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Identification and quantification of *Roseburia intestinalis* and *Escherichia coli* in hypertensive patients in Denpasar

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ABSTRACT

Background: Low-grade chronic inflammation may contribute to the development of atherosclerosis as well as hypertension by inducing the production of pro-inflammatory cytokines. Several studies have revealed a relationship between the microbiota living in the gut and the incidence of hypertension. Two of several gut microbiota that contributed, in this case, are *Escherichia coli* (*E. coli*) and *Roseburia intestinalis* (*R. intestinalis*). Therefore this study aimed to identify and quantify gut microbes namely *R. intestinalis* and *E.coli* in hypertensive patients in Bali, especially in Denpasar City.

Methods: This research is a cross-sectional study located at South Denpasar Health Center I, Bali, and the Integrated Biomedical Laboratory, Faculty of Medicine, Universitas Udayana. The sample in this study were hypertensive patients who had been registered at South Denpasar Health Center I and who met the inclusion criteria and did not meet the exclusion criteria. The *E. coli* and *R. intestinalis* concentrations were assessed by conducting DNA isolation and RT-qPCR. Statistical analysis using univariate analysis, bivariate analysis using the Independent T-Test; Mann-Whitney Test; Chi-Square, and ROC analysis

Results: There were 70 subjects included in this study. Thirty-one subjects were hypertensive patients and the other 39 patients were not hypertensive. The age of the subjects between the two groups was found statistically significant (p=0.007). We also found that comorbid were found mostly in hypertensive patients (p=0.009). Most of the comorbid experienced among them were cardiovascular diseases and diabetes mellitus. Hypertensive subjects had more *E. coli* concentration in their gut compared with non-hypertensive subjects (p=0.048), while *R. intestinalis* concentrations were similar in both groups (p>0.05). From the multivariate analysis, it was found that hypertensive patients tended to have a higher concentration of *E. coli* than non-hypertensive patients (p=0.012; 95% CI:0.055–0.417).

Conclusion: *E. coli* concentration in the gut is independently associated with the incidence of hypertension, while *R. intestinalis* concentration is not.

Keywords: *Roseburia intestinalis*, *Escherichia coli*, hypertension, gut microbiota.
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INTRODUCTION

Increased blood pressure or hypertension is one of the causes of coronary heart disease that can be prevented. Based on data from WHO, in 2015 it is estimated that hypertension occurs in one in four men and one in five women worldwide.[1] There were 7.8 million deaths caused by hypertension-related diseases in the same year. In the last two decades, the prevalence of hypertension has increased significantly in lower-middle-income countries. Cases of hypertension have increased from a total of 600 million cases in 2000 to 1 billion cases of hypertension in 2010 and are still increasing throughout the world.[2]

Low-grade chronic inflammation may contribute to the development of atherosclerosis as well as hypertension by inducing the production of pro-inflammatory cytokines. Several studies have revealed a relationship between the microbiota living in the gut and the incidence of hypertension. Studies using experimental animals show an increase in intestinal membrane permeability in hypertensive conditions. This is due to the low expression of gap junction proteins in the intestine, causing the translocation of metabolites produced in the intestine to occur more easily than under normal conditions. Lipopolysaccharide (LPS) produced by gram-negative bacteria will be able to enter the systemic blood circulation and trigger the production of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6). Gram-negative bacteria which are the source of LPS were found to be increased in subjects with hypertension compared to study subjects with normal blood pressure.[3] One study conducted in Indonesia found that *Escherichia coli* (*E.coli*), which is a gram-negative bacterium, was found to increase significantly in obese research subjects which is a risk factor for hypertension.[4,5]

Intestinal microbiota can also affect the risk of cardiovascular disease indirectly through the metabolites it produces, such as short-chain fatty acids (SCFA). High levels of SCFA in the stool are associated with high blood pressure, but high levels

of SCFA-producing bacteria in the stool are associated with normal or lower blood pressure.[6,7] Yan et al. (2017) found that one of the SCFA butyrate-producing bacteria, *Roseburia intestinalis* (*R. intestinalis*), was found at high levels in control patients compared to those with hypertension.[8] SCFA, in this case, butyrate, has an anti-inflammatory effect through the mechanism of histone deacetylation inhibition, suppresses the production of TNF-α, interleukin-12 (IL-12), and interferon-γ (IF-γ), and can increase the production of interleukin-10 (IL-10) which is an anti-inflammatory cytokine.[3]

Recently, many studies have shown an association between gut microbiota and diseases in humans, such as obesity, diabetes mellitus, and hypertension. However, in Indonesia, especially in Bali, studies on gut microbiota in hypertensive patients are still rare. Therefore, this study aimed to identify and quantify gut microbes namely *R. intestinalis* and *E. coli* in hypertensive patients in Bali, especially in Denpasar City.

METHODS

Design of Study

This research is a cross-sectional study located at South Denpasar Health Center I, Bali, and the Integrated Biomedical Laboratory, Faculty of Medicine, Udayana University, and starts from March 2021 to November 2021.

Samples

The sample in this study were hypertensive patients who had been registered at South Denpasar Health Center I and who met the inclusion criteria and did not meet the exclusion criteria. The inclusion criteria in this study included: 1) Adult patients aged >18 years who had routine checks for hypertension and/or health consultations at the South Denpasar Health Center I, 2) Domiciled in Denpasar, 3) Willing to participate in the study. Meanwhile, the exclusion criteria in this study included: 1) Patients with a history of chronic diseases such as cancer, heart failure, kidney failure, stroke, chronic inflammatory disease, peripheral artery disease, and patients who have a history of smoking and 2) Patients taking antibiotics, probiotics, prebiotics, or similar. Patients who are willing to take part in the study will take blood pressure data and other supporting data and are asked to provide a 5-gram stool sample for further examination at the Integrated Biomedical Laboratory, Faculty of Medicine, Udayana University.

Variables and Measurement

Variables assessed in this study included *E. coli* and *R. intestinalis* concentrations, age, gender, education, smoking history, height, weight, family history of hypertension, blood glucose levels, family history of DM, comorbidities, alcohol consumption, exercise history, and sedentary lifestyle habits. Variables of age, gender, education, smoking history, family history of hypertension, history of DM in comorbid families, alcohol consumption, sports history, and sedentary lifestyle habits were obtained through interviews and recorded in data collection sheets. Data on height, weight, blood pressure (systolic and diastolic), and blood glucose levels were measured according to standard measurements.

The concentration of *E. coli* and *R. intestinalis* was measured by RT-qPCR. The first process was preceded by the isolation of *E. coli* and *R. intestinalis* DNA using the QIAmp DNA stool Mini Kit (QIAGEN, Hilden, Germany), performed as recommended by the manufacturer.[9] After the DNA is isolated, it is followed by gel electrophoresis and nanodrop examination to ensure that the DNA has been completely isolated. After confirming the isolation of DNA, proceed with the RT-qPCR process with the A6020 GoTaq® 1-step RT-qPCR system (Promega, Madison, USA) using the primer *Roseburia intestinalis* (Forward 5'-GCACAGGGTCGCATGACCT-3; Reverse 5'- AACACATTACATGTTCT GTCATC-3 ')[10] and *Escherichia coli* (Forward 5'- GTGCTTTTGATATTTTT CCGA GTACATTGG– 3'; Reverse 5'- TTTATATCACGAAAACGTGAA ATTGCTGAT– 3').[11]

Statistical Analysis

Data analysis was performed using SPSS for Windows version 20.0 software. The statistical analysis was carried out in the form of univariate analysis, and bivariate analysis using the Independent T-Test; Mann-Whitney Test; Chi-Square, and ROC analysis. The P value is considered significant if P <0.05.

RESULTS

There were 70 subjects were included in this study. Thirty-one subjects were hypertensive patients and the other 39 patients were not hypertensive. The age of the subjects between the two groups was found statistically significant (p=0.007). There were more female subjects than males in both groups (p=0.571). Most of the subjects in both hypertensive (30%) and non-hypertensive (69.4%) patients were high school graduates (p=0.142). In clinical variables like BMI, we found no difference between the two groups (p=0.835). Based on the subjects' daily activity variables (smoking history, alcohol consumption, exercise, and sedentary lifestyle), there was no difference between the two groups (p>0.05). We also found no relationship in the family history of hypertension and diabetes mellitus between hypertensive and non-hypertensive subjects (p>0.05). The random blood sugar of hypertensive patients also tends to be similar to those who are not hypertensive (p>0.05). We also found that comorbid were found mostly in hypertensive patients (p=0.009). Most of the comorbid experienced among them were cardiovascular diseases and diabetes mellitus. Hypertensive subjects had more *E. coli* concentration in their gut compared with non-hypertensive subjects (p=0.048), while *R. intestinalis* concentrations were similar in both groups (p>0.05). The characteristics of the subjects are shown in **Table 1**.

The analysis was continued with linear regression analysis as a multivariate analysis to evaluate the relationship between the concentration of *E. coli* bacteria and the risk of hypertension (**Table 2**). From the results, it was found that hypertensive patients tended to have a higher concentration of *E. coli* than non-hypertensive patients (p = 0.012; 95% CI: 0.055 – 0.417).

ROC analysis was performed to assess the predictive value and identify the diagnostic value (sensitivity and specificity) of concentrations of *E. coli* and *R. intestinalis*. The ROC curve of *R. intestinalis* and *E. coli* concentration can

be seen in Figure 1 and the details of the analysis can be seen in Table 3. Based on the results of the ROC curve, the concentration of *E. coli* has not had very good potential as a predictive marker of hypertension because the value of the area under the curve (AUC) is in the range of 0.60 – 0.70. Meanwhile, the concentration of *R. intestinalis* bacteria cannot be used as a predictive marker of hypertension because the AUC value is <0.50. However, with a cut-off value of 0.0332 µM, the concentration of *E. coli* bacteria had fairly good sensitivity and specificity (71% and 48.7%) and was statistically significant (p = 0.048; 95% CI: 0.503 – 0.773). Meanwhile, the concentration of *R. intestinalis* bacteria with a cut-off value of 0.2577 µM, had a sensitivity and specificity that was not very good (61.3% and 33.3%) and was not statistically significant (p = 0.714; 95% CI: 0.335 – 0.614).

Table 1. Characteristics of the subjects

Variables	Hypertensive Status		p-value
	Hypertensive = 31	Non-Hypertensive = 39	
Age, median (range), years	55.00 (38 – 77)	49 (26 – 78)	**0.007*[b]**
Sex			
Men	9 (50.0%)	9 (50.0%)	0.571[c]
Women	22 (42.3%)	30 (57.7%)	
Education			
None	4 (66.7%)	2 (33.3%)	
Elementary School	4 (50.0%)	4 (50.0%)	0.142[c]
Middle School	5 (50.0%)	5 (50.0%)	
High School	11 (30.6%)	25 (69.4%)	
University	7 (70.0%)	3 (30.0%)	
Smoking History			
Yes	3 (75.0%)	1 (25.0%)	0.203[c]
No	28 (42.4%)	38 (57.6%)	
BMI, mean (SD), kg/m^2	25.00 (3.29)	25.19 (4.08)	0.835[a]
Family History of HT			
Yes	10 (43.5%)	13 (56.5%)	0.924[c]
No	21 (44.7%)	26 (55.3%)	
Blood Sugar, median (range), mg/dL	118 (69 – 348)	103 (75 – 213)	0.071[b]
Family History of DM			
Yes	3 (60.0%)	2 (40.0%)	0.463[c]
No	28 (43.1%)	37 (56.9%)	
***E. coli* Concentration**, median (range), µM	0.26 (0.01 – 4.98)	0.041 (0.00 – 1.51)	**0.048*[b]**
***R. intestinalis* Concentration**, median (range), µM	0.30 (0.00 – 1.23)	0.38 (0.00 – 2.81)	0.714[b]
Comorbid			
Yes	5 (100%)	0 (0%)	**0.009*[c]**
No	26 (40.0%)	39 (60.0%)	
Alcohol Consumption			
Yes	0 (0%)	2 (5.3%)	0.507[c]
No	27 (100%)	36 (94.7%)	
Exercise			
Not Routine	16 (42.1%)	22 (57.9%)	0.689[c]
Routine	15 (46.9%)	17 (53.1%)	
Sedentary Life Style			
Yes	2 (33.3%)	4 (66.7%)	0.572[c]
No	29 (45.3%)	35 (54.7%)	

Note: [a] analyzed with Independent T-Test; [b] analyzed with Mann-Whitney Test; [c] analyzed with Chi-Square Test; *statistically significant (p<0.05)

Table 2. Multivariate analysis of *E. coli* concentration

Variables	B	SE	Beta	t	p-value	95% CI
Constant	0.346	0.068		5.066	<0.001	0.210 – 0.483
E. coli concentration	0.236	0.091	0.300	2.597	**0.012***	0.055 – 0.417

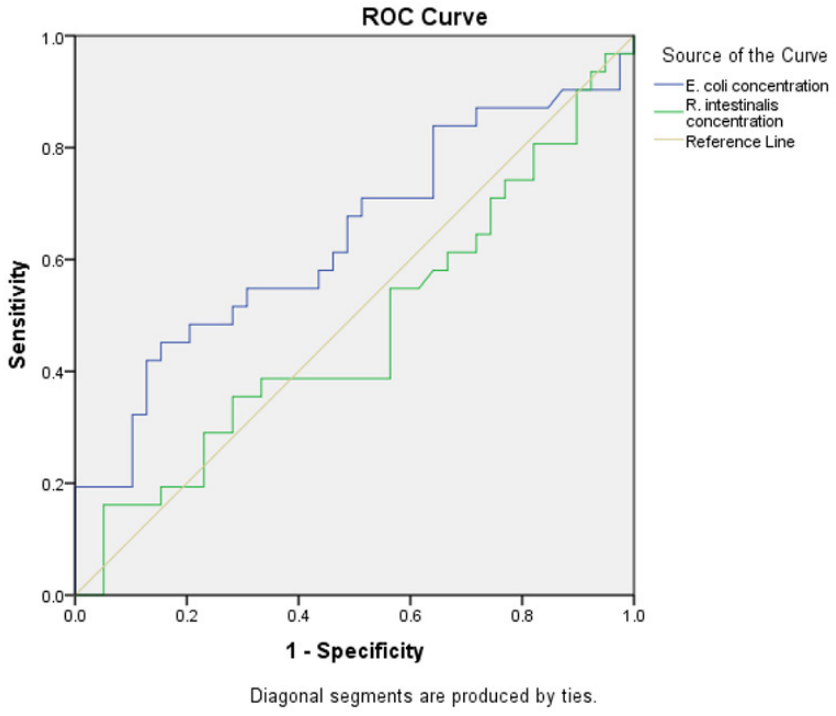

Figure 1. ROC curve of *R. intestinalis* and *E. coli* concentration

Table 3. ROC analysis of *R. intestinalis* and *E. coli* concentration

Variables	AUC	Cut-off Value	Sn	Sp	p-value	95% CI
E. coli concentration	0.638	0.0332	71.0%	48.7%	**0.048***	0.503 – 0.773
R. intestinalis concentration	0.474	0.2577	61.3%	33.3%	0.714	0.335 – 0.614

Note: *statistically significant (p<0.05)

DISCUSSION

The development of high blood pressure is a complex, multifactorial process that involves both genetic and environmental risk factors such as a sedentary lifestyle, increased sodium intake, unhealthy diet, and weight gain.[12] Currently, as the number of patients with HTN has increased, antihypertensive therapy has become a popular topic and a challenge in medical research. Recent studies have found associations between gut microbiota and the incidence of hypertension.[12–17]

E. coli is a bacterium from the Enterobacteriaceae family which is one of the most common bacteria found in the human digestive tract. *E. coli* is generally non-pathogenic in the human digestive tract, but several strains have high virulence and cause infections in humans and animals.[18] In hypertensive conditions, the permeability of the gastrointestinal tract increases as indicated by the lower gap junction mRNA expression in experimental rats. This increased permeability of the digestive tract causes increased translocation of methanolic compounds and endotoxins from the digestive tract to the systemic circulation system via the portal vein which can then cause various health problems such as low-grade inflammation.[19] As a gram-negative bacterium, this bacterium is capable of producing lipopolysaccharide (LPS) compounds derived from the outer membrane of gram-negative bacteria. The lipid A component of LPS is the main pathogen-associated molecular pattern (PAMP) that can interact with toll-like receptor 4 (TLR4). Furthermore, when LPS translocates from the digestive tract to the systemic circulation, it will form a complex with LPS binding protein (LBP) which can then bind to CD14 on the surface of mononuclear cells. This can induce the secretion of pro-inflammatory cytokines such as TNF- , interleukin-1 (IL-1), and interleukin-6 (IL-6), which are mediated by the MD2/TLR4 receptor complex.[19] Research by Masson et al (2015) showed that LPS administration in experimental rats could increase heart rate and norepinephrine levels, decrease baroreflex sensitivity, and increase neuroinflammation as indicated by the expression of TLR and TNF- in the paraventricular nucleus (PVN) which play a role in the blood pressure regulatory system.[20] Following the results obtained in this study, concentrations of *E. coli* were found to be higher in hypertensive subjects than in non-hypertensive subjects (p=0.048) and were found to influence the incidence of hypertension independently based on the results of multivariate analysis (p=0.012). Meanwhile, based on the results of the ROC curve, *E. coli* concentration has not had good potential as a predictive marker of hypertension because the value of the area under the curve (AUC) is in the range of 0.60 – 0.70 (p=0.048).

R. intestinalis is a gram-positive, anaerobic, rod-shaped, or slightly curved, motile bacterium using several sub-terminal flagella. This bacterium belongs to the Clostridium enteritidis XIVa family which regulates the development and differentiation of Treg cells and determines the homeostasis of intestinal mucosal tolerance.[21] The Roseburia genus has five well-characterized species (*Roseburia intestinalis*, *Roseburia hominis*, *Roseburia inulinivorans*, *Roseburia faecis*, and *Roseburia cecicola*), all of which produce short-chain fatty acids (SCFAs), such as acetate, propionate, and butyrate.[22] Previous studies have found that *R. intestinalis* and *Faecalibacterium prausnitzii* are the most abundant butyrate-producing bacteria in human feces.[23,24] Of note is that butyrate has been reported to

exert pervasive anti-inflammatory and metabolic modulation effects in different disease models. Therefore, *R. intestinalis* can be applied as a potential probiotic given its ability to produce butyrate. Nonetheless, the findings of this study were different in that the concentration of *R. intestinalis* was found not to differ significantly between the hypertensive and non-hypertensive subject groups (p=0.714). Our findings differ from other studies which state that *R. intestinalis* concentration is higher in normal non-hypertensive subjects.[6,25]

In the world, diabetes mellitus and hypertension are among the most prevalent diseases and cardiovascular risk factors, respectively, and as people become older, these conditions become more prevalent. Patients with type 2 diabetes mellitus (T2DM) frequently have elevated blood pressure (BP) levels, which are thought to at least partially reflect the effects of the underlying insulin resistance on the renal and vascular systems.[26,27] In this study, comorbidities such as heart disease and diabetes mellitus were found mostly in the group with hypertension (p=0.009). The findings in this study are in line with the theory that has been established so far. A significant difference was also found in the age variable between the two subject groups, with the hypertensive group having a higher median age than the non-hypertensive group (p=0.007). This is following previous research and postulated theories. It is known that as a person ages, inflammation and oxidative stress increase in the body which in turn causes endothelial dysfunction and causes hypertension in the elderly.[28–30]

This study also has several limitations. First, we did not analyze the food consumed by hypertensive and non-hypertensive subjects. However, the diversity and amount of certain gut microbiota are related to the food consumed by the subjects; this is also not limited to *E. coli* or *R. intestinalis*. Second, most subjects included in our study were also predominantly female, which may influence certain gut microbiota. This statement is supported by a study conducted by Haro et al. (2016) which analyze the abundance of gut microbiota from 39 men and 36 women.[31] Thus, a larger scale multicenter study taking into account the weaknesses in this study is needed to provide a more comprehensive picture of the condition of the gut microbiota in hypertensive subjects in Southeast Asia, especially in Bali, Indonesia.

CONCLUSION

E. coli concentration in the gut is independently associated with the incidence of hypertension, while *R. intestinalis* concentration is not. Although the concentration of E. coli in hypertensive subjects was found to be significantly higher than in the non-hypertensive group, the concentration of these bacteria cannot be used independently as a reference in diagnosing hypertension.

Conflict of Interest

All authors declare that there is no conflict of interest regarding the publication of this study.

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Author Contributions

All authors contribute equally regarding this study's execution and publication.

Ethical Clearance

This research was approved by the Research Ethics Commission Unit of the Faculty of Medicine, Universitas Udayana with the letter number: 1624/UN14.2.2.VII.14/LT/2021.

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