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RESEARCH ARTICLE

Antiviral Activity of Fecal Water Samples from HIV-1 Infected Subjects Treated with a Specific Probiotic Formulation

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Abstract: ***Objectives*:** The aim of the study was to investigate if the supplementation with multi-strain probiotics may be able to modulate T cell response in HIV-1 infected patients and to evaluate the anti-HIV activity of probiotic by studying fecal water (FW) samples.

***Methods*:** Three HIV-1-positive patients (Pt1, Pt2 and Pt3) on long-term suppressive combined antiretroviral therapy (cART) received a specific multi-strain probiotic supplementation (Vivomixx®), for six months (T6). Levels of T cell subsets were evaluated by flow cytometry. Anti-HIV activity of FW samples was evaluated *in vitro*.

***Results*:** CD4+ T cells levels increased in all HIV-1 infected patients whereas activation markers (CD38 and HLA-DR) were decreased both on CD4+ and CD8+ T cells. FW samples presented an increased inhibitory activity against HIV-1 compared to T0 (FW-Pt1: T0 =40%, T6 = 65% of reduction; FW Pt2: T0 = 26%, T6 = 46% of reduction; FW Pt3: T0 = 47%, T6 = 94% of reduction).

***Discussion*:** Our data suggest that the administration of the specific probiotic formulation improves the antiviral status of people living with HIV-1 under cART, also modulating T cell response.

***Conclusion*:** Anti-HIV activity of FW may have several public health and social implications for sexually transmitted diseases that need to be further explored.

Keywords: HIV-1, probiotics, fecal water, viral replication, immune activation, cART.

1. INTRODUCTION

Gut-associated lymphoid tissue (GALT) is the largest Human Immunodeficiency Virus type-1 (HIV-1) replication site and reservoir [1-4]. Destruction of $Th17^+$ and $Th22^+$ $CD4^+$ T cells in GALT during early-stages of HIV-1 infection promotes a decline in the immune system and mechanical barrier functions of the gut mucosa [5-9]. Consequently, in HIV-1 infected individuals, microbial translocation, chronic immune activation, and increased morbidity/mortality are observed [10-19], but the exact mechanisms underlying these effects are still poorly understood.

The rationale behind the use of oral bacteriotherapy in HIV-1 infected patients is based on the observation that the fecal flora is altered in these subjects for various reasons [20-32].

Supplementation with probiotic strains reinforces mucosal barrier function and modulates immune responses by affecting the intestinal epithelium and GALT [22, 33-44].

Furthermore, supplementation with probiotics seems to be able to promote beneficial effects also on the gut-brain axis and on the gut-heart axis [22, 45-51].

Probiotics have recently been shown to mediate the antiviral effect against certain viruses *in vitro* and *in vivo* [52-62]. The objective of this pilot study was to investigate if the oral bacteriotherapy administration may be able to modulate T cell response in HIV-1 infected patients and to assess what is its impact on *in vitro* HIV-1 replication.

2. MATERIALS AND METHODS

Three Caucasian HIV-1-positive patients (Pt1, Pt2 and Pt3) with a median age of 43 years (IQR 30-54) on long-term suppressive combined antiretroviral therapy (cART), and one healthy donor, as control, were recruited at the Department of Public Health and Infectious Diseases, "Sapienza" University of Rome. They received for 6 months two sachets daily of a multistrain probiotic formulation, each containing 450×10^9 billion bacteria (*L. palntarum* DSM24730, *S. thermophilus* DSM24731, *B. breve* DSM 24732, *L. paracasei* DSM 24733, *L. delbruckii ssp. Bulgaricus* DSM 24734, *L. acidophilus* DSM 24735, *B. longum* DSM 24736, *B. infantis* DSM 24737 - Trade name: Vivomixx® in EU, Vis-

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biome® in USA, DeSimone Formulation ® in Korea). During the 6 months supplementation period, all HIV-1 subjects continued to assume their antiretroviral treatment. Neither co-infections nor acute pathologies were observed during the period of supplementation in the subjects enrolled.

Subjects were followed every 3 months with blood examinations, including analysis of CD4+ and CD8+ T cell levels and plasma viral load. Immune activation markers on T cell subsets (CD38+ and HLA-DR+ on CD4+ and CD8+ T cells) were evaluated at baseline (T0) and after 6 months of probiotic administration (T6). Fecal samples were also collected at T0 and at T6 and stored at –20°C until analysis. Fecal water (FW) samples were extracted from feces by homogenizing them with sterile water (1:5, v/v) for 2 min followed by centrifugation (10,700 × g, 40 min, 4°C). The supernatant fractions were distributed to 1.5 ml Eppendorf tubes and stored at –20°C prior to analysis.

In addition, to confirm the *ex vivo* data, 10^5 bacteria from the multistrain probiotic formulation capsule were suspended in 10 ml of culture for thiocyanate. After 24h at 37°C, bacteria were centrifuged and the supernatant (SNT) was collected. C8166 cells and HIV-1-P1 [63], a T-tropic strain isolated from HIV positive patient, were used to study the antiviral activity of the SNT and FW samples. Cells were infected with HIV-1-P1 at a multiplicity of infection of 0.05 $TCID_{50/cell}$. After 1h of incubation at 37°C, cells were cultured with SNT or FW samples diluted 1/20 in RPMI 1640 medium for 24 h. HIV-RNA from cell culture supernatants was quantified by versant kPCR (Siemens Healthcare Diagnostics, Tarrytown, NY, USA). Experiments with SNT were separately repeated three times, FWs activity was assessed in two separate experiments.

Finally, fecal samples collected at T0 and T6 were analyzed in order to evaluate the efficacy of the multi-strain probiotic supplementation in changing the *Bifidobacteria* spp. levels and adherence to the treatment [39]. For this reason, the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) was used according to the manufacturer's instructions: 200 mg of frozen samples were suspended in 1.4 mL of ASL lysis buffer from the stool kit, added with glass beads (150-212 µm, Sigma–Aldrich, St. Louis, MO, USA), and homogenized. The suspension was incubated at 95 °C for 5 min, DNA was purified and eluted in 200 µL of AE buffer and the samples obtained were stored at −20 °C. Bacterial DNA from fecal samples were extracted and quantified by a real-time PCR, performed to evaluate *Bifidobacteria* levels. Briefly, PCR amplification and detection were performed on optical-grade 96-well plates, using the Applied Biosystems 7500 Real-Time PCR instrument (Applied Biosystems, Inc., Norwalk, CT, USA). The reaction mixture (25 µL) was composed of SensiMix SYBR Low-ROX (BIOLINE, Taunton, MA, USA), 500 nM primers for *Bifidobacterium* genus, and 2.5 µL of template DNA. A melting curve analysis was made after amplification, to distinguish target amplicons from aspecific non-target PCR products. Standard curves were made by using 10-fold dilutions of DNA, extracted from *Bifidobacterium breve*. All samples were analyzed in duplicate in two independent real-time PCR assays.

3. RESULTS

All patients completed the study and reported a good adherence to cART and to probiotic supplementation. Plasma HIV-1 viral load was undetectable for the 6 months of the observation. After six months of probiotic supplementation CD4+ T cells increased in all patients (mean value from 28,8% to 45,8%), whereas CD8+ T cells showed a slight decrease (mean value from 55,2% to 53,6%). Activation markers were decreased both on CD4+ and CD8+ T cells (Fig. **1**).

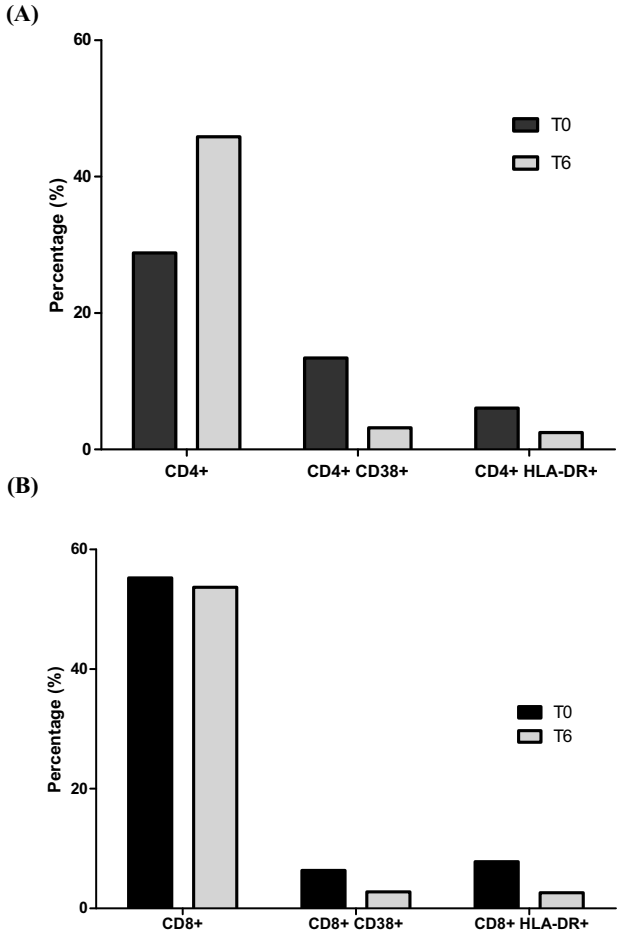

Fig. (1). Trends of CD4+ (**A**) and CD8+ (**B**) T cell percentages and activation markers levels (CD38+ and HLA-DR+) on both subsets of T cells. Means and standard deviation are shown.

No cytotoxicity was observed after treatment with FW samples when assessed by MTT assay (data not shown). SNT reduced HIV-1 replication by 47 ± 10%. FW samples presented an increased inhibitory activity against HIV-1. The percentage reduction of HIV-1 replication was higher in cells treated with FW samples collected at T6 than at T0 (FWPt1: T0 40% ± 8, T6 65% ± 5; FWPt2: T0 26% ± 10, T6 46% ± 6; FWPt3: T0 47% ± 10, T6 94% ± 4) (Fig. **2**).

A slight reduction of HIV-1 replication after probiotic administration was also observed in cells treated with donor FW samples (T0 46% ± 6, T6 58% ± 5).

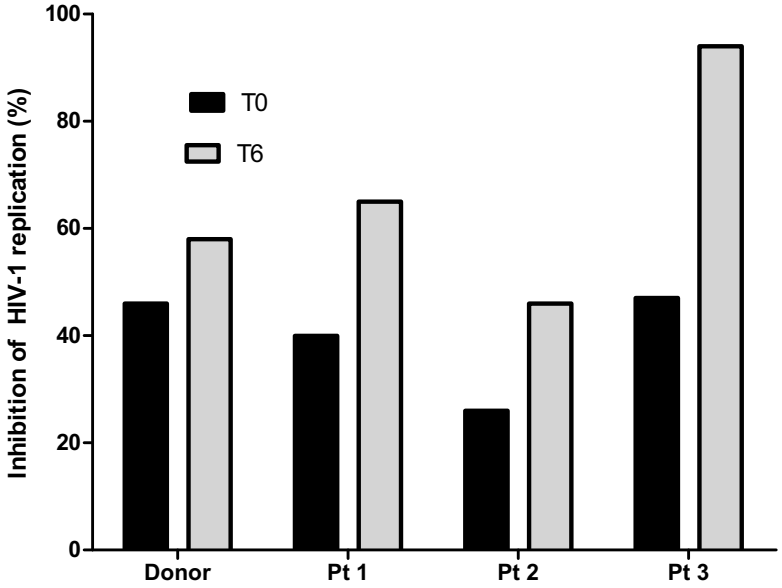

Fig. (2). Percentage of inhibition of HIV-1 replication in cells infected with HIV-P1 and treated with FW samples from donor and patients. Cells were treated with FW samples collected from donor and patients (Pts1-3) at T0 and after 6 months (T6) of probiotic supplementation. Means and standard deviation are shown.

The efficacy of the multi-strain probiotic supplementation in changing the bacterial composition and optimal adherence to the treatment was confirmed by the increase in *Bifidobacteria spp*. in fecal samples collected at T6 [Bifidobacteria DNA copies/g of feces; mean (log10): 8.3], compared to their basal level (T0) [Bifidobacteria DNA copies/g of feces; mean (log10): 7.6].

Finally, no side effects were observed over the course of 6 months of treatment in all patients enrolled.

4. DISCUSSION

The results of the present study (a) support the hypothesis of the antiviral properties of some probiotic strains against coated viruses [64-66], and (b) suggest that administration of this specific probiotic formulation can improve the antiviral status of HIV-1-positive patients, also modulating T cell response [22, 39, 42].

The evidences in favor of these hypotheses are that after probiotic administration the hosts' chronic inflammatory status was reduced since immune activation marker levels had decreased at the end of the treatment and CD4+ T cell proportion strongly increased. Furthermore, the fact that stool inhibitory activity against HIV-1 was enhanced following probiotic supplementation suggests that the probiotic has a direct anti-HIV-1 activity, and/or the host is further stimulated by the probiotic to produce molecules endowed with antiviral activity.

Indeed, the antiviral property of products released by bacteria has been reported in the literature. Produced by a wide variety of bacteria to fight other microorganisms in their competitive environments, bacteriocins form a heterogeneous group of peptides with great variations in size, structure and mode of action [67-69]. Bacteriocins have been isolated from Gram-positive bacteria, such as *Lactobacillus* and Gram-negative bacteria such as *Escherichia coli* and other enterobacteria living in gastrointestinal tract.

Inhibitory activity of bacteriocins against organisms that are closely related, including *Mycobacterium spp*, fungi and viruses has been reported [70, 71]. Authors described the antiviral activity of bacteriocin CRL35 produced by strain of *Enterococcus faecium*, which is capable of inhibiting the late stages of replication of a variety of HSV-1 and HSV-2 strains, also in a dose-dependent pattern [64, 72]. In addition, semi-purified bacteriocins GEn09, GEn12 and GEn17 produced by *Enterococcus durans* inhibit poliovirus-1 [65].

In addition, other peptides, such as Lantibiotics produced by bacteria, that contain the noncanonical amino acid lanthionine which exhibits antibacterial activities, displayed antiviral activity against HSV-1, HSV-2 and HIV-1 [66]. In our study, we found the antiviral activity of FW samples. It is reasonable to hypothesize that the combination of different bacteriocins as well as the peptides production by bacteria spp harboring in intestinal ambient could affect viral replication.

The gut milieu is extremely complex, and not all the effects that microbes have on the mucosa are detrimental. Microbes, mainly *Lactobacillus spp,* play a large role in developing mucosal immunity and maintaining barrier integrity [73-75]. One of the mechanisms by which *Lactobacillus spp* may blunt inflammatory responses is through the production of the lactic acid. Lactic acid appears to have antiviral activity against HIV-1 [76, 77].

In fact, it was found able i) to induce production of the anti-inflammatory mediator interleukin-1 receptor antagonist (IL-1RA), ii) to blunt production of cytokines, which attract HIV-1 target cells in response to toll-like receptors (TLR), suggesting a possible mechanism by which *Lactobacilli* are able to reduce the risk of HIV-1 acquisition [78].

Finally, mucosal microbes (mainly *Lactobacillus spp*) appear to play a significant role in modifying the risk of sexual HIV-1 acquisition in the absence of antiretrovirals [79, 80]. In fact, it has been shown that *L. crispatus* protected against inflammation and thus HIV-1 infection

CONCLUSION

This study has a number of limitations, the main of which are the small number of patients recruited (results were not sufficiently powered to detect statistical difference between groups) and the lack of a mechanistic model to explain the hypothetical functioning of FW. A further potential limitation is that the improvement in immune parameters observed, (*e.g.* increase in CD4+ T cells and reduction of activations markers), could be due to the efficiency of the antiretroviral treatment and not related to the probiotic treatment. However, we believe that these results are more likely attributable to probiotic supplementation, in fact they were observed in HIV-1 patients with a cronically suppressed viral load during cART, and a well-established immunological recovery. In addition to those limitations, the exact mechanism by which probiotics and FW samples can reduce HIV-1 replication and modulate T cell response remains to be defined.

Nonetheless, our observations can improve the debate on this topic and may have several social and public health implications for sexually transmitted diseases that need to be further explored.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The Ethics Committee at "Sapienza" University of Rome, Italy, approved the study, (Prot 813/18 Rif 2970).

HUMAN AND ANIMAL RIGHTS

No animals were used in this research. All humans research procedures were in accordance with the standards set forth in the Declaration of Helsinki principles of 1975, as revised in 2013 (http://ethics.iit.edu/ecodes/node/3931).

CONSENT FOR PUBLICATION

Written informed consents were obtained from all recruited subjects.

AVAILABILITY OF DATA AND MATERIALS

Datasets used in this study are available from the corresponding author [GdE] upon request.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

AUTHORS' CONTRIBUTIONS

Conceptualization, FF, GdE and OT; Data curation, FF, ENC, LS and GPI; Formal analysis, ENC and LS; Investigation, GC; Methodology, CS, IM and OT; Project administration, OT; Resources, LS; Supervision, VV and GdE; Validation, GdE; Writing – original draft, FF and OT; Writing – review & editing, GC, CS, IM, GdE and OT.

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