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Maraviroc modifies gut microbiota composition in a mouse model of obesity: a plausible therapeutic option to prevent metabolic disorders in HIV-infected patients

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ABSTRACT

Introduction. The proportion of HIV-infected patients with overweight/obesity has increased in recent years. These patients have an increased metabolic/cardiovascular risk compared with non-obese patients. Modulation of gut microbiota composition arises as a promising tool to prevent the development of obesity and associated disorders. The aim of this study was to investigate the impacts of maraviroc (MVC), a CCR5 antagonist approved for clinical use in HIV-infected patients, on gut microbiota composition in a mouse model of obesity.

Methods. Thirty two male C57BL/6 mice were assigned to: a) Control (chow diet), b) MVC (chow diet plus 300 mg/L MVC), c) High-fat diet (HFD) or d) HFD/MVC (HFD plus 300 mg/L MVC) groups. Body weight and food intake was recorded every 2-3 days. Mice were euthanized after 16 weeks of treatment and cecal contents were removed to analyse by real-time PCR four bacterial orders from the most dominant phyla in gut.

Results. Mice fed with a HFD showed a significant increase in *Enterobacteriales* ($p<0.001$ *vs.* control). MVC treatment induced a significant decrease in control ($p<0.05$) and HFD fed mice ($p<0.001$). Interestingly, this order was positively associated with body weight gain, insulin resistance and fatty liver. HFD induced a significant decrease in *Bacteroidales* and *Clostridiales* levels ($p<0.05$ and $p<0.01$, respectively). MVC decreased the presence of *Bacteroidales* ($p<0.05$ *vs.* control) while an increase was observed in HFD/MVC mice ($p=0.01$ *vs.* HFD). No direct effects of MVC were observed on *Clostridiales* and *Lactobacillales*.

Conclusions. MVC may constitute a new therapeutic option to prevent obesity and related disorders in HIV-infected patients.

Keywords: Maraviroc; Microbiota; Obesity; Feed Efficiency; Metabolism

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Maraviroc modifica la composición de la microbiota intestinal en un modelo animal de obesidad: una posible opción terapéutica para prevenir los trastornos metabólicos en pacientes infectados por el VIH

RESUMEN

Introducción. La proporción de pacientes VIH con sobrepeso/obesidad ha aumentado en los últimos años. Éstos tienen un mayor riesgo metabólico/cardiovascular que los no obesos. La modulación de la microbiota intestinal se considera una herramienta prometedora para prevenir el desarrollo de obesidad y de sus trastornos asociados. El objetivo de este estudio fue investigar el impacto de maraviroc (MVC), un antagonista de CCR5 empleado para el tratamiento de pacientes VIH, sobre la composición de la microbiota intestinal en un modelo murino de obesidad.

Métodos. 32 ratones macho C57BL/6 fueron asignados a los grupos: a) Control b) MVC (control más 300 mg/L de MVC), c) dieta alta en grasa (HFD) o d) HFD/MVC (HFD más 300 mg/L MVC). El peso corporal y la ingesta de alimentos se registraron cada 2-3 días. Los ratones fueron sacrificados a las 16 semanas. Se analizaron por qPCR cuatro órdenes bacterianos.

Resultados. Los ratones HFD mostraron un aumento significativo en *Enterobacteriales* ($p<0,001$ *vs.* control). El tratamiento con MVC disminuyó significativamente este orden ($p<0,05$ *vs.* Control y $p<0,001$ *vs.* HFD). *Enterobacteriales* se asoció positivamente con el aumento de peso, la resistencia a la insulina y el hígado graso. La ingesta de una HFD indujo una disminución significativa de *Bacteroidales* y *Clostridiales* ($p<0,05$ y $p<0,01$ respectivamente). MVC disminuyó la presencia *Bacteroidales* en animales control ($p<0,05$) e incrementó su presencia en HFD/MVC ($p=0,01$ *vs.* HFD). No se observaron efectos directos de MVC sobre *Clostridiales* y *Lactobacillales*.

Conclusiones. MVC podría constituir una nueva opción terapéutica para prevenir la obesidad y sus trastornos relacionados en pacientes infectados por el VIH.

Palabras clave: Maraviroc; Microbiota; Obesidad, Eficiencia energética; Metabolismo

INTRODUCTION

Gut microbiota refers to the trillions of microorganisms residing in the intestine that are involved in multiple physiological processes of the host. Recent research has shown that gut bacteria, through their effects on energy homeostasis and inflammation, could contribute to the development of several metabolic disorders such as obesity, diabetes, and cardiovascular diseases. Changes in gut microbiota may contribute to an increased intestinal permeability and, consequently, increased endotoxemia (increased lipopolysaccharides (LPS) plasma levels), which triggers inflammation and metabolic disorders[1]. In this context, several studies have demonstrated that high-fat diet-induced obesity or choline deficiency-induced fatty liver are associated with changes in gut microbiota composition[1-3]. The mechanisms by which gut microbiota affects obesity and related disorders have been brilliantly summarized elsewhere[4]. Thus, targeting microbiota may present new avenues for therapeutic interventions aim at preventing or reversing obesity.

The proportion of HIV-infected patients with overweight/obesity under antiretroviral treatment has significantly increased in recent years. These patients have an increased cardiovascular risk and metabolic abnormalities compared with non-obese and non-HIV infected patients. Thus, antiretroviral therapy-based molecules able to modulate gut microbiota are of great interest. Maraviroc (MVC), a CCR5 antagonist, is currently approved for clinical use in HIV-infected patients[5]. Beneficial actions of MVC in overweight/obese HIV-infected patients have been suggested since this drug does not alter adipocyte differentiation, but shows anti-inflammatory properties, neutral or even beneficial effects in glucose and lipid metabolism in human adipose cells[6,7]. The ability of MVC to ameliorate the development of hepatic steatosis in a mouse model of non-alcoholic fatty liver disease (NAFLD) associated with a high-fat diet (HFD) ingestion has also been described by our group[8]. However, MVC impacts on gut microbiota composition have not been analysed yet. Thus, the aim of this study was to investigate the effects of MVC on gut microbiota composition in a mouse model of obesity and to investigate if these changes could be associated with MVC actions on body weight gain and metabolism.

MATERIAL AND METHODS

Animals and animal model. Thirty two male C57BL/6 mice (5 weeks old) were purchased from Charles River (Barcelona, Spain). They were randomly assigned (n=8) to the following groups: a) Control: fed with a normal chow (Standard diet, 801010 RM1A (P), UK) and tap water; b) MVC: normal chow diet but receiving 300 mg/L of MVC (Pfizer, USA) in the drinking water. Mouse equivalent drug doses were calculated to get an equivalent to a human dose (300 mg/day)[8,9]; c) HFD: animals fed with a HFD (Research Diets, USA) and tap water; and d) MVC/HFD group: HFD but receiving MVC in the drinking water (same concentration than MVC Group). All animals had free access to food and water. Mice were weighted and food and water ingestion was recorded every 2-3 days per week. All mice were euthanized after 16 weeks of treatment. At that moment, blood samples were collected under anaesthesia after a 4 h fasting period. Liver, fat pads and cecal contents were removed for biochemical and molecular analyses. All procedures were carried out in accordance with the European Communities Council Directive on animal experiments (86/609/CEE and EU Directive 2010/63/EU) and with approval from the ethical committee on animal welfare of our institution (*Comité Ético de Experimentación Animal del CIBIR*).

Biochemical and hepatic analysis. Plasma levels of alanine aminotransferase (ALT), aspartato aminotransferasa (AST), glucose, triglycerides, HDL-and total cholesterol were measured using an automatic biochemical analyser (Cobas C711, Madrid, Spain). Insulin, leptin, adiponectin, TNFα and IL6 plasma levels were determined using commercial kits and following manufacturer's instructions. Insulin resistance was calculated using the homeostasis model assessment of insulin resistance (HOMA-IR). Atherogenic index was calculated as the ratio between total and HDL cholesterol. To quantify liver triglyceride content, 150 mg of liver was used as previously described[8,10]. Products of lipid peroxidation in liver (thiobarbituric acid reactive substances, TBARS) were analysed by ELISA following manufacturer´s instructions.

Real-time gene expression analysis. DNA from faecal microbiota was extracted using a specific kit (DNeasy Blood & Tissue Kit, Qiagen). Purity and concentration were subsequently determined by a Nanodrop spectrophotometer 1000 (ND-1000; Thermo Scientific, USA). Quantitative real time PCR (qPCR) was performed using Sybr Premix Ex Taq (Takara Bio Inc., Shiga, Japan) and with specific primers designed for *Enterobacteriales, Bacteroidales, Clostridiales and Lactobacillales* orders[11]. All procedures were performed according to manufacturers' instructions. Melting curves were obtained from 55 to 90ºC. All PCR reactions were performed in duplicate, and 16s gene was used to normalise gene expression.

Statistical analysis. All results are expressed as the mean ± standard error of the mean. Two different methods (Kolmogorov–Smirnov and Shapiro–Wilk tests) were used to check normal distribution. Data were analysed with one way ANOVA followed by a Bonferroni post-hoc test or with the Kruskal–Wallis test followed by Dunns post-tests depending on the normality of data. The association between variables was analysed by the Spearman rank-sum test. SPSS 17.0 software and GraphPad Prism 5 have been used for these statistical analyses.

RESULTS

Effects of MVC on microbiota composition (four bacterial orders). Mice fed with a HFD showed a significant increase in the abundance of *Enterobacteriales* ($p<0.001$ *vs.* control). MVC treatment induced a significant decrease in the abundance of this order in both control ($p<0.05$) and HFD mice ($p<0.001$) (figure 1A). HFD induced a significant decrease in *Bacteroidales* and *Clostridiales* levels ($p<0.05$ and $p<0.01$

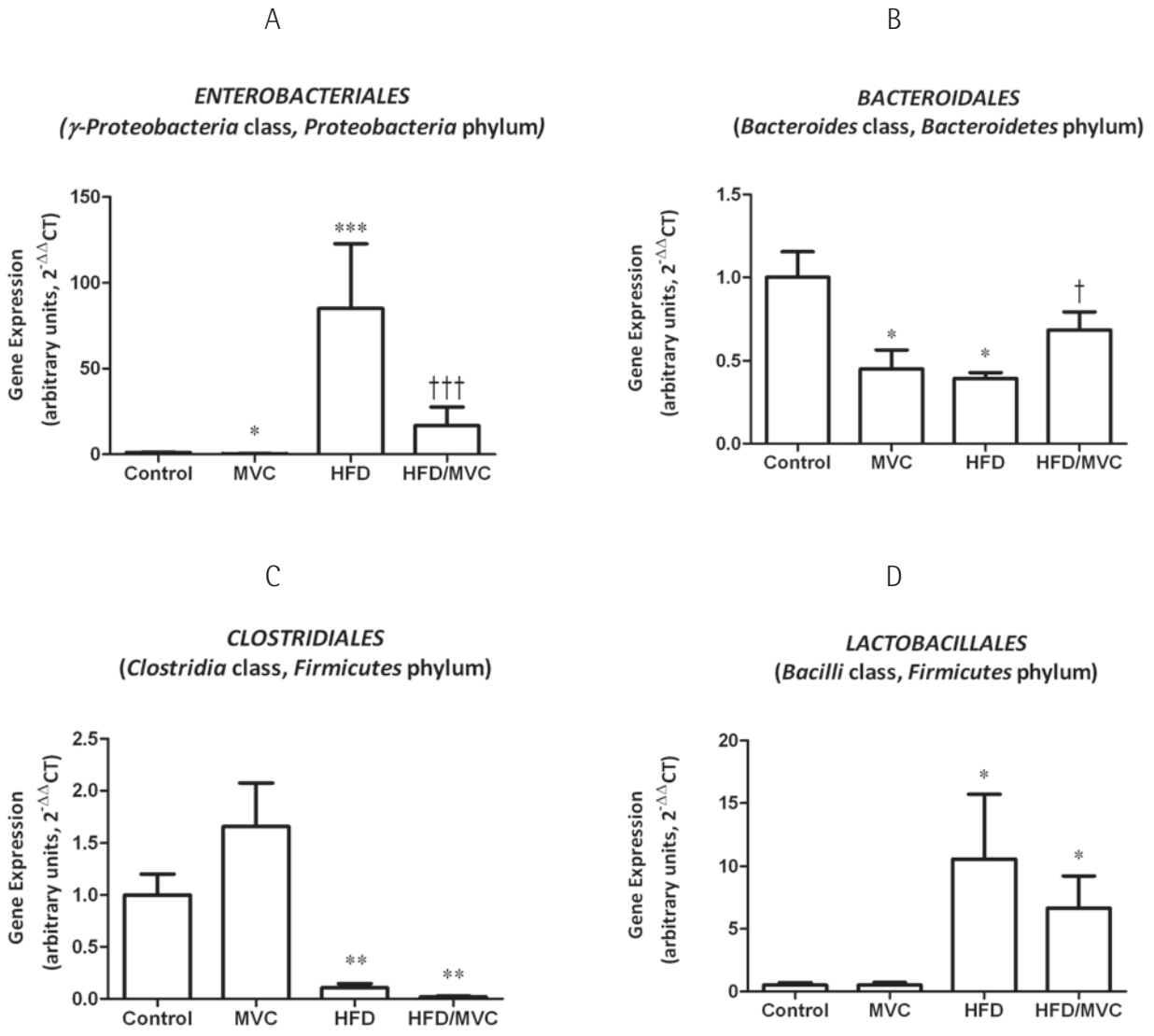

Figure 1 | **Effects of MVC on the abundance of four bacterial orders from the most dominant phyla in gut. *P<0.05, **P<0.01 and ***P<0.001 with respect to the control group. †P<0.05, †††P<0.001 with respect to the HFD group.**

respectively). MVC decreased the presence of *Bacteroidales* (p<0.05 *vs.* Control) while an increase was observed in HFD/MVC mice (p=0.01 *vs.* HFD). No direct effects were observed on *Clostridiales* after MVC supplementation although a slightly decrease was observed in HFD/MVC mice (figure 1B and 1C, respectively). HFD significantly increased the levels of *Lactobacillales* (p<0.05) while no significant effects were observed after MVC supplementation. However, a tendency to decrease the abundance of this order was observed in HFD/MVC mice (figure 1D).

Associations between the bacterial orders and physiological/biochemical parameters. HFD induced a significant increase in body weight gain (p<0.001). MVC showed a tendency to decrease this body weight gain despite the ingestion of the HFD (p=0.06). The same pattern was observed when glucose, insulin and HOMA index was analysed (table 1). The increase observed in body weight gain was accompanied by a significant increase in total fat (p <0.001). This increase was lower in the group supplemented with MVC (p <0.05 *vs.* HFD) being particularly significant in the subcutaneous depot (p <0.01 *vs.* HFD). The MVC group also showed a significant increase in the weight of the epididymal fat (p<0.05 *vs.* control). No significant effects were observed on leptin, adiponectin and IL-6 plasma levels. However, MVC reduced the TNFα plasma levels in both control and HFD mice (p<0.05 and p=0.09 respectively) (table 1). As previously reported by our group, MVC decreased liver weight, lipid accumulation in this organ and improved lipid me-

Tabla 1 **Effects of MVC on body weight gain, adipose and liver size, lipid and glucose metabolism, leptin, adiponectin, IL-6 and TNFα plasma levels in control and high-fat-fed mice.**

		Control	MVC	HFD	HFD+MVC	ANOVA
	Body weight gain (g)	7.70 ± 0.22	8.84 ± 0.34	28.65 ± 0.80***	24.98 ± 1.68***††	p<0.001
FAT PADS	Adipose Tissue (g)	0.63 ± 0.05	0.87 ± 0.10	5.68 ± 0.16***	4.97 ± 0.27***†	p<0.001
	Subcutaneous Adipose Tissue (g)	0.20 ± 0.01	0.27 ± 0.03	2.48 ± 0.13***	1.80 ± 0.14***††	p<0.001
	Epididymal Adipose Tissue (g)	0.34 ± 0.02	0.46 ± 0.04*	2.38 ± 0.09***	2.42 ± 0.17***	p<0.001
LIVER	Liver weight (g)	1.15 ± 0.04	1.13 ± 0.03	1.72 ± 0.10***	1.28 ± 0.03*†††	p<0.001
	Hepatic Triglyceride Content (mg/g tissue)	20.58 ± 1.72	23.58 ± 1.96	59.99 ± 7.02***	44.38 ± 5.49** †	p<0.001
	Hepatic Peroxidation (TBARs)	48.55 ± 2.01	55.00 ± 5.15	61.90 ± 6.62	77.11 ± 7.53*	p=0.01
LIPID METABOLISM	ALT (U/L)	34.50 ± 1.88	32.57 ± 1.78	66.0 ± 6.99**	43.71 ± 2.84†	p<0.001
	AST (U/L)	58.50 ± 4.50	63.75 ± 4.66	94.50 ± 6.58**	84.75 ± 13.20	p<0.01
	Triglycerides (mg/dL)	93.0 ± 6.31	99.75 ± 8.01	108.0 ± 5.07	101.3 ± 4.45	p=0.4924
	Total Cholesterol (mg/dL)	103.5 ± 5.86	95.25 ± 5.71	204.8 ± 8.96***	185.3 ± 5.25***[b]	p<0.001
	Total Cholesterol/HDL cholesterol	1.12 ± 0.01	1.10 ± 0.01	1.09 ± 0.01	1.12 ± 0.01	P=0.6726
GLUCOSE	Glucose (mmol/L)	289.7 ± 12.6	323.3 ± 25.4	413.3 ± 26.1	382.5 ± 11.9[c]	p<0.001
METABOLISM	Insulin (µU/mL)	0.6 ± 0.04	0.8 ± 0.08*	4.5 ± 1.1	3.3 ± 0.4	p<0.001
	HOMA index	9.8 ± 0.9	17.5 ± 1.9*	124.8 ± 36.2	69.0 ± 6.6	p<0.001
ADIPOKINES	Leptin (ng/mL)	2.11 ± 0.48	2.58 ± 1.12	82.74 ± 8.10***	81.75 ± 4.22***	p<0.001
	Adiponectin (µg/mL)	6.93 ± 0.22	7.26 ± 0.72	7.95 ± 0.61	7.68 ± 0.36	ns
INFLAMMATORY	TNFα (pg/mL)	4.27 ± 0.12	3.89 ± 0.06*	5.55 ± 0.28**	4.77 ± 0.32**[a]	p<0.001
CYTOKINES	Il-6 (pg/mL)	2.19 ± 0.14	2.11 ± 0.11	3.97 ± 0.35*	4.69 ± 0.87*	p=0,0008

*p<0.05; **p<0.01; ***p<0.001 vs. Control. †p<0.05;††p<0.01 vs. HFD group. [a]p=0.09; [b]p=0.08; [c]p=0.06 vs. HFD group.

tabolism (especially evident in ALT and total cholesterol levels) (table 1)[8]. Feed efficiency, an index that relates caloric intake with body weight gain, was significantly increased by the HFD (p<0.001), while significantly lower values were observed in the HFD/MVC group (p<0.01 *vs.* HFD). MVC did not change this parameter in animals fed the standard diet (figure 2A). Feed efficiency was positively and significantly associated with body weight gain (figure 2B) but also with the abundance of *Enterobacteriales* and *Lactobacillales* (table 2). *Enterobacteriales* and *Lactobacillales* orders were also significantly and positively associated (p<0.01) with body weight gain, fat pads, transaminases, total cholesterol, insulin and leptin levels as well as with the HOMA index. Furthermore, *Enterobacteriales* levels were positively associated with liver weight, TNFα and IL-6 plasma levels and *Lactobacillales* were positively associated with hepatic peroxidation (TBARs levels) and glucose plasma levels. In contrast, *Clostridiales* were significantly and negatively associated with body weight gain, food intake and feed efficiency, fat pads and liver size, hepatic TG content, transaminases, total cholesterol, glucose, insulin and HOMA index, leptin and TNFα plasma levels. Interestingly, no significant correlations were observed between *Bacteroidales* and any of the parameters analysed. None of the bacterial orders were associated with triglyceride plasma levels, the atherogenic index or adiponectin plasma levels (tables 1 and 2).

DISCUSSION

Microbial changes in human gut have been proposed as a possible cause of obesity[12-14]. Certain phyla and classes of bacteria are associated with improved transfer of calories from the diet to the host, and with changes in the host metabolism of absorbed calories[13,15]. We have demonstrated that MVC was able to counteract the increase in body weight gain induced by a HFD and to ameliorate the development of fatty liver and insulin resistance[8]. The lower energy efficiency found in the present study in the HFD/MVC group compared to the HFD could explain, at least in part, the lower body weight observed in these animals. Furthermore, the potent associations observed between this index and the microbiota (*Enterobacteriales* and *Lactobacillales* levels) underlines the key role of gut microbiota in the efficiency of energy extraction from the diet and, therefore, its involvement in the control of body weight.

We have herein demonstrated for the first time that the administration of MVC to mice fed with a control and/or HFD has a direct impact on gut microbiota composition. Changes observed at higher taxonomic levels (such as order-level), where the microbiota community structure is much more stable than lower-taxa levels (species), were unlikely to be due to day to day variability, and they are due to significant perturbations caused by MVC[11].

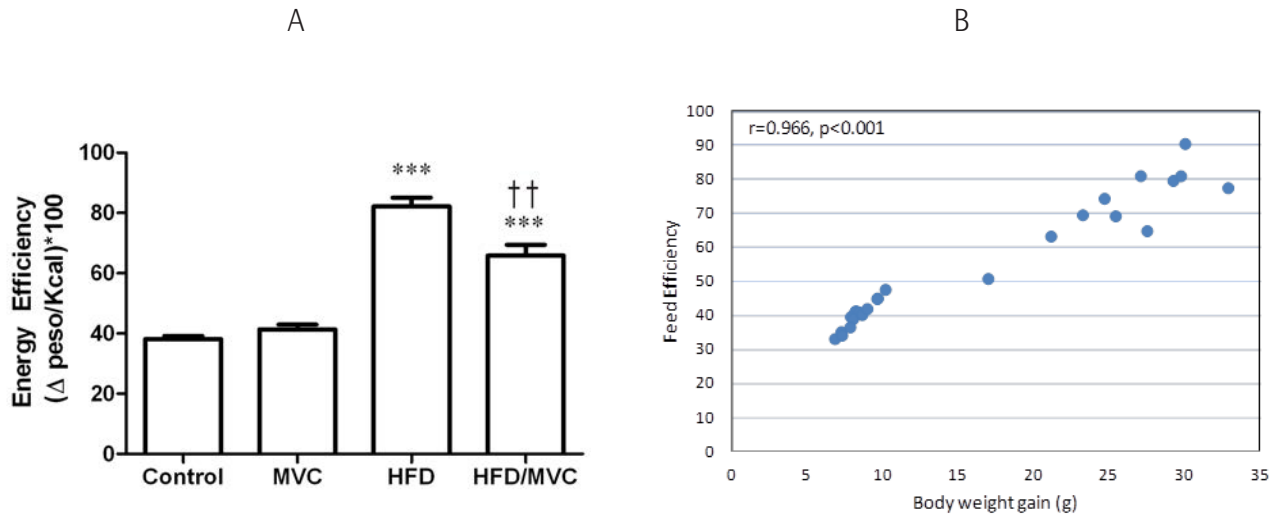

Figure 2 | **Effects of MVC on feed efficiency (A). Associations found between body weight gain and feed efficiency (B). ***P<0.001 with respect to the control group. ††P<0.01 with respect to the HFD group.**

We have demonstrated that the ingestion of a HFD induces an increase in the abundance of *Enterobacteroidales*, which agrees with previous results obtained in obese rats[2]. MVC reduced the abundance of these bacteria in both control and HFD-fed mice. *Enterobacteroidales* include a large amount of gram-negative and aerotolerant/facultative pathogenic bacteria. LPS are part of the outer membrane of the cell wall of gram-negative bacteria. Increased LPS levels triggers chronic to low-grade inflammation leading to obesity and diabetes. Our results could suggest that HFD feeding induces an increase in the abundance of gram-negative bacteria included in the *Enterobacteroidales* phylum, whereas MVC administration is able to counteract this effect. In addition, the positive association observed between the abundance of these bacteria and body weight gain, fat pads, liver weight, hepatic triglyceride content, transaminases, total cholesterol, insulin, HOMA index and inflammatory cytokines suggest that the inhibitory effects of MVC on this bacterial order could contribute to its positive actions on obesity, inflammation and related disorders (fatty liver and insulin resistance for instance).

It is generally accepted that obesity is accompanied by an increase in Firmicutes and a decrease in *Bacteroidales* phyla, although some controversial results have arisen in humans[16]. We have analysed two bacterial orders of the Firmicutes phylum: *Lactobacillales* and *Clostridiales*. HFD induced a significant increase in *Lactobacillales*. These results are in agreement with a recent study that confirms the correlation between concentration of certain lactobacillus species and obesity[17]. MVC showed a tendency to decrease the presence of these bacteria in control and HFD-fed groups. A similar positive association than that observed with *Enterobacteroidales* was obtained between this order and several metabolic parameters. Of interest, the very high correlation observed with liver weight, hepatic triglyceride content and hepatic peroxidation. In this context, a very recent study has demonstrated that fatty liver accompanies an increase in Lactobacillus spp. in the hind gut of C57BL/6 mice fed a HFD[18]. Thus, the associations that we have found could suggest that the decrease observed in *Lactobacillales* after MVC treatment could underline the previously described protective effects of MVC in hepatic steatosis[8].

Clostridiales levels were diminished by HFD feeding whereas MVC did not exert any effect. However, potent negative associations were observed with almost all the parameters studied suggesting a potential protective role of this order in preventing the development of obesity. Thus, the more *Clostridiales* you have, the more protection against obesity. However, a deeper analysis is needed to support this hypothesis since some members of this order have anti-inflammatory effects but others just the opposite[19].

Bacteroidales levels were decreased in HFD mice as previously described[20]. The present results showed opposite effects of MVC administration in control mice in comparison with the obese group. These results could suggest that MVC actions are dependent on a number of factors such as diet composition and energy balance. In fact, other studies have also observed differential actions of some anti-obesity treatments (such as β-adrenergic agonists or Omega-3 fatty acids) between control and HFD-induced overweight/obese animals[21,22]. A very recent study found that administration of *Bacteroides uniformis* CECT 7771 ameliorates HFD-induced metabolic and immune dysfunction associated with intestinal dysbiosis in obese mice[23]. Our results showed a significant increase in the abundance of Bacteroidales (which includes the *B. uniformis* among other bacteria) in the HFD/MVC group compared to the HFD, and this increase could be responsible, at least in part, to the beneficial effects of MVC on obesity. However, the absence of associations found with any of the physiological parameters

Table 2 **Associations (Spearman's rank correlation coefficients) found between the abundance of the four bacterial orders analysed and several biochemical and physiological variables.**

		ENTEROBACTERIALES		BACTEROIDALES		CLOSTRIDIALES		LACTOBACILLALES	
		Spearman Rho	Significance (two tail)	Spearman Rho	Significance (two tail)	Spearman Rho	Significance (two tail)	Spearman Rho	Significance (two tail)
	Body weight gain (g)	0.570	0.002	-0.212	0.298	-0.702	P<0.001	0.557	0.003
	Food Intake (calories)	0.572	0.002	-0.277	0.171	-0.642	P<0.001	0.486	0.012
	Feed Efficiency	0.566	0.003	-0.196	0.338	-0.715	P<0.001	0.610	0.001
FAT PADS	Adipose Tissue (g)	0.643	P<0.001	-0.177	0.386	-0.672	P<0.001	0.653	P<0.001
	Subcutaneous Adipose Tissue (g)	0.613	0.001	-0.174	0.396	-0.702	P<0.001	0.644	P<0.001
	Epididymal Adipose Tissue (g)	0.660	P<0.001	-0.146	0.478	-0.648	P<0.001	0.660	P<0.001
LIVER	Liver weight (g)	0.533	0.005	-0.234	0.249	-0.480	0.013	0.359	0.071
	Hepatic Triglyceride Content (mg/g tissue)	0.594	0.001	-0.335	0.0094	-0.655	P<0.001	0.547	0.004
	Hepatic Peroxidation (TBARs)	0.425	0.070	-0.035	0.887	-0.400	0.090	0.605	0.006
LIPID METABOLISM	ALT (U/L)	0.594	0.001	-0.335	0.0094	-0.655	P<0.001	0.547	0.004
	AST (U/L)	0.594	0.001	-0.335	0.0094	-0.655	P<0.001	0.547	0.004
	Triglycerides (mg/dL)	0.106	0.608	-0.123	0.550	-0.302	0.134	0.118	0.566
	Total Cholesterol (mg/dL)	0.711	P<0.001	-0.150	0.465	-0.757	P<0.001	0.647	P<0.001
	Total Cholesterol/HDL cholesterol	0.245	0.245	0.109	0.597	-0.093	0.652	0.236	0.245
GLUCOSE	Glucose (mmol/L)	0.372	0.062	-0.144	0.483	-0.675	P<0.001	0.528	0.006
METABOLISM	Insulin (µU/mL)	0.595	0.001	-0.168	0.412	-0.712	P<0.001	0.614	0.001
	HOMA index	0.563	0.003	-0.132	0.519	-0.757	P<0.001	0.630	0.001
ADIPOKINES	Leptin (ng/mL)	0.693	P<0.001	-0.042	0.837	-0.779	P<0.001	0.689	P<0.001
	Adiponectin (µg/mL)	0.074	0.719	-0.074	0.720	0.019	0.925	-0.118	0.565
INFLAMMATORY	TNFα (pg/mL)	0.533	0.005	-0.133	0.517	-0.407	0.039	0.353	0.077
CYTOKINES	Il-6 (pg/mL)	0.443	0.023	-0.246	0.225	-0.183	0.371	0.127	0.535

studied makes this assumption difficult to test. It is plausible that specific bacteria included in this order (such as *B. uniformis*) could be associated with obesity and metabolism but these results do not translate into differences at the order level. More complex metagenomic analyses should be needed to test this hypothesis.

In conclusion, this is the first study demonstrating the ability of MVC to alter gut microbiota composition (at least at the level of bacterial orders). Although some of its actions are dependent on diet composition and animal metabolic status, MVC may constitute a therapeutic option for modulating the obesity-induced changes in gut microbiota in HIV-infected patients. This should be taken into account when designing antiretroviral regimens.

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