UNIVERSITI PUTRA MALAYSIA

BACTERIAL AND SHORT-CHAIN FATTY ACID PROFILING OF FAECAL SAMPLES

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by

NUR HUDA BINTI FAUJAN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirement for the degree of Doctor of Philosophy

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This thesis is dedicated to …..

- teachers who teach people for a better tomorrow.

- my parents, my husband and my daughter, all my siblings and family-in-laws for their moral support, love, patience and understand

Abstract of the thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

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Human gastrointestinal tract consists of an extremely complex microbiota mainly the anaerobic organisms. Many studies have been done to investigate the human intestinal microbiota. The use of molecular techniques based on DNA for microbial studies is of interest due its reliability, rapid and sensitivity method. Real-time polymerase chain reaction (PCR) has become one of the most widely used methods and an important tool for analysis of bacterial quantification as well as for detection and identification of bacterial species.

In this study, real-time PCR has been used to investigate and quantify bacterial species in human faecal samples. Prior to the analysis, the extraction of DNA from human faeces had been optimised to produce a high quality and yield of DNA. Based on this purpose, DNA from faecal samples was extracted using QIAamp® DNA Stool Mini Kit. However, to give maximum DNA yields, four different procedures of extraction using this kit have been modified by adding 1 mL of 1x PBS buffer in

preheating step or 600 µL of 1x PBS buffer incorporated with InhibitEx (inhibitor-adsorption) tablet. Two different amounts of 20 mg/mL proteinase K (15 µL or 25 µL) were used to digest protein and remove protein contamination. From this study, results showed that the addition of 600 µL of 1x PBS buffer incorporated with the InhibitEX tablet increase removal of inhibitors in faecal samples. Addition of 25 µL of proteinase K also produced higher DNA yield compared to the addition of 15 µL of proteinase K for protein digestion. Optimisation of DNA extraction is a crucial factor in molecular analysis of faecal samples due to the presence of many inhibitors in faecal samples that can interfere with the accuracy of real-time PCR analysis.

Next, specific primer based on 16S rRNA gene was used to analyse and amplify bacterial DNA from faecal samples. The specificity of all primers used were tested by conventional polymerase chain reaction (PCR). Results showed that all primers were highly specific with the target bacteria and amplified DNA with the expected PCR products size. In order to apply real-time PCR for quantitative analysis, the real-time PCR conditions have been optimised to provide an accurate analysis. Standard calibration curve for quantitative analysis was constructed using plasmid DNA. All standard calibration curves plotted from plasmid DNA produced slope between -3.84 and -3.29, which highlighted the efficiency of reaction assay between 81.8% and 101.4%. Efficiency of real-time PCR assay is a crucial factor in order to provide accurate and precise result for quantification.

In order to apply real-time PCR for analysis of intestinal microbiota, faecal samples were obtained from subjects with inflammatory bowel disease (IBD) and also healthy condition. From this study, results showed that *Bacteroides* and *B. fragilis* in faecal samples of IBD patients were significantly higher (P<0.05) than that of healthy persons. The mean for *Bacteroides* in faecal samples of IBD patients and healthy persons were 10.9 $\log_{10}$ cells/g and 10.3 $\log_{10}$ cells/g, respectively. The mean for *B. fragilis* in faecal samples of IBD patients and healthy person were 5.9 $\log_{10}$ cells/g and 4.6 $\log_{10}$ cells/g, respectively. *B. fragilis* was not even detected in some faecal samples of healthy persons. Other bacteria such as *Desulfovibrios*, *Clostridium coccoides*, *Escherichia coli* and *Enterococcus faecalis* did not show any significant difference (P>0.05) in faecal samples between two groups of subjects.

The concentration of short-chain fatty acids (SCFAs) of faecal samples in both groups was also analysed using high performance liquid chromatography (HPLC) method. The SCFAs in human colon are produced by fermentation of carbohydrates, proteins and peptides by human colonic bacteria. Results showed that acetic, butyric and propionic acids concentration was lower in faecal samples of IBD patients as compared to that of healthy persons. However, only butyric and propionic acids were significantly lower (P<0.05) in faecal samples of IBD patients as compared to that of healthy persons. The mean concentration of butyric acid in faecal samples of IBD patients was 66.2 μmol/g and in healthy person was 175.0 μmol/g. The mean concentration of propionic acid in faecal samples of IBD patients was 52.8 μmol/g and in healthy persons was 93.3 μmol/g. Results also showed that concentration of lactic acid in faecal samples of IBD patients was higher than that of healthy persons. The concentration of formic, pyruvic and isobutyric acids in faecal samples were also

analysed. In faecal samples of IBD patients, the concentration of pyruvic and isobutyric acids were higher than that of healthy person but not significant (P>0.05).

In conclusion, DNA extraction from faecal samples using QIAamp® DNA Stool Mini Kit with addition of 600 mL of 1x PBS buffer in removal inhibitors step and higher amount of 20 mg/mL proteinase K in eliminating contaminated protein resulted in increased quality and yield of faecal DNA. By using real-time PCR, this study showed that the composition of certain bacterial species in IBD faecal samples differed from that in faecal samples of healthy persons. The concentration of certain SCFAs also changed in faecal samples of IBD patients and which might be attributed to difference in certain bacterial species. Furthermore, this study also showed that real-time PCR assay was an efficient and rapid method for studying bacterial profile in faecal samples.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan Ijazah Doktor Falsafah

PEMPROFILAN BAKTERIA DAN ASID LEMAK BERANTAI-PENDEK DALAM SAMPEL NAJIS

Oleh

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Saluran pencernaan makanan manusia mengandungi mikrobiota yang sangat kompleks terutamanya daripada organisma anaerob. Banyak kajian telah dilakukan untuk menyelidik mikrobiota usus manusia. Penggunaan teknik molekular yang berdasarkan penggunaan DNA untuk kajian mikrob telah menjadi perhatian kerana keputusan yang lebih dipercayai, cepat dan sensitif. Kaedah tindak balas rantaian polimerase (PCR) masa-nyata telah menjadi salah satu kaedah yang paling banyak digunakan dengan meluas dan menjadi satu alat yang penting untuk analisis pengiraan bakteria serta pengesanan dan pengenalpastian bakteria.

Dalam kajian ini, PCR masa-nyata telah digunakan untuk menyelidik dan mengira spesis bakteria dalam sampel najis manusia. Sebelum analisis dijalankan, pengekstrakan DNA daripada najis manusia telah dioptimakan untuk menghasilkan perolehan DNA yang banyak dan berkualiti. Berdasarkan tujuan ini, DNA telah diekstrak menggunakan Kit Mini QIAmp® DNA Stool. Bagaimanapun, untuk

memberikan perolehan DNA yang maksimum, empat prosedur pengekstrakan yang berbeza telah diubahsuai semasa penggunaan kit ini dengan penambahan 1 mL larutan penimbal PBS pada langkah prapemanasan sampel atau dengan menambah 600 µL larutan penimbal 1x PBS yang digabungkan dengan tablet InhibitEX (penjerapan-perencat). Dua jumlah 20 mg/mL proteinase K yang berbeza (15 µL atau 25 µL) juga telah digunakan untuk mencernakan protein dan menyingkirkan pencemaran protein. Dari kajian ini, keputusan menunjukkan bahawa penambahan 600 µL larutan penimbal 1x PBS yang digabungkan dengan tablet InhibitEX telah meningkatkan penyingkiran bahan perencat dalam sampel najis. Penambahan 25 µL protenaise K juga telah menghasilkan DNA yang lebih banyak berbanding dengan penambahan hanya 15 µL protenaise K bagi pencernaan protein. Pengoptimuman pengekstrakan DNA merupakan faktor yang sangat penting dalam analisis molekular sampel najis kerana kehadiran banyak bahan perencat dalam sampel najis yang boleh mengganggu ketepatan analisis PCR masa-nyata.

Seterusnya, primer spesifik yang berdasarkan gen 16S rRNA telah digunakan untuk menganalisis dan menggandakan DNA bakteria daripada sampel najis. Kespesifikan semua primer yang digunakan telah diuji melalui PCR konvensional. Keputusan kajian menunjukkan bahawa semua primer adalah spesifik dengan bakteria yang disasarkan dan menggandakan DNA dengan saiz produk yang telah dijangkakan. Untuk mengaplikasikan penggunaan PCR masa-nyata sebagai analisis kuantitatif, keadaan PCR masa-nyata telah dioptimumkan untuk memberikan analisis yang tepat. Lengkuk kalibrasi piawai yang digunakan untuk analisis kuantitatif telah dihasilkan dengan menggunakan DNA plasmid. Semua lengkuk kalibrasi piawai yang telah diplot daripada DNA plasmid menghasilkan kecerunan diantara -3.84 hingga -3.29,

yang mana ini telah memberikan kecekapan asai tindak balas di antara 81.8% hingga 101.4%. Kecekapan asai tindak balas rantaian polymerase masa-nyata merupakan faktor yang sangat penting untuk memberikan keputusan pengiraan yang tepat dan jitu.

Untuk mengaplikasikan penggunaan PCR masa-nyata untuk analisis mikrobiota usus, sampel najis telah diperolehi daripada subjek dengan penyakit radang usus (IBD) dan juga yang sihat. Dari kajian ini, hasil kajian menunjukkan bahawa *Bacteroides* dan *B. fragilis* dalam sampel najis pesakit IBD adalah lebih tinggi secara signifikan (P<0.05) daripada sampel najis manusia sihat. Purata bacaan *Bacteroides* dalam sampel najis pesakit IBD dan manusia sihat adalah 10.9 $\log_{10}$ cells/g dan 10.3 $\log_{10}$ cells/g. Purata bacaan *B. fragilis* dalam sampel najis pesakit IBD dan manusia sihat adalah masing-masing 5.9 $\log_{10}$ cells/g dan 4.6 $\log_{10}$ cells/g. Pada beberapa sampel najis manusia sihat, *B. fragilis* juga tidak dikesan. Bakteria lain seperti *Desulfovibrios*, *Clostridium coccoides, Escherichia coli* dan *Enterococcus faecalis* tidak menunjukkan perbezaan yang signifikan (P>0.05) antara dua kumpulan subjek.

Kepekatan asid lemak rantai pendek (SCFAs) dalam sampel najis pada kedua-dua kumpulan juga telah dianalisis menggunakan kaedah kromatografi cecair berprestasi tinggi (HPLC). SCFAs dalam kolon manusia adalah merupakan produk hasil penapaian karbohidrat, protein dan peptida oleh bakteria usus manusia. Hasil kajian menunjukkan bahawa kandungan asid asetik, asid butirik dan asid propionik pada sampel najis pesakit IBD adalah lebih rendah jika dibandingkan dengan sampel najis manusis sihat. Namun, hanya kepekatan asid butirik dan asid propionik sahaja yang lebih rendah secara signifikan (P<0.05) dalam sampel najis pesakit IBD berbanding

dalam najis manusia sihat. Purata kepekatan asid butirik dalam sampel najis pesakit IBD adalah adalah 66.2 μmol/g manakala pada manusia sihat adalah 175.0 μmol/g. Purata kepekatan asid propionik pada najis pesakit IBD adalah 52.8 μmol/g manakala pada manusia sihat adalah 93.3 μmol/g. Keputusan juga menunjukkan bahawa kepekatan asid laktik dalam sampel najis pesakit IBD lebih tinggi daripada dalam sampel najis manusia sihat. Kepekatan asid formik, asid piruvik dan asid isobutirik dalam sampel najis juga telah dianalisis. Dalam sampel najis pesakit IBD, kepekatan asid piruvik dan asid isobutirik adalah lebih tinggi daripada manusia sihat tetapi perbezaan antara dua sampel ini tidak berbeza secara signifikan (P>0.05).

Sebagai kesimpulan, pengekstrakan DNA dari sampel najis menggunakan Kit Mini QIAmp® DNA Stool dengan penambahan 600 mL larutan penimbal 1x PBS pada langkah penyingkiran bahan perencat dan penggunaan jumlah 20 mg/mL proteinase K yang tinggi telah menghasilkan DNA yang lebih berkualiti dan banyak. Dengan menggunakan PCR masa-nyata, kajian ini telah menunjukkan bahawa komposisi bakteria tertentu dalam sampel najis pesakit IBD berbeza berbanding dalam sampel najis manusia sihat. Kepekatan SCFAs tertentu juga telah berubah dalam sampel najis pesakit IBD dan menunjukkan ada perkaitan dengan perubahan komposisi beberapa spesies bakteria tertentu. Seterusnya, kajian ini juga telah menunjukkan bahawa penggunaan kaedah asai PCR masa-nyata dalam mengkaji pemprofilan bakteria dalam sampel najis adalah cekap dan cepat.

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