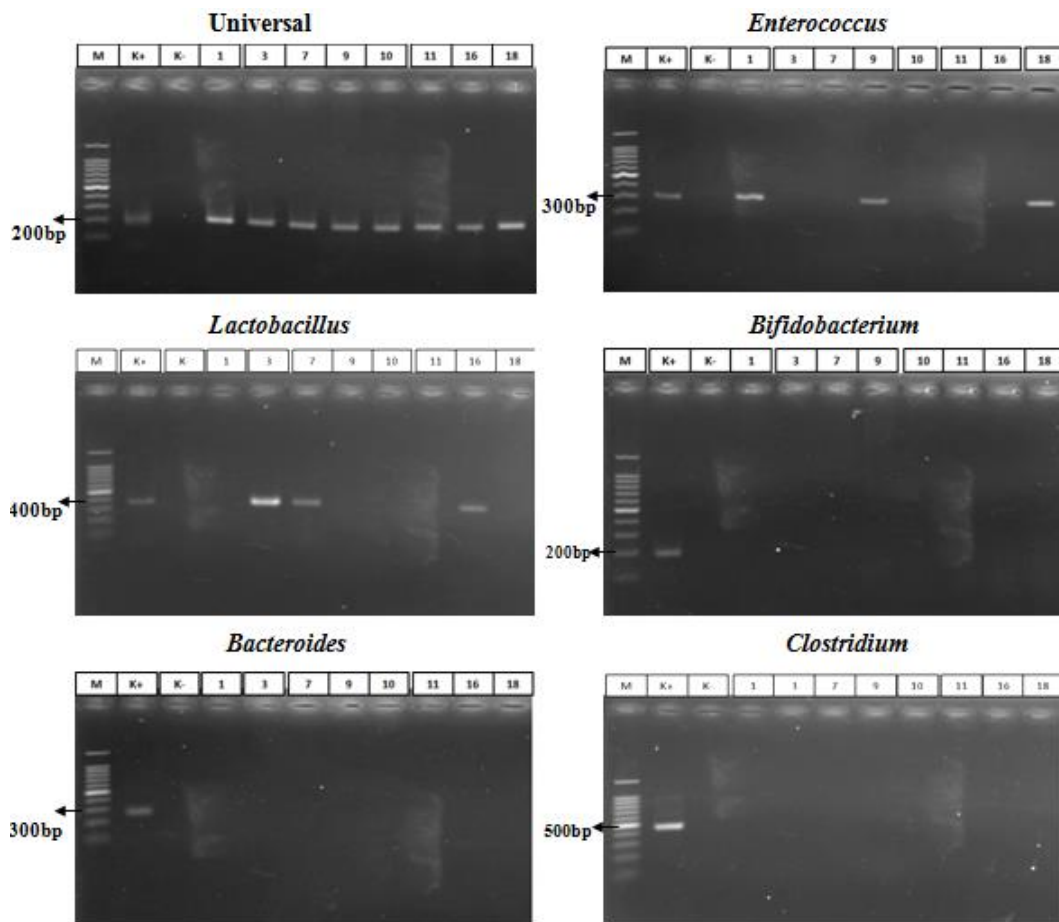

Figure 2. DNA amplification showed bacterial profiling of Universal, *Enterococcus*, *Lactobacillus*, *Bifidobacterium*, *Bacteroides*, and *Clostridium*.

Table 5. Interpretation of 16S rRNA PCR products in selected isolates

Isolate	Universal Bacteria	*Enterococcus*	*Lactobacillus*	*Bifidobacterium*	*Bacteroides*	*Clostridium*
	±200bp	±300bp	±400bp	±200bp	±300bp	±500bp
Isolate 1	+	+	-	-	-	-
Isolate 3	+	-	+	-	-	-
Isolate 7	+	-	+	-	-	-
Isolate 9	+	+	-	-	-	-
Isolate 10	+	-	-	-	-	-
Isolate 11	+	-	-	-	-	-
Isolate 16	+	-	+	-	-	-
Isolate 18	+	+	-	-	-	-

Description: + = present; - = absent

In patients with RA, dysregulation of immune system is characterized by the appearance of autoantibodies and autoreactive T cells. RA patients experience dysfunction in circulating regulatory T (Treg) cells as well as an increase of T helper 17 (Th17) in synovial tissue [42]. In this case, macrophages and dendritic cells provide an environment that supports Th17 differentiation and suppresses Treg cell differentiation, which can shift T cell homeostasis toward inflammation [43]. Increasing autoantibody production triggers the formation of more proinflammatory cytokines. The secretion of proinflammatory cytokines stimulates B cells to produce antibodies [44].

One of the factors causing damage to mucosal sites is changes in diversity and abundance of microbes [45]. This triggers inflammation through an imbalance of T cell subpopulations (Th1, Th2, Th17 and Treg cells) [46]. The diversity and abundance of microbes can be analyzed using the Simpson index [47]. The Simpson index analysis results indicate the presence of microbial dominance in the RA and control groups. Overall, the control group had a higher dominance value than the RA group. This showed that the types of microbes in the digestive tract of the RA group were more diverse. High bile acid concentration in the digestive tract is one of the factors that cause the diversity of bacteria. The anatomical structure of the intestine, pH, oxygen pressure, availability of substrate and food flow rate are also the elements that lead to the diversity of bacteria in the digestive tract [48]. The data also showed that the structure of the intestinal microbiota could change periodically.

Changes in microbial diversity in the control and RA group can be evaluated through 16S rRNA gene analysis using PCR. The results of the 16S rRNA gene analysis showed a group of *Enterococcus* found in both the RA and control group (Table 5). The abundance of *Enterococcus* in the control group was lower than in the RA group. *Enterococcus* is one of the pathogenic bacteria that often causes bloodstream infections (BSI) [49]. The common species of *Enterococcus* found in the digestive tract are *E. faecalis* and *E. faecium*. However, *E. faecium* was reported to have a higher level of antibiotic resistance [50]. Previous studies had also found that patients with inflammatory bowel disease and rheumatoid arthritis have a high risk of being infected with *E. faecium* [51]. Infection by various bacteria and viruses often manifests arthritis. Other bacteria associated with infections of the digestive tract are *Salmonella* and *Shigella,* which can cause oligoarticular inflammation or polyarticular arthritis within four weeks of infection [52].

The results of this study reported that *Lactobacillus* bacteria dominated the control group, which was not found in the RA group. Some studies showed the opposite, where *Lactobacillus* was more common in RA patients than in the healthy control group [53,54]. Previous studies mentioned several species of *Lactobacillus*, such as *L. bifidus*, can cause joint swelling in mice [55]. *Lactobacillus* is a normal bacterial flora that has a role as a probiotic. The presence of probiotic microorganisms in the intestinal mucosal layer can prevent the presence of pathogenic bacterial colonization [56]. The presence of *Lactobacillus* bacteria, which acts as probiotic bacteria, causes the *Enterococcus* bacterial group in control patients to be found in small amounts. This bacterium has an immunoregulatory function and plays a role in maintaining intestinal microbiota homeostasis by secreting immunomodulatory agents [57]. Our study found isolate 10 in the control and RA group. Isolate 10 was not identified in all markers. Previous studies showed the presence of other bacteria that could cause inflammation,

namely *Enterobacter cloacae*. *E. cloacae* is one of the LPS-producing gram-negative bacteria [58]. A high LPS number stimulates the inflammatory response by macrophages, which weakens the intestinal epithelial barrier triggering chronic inflammation in both rats and humans [59]. The number of pathogenic bacteria that is not offset by probiotic bacteria exacerbates damage to the intestinal epithelial barrier [60]. The loss of the *Lactobacillus* bacteria group in RA patients is thought to be related to competition with pathogenic bacteria. Previous studies had shown that supplementation with vegetarian foods enriched with *L. plantarum* or *L. casei* could improve health and reduce the level of inflammation in RA patients [61,62]. Therefore, it can be said that different *Lactobacillus* species have different effects in RA patients. It is not yet known with certainty whether RA disease is initiated by the presence of specific pathogenic bacteria [63].

Isolate 11 was the only bacteria found specifically in the RA group, which was not amplified using the primer set used in this study. Isolate 11 is not supposed to be included in the bacterial family *Bacteroides*, *Clostridium*, *Bifidobacterium*, *Enterococcus*, or *Lactobacillus*. *Bifidobacterium*, *Eubacterium rectale*, and *Clostridium coccoides* bacterial groups were reported to decrease in RA patients [64]. Moreover, the *Bacteroides* in the RA group also decreased [65]. Several studies also revealed the presence of other bacteria associated with RA morbidity, including *Mycoplasma fermentans* [66], *E. coli* [67], and *Proteus mirabilis* [68], so that further research is needed to determine the family of bacterial isolate 11.

CONCLUSION

This study found that *Enterococcus* was the bacterial group found in abundance in the control (Non-RA) and RA patients in Malang. The abundance of *Enterococcus* in RA patients was higher than in the control patients. The bacterial group in control patients was dominated by the *Lactobacillus* bacteria, which was not found in the RA patients. The diversity of *Lactobacillus* species can have positive (symbiotic) effects that inhibit the presence of *Enteroccocus* bacteria in RA patients. Further analysis is required to determine the bacterial group of isolate 11 in the RA patients.

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