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The effect of Ajwa dates extract (*Phoenix dactylifera L.*) on gut microbiota of Sprague Dawley rats induced by a high-fat diet

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Abstract

Excessive intake of nutrients will lead to obesity associated with changes in the gut microbiota composition and inflammatory-metabolic disorders. Ajwa dates have a high fibre content to promote the growth of beneficial bacteria and inhibit pathogenic bacteria that trigger metabolic diseases. This study aimed to determine the effect of Ajwa date extract (ADE) on the gut microbiota of Sprague dawley rats induced by a high-fat diet (HFD). The research design was a pre-post test with a control group design. A total of fifteen male rats were divided into 3 groups, 5 rats in the control group were only given standard feed (control group, CG), 5 rats were induced standard feed and HFD (treatment group I, TG I), and 5 rats that were treated with standard feed, HFD, and the intervention of ADE (treatment group II, TG II). HFD induction for 28 days and ADE for 7 days. The microbiota in the faeces samples were extracted and determined by qPCR (quantitative-Polymerase Chain Reaction) method. After the intervention of ADE showed that CG and TG I did not experience a significant change in microbiota composition, while in TG II there was a significant change in the genus *Bifidobacterium* ($p<0.01$), *Bacteroides fragilis* ($p<0.043$), *Clostridium* ($p<0.036$), *Lactobacillus* ($p<0.043$), and *Escherichia coli* ($p<0.043$). In conclusion, ADE was able to modulate the gut microbiota of Sprague Dawley rats induced by a high-fat diet.

1. Introduction

With the increasing age, there are increasing risk factors for humans to develop several chronic diseases, namely obesity, type 2 DM, Alzheimer's disease, cardiovascular disorders, and cancer. Obesity is known to be an excess of adipose tissue and occurs when there is an imbalance between energy intake and energy expenditure (Jura and Kozak, 2016) and is associated with a diet high in fat, sugar and salt, and low in fiber (P2PTM Kemenkes RI, 2020). 2016 WHO data states that more than 650 million (13%) of the world's adult population are obese. Compared with underweight, obesity and overweight kill more people worldwide (World Health Organization, 2021). By 2020, approximately 29% of people 65 and older are obese in the United States. This percentage has continued to increase since 2013 when it was discovered that 25% of America's elderly were obese (Statista, 2020).

Weight gain is associated with the aging process caused by excessive production of ROS (reactive oxygen species) and causes an inflammatory process that will affect telomeres. In general, obesity and aging share a spectrum of phenotypes, namely redox imbalance, mitochondrial dysfunction, macromolecular accumulation, weakened immunity, and systemic inflammation. Thus, obesity may accelerate aging at multiple levels from cells to systems (Tam *et al*., 2020).

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The human gut is capable of hosting trillions of microorganisms, including more than 10^{14} bacteria from 2000 species (Kau *et al*., 2011). The gut microbiota consists of more than 50 bacterial phyla, but there are five bacterial phyla that dominate the human gut microbiota, that is *Firmicutes* and *Actinobacteria* - gram-positive, *Bacteroidetes* - gram-negative, *Proteobacteria* and *Verrucomicrobia* (D'Aversa *et al*., 2013).

Food is the main element that affects the gut microbiota. Switching from a plant-based diet rich in polysaccharides to a "Western" diet high in fat and high in sugar alters microbiota structure in a single day, representation of metabolic pathways, and microbiome gene expression (Turnbaugh *et al*., 2009). Altered gut microbiota composition (dysbiosis) is associated with age-related inflammation and associated morbidity. Therefore, modulating the gut microbiota is considered an important measure to control age-related inflammatory processes (D'Aversa *et al*., 2013).

Dates are a good source of energy and rich in nutrients containing 44-88% carbohydrates, 6.4-11.5% dietary fiber, 2.3-5.6% protein, 0.2-0.5% fat, and 15 grams of minerals and vitamins (Khan *et al*., 2016). Based on the total phenolic content of various types of dates, Ajwa Al Madinah dates had the first highest phenolic content of 22.11 mg/100 g (Assirey, 2015). Meanwhile, the total flavonoid content of Ajwa dates was the second highest after the Saffawy variety, which was 2.78 mg/100 g (Hamad *et al*., 2015).

There were changes in intestinal bacteria caused by dates, whether consumed directly or with extracts rich in polyphenols. Consumption of dates can provide health in the large intestine by increasing the growth of beneficial bacteria and inhibiting pathogenic bacteria that trigger metabolic diseases (Eid *et al*., 2021).

In recent years, the gut microbiota which plays an important role in human health has attracted much attention and is developing very rapidly (Li *et al*., 2019). However, not many studies have used Ajwa dates to see changes in the gut microbiota. Therefore, the authors wanted to conduct this research to determine the effect of Ajwa date extract (ADE) on the gut microbiota of Sprague Dawley rats induced by a high-fat diet (HFD).

2. Materials and methods

2.1 Research design and target

This research is an experimental study on male white rats *Rattus norvegicus* Sprague Dawley strain aged 6-12 months weighing between 150-200 grams in healthy conditions with a pre-post test design with control group design by comparing the results of observations in the experimental and control groups. This research has received an Ethics Approval Letter No: 275/ UN4.6.4.5.31/PP36/2021 from the Health Research Ethics Committee, Faculty of Medicine, Hasanuddin University. The research was carried out by the research code of ethics.

Animal care and intervention are carried out at the Biopharmaceutical Laboratory of the Faculty of Pharmacy, Hasanuddin University, Makassar. The manufacture of ADE was carried out at the Phytochemical Laboratory of the Faculty of Pharmacy, Hasanuddin University, Makassar. Analysis of high-fat feed was carried out at the Faculty of Animal Husbandry, Hasanuddin University, Makassar (Table 1). Analysis of antioxidant levels was carried out at the Biopharmaceutical Laboratory, Faculty of Pharmacy, Hasanuddin University, Makassar (Table 2). Observation and examination of the gut microbiota using qPCR method were carried out in the research unit of the HUMRC Laboratory of the Hasanuddin University Teaching Hospital, Makassar.

Table 1. Results of HFD analysis.

HFD	Crude Fat %	Crude Protein %	Carbohydrate %
I	66.27	3.47	11.65
II	66.51	3.88	11.28

Table 2. Phytochemical analysis results.

Parameters	Average rate
Total Flavonoids (mg/1g)	0.35
Total Polyphenols (mg/1g)	2.77
Total Tannins (mg/1g)	6.819
Antioxidant IC_{50}	654.974 ppm

The HFD used consisted of 65 mL of cow's milk and 35 g of margarine. This feed contains 11.28% carbohydrates, 3.88% crude protein, and 66.51% crude fat. To get the HFD formula as much as 100 mL of margarine, which is still solid is heated in a flame with a temperature of 45°C to a liquid form and then mixed with cow's milk (Getz and Reardon, 2012).

2.2 Procedures

The cage adaptation (acclimatization) was carried out for 7 days. During the adaptation period, all groups of rats were given a standard feed of approximately 30 g/ day and given adequate drinking. The cage is cleaned every day. To maintain a stable environment, the rats were placed in a room with adequate air circulation and maintained at room temperature at a standard temperature (20-28°C) with a humidity of 50-10% and the room lights were set in a 12-hour dark and light cycle. The body weight of the whole group of rats was weighed every week.

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A total of fifteen rats were divided into three groups, with a total sample of five in each group consisting of a CG which was only given standard feed, TG I which was given standard feed and induced HFD, and TG II given standard feed, HFD intervention, and ADE. After the adaptation period, the CG was still given standard feed only, while TG I and TG II were given standard feed and HFD for 28 days as much as 2 mL/200 g of body weight of rats per day per gastric feeding tube every morning before giving standard feed to avoid rejection in rats due to satiety.

After 28 days, the intervention of ADE on TG II was 1.62 mL/200 g BW rats per day per gastric feeding tube for 7 consecutive days. The treatment of ADE was done by feeding tube.

2.3 Data collection

The parameters measured in this study were blood glucose levels and gut microbiota. To measure blood glucose levels, blood was taken from the tip of the rat's tail with a lancet and measured with a glucometer. Blood glucose levels were measured before treatment of HFD, every week for 28 days of HFD induction, and after 7 days after treatment of ADE.

For microbiota extraction, all faecal samples were examined and performed using qPCR technique. Faecal samples were taken pre-post HFD intervention and after ADE intervention. Total faecal DNA was extracted using an independent culture stool DNA Isolation Kit (Norgen, Canada) as per the manufacturer's protocol. The target bacteria to be studied are *Lactobacillus, Bacteroides fragilis, Clostridium, Bifidobacterium* and *Escherichia coli.*

2.4 Statistical analysis

The data processing technique used the SPSS software version 25.0 with a significance of ≤ 0.05. The measurement results are presented in the form of narratives and tables.

3. Results and discussion

3.1 Test results of tested animal blood glucose levels

A HFD intervention was given to TG I and TG II. Measurement of blood glucose levels of experimental rats was obtained from three different measurement times: day 0 before treatment (pre-test), day 35 after feeding a HFD (post-test I), and day 42 after intervention of ADE (post-test II). After feeding a HFD, there was a significant increase in blood glucose levels in TG I and TG II, while CG which was not given a HFD intervention decreased by 2.7%. (Table 3)

3.2 Results of tests for intestinal microbiota of experimental animals after hfd feeding

The changes of mean microbiota profile in pre-test, post-test I are shown in Figure 1. On the 0^{th} and 35^{th} days, the CG was still given standard feed, while the TG I and TG II were given HFD induction. There were no significant p-values for the genus and species of bacteria, except for *B. fragilis* species (an increase of 3%). It can be concluded that there was a difference in the average number of *B. fragilis* on days 0 and 35 on CG (Table 4).

On the 0^{th} and 35^{th} days of TG I and TG II, there were differences in the average number of *Bifidobacterium, Clostridium, Lactobacillus* and *E. coli* on day 0 and 35 after the intervention of HFD on TG I (Table 5 and Table 6).

3.3 Results of statistical test of the intestinal microbiota of experimental animals after ADE treatment

Based on Figure 1, on the 35^{th} and 42^{nd} days, CG and TG I were only given standard feed and not given ADE. There were no significant p-values for the genus or species of bacteria, hence, it was concluded that there were no differences in the average number of *B. fragilis* on days 35 and 42 on CG and TG I (Table 4 and Table 5).

Meanwhile, in TG II after the intervention of the 35^{th} and 42^{nd} days of ADE, a significant p-value in the genus *Bifidobacterium, B. fragilis, Clostridium, Lactobacillu*s and *E. coli* on the 35^{th} and 42^{nd} day after the intervention of ADE in TG II (Table 6).

Table 3. Statistical test results of rat blood glucose levels in each group on day 0, day 35, and day 42.

Group	Average feed consumption/day (g)	Mean GD (ml/dL) ± SD			Δ Average GD %		P-value*	
		Day 0	Day 35	Day 42	Day 0 and 35	Day 35 and 42	Day 0 and 35	Day 35 and 42
Control	28.2	95.2±4.1	92.6±5.0	90.6±2.7	↓2.7%	↓2.2%	0.179	0.489
Treatment I	28.6	89.8±7.0	119.4±8.6	115.2±10	↑33%	↓3.5%	0.001	0.389
Treatment II	28.3	102.8±13.6	124±18.3	100.4±15.1	↑20.6%	↓19%	0.026	0.001

CG: Control Group – standard feed only, TG I: Treatment Group I — standard feed and HFD intervention, TG II: Treatment Group II — standard feed, HFD, and Ajwa dates extract intervention.
*p<0.05 significantly different.

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Table 4. Intestinal microbiota test results of the control group (CG).

Microbiota	Mean (log DNA copies/g) ± SD			Δ Average %		p Value*	
	Pre-Test (D-0)	Post Test I (D-35)	Post Test II (D-42)	Day 0 and 35	Day 35 and 42	Day 0 and 35	Day 35 and 42
Bifidobacterium	4.79±0.37	4.81±0.31	4.77±0.51	↑0.5%	↓0.8%	0.8	0.84
Bacteroides	0.00±0.00	3.03±1.50	2.45±0.44	↑3.0%	↓19%	0.043	0.345
Clostridium	6.73±0.46	6.59±0.60	6.60±0.56	↓2%	↑0.1%	0.432	0.822
Lactobacillus	6.79±0.54	6.83±0.57	6.85±0.30	↑0.6%	↑0.3%	0.884	0.914
E. coli	5.48±1.22	5.63±2.30	5.48±1.97	↑2.8%	↓2.7%	0.774	0.38

CG: Control Group – standard feed only
*p<0.05 significantly different.

Table 5. Intestinal microbiota statistics test results treatment group I.

Microbiota	Mean (log DNA copies/g) ± SD			Δ Average %		p Value*	
	Pre-Test (D-0)	Post Test I (D-35)	Post Test II (D-42)	Day 0 and 35	Day 35 and 42	Day 0 and 35	Day 35 and 42
Bifidobacterium	5.36±0.53	4.15±0.22	4.20±0.27	↓22.5%	↑1.2%	0.003	0.665
Bacteroides	0.53±0.73	0.00±0.00	0.00±0.00	↓100%	0%	0.180	1.000
Clostridium	6.17±0.81	7.40±0.32	7.74±0.17	↑19.9%	↑4.6%	0.013	0.095
Lactobacillus	6.89±0.71	7.83±0.25	7.64±0.54	↑13.7%	↓2.5%	0.034	0.480
E. coli	3.71±1.24	6.91±2.56	6.54±2.70	↑86.4%	↓5.4%	0.043	0.138

TG I: Treatment Group I — standard feed and HFD intervention
*p<0.05 significantly different

Table 6. Statistical test results of intestinal microbiota treatment group II.

Microbiota	Mean (log DNA copies/g) ± SD			Δ Average %		p Value*	
	Pre-Test (D-0)	Post Test I (D-35)	Post Test II (D-42)	Day 0 and 35	Day 35 and 42	Day 0 and 35	Day 35 and 42
Bifidobacterium	5.07±0.33	4.25±0.31	5.99±0.98	↓16.2%	↑41%	0.011	0.013
Bacteroides	1.19±1.54	0.00±0.00	1.41±0.90	↓100%	↑100%	0.068	0.043
Clostridium	6.16±0.81	8.03±0.71	7.51±0.66	↑30.4%	↓6.3%	0.012	0.036
Lactobacillus	7.74±0.72	8.23±0.61	8.86±0.40	↑6.3%	↑7.6%	0.006	0.043
E. coli	3.64±1.10	7.22±2.42	5.60±2.32	↑98.4	↓22.5%	0.043	0.043

TG II: Treatment Group II — standard feed, HFD, and ADE intervention.
*p<0.05 significantly different

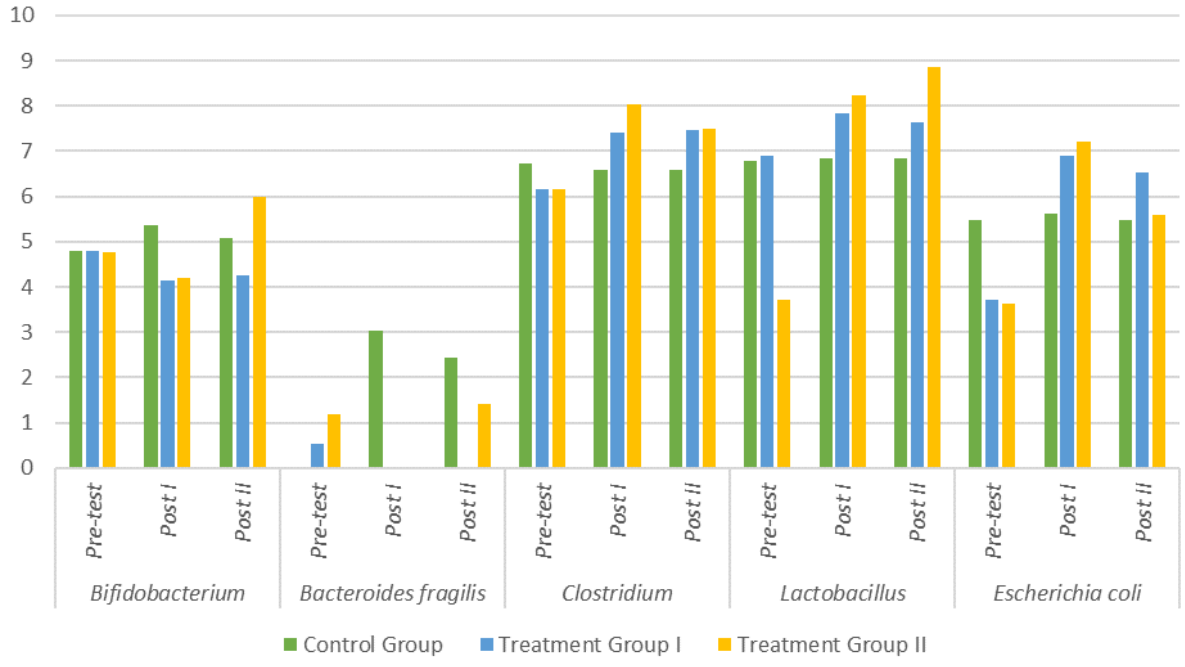

Figure 1. Microbiota profile of all groups.

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HFD interventions can lead to obesity which is a risk factor for diabetes mellitus. This study is in agreement with the research conducted by Antonioli *et al*. (2017) by comparing rats given a HFD and the Normocaloric Diet (NCD). It was found that the increase in blood glucose levels was higher in rats fed with HFD than in rats given NCD only.

After 7 days of intervention of ADE, there was a decrease in blood glucose levels in rats. The mechanism by which Ajwa dates can inhibit a decrease in blood glucose levels is not known with certainty, but this may be influenced by the fiber and antioxidant content in Ajwa dates which can reduce free radical activity and low-grade inflammation from a HFD. Other studies have also proven the benefits of Ajwa date seed extract and found a significant decrease in blood glucose levels in normal rats and diabetic rats (Hasan and Mohieldein, 2016).

This is also described by Zhang *et al*. (2013) about the main metabolites in Ajwa dates are primary metabolites, sugars, and proteins. But in sugar, only monosaccharides are found in Ajwa dates, which consist of a mixture of isomers of fructose and glucose, accounting for a total of about 65% of the total weight of the fruit. Because fructose was found to be higher than glucose in its total sugar content, consumption of dates is not risky for people who have problems with sugar modulation, such as type 2 DM patients.

Consumption of HFD in both animals and humans triggers changes in the composition of the gut microbiota which is characterized by an increase in the number of *Firmicutes* and a decrease in the number of *Bacteroidetes*. This study showed that the species ratio of *B. fragilis* disappeared after HFD intervention. Associated with the mechanism of metabolic endotoxemia (Cani *et al*., 2007), this ratio is not appropriate because *Bacteroides* belongs to the phylum *Bacteroidetes* which is the main group of Gram-negative bacteria in the gut microbiota. This can be explained by the fact that the LPS endotoxic activity of bacteria belonging to the phylum *Bacteroidetes* is considered to be lower than that of other Gram-negative bacteria such as those belonging to the phylum *Proteobacteria* which increases in obesity (Magne *et al*., 2020). This research is in accordance with previous research conducted by Ley *et al*. (2006) by comparing wild-type mice and genetically obese (ob/ob) mice, the results showed an increase in the ratio in the *Firmicutes* phylum and a decrease in *Bacteroidetes* (Schwiertz *et al*., 2010).

Meanwhile, the increase in *Firmicutes* ratio after intervention of HFD is also supported by the explanation of Lee *et al*. (2021) that *Lactobacillus* has a high diversity in its resistance to oxidative stress. One of them is *Lactobacillus sakei*, which is one of the *Lactobacillus* strains that is correlated with obesity, highly resistant to oxidative stress through catalase activity and showed a higher survival rate than *L. sakei* DSM 20017 in vitro and higher colonization in the colon of rats fed with HFD. Although *Lactobacillus* is reported to be correlated with the occurrence of ROS caused by the HFD, to date, there has been no correlation between *Lactobacillus* and obesity.

According to the research conducted by Marques *et al*. (2016), by modulating the gut microbiota of rats with HFD, the composition of the microbiota was similar in both Wistar and Sprague Dawley rats, except for *Clostridium*, one of which, *Clostridium leptum* species, also was found to be increased in Sprague Dawley rats induced by HFD.

In the *Actinobacteria* genus, there is a decrease in *Bifidobacterium* species that function to maintain the intestinal barrier on HFD (Cani *et al*., 2012). It is known that *Bifidobacterium* is negatively correlated with increased body weight, adipose tissue, glucose intolerance, and inflammatory markers and vice versa (Cani *et al*., 2007).

Meanwhile, the study found an increase in the *E. coli* species which is included in the phylum *Proteobacteria* after the HFD intervention was carried out, where it was said that the decrease in microbial diversity and growth of *Proteobacteria* were the main characteristics of dysbiosis (Weiss and Hennet, 2017).

In addition, Muñoz-Garach *et al.* (2016) described bacterial species that were positively correlated with the occurrence of insulin resistance, such as *Lactobacillus gasseri, Streptococcus mutans* and *E. coli*.

Our results concurred with previous research conducted by Karim *et al.* (2022) that there was a decrease in body weight in experimental rats after the intervention of ADE for 7 days and an abundant change in the composition of the gut microbiota, which can be seen in this study.

Furthermore, the study by Wu *et al*. (2011) also supported our result, who explained after giving a HFD for 10 days, an increase in the number of *Bifidobacterium*. It was also known that ADE has a good fiber content which can modulate the growth of *Bifidobacterium*. The previous study by Cani *et al*. (2007) reported that *Bifidobacterium* was recognized as the main gut bacteria involved giving the positive effects observed after prebiotic treatment and causing a decrease in the activation of inflammatory cascades.

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In this study, there was an increase in *Bacteroides* after treatment of ADE. Our findings are in accordance with a study conducted by Wang *et al*. (2018) which revealed that the relative abundance of the phyla *Bacteroidetes* in HFD and fiber mice was increased, suggesting that soluble dietary fiber selectively promoted the growth of the phyla *Bacteroidetes*.

This study's results agreed with those of Graf *et al*. (2015) who reported that there was an abundance of *Bifidobacterium* and *Lactobacillus* after the intake of fiber. Turns out that *Lactobacillus* spp. associated with BMI is divided into 2 species (species that cause weight gain, and species that cause weight lose) (Million *et al*., 2012). Meanwhile, *Clostridium* decreased after being given ADE. Another study by Zheng *et al*. (2018) which compared control rats and rats fed with inulin fiber, found a significant decrease in *Clostridium XI* during 8 weeks of fiber treatment. The decrease in *Clostridium* has been seen in the first week of fiber treatment.

In this study, there was a decrease in *E. coli* after being given ADE. This is due to the presence of antibacterial properties in ADE which inhibits the growth of *E. coli* bacteria. The presence of antimicrobial properties in dates is most likely due to the presence of antioxidant compounds, phenolic compounds, tannins, alkaloids, flavonoids, and steroids. All these compounds can inhibit the growth of microorganisms and fight bacterial infections (Samad *et al*., 2016).

5. Conclusion

It can be concluded that Ajwa dates extract *(Phoenix dactylifera L.)* can increase beneficial bacteria and reduce pathogenic bacteria in the gut microbiota, as well as being able to reduce body weight and blood glucose levels.

Conflict of interest

The authors declare no conflict of interest.

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