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UNIDAD ACADÉMICA ESPECIALIZADA EN MICROBIOLOGÍA

UNIDAD ACADÉMICA DE MEDICINA

"ANÁLISIS EN LA COMPOSICIÓN DE LA MICROBIOTA INTESTINAL EN PACIENTES CON ENFERMEDAD RENAL CRÓNICA"

T E S I S

QUE PARA OBTENER EL GRADO DE

MAESTRÍA EN CIENCIAS BIOMÉDICAS

P R E S E N T A:

J O S É C R U Z M O R A

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UNIVERSIDAD AUTÓNOMA DE GUERRERO
UNIDAD ACADÉMICA DE CIENCIAS QUÍMICO BIOLÓGICAS
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UNIDAD DE INVESTIGACIÓN ESPECIALIZADA EN MICROBIOLOGÍA
MAESTRÍA EN CIENCIAS BIOMÉDICAS

APROBACIÓN DE TESIS

En la ciudad de Chilpancingo, Guerrero, siendo los 22 días del mes de junio de dos mil doce, se reunieron los miembros del Comité Tutoral designado por la Academia de Posgrado de la Maestría en Ciencias Biomédicas, para examinar la tesis titulada **"Análisis en la composición de la microbiota intestinal en pacientes con enfermedad renal crónica"**, presentada por el alumno José Cruz Mora, para obtener el Grado de Maestría en Ciencias Biomédicas. Después del análisis correspondiente, los miembros del comité manifiestan su aprobación de la tesis, autorizan la impresión final de la misma y aceptan que, cuando se satisfagan los requisitos señalados en el Reglamento General de Estudios de Posgrado e Investigación Vigente, se proceda a la presentación del examen de grado.

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ÍNDICE GENERAL

Effects of a symbiotic base diet on gut microbiota in Mexican patients with end-stage renal disease.

ABSTRACT

Background: Gut microbiota provides beneficial effects under normal physiological conditions, but is able to contribute to inflammatory diseases in susceptible individuals.

Objective: We wanted to test whether a symbiotic gelatin was associated with specific modifications of the gut microbiota in patients with end-stage renal disease (ESRD).

Design. Eighteen patients with ESRD´s diagnosis with renal replacement therapy (hemodialysis) were included in this study. They were randomly assigned to two treatment groups: a) test group (nutritional counseling + symbiotic), or b) control group (nutritional counseling + placebo). Clinical history and the evaluation of Gastrointestinal Symptom Rating Scale were performed. Gut microbiota composition was analyzed by real-time PCR from fecal samples. All subjects were followed for 2 months.

Results: Gastrointestinal symptoms scores (scale 8-36) were significantly reduced in the test group (start 12(10-14), end 9(8-10)) compared with control group (start 11(8-20), end 12.5 (9-22.5)) ($p = 0.022$). Bifidobacterial counts were higher in the second samples (mean: 5.53± 1.72 $\log_{10}$ cells/g) than in first samples (4.18±0.88 $\log_{10}$ cells/g); $p = 0.0344$ in the patients of the test group. Also, lactobacilli counts had a little decrease in the test group (2.37(1.8-2.69) and 1.86(1.59-2.6)) that in the control group (2.3(2.2-2.53) and 1.53(1.14-1.76), between the first and second samples.

Conclusion: Short term symbiotic treatment in patients with ESRD resulted in improvement of the gastrointestinal symptoms; also we found that intervention with a symbiotic therapy can lead the increase of *Bifidobacterium* counts.

KEY WORDS: Gut microbiota, probiotics, prebiotics, bifidobacteria, gastrointestinal symptoms and patients with ESRD.

INTRODUCTION

At present, chronic kidney disease (CKD) is broadly defined on the basis of changes in the glomerular filtration rate and/or parenchymal damage presence (1). Untreated or poorly managed CKD can lead to numerous health problems, particularly cardiovascular disease and kidney failure, also known as end-stage renal disease (ESRD), requiring treatment with dialysis or a kidney transplant for survival (2). Dietary modification represents an attractive intervention to prevent kidney function declination. Individual nutrients of diet as protein, fat, cholesterol, fiber, antioxidant vitamins, vitamin D, folate, fructose, sodium, and potassium are associated with microalbuminuria and glomerular filtration rate declination (3); however the nutrients assimilation is affected by gut microbiota.

The gut microbiota harbors large bacterial populations in the intestine and colon, approximately $10^{11–12}$ microorganisms per gram of content, and are comprised of mainly anaerobes. Gut microbiota studies assigned 98% of all species to only four bacterial phyla: *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria* (4). With the development of methods for identifying gut microbiota that do not require culturing, a much more thorough and reliable assessment of the gut microbiota is now possible (5). Specifically, the sequencing of 16S ribosomal RNA genes from amplified bacterial nucleic acid extracted from fecal material or mucosal samples has greatly facilitated the identification and classification of bacteria (6). Gut microbes are considered to contribute to body weight regulation and related disorders by influencing metabolic and immune host functions (7).

Modulation of gut microbiota by antibiotic treatment or probiotics (*Bifidobacterium* and *Lactobacillus*) with oligofructoses, reduced glucose intolerance, decreased body weight gain and inhibited inflammation in mice (8, 9). Bacterial lipopolysaccharide is a gut microbiota-related factor that triggers secretion of pro-inflammatory cytokines (10, 11) (Figure 1). The mechanisms of the beneficial effect of essential fatty acids are thought to be a result of anti-inflammatory, antithrombotic, antiarrhythmic, hypolipidemic, and vasodilatory properties (12, 13). With consumption of grain sorghum lipid extract, population of bifidobacteria increased and showed a strong positive association with rise in

HDL cholesterol levels (14). Moreover, high counts of bifidobacteria may provide protection against overweight and obesity development (15). The objective of this study was to investigate if the symbiotic supplementation modified the gut microbiota in patients with ESRD. On this study, real-time PCR was standardized as methodology to quantify the main group of gut microbiota in patients with ESRD to evaluate the effect of symbiotic supplementation, it establish the basis for subsequent studies, focusing on the gut microbiota composition; it could be a novel target for treating chronic diseases.

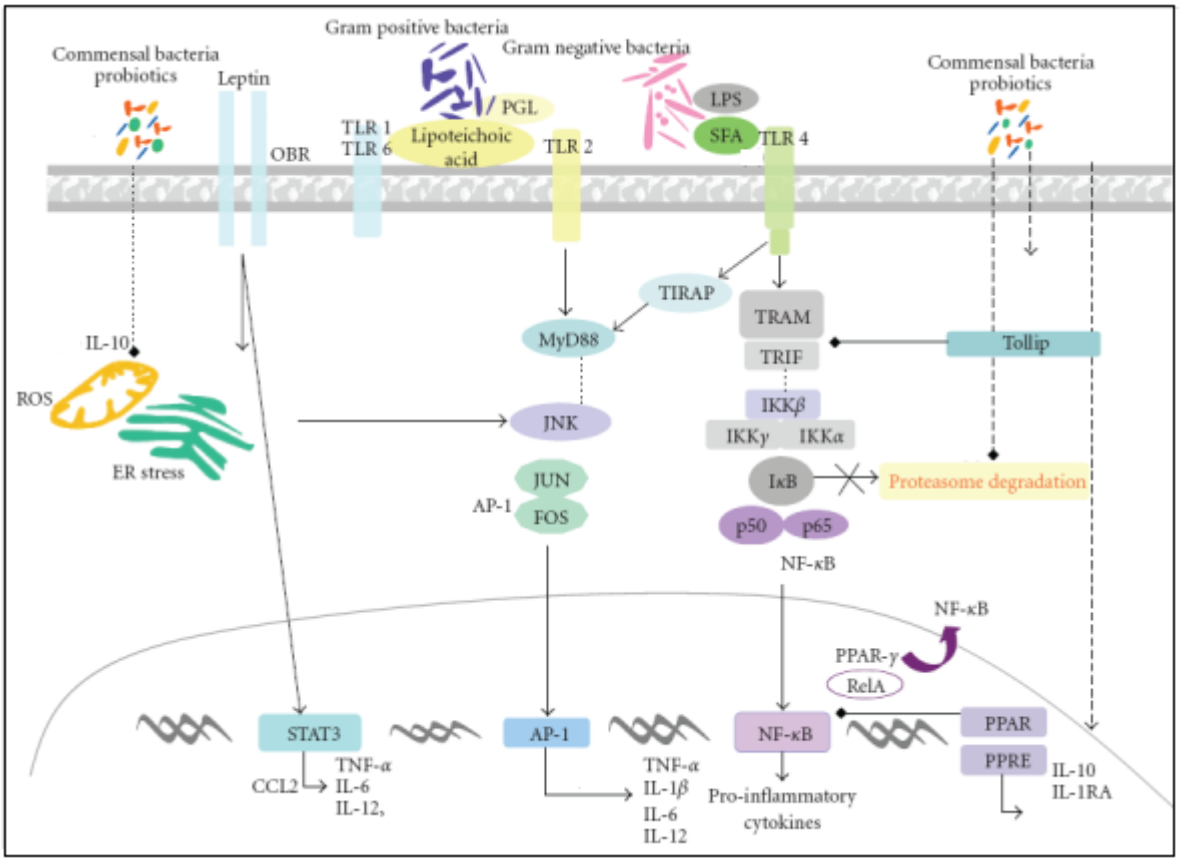

Figure 1. Schematic diagram of signaling pathways triggered by bacterial components, saturated fatty acids, and adipokines in epithelial and innate immune cells leading to either activation or negative regulation of proinflammatory pathways related to obesity and insulin resistance (7).

SUBJECTS AND METHODS

Design. Subjects were recruited from Dialysis Clinic, at the city of Guadalajara, Jalisco, included 18 patients with ESRD diagnosis, also renal replacement therapy (hemodialysis), for at least three months prior to baseline. Study was approved for bioethics committee and all patients accept to participate at the study by mean of informed consent in writing. They were randomly assigned to two treatment groups: a) test group (nutritional counseling + symbiotic (*Lactobacillus acidophilus* and *Bifidobacterium* as probiotic, inulin as prebiotic with vitamin E, C, B1, B2, B6, B12 and omega 3 (eicosapentaenoic acid and docohexaenoic acid)), or b) control group (nutritional counseling + placebo). Clinical history and the evaluation of Gastrointestinal Symptom Rating Scale were performed and standardized by trained personnel. Gut microbiota composition was analyzed by real-time PCR from fecal samples. All subjects were followed for 2 months.

DNA extraction and specificity of the primers used. The DNA extractions from pure cultures of the control strain and fecal samples were extracted with the use of the QIAamp DNA stool Mini kit (Qiagen) following the manufacturer's instructions. To characterized gut microbiota, PCR primers were used to target different species or groups of gut microbiota (Table 1). PCR products of *Clostridium leptum, Bacteroides fragilis, Bifidobacterium* and *Prevotella* were purify with PureLink PCR Purification Kit and sequenced in an APIPRISMA 310 Genetiz Analizer (Applied Biosystems), then were realized a BLAST (Basic Local Alignment Search Tool) with the sequence of PCR products, to verify the specificity of the primer used.

Gut microbiota analysis. Quantitative polymerase chain reaction (qPCR) was conducted as previously described (15). Briefly, the amplification and detection were performed with a 7500 Fast Real-Time PCR System (Applied Biosystems). Each reaction mixture of 25 μL was composed of Fast-Plus EvaGreen qPCR Master Mix (Biotium), 1 μL of each of the specific primers at a concentration of 0.25μmol/L, and 2μL of template DNA. The fluorescent products were detected at the last step of each cycle. A melting curve analysis was made after amplification to distinguish the targeted PCR product from the nontargeted

PCR product. Curve standard was done with DNA extracted from a known amount of *Escherichia coli* ATCC 25922 was added in serial dilutions from $5x10^6$ to $5x10^2$ cells to a series of PCR mixtures with *E. coli* specific primers.

Statistics. The descriptive analysis was performed using frequencies and chi-square test for categorical variables; asymmetry variables with Meanwhile, we used 25th, 50th and 75th percentiles and the Two-sample Wilcoxon rank-sum (Mann-Whitney) test to find differences between median values, finally symmetry variables were reported with mean ± std. dev. and test of *t* Student to obtain the *p* value. Statistical analysis was performed using STATA software (V.9) and $p<0.05$ was reported as statistically significant.

Table 1. Sequence of primers for real-time PCR.

Bacterial group	Sequence	Size (pb)	Tm (°C)	Ref
Clostridium leptum	GCACAAGCAGTGGAGT	239	50	15
	CTTCCTCCGTTTTGTCAA			
Bacteroides fragilis	ATAGCCTTTCGAAAGRAAGAT	495	50	15
	CCAGTATCAACTGCAATTTTA			
Bifidobacterium	CTCCTGGAAACGGGTGG	550	55	15
	GGTGTTCTTCCCGATATCTACA			
Prevotella	CACRGTAAACGATGGATGCC	513	55	15
	GGTCGGGTTGCAGACC			
Lactobacillos	TACATCCCAACTCCAGAACG	90	55	16
	AAGCAACAGTACCACGACC			
Escherichia coli	CATGCCGCGTGTATGAAGAA	95	55	17
	CGGGTAACGTCAATGAGCAAA			

Tm: Annealing temperature.

RESULTS

The general characteristics of patients with ESRD are presented in Table 2. Eight of the 18 patients with ESRD, were assigned to test group and 10 to the control group. In the analysis by group, some variables as age, gender, CKD diagnosis time, personal history of chronic diseases, not presented significant differences, indicated that the study groups are homogeneous. Gastrointestinal symptoms scores in the intervention group, showed a significant decrease, before and after treatment.

Melting curve were realized with the used primer to *C. leptum, B. fragilis, Bifidobacterium, Prevotella, Lactobacillus* and *E. coli*, it shows the presence of a single peak, indicating absence of nonspecific products of PCR (Figure 2a). Standard curve was realized with DNA extracted from a known amount of the control strain, obtain a r^2 of 0.99 and a slope of -3.3, in the analysis by real-time PCR to cuantify the bacterial group of gut microbiota in the patients with ESRD (Figure 2b).

Figure 3 shows the composition of the gut microbiota from the first and second fecal samples in both groups. Control group do not show significant differences between the second and first fecal samples, however, they show a lightly decrease of *C. leptum* group. While in the test group a significantly increase of *Bifidobacterium* (p = 0.0344) were observed. Also, *Lactobacillus* counts had a little decrease in the test group that in the control group between the first and second samples.

Table 2. General characteristics of the study groups.

	Control group (n=10)	Test group (n=8)	*p* value
Age (years)*	30.6±9.5	34±10	0.236
Gender, n (%)[&]			
Female	1 (10)	2 (25)	0.559
Male	9 (90)	6 (75)	
CKD diagnosis Time (years)*	7.15±3.9	5.0 ± 3.7	0.134
Duration of kidney dialysis (years)*	5.7±3.2	4.1± 2.2	0.119
Personal history of DM2, n (%)[&]			
Yes	1 (10)	1 (12.5)	1.000
No	9 (90)	7 (87.5)	
Personal history of hypertension, n (%)[&]			
Yes	4(40)	5(62.5)	0.637
No	6(60)	3(37.5)	
Personal history of obesity, n (%)[&]			
Yes	1 (10)	1 (12.5)	1.000
No	9 (90)	7 (87.5)	
Gastrointestinal symptoms (score)			
Before treatment	14.8±9.4[a]	12.87±4.1[b]	0.299
After treatment	14.1±8.7[a]	9.28±1.5[b]	0.086

*Mean ± s.d., *t* Student test
[&]Chi-square test.
[a]Statistical differences between before and after intervention in the control group (p= 0.442).
[b]Statistical differences between before and after intervention in the test group (p= 0.025).

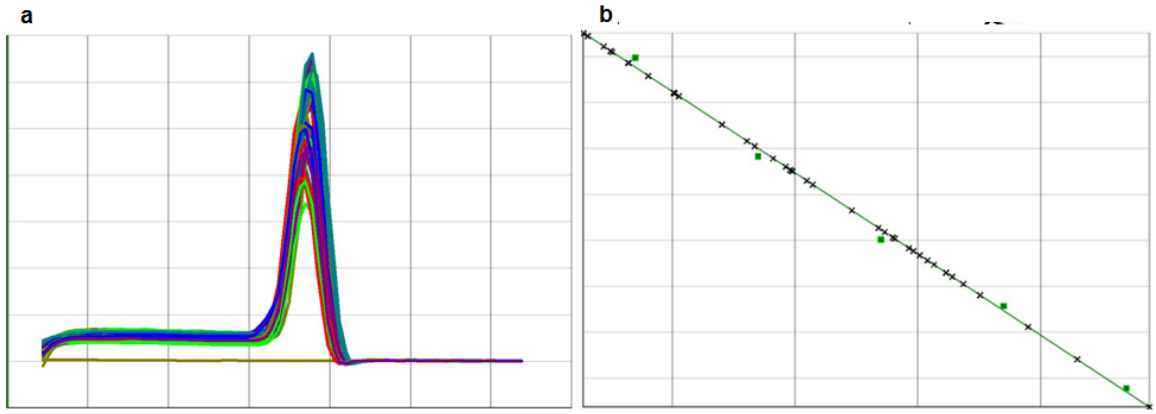

Figure 2. Real-time PCR validation. a) Melting curve with specific primer for *E. coli*, using DNA of control strain and samples of fecal matter of patients with ESRD. b) Standard curve with specific primer for *E. coli*, using dilutions of DNA of control strain and samples fecal matter of patients with ESRD, obtaining a r^2 of 0.99 and slop of -3.5.

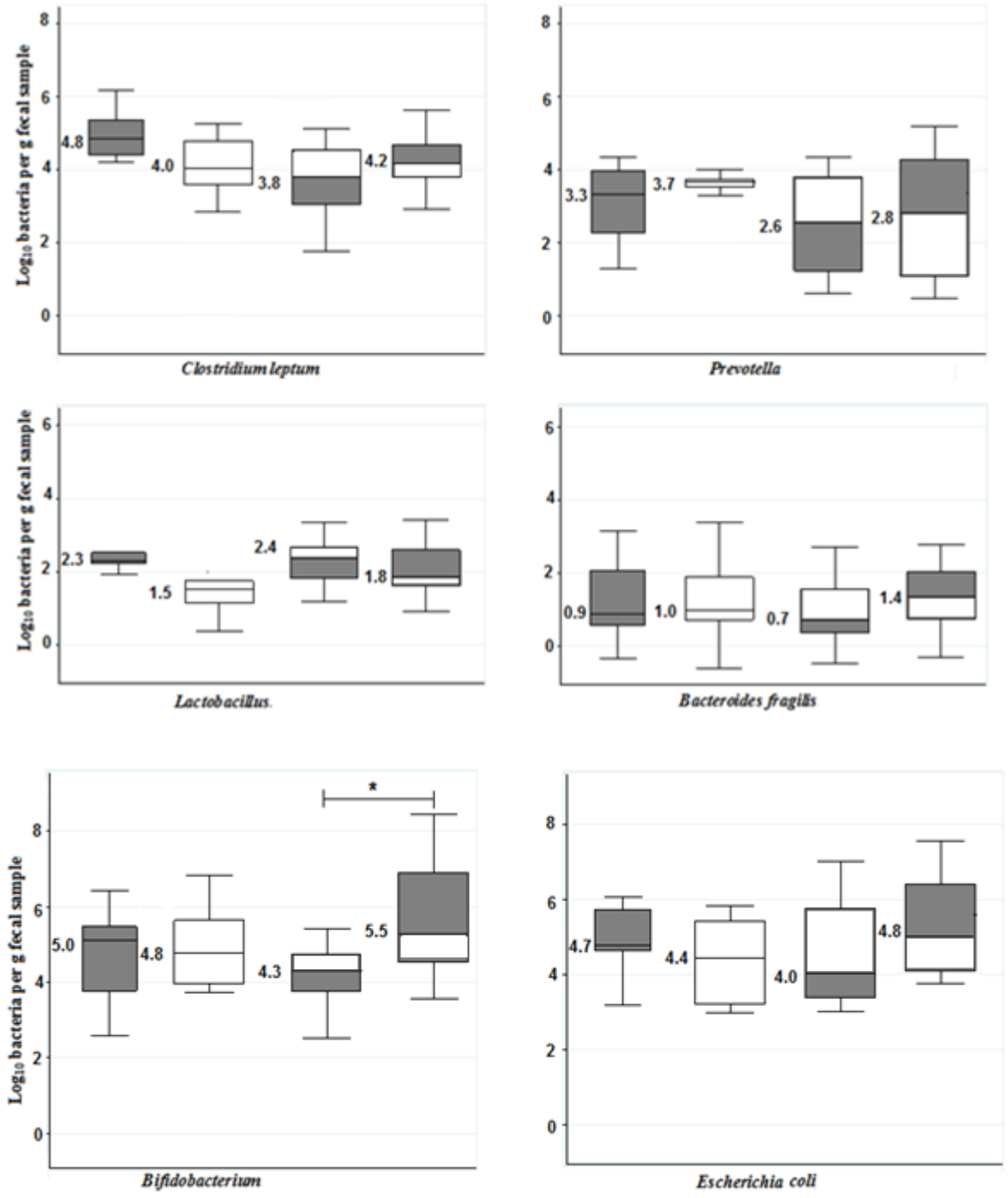

Figure 3. Box plots of bacterial groups quantified by EvaGreen real-time PCR in patient with end-stage renal disease. First sample of control group (black; $n = 10$), second sample of control group (white; $n = 10$), First sample of test group (black and white; $n = 8$) and second sample of test group (white and black; $n = 8$). The median counts are presented by numbers. Boxes show the upper (75%) and the lower (25%) percentiles of the data. Whiskers indicate the highest and the smallest values. * $p < 0.05$, Two-sample Wilcoxon rank-sum (Mann-Whitney) test.

DISCUSSION

Symbiotic supplementation for 2 month significantly reduced the gastrointestinal symptoms in patients with ESRD. This reduction can be due to the contents of gut microbiota. Symbiotic therapy can be potentially improved through combination with specific probiotics (live microbes that can provide a health benefit to the host), prebiotics (selectively fermentable substances that confer benefits to the host). A further advantage of using a symbiotic is that the prebiotic component would promote the growth of indigenous organisms in the gut with probiotic properties (19).

In order to clarify the population structure of the predominant phylogenetic groups in the human gut microbiota, they were standardized the real-time PCR with group-specific primers for the anaerobic bacterial and the species of *E. coli* with specific primer for the analysis of the gut microbiota. DNA extraction was realized using the QIAamp DNA Stool Mini Kit, it allows getting DNA in appropriately amount and purity to perform the PCR on fecal samples and control strain culture. Li *et al*. (20), use PCR-denaturing gradient gel electrophoresis (DGGE) DNA profiling, random cloning and sequence analysis of 16S rRNA genes to compare the QIAamp DNA Stool Mini Kit with the bead beating technique in the preparation of DNA extracts from gut microbiota of pigs, both the QIAamp kit and bead beating method lysed approximately 95% of bacterial cells. A random cloning and sequence analysis also demonstrated the high quality of DNA extracts using the two methods; suggest the appropriateness of the QIAamp DNA Stool Mini Kit for the studies of gut microbial ecology and the effectiveness of the QIAamp kit in processing multiple samples for cell lysis and DNA extraction.

A detection and identification of predominant bacteria in the intestinal microbiota was performed by end-point PCR amplification with specific group primers for anaerobic bacteria (*B. fragilis, Bifidobacterium, Prevotella* and *C. leptum*), which were designed and tested with control strains to determine his specificity in the study perform by Matsuki *et al.* (16). In the study the PCR products amplified with specific primers group for anaerobic bacteria were purified and sequenced. Using the Basic Local Alignment Search Tool

(BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi), the obtained sequences of PCR products were compared against the sequence database, obtaining a maximum identification of 95%, in this way, we demonstrated the specificity of the primers used for anaerobic bacteria. Whereas the group-specific primers for *Lactobacillus* were designed y tested by Menard *et al*, (17), in this study, we used the strain of *L. casei* Shirota of Yakult to verify their specificity. The primers for *E. coli*, as well as of the anaerobic bacteria are specific for the 16S RNA gene that has highly conserved sequence; we used the control strain *E. coli* ATCC 25922. Finally, the real-time PCR was performed using the EvaGreen, a new DNA intercalating, which is more stable and sensitive than SYBR Green I (according to the manufacturer's product and safety data sheet of EvaGreen), however like the SYBR Green I, the EvaGreen is an intercalating DNA that binds to nonspecific manner, thus to validate the method, it was necessary to melting curves for the used primers, showing the formation of a single peak, indicating the absence of nonspecific PCR products.

Analysis of the composition of gut microbiota by real-time PCR in patients with ESRD, no observed significant difference in the bacterial groups in the control group compared with the intervention group (data not shown). Subsequently was perform the comparison between the first and second stool sample in both groups, in the control group showed no significant differences in the groups of bacteria from the intestinal microbiota in patients with ESRD. While the intervention group showed a significant increase in the *Bifidobacterium* group (p = 0.0344) between the first and second stool sample. Also, Lactobacillus numbers had a smaller decrease in the test group between the first and second samples. Symbiotic supplementation facilitated the increase in population of *Bifidobacterium* and the preservation of *Lactobacillus sp* numbers. Indeed, *Bifidobacterium sp* and *Lactobacillus sp* represent an important and complex group of bacteria whose presence is often associated with beneficial health effects (21-23). Postulated health advantages associated to bifidobacteria include the inhibition of pathogenic microorganisms, improvement of lactose digestion, and reduction of serum cholesterol levels, prevention of cancer and enhancement of the host's immune system (24, 25). Intake of prebiotics can increase the counts of bifidobacteria (26) and exert positive effects on absorption of nutrients and minerals, synthesis of vitamins, prevention of constipation,

colon cancer, and improvement of blood sugar and lipid profile (27). Taki *et al*. (28), they showed that the oral administration of *Bifidobacterium longum* in a gastroresistant seamless capsule to hemodialysis (HD) patients is effective in decreasing the pre-HD serum levels of homocysteine, indoxyl sulfate, and triglyceride. The reduction in the serum level of homocysteine is mainly attributable to the supply of folate produced by *Bifidobacterium longum* in the human intestines.

A limitation of this study was that biochemical parameters (urea, serum creatinine, cholesterol, triglycerides and HDL-cholesterol) were not measured, due that patients presented nofasting, the association with gut microbiota remains uncertain.

In conclusion, in this work we have confirmed that Real-Time PCR is a powerful technique in studying the fecal microbiota. In this short term study has provided the first evidence that symbiotic have the potential to be developed into acceptable therapies for patients with renal disease, it can facilitated the increase in population of bifidobacteria.

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ANEXOS

ANEXO No. 1 "Estandarización de la PCR en tiempo final"

Se realizó la amplificación con los iniciadores grupo específicos para bacterias anaerobias, obteniendo los tamaños de productos de PCR esperados: *Bacteroides fragilis* (495 pb), *Clostridium leptum* (239 pb), *Prevotella* (513 pb) y *Bifidobacterium* (550 pb), a partir de DNA extraído de materia fecal (Figura 4a). Para los géneros bacterianos de *Lactobacillus* y *E. coli* se utilizó DNA extraído de cultivos puros de las cepas control de *E. coli* ATCC 25922 con un producto amplificado de 95 pb y *L. casei* Shirota de Yakult obteniendo un producto amplificado de 90 pb (Figura 4b).

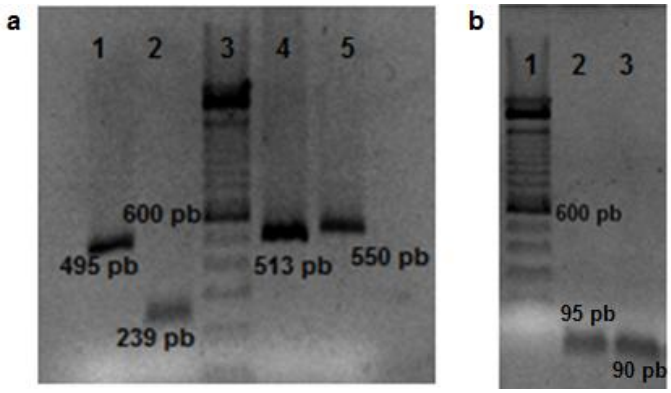

Figura 4. Productos de PCR amplificados con iniciadores grupo específicos bacterianos de la microbiota intestinal. a) Productos amplificados de bacterias anaerobias a partir de DNA extraídos de materia fecal. Carril 1, *Bacteroides fragilis*; Carril 2, *Clostridium leptum*; Carril 3, Marcador de PM (100 pb); Carril 4, *Prevotella sp*; Carril 5, *Bifidobacterium sp.* b) Productos amplificados del género de *Lactobacillus* y *Escherichia coli*. Carril 1, MPM (100 pb); Carril 2, *E. coli* ATCC 25922; Carril 3, *L. casei* Shirota de Yakult.

ANEXO No. 2 "Especificidad de los iniciadores utilizados para bacterias anaerobias"

Los productos de PCR amplificados con los iniciadores grupos específicos para bacterias anaerobias (*Bacteroides fragilis* (495 pb), *Clostridium leptum* (239 pb), *Prevotella* (513 pb) y *Bifidobacterium* (550 pb)), a partir de DNA extraído de materia fecal, se purificaron y secuenciaron y con la secuencia se realizó un BLAST, obteniendo una identificación máxima en promedio del 95% (Figura 5-8).

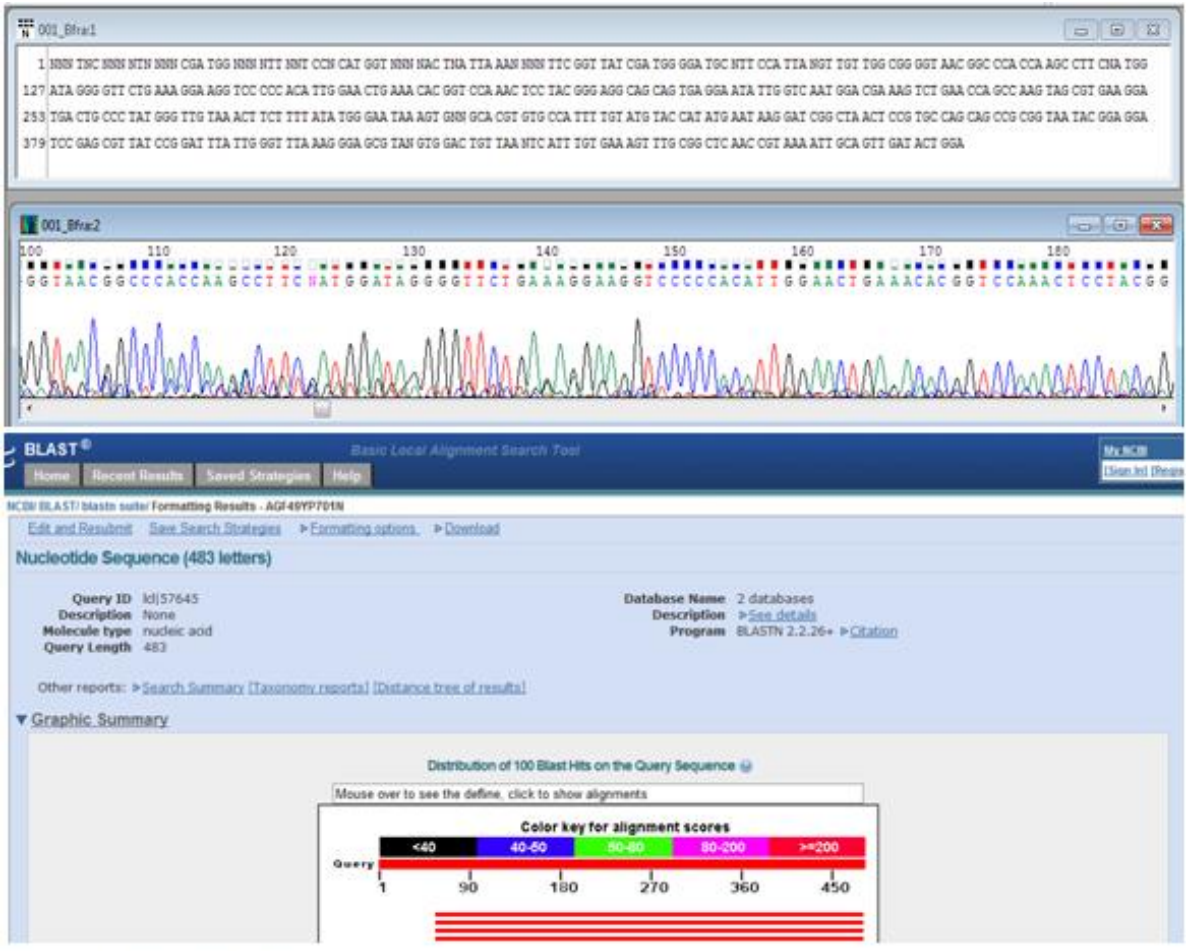

Figura 5. Secuencia y BLAST del producto amplificado con iniciadores grupo especifico para *Bacteroides fragilis*.

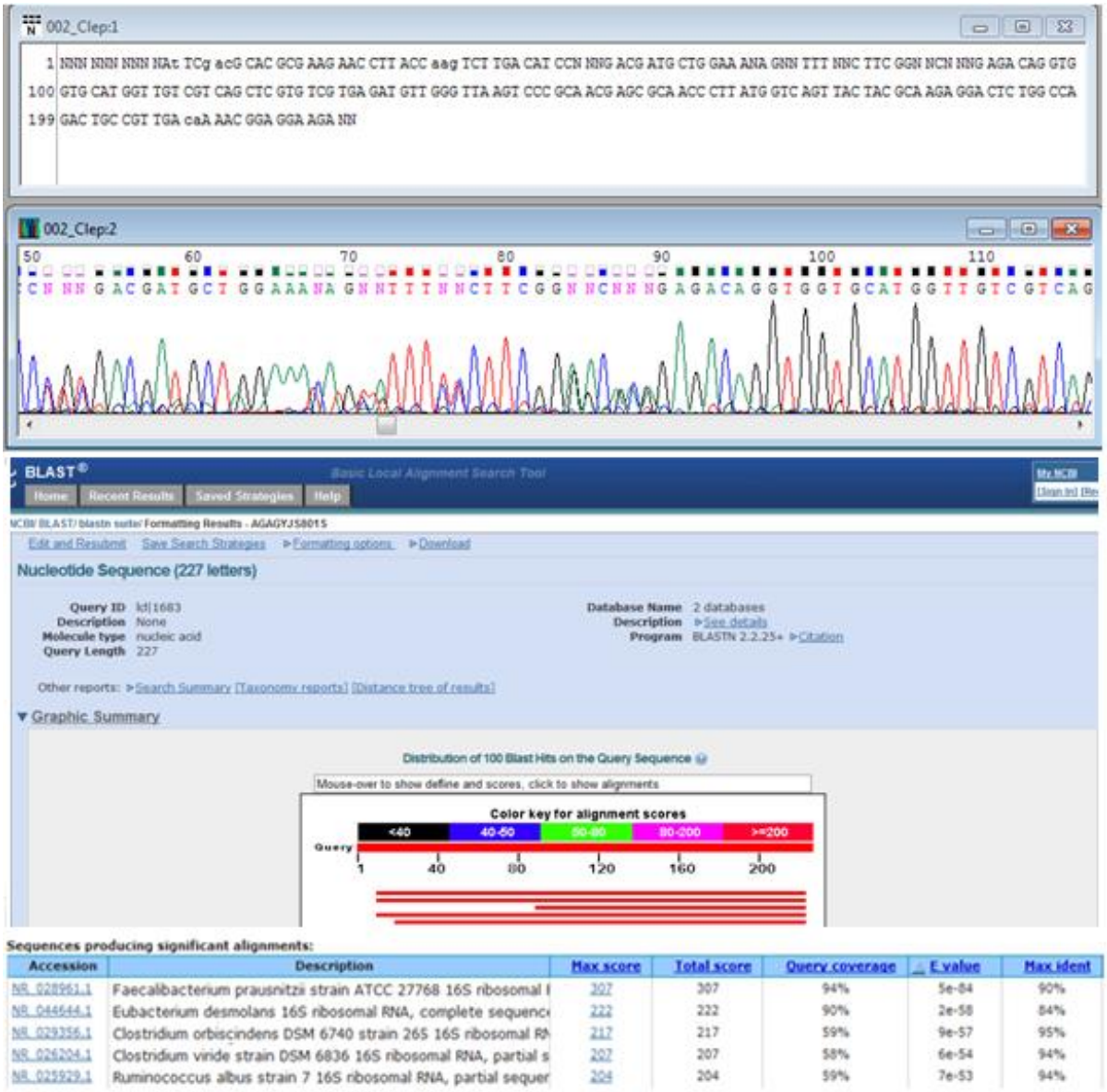

Figura 6. Secuencia y BLAST del producto amplificado con iniciadores grupo especifico para *Clostridium leptum*.

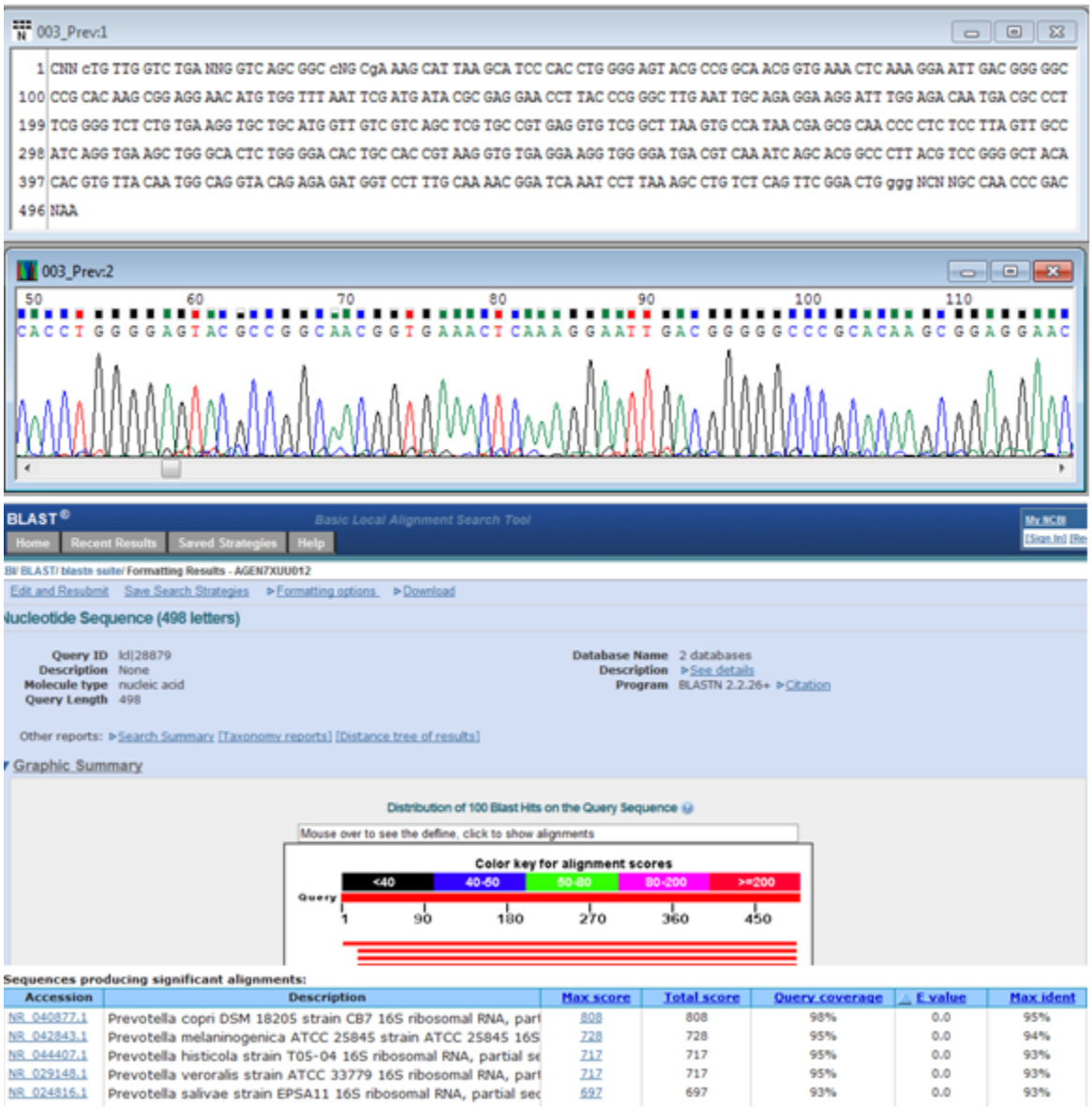

Figura 7. Secuencia y BLAST del producto amplificado con iniciadores grupo especifico para *Prevotella*.

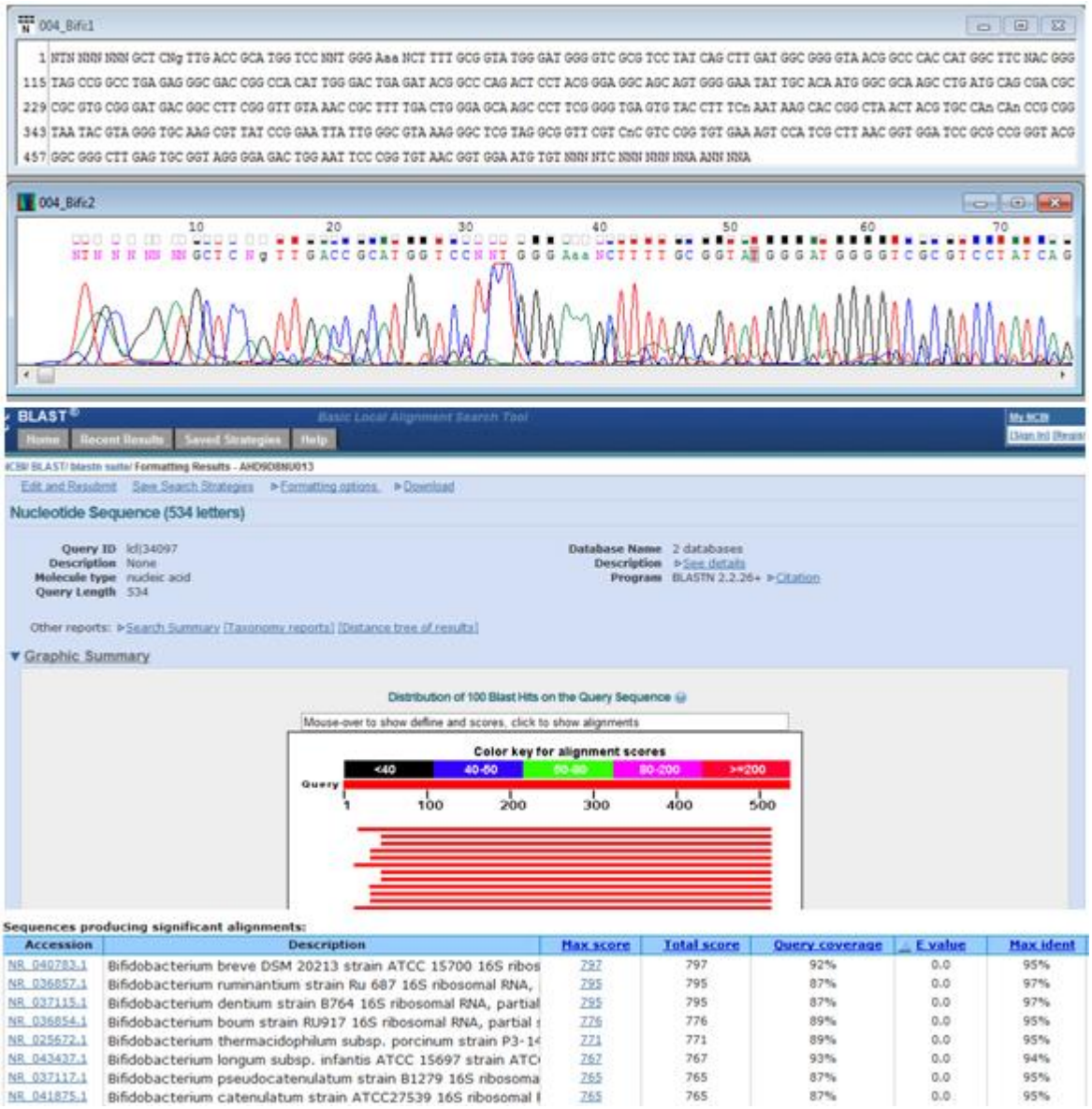

Figura 8. Secuencia y BLAST del producto amplificado con iniciadores grupo especifico para *Bifidobacterium*.

ANEXO No. 3 "Cuantificación de microorganismos para la curva estándar"

Para realizar la curva estándar, fue necesario cuantificar la cepa control de *E. coli* ATCC 25922 por mililitro, mediante la comparación de la turbidez del cultivo bacteriano con la escala de McFarland, la determinación de la densidad óptica por espectrofotometría y el recuento de unidades formadoras de colonias por mililitro (UFC/mL) mediante la técnica de extendido en placa (Tabla 3).

Tabla 3. Cuantificación de la cepa control *E. coli* ATCC 25922 por mL.

Cepa control	Escala de McFarland	Densidad óptica	Recuento en placa (UFC/mL)			No. de m.o. por mL
			Dilución -5	Dilución – 6	Dilución - 7	
E. coli ATCC 25922	Tubo No. 4	0.596	Más de 300 UFC/mL	90 UFC/mL	10 UFC/mL	$10x10^8$

En la extracción con el QIAamp DNA stool Mini kit (Qiagen), el proceso de elución se realizó con 200 μL de reactivo, por lo cual todo el DNA quedó en un volumen final de 200 μL. Por lo tanto cada microlitro de DNA contiene $5x10^6$ copias de DNA de la cepa control de *E. coli* ATCC 25922.

ANEXO No. 3 "Curvas de disociación"

Se realizaron curvas de disociación para demostrar la ausencia de productos inespecíficos amplificados por los iniciadores grupo específicos utilizados en el estudio (Figura 9).

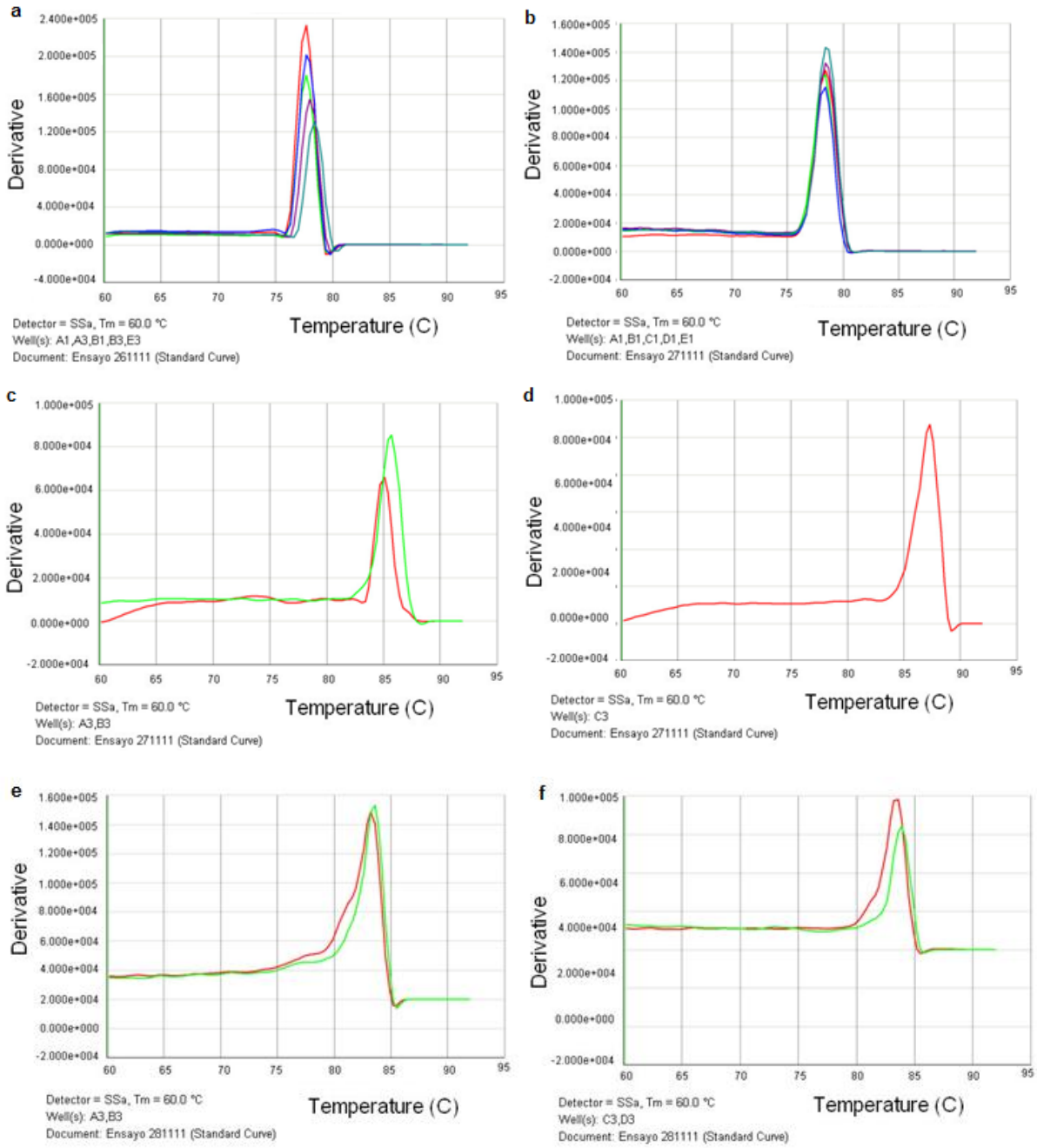

Figura 9. Curvas de disociación obtenidas en la amplificación del DNA por PCR en tiempo real. a) Grupo de *Lactobacillus*, b) *Eschericha coli*, c) Grupo de *Prevotella*, d) Grupo de *Bifidobacterium*, e) Grupo de *Bacteroides fragilis* y f) Grupo de *Clostridium leptum.*

ANEXO No. 4 "Curva estándar"

Se estandarizó la curva estándar con diluciones de la cepa control *E. coli* ATCC 25922 (5x106 a 5x102) como se muestra en la Figura 10a, posteriormente se cuantificaron las muestras de heces de los pacientes con enfermedad renal en estado terminal utilizando como referencia la curva estándar (Figura 10b).

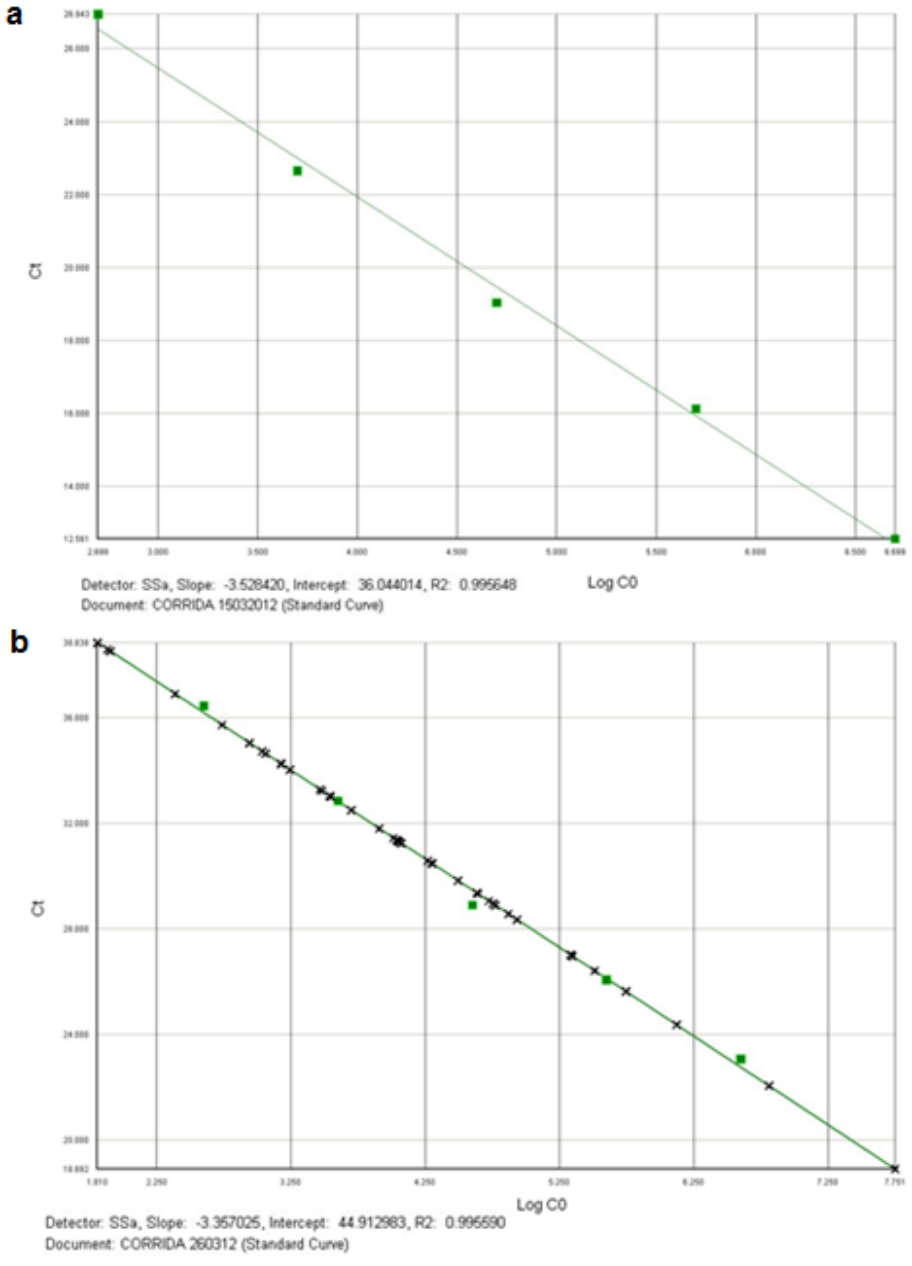

Figura 10. Curva estándar utilizando la cepa control *E. coli* ATCC 25922. a) Diluciones de DNA ($5x10^6$ a $5x10^2$) de la cepa control. b) Cuantificación de las muestras fecales de los pacientes con ERET.

ANEXO No. 5 "Cuantificación de la microbiota intestinal en ambos grupos"

Se realizó la cuantificación de la composición de la microbiota intestinal en pacientes con enfermedad renal en estado terminal por PCR en tiempo real, posteriormente se realizó la comparación de las cuantas de los grupos bacterianos en ambos grupos (grupo control y de intervención) como se muestra en la tabla 4.

Tabla 4. Composición de la microbiota intestinal de grupo control (n=10) y del grupo de intervención (n=8).

Población	Grupo control	Grupo de intervención	Valor de *p*
Clostridium leptum	4.35 ± 1.12	3.96 ± 0.99	0.1425
Bacteroides fragilis	1.12 ± 1.11	1.14 ± 1.0	0.4140
Bifidobacterium	4.83 ± 1.12	4.85 ± 1.49	0.4788
Prevotella	3.59 ± 1.08	3.04±1.62	0.2652
Escherichia coli	4.63 ± 1.05	4.98 ± 1.45	0.2054
Lactobacillus	2.0±1.13	2.17±0.79	0.3319

Media Log $_{10}$ celulas/g de materia fecal ± desviación estándar y la prueba de *t* Student