Original Research Article

EFFECTS OF PROBIOTICS ON INTESTINAL MICROFLORA OF HIV-INFECTED INDIVIDUALS

ABSTRACT

Aims: HIV-1 infection results in structural damage to the intestinal mucosa and changes of gut microflora following dysfunction of the gastrointestinal system, including compromised barrier function. Known properties of probiotics suggest that they may be useful tools in restoring normal intestinal flora. Our study goal was to determine whether the use of a probiotics can recover normal gut flora in chronically HIV-infected adults.
Study design: Cohort Design
Place and Duration of Study: Sumy State University, Medical Institute. Department of Microbiology and Clinical Immunology
Methodology: The study involved 40 HIV-1-infected patients of the regional center of prevention and control of AIDS in Kharkov. All the patients were informed about the purpose and plan of study and gave their written agreement to participate in the study. All the patients had been diagnosed according to the criteria of WHO with the III-IV stage of HIV infection. During the month before the survey the patients did not take any antibiotics. Dysbiosis correction circuit was designed for one month taking of probiotic preparations. Six weeks later the follow-up study was conducted to investigate gut microflora of 20 HIV-infected patients.
Results: Changes of intestinal microbiota were found in all of the patients. In the most cases the decrease of obligatory microorganisms, especially *Bifidobacterium* spp. (in 90 % of patients) was found. Overgrowth of major opportunistic pathogens (*S. aureus* and *Candida spp*.) was registered in only a minority of patients. The probiotic interventions resulted in significantly elevated levels of beneficial bacteria load (such as *Bifidobacterium spp*, *Lactobacillus spp*.) and a decrease in patogenic bacteria load (such as *Clostridium spp, Candida spp*).
Conclusion: Probiotic preparations can successfully augment the levels of beneficial species in the gut during chronic HIV-1 infection. These findings may help inform future studies aimed at testing pre- and probiotic approaches to improve gut function and mucosal immunity in chronic HIV-1 infection.

Keywords: microbial translocation; inflammation; probiotic bacteria; lactobacillus; HIV-1; anti-retroviral therapy (ART).

1. INTRODUCTION

It is known that the total number of microorganisms inhabit the human gut (10^{14}), on two orders exceeds total number of the cells. Besides, a large number of exogenous xenobiotic including pathobionts and food antigens passes through the intestine daily. It's not a surprise that up to 80 % of the lymphoid tissue is associated with the intestine region (GALT).
Indigenous intestinal microflora has a symbiotic relationship with the intestinal mucosa and is an integral part of the gastrointestinal tract. Close interaction between the microbiota and mucosa is a major imperative of intestinal homeostasis [1, 2]. It has been found out recently that dysbiotic changes in the gut (dysbiosis) accompany not only various intestinal disorders, but are also associated with a wide range of multi-organ pathologies, including HIV infection [3, 4].
It is shown that after penetrating into the mucosa HIV infects 60% of resting Ki67-CD4+ T-cells, leading to their activation. Activated CD4 + T-cells actively produce virus which infects cells via the neighbor cell contacts and circulates through the bloodstream to distant organs and tissues. As a result, the body forms a large reservoir of active infectious virus that can't be neutralized so far. [5]

Equally important is the direct impact of the virus on enterocytes. HIV has been established to infect and destroy vast amount of GALT CD4+ T cells and dendritic cells, as well as affect directly enterocytes: tat protein of HIV inhibits glucose uptake by enterocytes, impairing their function, gp120 protein increases the amount of calcium in the cells, which causes depolymerization of tubulin and, consequently, dysfunction of cytoskeleton. This leads to disruption of intercellular interaction and increased permeability of the intestinal barrier. At the same time the expression of genes that control the integrity of epithelium is suppressed [6].

Enteropathy accompanied by HIV is characterized by villous atrophy, crypt hyperplasia, malabsorption of several important nutrients, apoptosis of enterocytes, and increased permeability of epithelium. Mass deaths of the immune effector cells in the lamina propria, destruction of Peyer's patches, and a sharp reduction of secretory IgA and defensins levels create favorable conditions for the breeding of excessive microflora including pathogenic one in the intestinal lumen [7].

These factors lead to the penetration of lipopolysaccharide (LPS) and other bacterial components through the intestinal barrier into the blood circulation although bacteremia is not observed as a rule. Translocation of LPS and chronic exposure to peripheral lymphocytes result in persistent systemic immune response accompanied by high level of proinflammatory cytokines, which fairly soon leads to the depletion of the immune system. It is believed that translocations and chronic immune activation play a key role in the development and progress of opportunistic complications [8, 9].

Although it is not clear whether the dysbiosis of intestinal microbiota in HIV infection is a primary factor leading to the development of the disease or secondary response to other factors; but it is evident that it plays a significant role in the chronic phase of infection and the appearance of opportunistic complications.

This opens up the prospect of influence on the infectious process by correcting dysbiotic changes in HIV-infected patients. In this regard, the aim of the present study was to evaluate changes in microflora of the large intestine in chronic HIV infection and the possibility of correction by means of bacterial preparations (probiotics).

2. MATERIAL AND METHODS / EXPERIMENTAL DETAILS / METHODOLOGY

2.1 Patients

The study involved 40 HIV-1-infected patients of the regional center of prevention and control of AIDS in Kharkov. The study was conducted on an outpatient basis of the Department of Microbiology and Clinical Immunology in Kharkiv Medical Academy of Postgraduate Education (KhMAPE). All the patients were informed about the purpose and plan of study and gave their written agreement to participate in the study. All the patients had been diagnosed according to the criteria of WHO with the III-IV stage of HIV infection. Patients were divided into the two groups:

Group 1 (n=24) (60% - patients) - CD4 + cells counts at the time of the study was less than <350 cells / µl.

Group 2 (n=16) - CD4 + cells counts at the time of the study was higher than <350 cells / µl.

Dysbiosis correction circuit was designed for one month taking of probiotic preparations. Six weeks later the follow-up study was conducted to investigate gut microflora of 20 HIV-infected patients.

2.2 Fecal Bacteriologic Culture

The contents of the colon in an amount of 2-3 g was taken to the laboratory and processed within 2 hours in a sterile vial without preservative. Collection of material was carried out before the use of antibiotics and bacterial preparations (probiotics, prebiotics et al.)[10, 11].

The study of qualitative and quantitative composition of microflora of the colon was carried out by plating ten-fold dilutions of faeces samples (10^1-10^9) on a standard set of selective and differential diagnostic medium for the selection of intestinal microorganisms. Ten-fold serial dilutions of each fecal sample were performed and plated on selective and non-selective media for enumeration of the members of the intestinal microflora. Stool samples were placed on solid media (Bismuth Sulphite Agar, EMB Agar (Levine), Endo Agar, Blood Agar, Baird-Parker Agar, Sabouraud Dextrose Agar, Clostridial Agar, Rogosa SL Agar, Bifidobacterium Agar, HiMedia Lab., India). The plates were incubated at 37 'C for 24 or for 48 h. The incubated microorganizms were then counted and identified with accordance to standard procedures [10, 11]. Summarized data of control group (10 healthy adult's) microflora contents served as a normal standard.

During the survey, patients did not receive medications with potentially possible effects on the gastrointestinal tract, including antibiotics.

Correction of dysbiotic disorders was carried out by taking into account the individual personified the intestinal flora changes. The structure included cocktail commercial preparations of Probiotic Complex ("Santegra", USA); Enterol 250 ("Biocodex", Ukraine); Bifikol ("Biopharma", Ukraine); Laktiale ("Farmak", Ukraine).). All patients received probiotic drugs, depending on their microbial content. Thus, patients with a lack of lactobacilli, bifidobacteria and enterococci in feces samples received "Lactiale" according to the instructions. Due to the composition of preparations, "Probiotic Complex" was administered at reducing the number of bifidobacteria and lactobacilli; "Bifikol" was prescribed in cases with a deficit of *E.coli*.

The scheme of correction was calculated for 1 month of taking probiotics. Clinical and microbiological changes were evaluated before and after correction by probiotics.

All bacterial counts (colony-forming units (CFU)/g of wet feces) were transformed to logarithm (log_{10}CFU) for ease of statistical analysis.

2.3 Statistical Analysis

The results are presented in the form of averages, standard deviation and median assuming a normal distribution of data. Normal distribution of quantitative traits was verified by the Shapiro-Wilk test. The research results are processed using "STATISTICA 10.0" (StatSoft Inc., USA, version 10.0.1011.6) and spreadsheet editor Microsoft Excel 2013.

3. RESULTS AND DISCUSSION

27 of 40 (67.5%) HIV-infected patients participated in the study were women and 13 (32.5%) men. The average age of patients was 35.6 ± 8.2 years. The average number (M ± m) of CD4+ T cells before the study was 426 ± 264 in 1 μl (Table 1).

Table 1. Patients characteristics of the study groups (n=40)

Characteristic	Value	HAART treatment	
		More than 1 year n=24	Less than 1 year n=16
Sex n(%)			
-male	13(32.5%)	6(25%)	7(44%)
-female	27(67.5%)	18(75%)	9(56%)
Age ((year)			
M±SD;*	35.6±8.2	37,4± 7,8	33±8,2
Median))	34	36	31
Blood CD4 cell count (cells/ml)			
- all(n=40)			
M±SD;	426±264	281±111	600±307
Median	416	243	479
- <350 cells / μl			
M±SD	223±99	235±100	201±108
Median	220	299	199
- >350 cells / μl			
M±SD	462±280	300±113	691±289
Median	424	239	595

**Mean ± standard deviation (SD)*

Table 2. Fecal flora in HIV-1 infected patients before and after the correction of probiotics

Covariates	Fecal flora before correction (log_{10}CFU)	Patients (n=40)	Fecal flora after correction (log_{10}CFU)	Patients (20)	Normal
Bifidobacterium spp.	5.9 ± 0.9 7.0 ± 1.1	36 4	5.9 ± 1.4 8.0 ± 1.8	19 1	9.7 ± 1.4
Lactobacillus spp.	5.0 ± 0.8 6.7 ± 1.07	35 5	5.0 ± 1.1 7.7 ± 1.7	12 8	7.7 ± 1.2
E. coli (lac+)	5.9 ± 1.2 8.7 ± 1.4	24 16	8.7 ± 1.9	20	8.0 ± 1.3
E. faecalis	5.0 ± 1.02 7.7 ± 0.8	25 15	5.0 ± 1.1 7.7 ± 1.7	6 14	7.74 ± 1.2
E. faecium	5.0 ± 0.8 5.9 ± 0.9	34 6	5.0 ± 0.9	20	7.7 ± 1.2
E. coli Hly	ND 5.0 ± 0.8	2 38	ND 5.0 ± 1.1	19 1	ND
S.aureus	ND 4.0 ± 1.6	33 7	ND	20	ND
S. epidermidis	4.0 ± 0.8 5.0 ± 1.4	27 13	ND	20	4.0 ± 0.6
Candida spp.	2.9 ± 0.5 4.0 ± 1.5	32 8	ND	20	4.0 ± 0.6
Cl. perfringens	2.0 ± 0.3 2.9 ± 0.4	38 2	2.0 ± 0.5 2.9 ± 0.7	16 4	2.9 ± 0.5

Data as mean ± standard deviation (Log_{10}counts/g feces)
ND not detected

As it can be seen from *Table 2*, the quantitative and qualitative composition of the normal microflora of the large intestine has been altered in all patients.

In studyng the microbial gut content, there were discovered violations of the qualitative and quantitative composition of anaerobic and facultative anaerobic bacteria. Reduced number of bacteria concerned primarily *bifidobacteria,* which dominated in the anaerobic flora and accounted for about 95% of the gut microflora. According to our data, in 90 % of cases the number of *bifidobacteria* was less than 5.9 ± 0.9

and in 10 % of cases it was about 7.0 ± 1.1. The number of very important *lactobacilli* at HIV infection is significantly reduced against healthy controls accounting less than 5.0 ± 0.8 in 87.5 % and 6.7 ± 1.07 in 12.5 % of patients against 7.7 ± 1.23 respectively ($p<0.05$).

The group of anaerobic bacteria, *Bacteroides*, wasn't detected in patients. The leading representative of the facultative anaerobic bacteria belonging to the indigenous microflora is *E. coli*. About half of all patients, the number of facultative anaerobes has remained constant, and the other it decreased by 1-2 orders. Hemolytic *E. coli* strains in small concentrations were presented in only 5% of the patients.

The same trend is observed in relation to other pathobionts: *S. aureus, S. epidermidis,* and *C. albicans* in low titers are found in only a minority of infected patients ($p<0.05$). Only in one patient *Clostridium spp.* were isolated in very low concentrations. In addition, a case of a serious intestinal dysbiosis in HIV-infected patients was accompanied by falling down on 1-2 orders of the obligate commensals *E. faecalis,* and *E. faecium* presented in large numbers in the faeces of healthy adults.

Thus, HIV infection, regardless of the duration of the course, the clinical stage of the disease and antiviral managing manifests a profound violation of the gut homeostasis accompanied by a simultaneous decrease in quantitative anaerobic (*bifidobacteria* and *lactobacilli*) and facultative anaerobic flora (*E. coli*).

In our study, the use of probiotic bacterial preparations on the background of the microbiome dysbiosis in HIV-infected patients resulted in a significant mitigation of these violations, but complete restoration was not also observed (*Table 2*).

Serious changes of the intestine microflora in chronic HIV infection have been identified by other researchers, too [12,13]. Significant changes of intestinal microbiota is accompanied by the appearance of communities of enteropathogenic bacteria capable of converting tryptophan to kynurenine immunomodulatory derivatives, which correlates with the progression of the disease and contributes to the violation of mucosal immunity. At the same time ART-naïve patients increases the levels of some bacterial taxa, and the suppression of 45 taxa. The most significant enrichment was mentioned for Erysipelotrichaceae, which often accompanies obesity and is associated with increased incidence of cardiovascular system disorders. Such types as *Proteobacteria* are part of the most enriched genera of ART-naïve patients. Among them is the species included in the genera of *Salmonella, Escherichia, Serratia, Shigella* and *Klebsiella* of the *Enterobacteriaceae* family, known as pro-inflammatory pathobionts. The gut content of ART-naÏve HIV-carriers is enriched with *Staphylococcus, Pseudomonas, Campylobacter spp., Candida albicans*, which often cause opportunistic infections and bacteremia, with a significant decrease in the content of *bifidobacteria* and *lactobacilli, Clostridia* and *Bacteroides* with particularly strong suppression of *Bacteroides* and *Alistipes* genera. The studies showed the dramatic reduction in the levels of lacto-and bifidobacteria and increases in the concentration of pathogenic species, including *Candida albicans* and *Pseudomonas aeruginosa* in HIV-carries [14, 15].

Thus, HIV-induced dysbiosis appears to be characterized by decreased abundances of bacteria that are regarded as commensal or protective accompanied by an expansion of bacteria that are potentially inflammatory or pathogenic, which agrees with mucosal inflammation in HIV infection.

Such probiotics as *Lactobacillus rhamnosus* GR-1 have a beneficial effect on preservation of immunity in HIV infection [16].

Recently, it has been found that the balance between the two subpopulations of CD4 + regulatory T cells, Th17 and CD25 + FoxP3 + (Treg) is responsible immune mechanisms which protect against infections and autoimmune disorders.

Treg-cells (regulatory T cells) express toll-like receptor 4 (TLR-4) and activated by LPS. Some *Lactobacillus* species (*L. reuteri* and *L. casei,* but not *L. plantarum*) also activate these cells [17]. The number of HIV-specific Treg-cells is increased in patients responding to ART.

While significant depletion of Th17-cells and reduction of CD4+ CD161+ cells associated with progressive loss of Treg-cells, increased immune activation and progression of the disease [18]. The combination of probiotics in the model system can increase the content of Treg-cells, and suppress the development of the disease [19]. Activity had only a mixture of several species of *Lactobacilli*. Suppressive activity was accompanied by increased secretion of IL-10 Treg-cells, which led to a weakening of the secretion of pro-inflammatory cytokines by cells Th1 and Th17. Model system showed that taking of probiotics (*L. acidophilus, L. casei, L. reuteri, Bifidobacterium bifidium* and *Streptococcus thermophilus*) induced a low response of T and B cells, reduced the secretion of Th1, Th2 and Th17 cytokines, inhibited apoptosis and caused migration of Treg-cell into the inflammation area [20]. Probiotics have a beneficial effect on the HIV-infection. [16]. Gori et al. have shown that simultaneous use

of probiotics results in a significantly increased number of *bifidobacteria* and reduction of *Clostridium coccoides, Eubacterium rectale, Clostridium lituseburense* and *Clostridium histolyticum* [20]. It is also shown that the oligosaccharide mixture as a prebiotic reduces the level of sCD14, decreases activation of CD4+ T-cells and enhances the NK-cell activity in ART-naïve HIV-infected adults [21].
Thus, the beneficial effect of probiotic bacteria may include effects on the immune status and immune activation, course of HIV infection, on translocation and the balance of regulatory T-cells. These findings thus suggest that the correction of dysbiosis can have desirable effects in the restoration of intestinal function and repair.

4. CONCLUSION

In conclusion, a decrease in total obligate anaerobes and an increase in pathogenic bacteria in the gut are indicated in patients with HIV-1 and probiotic preparations can successfully augment the levels of beneficial species in the gut. These findings may help inform future studies aimed at testing pre- and probiotic approaches to improve gut function and mucosal immunity in chronic HIV-1 infection.

ETHICAL APPROVAL

Study protocol was approved by the Ethics Committee of the regional center of prevention and control of AIDS in Kharkov.

REFERENCES

1. Hooper LV, Macpherson AJ. Immune adaptations that maintain homeostasis with the intestinal microbiota. Nat Rev Immunol. 2010;10:159–69.doi:10.1038/nri2710.

2. Pabst R, Russell MW, Brandtzaeg P. Tissue distribution of lymphocytes and plasma cells and the role of the gut. Trends Immunol. 2008;29:206–8. doi:10.1016/j.it.2008.02.006.

3. Henao-Mejia J, Elinav E, Jin C, Hao L, Mehal WZ, Strowig T, et al. Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. Nature. 2012;482:179–85. doi:10.1038/nature10809nature10809.

4. Vujkovic-Cvijin I, Dunham RM, Iwai S, Maher MC, et al. Dysbiosis of the gut microbiota is associated with HIV disease progression and tryptophan catabolism. Sci. Transl. Med. 2013;5: doi:10.1126/scitranslmed.3006438

5. Turville SG, Peretti S, Pope M: Lymphocyte-dendritic cell interactions and mucosal acquisition of SIV/HIV infection. Curr Opin HIV AIDS. 2006;1(1):3–9.

6. Guadalupe M, Sankaran S, George MD, Reay E, Verhoeven D, Shacklett BL, et al: Viral suppression and immune restoration in the gastrointestinal mucosa of human immunodeficiency virus type 1-infected patients initiating therapy during primary or chronic infection. J Virol. 2006;80 (16):8236–8247.

7. Saxena D, Li Y, Yang L. Human Microbiome and HIV/AIDS // Curr HIV/AIDS Rep. 2012;9(1):44–51. doi:10.1007/s11904-011-0103-7

8. Brenchley JM, Price DA, Schacker TW. Microbial translocation is a cause of systemic immune activation in chronic HIV infection. Nat Med. 2006;12:1365–71.

9. Mehandru S, Poles MA, Tenner-Racz K. Mechanisms of gastrointestinal CD4+ T-cell depletion during acute and early human immunodeficiency virus type 1 infection. J Virol. 2007;81:599–612.

10. The principles of correction of disorder of microecology of intestine: methodic recommendations / Moscow Research Institute of Pediatrics and Pediatric Surgery. – Moscow. 2004;38.

11. The current approaches of the correction of intestinal dysbiosis: methodic recommendations / Ukrainian Centre for Scientific Medical Information and patent-license work. 2000;27.

12. Saxena D, Li Y, Yang L. et al. Human Microbiome and HIV/AIDS // Curr HIV/AIDS Rep. 2012;9(1):44–51. doi:10.1007/s11904-011-0103-7.

13. Dillon SM, Lee EJ, Kotter CV, Austin GL, Dong Z, Hecht DK, Gianella S, Siewe B, Smith DM, Landay AL, Robertson CE, Frank DN, Wilson CC An altered intestinal mucosal microbiome in HIV-1 infection is associated with mucosal and systemic immune activation and endotoxemia. Mucosal Immunol. 2014; 7(4):983-94.

14. Mutlu EA, Keshavarzian A, Losurdo J, Swanson G, Siewe B, Forsyth C, et al. A compositional look at the human gastrointestinal microbiome and immune activation parameters in HIV infected subjects. PLoS Pathog. 2014;10(2):e1003829.

15. Gori A, Tincati C, Rizzardini G, Torti C, Quirino T, Haarman M, et al. Early impairment of gut function and gut flora supporting a role for alteration of gastrointestinal mucosa in human immunodeficiency virus pathogenesis. J. Clin. Microbiol. 2008;46:757–758.

16. Das JK, Mishra D, Ray P, Tripathy P, Beuria TK, et al. In vitro evaluation of anti-infective activity of a Lactobacillus plantarum strain against Salmonella enterica serovar Enteritidis. Gut Pathog. 2013;5: 11. doi: 10.1186/1757-4749-5-11.

17. Hemsworth J, Hekmat S, Reid G. The development of micronutrient supplemented probiotic yogurt for people living with HIV: Laboratory testing and sensory evaluation. Innov Food Sci Emerg Technol. 2011;12: 79–84.

18. Smits HH, Engering A, van der Kleij D, de Jong EC, Schipper K, et al. Selective probiotic bacteria induce IL-10-producing regulatory T cells in vitro by modulating dendritic cell function through dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin. J. Allergy Clin. Immunol. 2005;115:1260-1267.

19. Prendergast A, Prado JG, Kang YH, Chen F. HIV-1 infection is characterized by profound depletion of CD161 + Th17 cells and gradual decline in regulatory T cells. Aids. 2010;24:491-502.

20. Lavasani S, Dzhambazov B, Nouri M, Fak F, Buske S. A novel probiotic mixture exerts a therapeutic effect on experimental autoimmune encephalomyelitis mediated by IL-10 producing regulatory T cells. PLoS One. 2010; 5: doi: 10.1371 / journal.pone.0009009

21. De Roock S, van Elk M, van Dijk ME, Timmerman HM, Rijkers GT, Prakken BJ, et al. Lactic acid bacteria differ in their ability to induce functional regulatory T cells in humans. Clin. Exp Allergy. 2010;40:103–110.

22. Gori, A, Rizzardini G, Van'T Land B, Amor KB, van Schaik J, Torti C, et al. Specific prebiotics modulate gut microbiota and immune activation in HAART-naive HIV-infected adults: Results of the "COPA" pilot randomized trial. Mucosal Immunol. 2011;4:554-563.

265