

Effects of *Dioscorea esculenta* and *Eubacterium rectale* on insulin receptor substrate 1 (Irs1) expression in skeletal muscle and homeostatic model assessment-insulin resistance (HOMA-IR) in diabetic rats

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ABSTRACT

Low expression of insulin receptor substrate 1 (Irs1) is associated with insulin resistance and Type 2 Diabetes Mellitus (T2DM). This study was performed to evaluate the effects of *Dioscorea esculenta* and *Eubacterium rectale* on the Irs1 expression in the skeletal muscle and the homeostatic model assessment-insulin resistance (HOMA-IR) of diabetic rats. Twenty-five male Wistar rats were divided into five groups i.e. non diabetic rats Group 1; diabetic rats as Group 2; diabetic rats + *D. esculenta* as Group 3; diabetic rats + *E. rectale* as Group 4 and diabetic rats + both *E. rectale* and *D. esculenta* as Group 5. Rats were made diabetic with induction of intraperitoneally injection of nicotinamide and streptozotocin. After four weeks of the interventions, the blood and skeletal muscles were taken. The Irs1 expression was analyzed with immunohistochemical staining, plasma glucose levels were analyzed using a spectrophotometer, and insulin was analyzed using ELISA methods. All intervention groups showed reduced plasma glucose levels and HOMA-IRs ($p < 0.001$) and increased Irs1 expression. The greatest reduction of plasma glucose levels and increase of Irs1 expression in the skeletal muscle were found in Group 4, however, the lowest of HOMA-IR was seen in Group 5. These results suggested that *D. esculenta*, *E. rectale*, and the combination of reduced plasma glucose levels and HOMA-IR was due to increasing Irs1 expression in skeletal muscle.

ABSTRAK

Eksresi Irs1 yang rendah berhubungan erat dengan resistensi insulin dan diabetes mellitus tipe 2 (DM tipe 2). Tujuan penelitian ini adalah mengkaji pengaruh pati gembili (*Dioscorea esculenta*) dan *Eubacterium rectale* terhadap ekspresi protein Irs1 dan *homeostatic model assessment-insulin resistance* (HOMA-IR) pada tikus model DM tipe 2. Duapuluh lima

tikus jantan Wistar dibagi 5 kelompok yaitu tikus kontrol normal tidak diabetes sebagai Kelompok 1; tikus DM sebagai Kelompok 2; tikus DM + gembili sebagai Kelompok 3; tikus DM + *E. rectale* sebagai Kelompok 4; tikus DM + gembili dan *E. rectale* sebagai Kelompok 5. Induksi DM dilakukan pada tikus menggunakan nikotinamid dan streptozotosin secara intraperitoneal. Kadar glukosa dianalisa sebanyak 2 kali sebelum dan sesudah perlakuan. Setelah perlakuan selama 28 hari, jaringan otot skelet digunakan untuk analisis ekspresi protein Irs1 dengan metode *immunohistochemical staining*. Plasma darah juga digunakan untuk analisis kadar insulin menggunakan metode ELISA. Pada tikus DM yang diberi perlakuan menunjukkan adanya penurunan kadar glukosa dan nilai HOMA-IR secara signifikan ($p < 0,001$), sedangkan ekspresi protein Irs1 meningkat. Penurunan glukosa dan peningkatan ekspresi Irs1 terbanyak ditemukan pada tikus DM yang diberi *E. rectale* pada Kelompok 4 sedangkan nilai HOMA-IR terendah ditemukan pada tikus DM yang diberi gembili dan *E. rectale* pada kelompok 5. Hasil ini menunjukkan bahwa pemberian gembili, *E. rectale*, serta kombinasi keduanya dapat menurunkan kadar glukosa dan nilai HOMA-IR melalui peningkatan ekspresi Irs1 pada otot skelet.

Keywords: *Dioscorea esculenta* - *Eubacterium rectale* - insulin resistance - Irs1 - type 2 diabetes mellitus

INTRODUCTION

Skeletal muscle is the predominant site of insulin-mediated glucose uptake in the postprandial state in humans. In skeletal muscle, insulin binds to the insulin receptors leading to phosphorylation of the insulin receptors. Once the insulin receptor has been phosphorylated, insulin receptor substrate-1 (Irs1) moves to the cell membrane and becomes phosphorylated on contiguous tyrosine molecules. Insulin resistance in skeletal muscle is considered to be the initiating or primary defect before β -cell failure and hyperglycemia develops.¹

Insulin receptor substrate-1 plays a major role in regulating insulin sensitivity and glucose metabolism in skeletal muscle and adipose tissue.² Systemic dyslipidemia and intramuscular lipid accumulation are closely associated with the development of skeletal muscle insulin resistance.³ High fatty acids increase intracellular fatty acyl-CoA and 1,2-diacylglycerol which reduce insulin-stimulated glucose transport activity.⁴

However, the dietary short-chain fatty acids (SCFAs) can reduce blood glucose level by increasing adenosine monophosphate-activated protein kinase (AMPK) activity in the liver and muscle tissue.^{5,6} AMPK increases the translocation of the glucose transporter GLUT4 to the membrane and thus increases glucose uptake in the muscle.^{7,8}

Short-chain fatty acids such as acetate, propionate, and butyrate are the end products of the dietary fibers fermentation by the anaerobic intestinal microbiota.^{9,10} *Dioscorea esculenta* is a tuber yam that contains high insoluble fibers, such as resistant starches¹¹ whereas *Eubacterium rectale* is one of butyrogenic bacteria¹² that can ferment fibers to produce butyrate. Administration of butyrate was reported reducing 60% of homeostatic model assessment for insulin resistance (HOMA-IR) in mice.⁵ This study was conducted to evaluate the effects of dietary *D. esculenta* and *E. rectal* on the Irs1 expression in the skeletal muscle and the HOMA-IR of male diabetic rats.

MATERIALS AND METHODS

Animal and preparation of diet

Dioscorea esculenta starch was prepared according to Richana *et al.*¹³ *Eubacterium rectale* bacteria (DSMZ, *E. rectale* 17629, Germany) was cultured to get 10⁹ CFU/mL. Twenty-five male Wistar rats, (200-300 g, 3 months old), from the Faculty of Veterinary Medicine, the *Institut Pertanian Bogor*, Bogor, Indonesia, were housed in cages in an animal room (22-25°C room temperature on a 12-hour day/light cycle) with free access to food and water during the experimental period. The semi-purified rat diet was slightly modified (alpha cell changed with carboxymethyl cellulose).¹⁴ The intervention diet was identical to the standard diet with the exception that 170 g *D. esculenta* starch was substituted for the 170 g of corn starch. The experimental procedure has been approved by the Medical and Health Research Ethics Committee, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta.

Experimental study

This study was performed at laboratory of the Center of Food and Nutrition Studies, Universitas Gadjah Mada, Yogyakarta, Indonesia. Rats were divided into 5 groups with 5 rats in each group. Group 1 as non-diabetic rats control were given standard diet; Group 2 as diabetic rats control were given standard diet; Group 3 were diabetic rats were given *D. esculenta* (0.35 g/day in the diet); Group 4 were diabetic rats given *E. rectale* (1 x 10⁹ CFU/mL/day by gavage), and Group 5 were diabetic rats given the combination of *D. esculenta* and *E. rectale*. The diet of *D. esculenta*, *E. rectale* or its combination was given for 28 days.

Rats were made diabetic with induction of intraperitoneally injection of nicotinamide at

the dose of 120 mg/Kg. BW (Sigma-Aldrich, USA) 15 min before intraperitoneally injection of streptozotocin at the dose of 60 mg/Kg. BW (Nacalai Tesque, Inc., Japan).¹⁵ Five days after the injection, the glucose level was measured (DiaSys, Holzheim, Germany) and the rats with blood glucose level more than 170 mg/dL were considered to be diabetes. The plasma glucose level was measured twice before and after the treatments. The HOMA-IR was analyzed based on formula of Jeong *et al.*¹⁶ as follows: HOMA-IR= fasting insulin (µg/L) x fasting glucose (mg/dL)/22.5. Insulin test kits were obtained from DRG International, USA.

After 28 days of intervention, the skeletal muscle from the soleus muscle was removed under anesthesia for *Irs1* expression analysis using the immunohistochemical staining method. Immunohistochemistry of *Irs1* expression on the skeletal muscle tissues was performed based on the standard streptavidin-biotin labeling technique. Briefly, the waxes of sections were removed by xylene and rehydrated in graded alcohols, then rinsed with phosphate-buffered saline (PBS). The tissues were treated by H₂O₂ and incubated for 10 min in 90°C and then rinsed for 15 min in 0.01 M PBS (pH 7.40). The tissues were then blocked with universal tracking antibody (primer *Irs1* antibody, Sigma-Aldrich, USA) and horse radish peroxidase (HRP), incubated for 1 hour, and rinsed for 15 min with 0.01 M PBS.

After washing, the secondary antibody (biotinylated) was added to the tissues for 10 min followed by a rinse for 15 min with 0.01 M PBS. Streptavidin peroxidase was added to the tissues for 5 min, and the tissues were then rinsed for 15 min with 0.01 M PBS. After washing in PBS, diaminobenzidine was added to the tissues for 5-10 min for staining, and the tissues were then rinsed with tap water for 10-15 min. After that, the tissues were

counterstained with Mayer’s hematoxylin solution for 3-4 min and subsequently rinsed with tap water. The tissues were then placed in graded alcohols and xylene once or twice and mounted using E-Z mount.

Statistical analysis

Data were presented as mean ± SEM (standard error of the mean). Plasma glucose levels, HOMA-IR, and Irs1 expression in the skeletal muscle were analyzed with one-way analysis of variance (ANOVA) followed by Tukey’s honest significant difference (HSD) tests. A p value < 0.05 was considered as statistically significant.

RESULTS

Diet of *D. esculenta* or *E. rectale* or its combination for 28 days significantly decreased the plasma glucose level and HOMA-IR in diabetic rats (TABLE 1 and 2). The highest reduction in the plasma glucose level was observed in diabetic rats treated with *E. rectale* (Group 4), whereas the highest reduction the HOMA-IR value was observed in diabetic rats treated with the combination of *D. esculenta* and *E. rectale* (Group 5) (p<0.05).

TABLE 1. Plasma glucose levels of the diabetic rats before and after the diet of *D. esculenta*, *E. rectale* or its combination

Group	Intervention	Plasma glucose (mg/dL)		Mean Difference 95% CI	P
		Before	After		
1	Non-diabetic rats	70.49 ± 3.55	73.36 ± 1.25	-2.87 (-15.86;10.12)	0.573
2	Diabetic rats	214.22 ± 17.72	230.45 ± 2.06	16.24 (-33.27;65.74)	0.414
3	Diabetic rats + <i>D. esculenta</i> (0.35/day)	217.52 ± 10.15 ^a	159.47 ± 2.19 ^{c,d}	-58.05 (-88.03;-28.07)	0.006
4	Diabetic rats + <i>E. rectale</i> (1x 10 ⁹ CFU/mL/day)	203.36 ± 19.29 ^a	116.36 ± 2.15 ^{c,e}	-87.00 (-138.45;-35.57)	0.009
5	Diabetic rats + combination of both	171.86 ± 3.55 ^{a,b}	106.13 ± 1.61 ^c	-65.72 (-78.28; -53.16)	<0.001
	p	<0.001	<0.001		

^aSignificantly different compared to Group 1 and 2 (p<0.05); ^bSignificantly different compared to Group 3 and 4 (p<0.05); ^cSignificantly different compared to Group 1 and 2 (p<0.05); ^dSignificantly different compared to Group 4 and 5; ^eSignificantly different compared to Group 5 (p<0.05). One way ANOVA followed by by Tukey’s HSD tests.

TABLE 2. Insulin levels and HOMA-IR of the diabetic rats after the diet of *D. esculenta*, *E. rectale*, or their combination

Group	Intervention	HOMA-IR
1	Non-diabetic rats	5.19 ± 0.23
2	Diabetic rats	16.92 ± 0.23
3	Diabetic rats + <i>D. esculenta</i> (0.35 g/day)	3.16 ± 0.35 ^{a,b}
4	Diabetic rats + <i>E. rectale</i> (1 x 10 ⁹ CFU/mL/day)	2.16 ± 0.11 ^{a,c}
5	Diabetic rats + combination of both	1.74 ± 0.08 ^a
	p	<0.001

^aSignificantly different compared to Group 1 and 2 (p<0.05); ^bSignificantly different compared to Group 4 and 5 (p<0.05); ^cSignificantly different compared to Group 5 (p<0.05). One way ANOVA followed by Tukey's HSD tests.

FIGURE 1 shows immunohistochemical analysis of the *Irs1* expression in the skeletal muscle of all groups. The positive *Irs1* expression is presented by brown cytoplasm of

the skeletal muscle (yellow arrows), whereas the negative *Irs1* expression is presented by blue cytoplasm of the skeletal muscle (blue arrows).

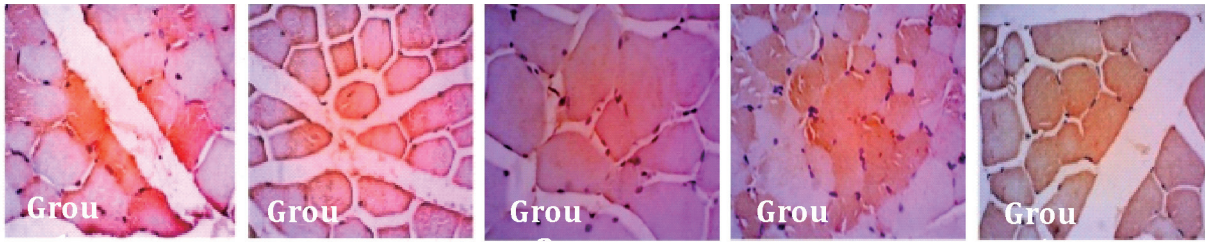


FIGURE 1. *Irs1* expression in the skeletal muscle cells of the rats. Positive *Irs1* expression is presented by brown cytoplasm (yellow arrows), and negative expression is presented by blue cytoplasm (blue arrows). Group 1: non-diabetic rats; Group 2: diabetic rats; Group 3: diabetic rats + *D. esculenta* (0.35 g/day); Group 4: diabetic rats + *E. rectale* (1 x 10⁹ CFU/mL/day); Group 5: diabetic rats + combination of both.

The effect of *D. esculenta*, *E. rectale*, and its combination diet on *Irs1* expression in diabetic rats is presented in FIGURE 2. The *Irs1* expression in skeletal muscle cells of diabetic rats (Group 2: 8.18 ± 2.50%) was lower than that of rat received *D. esculenta* (Group 3: 12.7 ± 4.11%), however it was not significantly different. In contrast, the *Irs1*

expression in skeletal muscle cells of diabetic rats received *E. rectale* (Group 4: 19.16 ± 5.32%) or combination of *D. esculenta* and *E. rectale* (Group 5: 17.45 ± 2.64%) were significantly higher than that in diabetic rats or Group 2 (p <0.05). The highest *Irs1* expression was observed in non-diabetic rats (28.69 ± 5.51%).

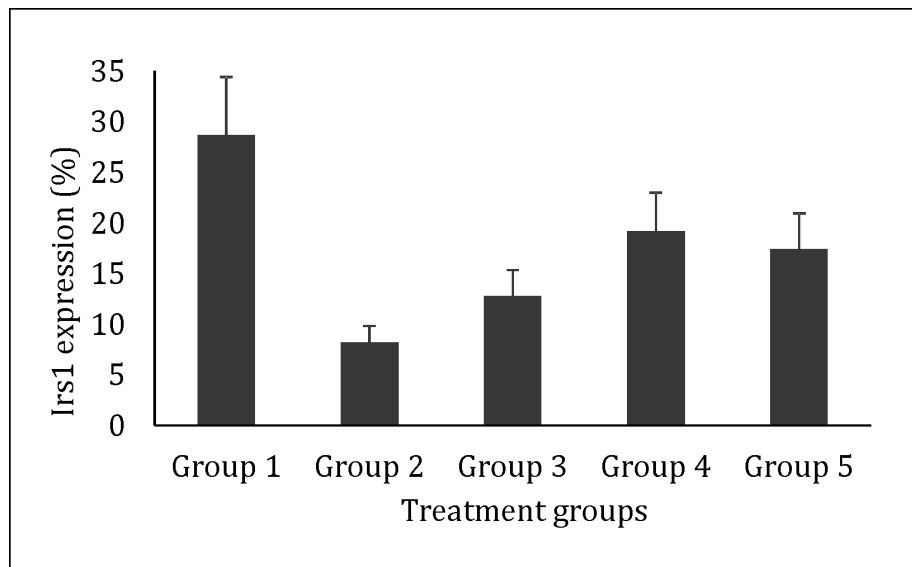


FIGURE 2. Effects of *D. esculenta*, *E. rectale*, and their combination diet on Irs1 expression in diabetic rats. Group 1: non-diabetic rats; Group 2: diabetic rats; Group 3: diabetic rats + *D. esculenta* (0.35 g/day); Group 4: diabetic rats + *E. rectale* (1×10^9 CFU/mL/day); Group 5: diabetic rats + combination of both.

DISCUSSION

Insulin resistance is associated with the development of type 2 diabetes mellitus (type 2 DM). Insulin stimulates glucose uptake, utilizes and promotes glucose storage in the cells by inducing multiple signaling pathways in all tissues that express transmembrane insulin receptors (e.g. adipose tissue, muscle, and the liver).¹⁷ Skeletal muscle has been identified as the major tissue of glucose metabolism and accounts for approximately 75% of the whole-body insulin-stimulated glucose uptake.¹⁸ Insulin resistance in skeletal muscle is manifested by decreased insulin-stimulated glucose uptake resulting from impaired insulin signaling and multiple post-receptor intracellular defects, including impairments in glucose transport.¹⁹ One of the insulin effects is mediated by Irs1.²⁰

Low expression of Irs1 is characterized by insulin resistance and its hallmarks, which include higher levels of insulin, glucose,

and triglycerides, and the low Irs1 protein expression is associated with low Irs1 mRNA levels.²¹ In this study, the expression of the Irs1 protein in the skeletal muscle was lower in the diabetic rats than the diabetic rat groups that received treatment. The administration of *D. esculenta*, *E. rectale* or the combination of both *D. esculenta* and *E. rectale* increased the protein expression of Irs1; however, *D. esculenta* alone did not significantly improve Irs1 protein expression. In the group that was treated with *E. rectale*, the Irs1 expression was slightly higher than that in the combination group.

Eubacterium rectale is a butyrogenic bacteria¹² that has the ability to induce the fermentation of resistant starch to produce methane and metabolically active SCFAs, primarily acetate, propionate, and butyrate.⁶ Butyrate has been suggested to improve insulin sensitivity and energy expenditure in mice. The HOMA-IR is 60% lower in mice that are treated with butyrate.⁵ *Dioscorea*

esculenta has been reported to have a highly resistant starch content of approximately 10.4 mg per dry weight,¹¹ and resistant starch has been suggested to act as a prebiotic that promotes bacterial growth.²² Additionally Winarti et al.²³ reported that the *D. esculenta* content of inulin is 14.77% (dry weight). Inulin is a soluble dietary fiber that cannot be hydrolyzed by enzymes in digestive tracts, but it can be fermented and selectively stimulates the growth or activity of a number of intestinal bacteria that potentially benefit health; therefore, inulin has been called a prebiotic.²⁴

A study showed that inulin can stimulate the growth of bacteria, especially bacteria of the phylum *Firmicutes* cluster XIVa (strain *E. rectale* A1-86). Moreover, Scott et al.²⁵ reported that the *E. rectale* A1-86 can produce butyrate in media with starch and inulin substrates. However, in the present study, the *D. esculenta* reduced the potential effects of the *E. rectale*. This result may be because the resistant starch of *D. esculenta* is not suitable for these bacteria or may inhibit the growth of these bacteria.

The development of skeletal muscle insulin resistance is intimately associated with systemic dyslipidemia and intramuscular lipid accumulation.²⁶ Dysregulation of fatty acid metabolism plays a pivotal role in the pathogenesis of insulin resistance in skeletal muscle.¹⁹ Fatty acids induce insulin resistance in skeletal muscle by blocking the insulin-mediated activation of Irs1-associated phosphatidylinositol 3-kinase (PI3-Kinase). High plasma fatty acid levels increase intracellular fatty acyl-CoA and 1,2-diacylglycerol levels, and these latter factors activate protein kinase c (PKC)- θ , which increases Irs1 Ser307 phosphorylation.⁴

However, other fatty acid types have beneficial effects against insulin resistance. High dietary intakes of monounsaturated

fatty acids, such as oleic acid, have been associated with improved insulin sensitivity in general populations.²⁷ It has been reported that SCFAs, such as butyrate, improve insulin sensitivity, and increase energy expenditure in mice. SCFAs can be utilized for the de novo synthesis of lipids and glucose, which serve as the main energy sources.²⁸ Between 10% and 20% of ingested dietary carbohydrates are resistant to small intestinal digestion. These non-digestible dietary carbohydrates enter the colon where resistant starch and fermentable non-starch polysaccharides are fermented by colonic bacteria to SCFAs, lactate, and gases, such as CO₂, H₂, and methane. Butyrate provides the major energy source for colonic epithelial cells. Acetate and propionate are absorbed into the portal circulation and metabolized in the liver and are also metabolized in other tissues including adipose tissue. Propionate is primarily metabolized in the liver, where it also acts to reduce serum cholesterol and blood glucose.²⁹

This study revealed that the *E. rectale* and the combination of *D. esculenta* and *E. rectale* increased Irs1 expression and decreased HOMA-IR. *E. rectale* appears to ferment the dietary fiber or resistant starch of *D. esculenta* to produce SCFAs. SCFAs can influence blood glucose levels by increasing AMPK activities in the liver and muscle tissues.⁵ Recent research suggested that the activation of AMPK increases the translocation of the glucose transporter GLUT4 to the plasma membrane and thus increases muscle glucose uptake.^{7,8}

CONCLUSIONS

We conclude that *E. rectale* and the combination of *E. rectale* and *D. esculenta* reduce the HOMA-IR of type 2 diabetic rats by increasing Irs1 protein expression. Further

studies are recommended to compare these results with other probiotics.

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