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In-vitro cytokine production and nasopharyngeal microbiota composition in the early stage of COVID-19 infection

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ABSTRACT

Background: To determine and compare nasopharyngeal microbiota (NM) composition, in vitro basal (Nil tube), provoked (Mitogen tube) production of cytokines at the early stage of COVID-19.

Methods: This cross-sectional study included 4 age and sex-matched study groups; group 1 (recovered COVID-19) (n = 26), group 2 (mild COVID-19) (n = 24), group 3 (severe COVID-19) (n = 25), and group 4 (healthy controls) (n = 25). The study parameters obtained from the COVID-19 (group 2, and 3) at the early phase of hospital admission.

Results: The results from the research depicted that the Mean ± SD age was 53.09 ± 14.51 years. Some of the in vitro cytokines production was significantly different between the study groups. Some of the findings on cytokines depicted a significant differences between study groups were interleukin (IL)-1α, IL-1β Mitogen, and their subtraction (i.e Mitogen-Nil). Regarding IL-10, and IL-17a levels, Mitogen, and Mitogen-Nil tube production levels were significantly different between the groups. Surprisingly, most of these measures were lowest in the severe COVID-19 patients’ group. Using discriminant analysis effect size (LEfSe), Taxa of NM with significant abundance was determined. About 20 taxa with an LDA score > 4 were identified as candidate biomarkers. Some of these taxa showed a significant correlation with IL-1β and IL-10 Mitogen and Mitogen-Nil levels (R > 0.3 or < -0.3, p < 0.05).

Conclusions: The findings of this particular study regarding the early stage of COVID-19 showed that in vitro cytokines production, studies might be more useful than the ordinary cytokines blood level measurement. Besides, the study identified some NM species that could be candidate biomarkers in managing this infection. However, further detailed studies are needed in these fields.

Keywords: Cytokine, COVID-19, Dysbiosis, In-vitro, Microbiota, Quantiferon test, Virus

1. Introduction

The Coronavirus (COVID-19) is a new and highly contagious pandemic viral infection that has posed a threat across the world since the end of 2019. The virus has a high rate of morbidity and/or mortality. To get over this pandemic, we have to hit the books and acquire relevant scientific knowledge that will assist in curbing its spread. During viral infections, cytokines’ responses to secondary bacterial infections is usually disregulated. Some of these cytokines (Interferons [INF], tumour necrosis factor [TNF]-α, interleukin [IL]-6) may have a protective role in some of the viral infections (Such as influenza and Herpes Simplex) [1–3]. In a recent study by Han H, et al. [4], COVID-19 patients had a higher serum level of cytokines (TNF-α, IFN-γ, IL-4, IL-6 and IL-10). Additionally, serum IL-6 and IL-10 levels were found to be predictive of disease severity. Interleukin-6 and its receptor monoclonal antibody (i.e. tocilizumab) are widely evaluated in COVID-19 patients [3,5–9]. Although this treatment approach was promising initially, the last randomized clinical trials showed the ineffectiveness of the IL-6 based therapy in preventing intubation and/or mortality of COVID-19 [10]. So, the above paradoxes regarding IL-6 and its system necessitate new approaches and horizons in evaluating COVID-19 infection-related cytokine(s) production. In-vitro cytokine production is studied in
### Table 1
Comparison of anamnestic data, oxidative stress parameters, and nasopharyngeal microbiota indices of the study groups.

| Age (years) | Recovered (N = 26) | Mild (N = 24) | Severe (N = 25) | Control (N = 25) | Total (N = 100) | value |
|-------------|--------------------|--------------|----------------|-----------------|-----------------|-------|
| Mean (SD)   | 51.346 (16.314)    | 53.500 (14.271) | 59.040 (13.609) | 59.460 (12.227) | 53.090 (14.513) | NS    |
| Range       | 25.000-89.000      | 25.000-87.000 | 25.000-85.000 | 29.000-80.000 | 25.000-89.000 |       |
| Post-hoc    | a                  | a             | a              | a               | a               |       |
| Gender      |                    |               |                |                 |                 |       |
| Male        | 13 (50.0%)         | 13 (54.2%)    | 12 (48.0%)     | 13 (52.0%)      | 12 (48.0%)      | 51(51.0%) |
| Post-hoc    | a                  | a             | a              | a               | a               |       |
| Female      | 13 (50.0%)         | 11 (45.8%)    | 13 (48.0%)     | 13 (52.0%)      | 13 (52.0%)      | 49(49.0%) |
| Comorbidities |                  |               |                |                 |                 | 0.001 |
| Absent      | 25(96.1%)          | 21(87.3%)     | 19(76.0%)      | 16(64.0%)       | 81(81.0%)       | 0.022 |
| Present     | 1(3.9%)            | 3(12.5%)      | 6(24.0%)       | 9(36.0%)        | 41(41.0%)       |       |
| Smoking     |                    |               |                |                 |                 |       |
| Absent      | 25(96.1%)          | 21(87.3%)     | 19(76.0%)      | 16(64.0%)       | 81(81.0%)       | 0.011 |
| Present     | 1(3.9%)            | 3(12.5%)      | 6(24.0%)       | 9(36.0%)        | 41(41.0%)       |       |
| Tobacco use |                    |               |                |                 |                 |       |
| Absent      | 25(92.0%)          | 23(95.8%)     | 23(92.0%)      | 23(92.0%)       | 94(94.0%)       |       |
| Present     | 1(0.8%)            | 1(0.4%)       | 2(0.8%)        | 2(0.8%)         | 6(6.0%)         |       |
| BMI (Kg/m²) |                    |               |                |                 |                 |       |
| Median      | 27.200             | 28.700        | 29.800         | 26.600          | 27.700          |       |
| Q1, Q3      | 20.500-39.100      | 25.400-46.900 | 26.500-41.600 | 29.100-31.000  | 31.000-36.280   |       |
| Alcohol use |                    |               |                |                 |                 |       |
| Absent      | 85.000             | 61.700        | 63.140         | 59.600          | 59.000          |       |
| Present     | 0.022              | 0.165         | 2.970          | 5.500           | 5.000           |       |

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some disease conditions [11,12]. As far as we know, there is no study evaluating in vitro cytokine(s) production in COVID-19 infection. Even if no clinical studies in this field, oxidative stress is expected to have a major role in COVID-19 infection [13]. One of the simplest and practical ways to study the oxidative stress status of the host is total oxidant status (TOS), total antioxidant status (TAS), and their ratio [i.e. oxidative stress index (OSI)] [14]. Whether in vitro above-mentioned oxidative stress parameters could help diagnose or manage COVID-19 infection needs to be studied.

Another entity that has an emerging role in immunity and inflammation is host-microbiota. This proper interaction between the immune system and host-microbiota helps develop effective protective measures against pathogens [15]. Furtherly, studies showed the effect of gut microbiota dysbiosis on the production of inflammatory cytokines [16]. Just recently published studies showed alteration of gut microbiota composition in COVID-19 patients [17,18]. Microbiome species with known immunomodulatory potential, such as Faecalibacterium prausnitzii, Eubacterium rectale and bifidobacteria were underrepresented in COVID-19 patients [18]. As we know, the main (maybe the sole) route of entry of Coronavirus to the host is the respiratory tract. There was a dysbiosis in NM compositions in some of the respiratory viral infections (especially the symptomatic one) [19]. According to our knowledge, there are only 2 studies that evaluated the NM composition in COVID-19. As mentioned by the authors, both studies had a limitation of a small sample size [20,21]. The main reason for this limitation would be the difficulty of conducting such a study in the pandemic condition! One of them showed no alteration in COVID-19 infection [20]. The other one showed reduced Fusobacterium periodonticum microbiota species in COVID-19 patients (compared to healthy controls) [20]. There are no studies yet comparing NM composition in different COVID-19 patients (mild, severe, and recovered), and most importantly, in combination with in vitro inflammatory cytokines and/or oxidative stress parameters production. Therfore, the reaserch study in this context will focus on analyzing and interpreting in vitro cytokines and oxidative stress parameters production levels and NM composition of COVID-19 patients. Also, we will try to compare these mentioned parameters with recently recovered COVID-19 patients and healthy controls same parameters. Additionally, we will try to see if there is any relation between these study parameters.

2. Materials and methods

This prospective cross-sectional study has been approved by the Bakirkyor Dr.sadi Konuk training & research hospital’s ethical committee (Decision No. 2020–07). Before enrollment, written consent was obtained from all participants (or their 1st-degree relatives).

2.1. Study populations and groups

Group 1 participants consisted of recovered COVID-19 persons. The second and 3rd groups consisted of newly diagnosed mild and severe COVID-19 patients, respectively. The last 4th group consisted of healthy controls (supplementary [s]Fig. 1). An age and sex group matching was applied.

Inclusion criteria;
- Age ≥ 18 years old (all groups),
- Complete cure and elapsing more than one month from 1st symptom of COVID-19 infection (group 1).
- Recent diagnosis of mild (group 2) or severe (group 3) COVID-19 infection,
- No history of current or previous COVID-19 disease (group 4).

Exclusion criteria;
- Inability to give written consent (all groups),
- History of malignancy or chronic infections (such as TB) (all groups),
- Using of medications at the time of enrollment that may affect study parameters (such as antibiotics, probiotics, steroids, immunosuppressive drugs, etc.) (all groups),
- Need of intubation and mechanical ventilation at the time of enrollment and sampling (all groups).

Classification criteria of COVID-19 patients were as follow [22];

1. Mild (no or mild pneumonia).
2. Severe disease (e.g. dyspnea, hypoxia, or > 50% lung involvement on imaging within 24 to 48 h).
3. Critical disease (e.g. respiratory failure, shock, or multigorgan dysfunction).

Within 24 h of admission or application to the hospital, a nasopharyngeal swab for microbiota analysis (using DNA/RNA Shield Collection Tube w/ Swab [Zymo Research, Irvine, CA]) and fasting blood samples were taken into the Nil (no antigen), and Mitogen (phytobhemoglutinin) of the known QuantiFERON®-TB Gold In-Tube test’s Nil and Mitogen test tubes. These two tubes were incubated for 24 h at 37 °C. Then the tubes were centrifuged, and plasma was removed from each tube and placed in a plasma storage container. These containers were then frozen at ~80 °C until the day of analysis of the laboratory

| Table 1 (continued) | Recovered (N = 26) | Mild (N = 24) | Severe (N = 25) | Control (N = 25) | Total (N = 100) |
|---------------------|------------------|--------------|----------------|-----------------|-----------------|
| Q1, Q3              | 5.411, 6.172     | 5.001, 5.884 | 3.982, 5.813   | 5.359, 5.862    | 5.007, 5.973    |
| Range               | 24-555, 6-845    | 3.868-6.591  | 2.305-6.559    | 2.714-6.478     | 2.305-6.845     |
| Simpson index       |                  |              |                |                 |                 |
| Median              |                  |              |                |                 |                 |
| Q1, Q3              | 149.500          | 148.832      | 113.000        | 153.000         | 143.920         |
| Range               | 51.900-290.000   | 72.000-274.909 | 41.000-260.069 | 111.000-211.976 | 41.000-290.000 |
| Post-hoc            | a                | a            | a              | a               | a               |
| Chao1 index         |                  |              |                |                 |                 |
| Median              |                  |              |                |                 |                 |
| Q1, Q3              | 0.981            | 0.611        | 0.980          | 0.551           | 0.981           |
| Range               | 6.591            | 2.305        | 18.827         | 13.714          | 14.973          |
| Post-hoc            | ab               | a            | b              | a               | a               |
| PD_whole_tree index |                  |              |                |                 |                 |
| Median              |                  |              |                |                 |                 |
| Q1, Q3              | 0.968            | 0.954        | 0.935          | 0.962           | 0.955           |
| Range               | 0.949-0.973      | 0.925-0.964  | 0.880-0.969    | 0.946-0.967     | 0.930-0.969     |
| Post-hoc            | a                | ab           | b              | ab              | ab              |

NS: Not significant,
Post-hoc: If same letters p value is > 0.05, otherwise p < 0.05.
| Table 2                                                                 |
|----------------------------------------------------------------------|
| Study groups’ *in vitro* cytokines production profiles (Nil, Mitogen, and Mitogen -Nil tubes). |

| CCL2 Nil(pg/mL) | NS |
|-----------------|----|
| Median          | 3702.365 |
| Q1, Q3          | 2503.595, 5349.085 |
| Range           | 821.560 - 21325.980 |
| Post-hoc        | a a a a |

| CCL2 Mitogen(pg/mL) | NS |
|---------------------|----|
| Median              | 4518.840 |
| Q1, Q3              | 3012.578, 9105.382 |
| Range               | 1499.470 73092.610 |
| Post-hoc            | a a a a |

| CCL2 Mitogen-Nil(pg/mL) | NS |
|-------------------------|----|
| Median                  | 1145.805 |
| Q1, Q3                  | -259.405, 4428.573 |
| Range                   | -1358.590 a |
| Post-hoc                | a a a a |

| GCSF Nil (pg/mL) | 0.004 |
|------------------|------|
| Median           | 36.510 |
| Q1, Q3           | 28.290, 60.365 |
| Range            | 13.030-181.970 |
| Post-hoc         | a ab b ab |

| GCSF Mitogen (pg/mL) | <0.001 |
|----------------------|-------|
| Median               | 356.980 |
| Q1, Q3               | 106.103, 608.765 |
| Range                | 53.400 -1843.060 |
| Post-hoc             | ab c a b |

| GCSF Mitogen-Nil (pg/mL) | <0.001 |
|--------------------------|-------|
| Median                   | 275.630 |
| Q1, Q3                   | 52.472, 551.035 |
| Range                    | -47.340 – 1806.550 |
| Post-hoc                 | ab c c ac b |

| CXCL10 Nil(pg/mL) | 0.003 |
|-------------------|------|
| Median            | 670.180 |
| Q1, Q3            | 220.905, 4914.793 |
| Range             | 13.490-9941.820 |
| Post-hoc          | a a a a b |

| CXCL10 Mitogen(pg/mL) | NS |
|-----------------------|----|
| Median                | 1399.085 |
| Q1, Q3                | 1143.340, 4863.500 |
| Range                 | 250.000 -21292.430 |
| Post-hoc              | a a a a a |

| CXCL10 Mitogen-Nil(pg/mL) | NS |
|---------------------------|----|
| Median                    | 116.905 |
| Q1, Q3                    | -3916.352,3771.782 |
| Range                     | -9034.740 -21042.430 |
| Post-hoc                  | a a a a a |

| IFN-γ Nil(pg/mL) | NS |
|------------------|----|
| Median           | 28.935 |
| Q1, Q3           | 7.400, 63.475 |
| Range            | 2.020-225.230 |
| Post-hoc         | a a a a |

| IFN-γ Mitogen(pg/mL) | NS |
|----------------------|----|
| Median               | 198.630 |
| Q1, Q3               | 113.175, 241.330 |
| Range                | 6.160-2000.000 |
| Post-hoc             | a a a a |

| IFN-γ Mitogen-Nil(pg/mL) | NS |
|--------------------------|----|
| Median                   | 165.620 |
| Q1, Q3                   | 70.507, 202.870 |
| Range                    | -53.770-1909.990 |
| Post-hoc                 | a a a a |

| IL-1β Nil(pg/mL) | 0.012 |
|------------------|------|
| Median           | 130.950 |
| Q1, Q3           | 75.825, 289.772 |
| Range            | 1.430-613.940 |
| Post-hoc         | ab a ab b |

| IL-1β Mitogen(pg/mL) | <0.001 |
|----------------------|-------|
| Median               | 422.015 |
| Q1, Q3               | 282.423, 889.540 |
| Range                | 116.640-889.590 |
| Post-hoc             | ab a a b |

(continued on next page)
Table 2 (continued)

| Post-hoc | a | b | bc | c | <0.001 |
|----------|---|---|----|---|--------|
| IL-17 Mitogen-Nil(pg/mL) | 258.635 | 47.650 | 55.400 | 12.900 | 87.745 |
| Median | 146.310, 546.477 | –10,860, 90.053 | –5,010, 191.100 | –58,150, 291.200 | –2,355, 276.870 |
| Range | –433,990, 465.140 | –228,700, 612,780 | –262,470, 584.980 | –329,020, 442.550 | –433,990, 865.140 |
| Post-hoc | a | b | b | b | b |
| IL-7 Nil(pg/mL) | 17.240 | 14.590 | 16.530 | 20.050 | 17.220 |
| Median | 14,082, 19.310 | 10,777, 17.863 | 15,120, 18.460 | 19,090, 22.450 | 14,087, 20.020 |
| Range | 9,250, 26,060 | 3,630, 26,400 | 4,400, 24,650 | 10,050, 29,210 | 3,630, 29,210 |
| Post-hoc | a | a | a | b | b |
| IL-7 Mitogen(pg/mL) | 18.460 | 15.300 | 17.150 | 18.260 | 17.740 |
| Median | 17,220, 20,665 | 12,547, 17,203 | 13,540, 18,880 | 17,570, 19,230 | 15,300, 19,300 |
| Range | 12,470, 26,460 | 6,310, 27,020 | 5,890, 27,980 | 13,000, 20,330 | 5,890, 27,980 |
| Post-hoc | a | b | ab | a | a |
| IL-7 Mitogen-Nil(pg/mL) | 2.265 | 0.275 | 0.390 | –2.280 | 0.055 |
| Median | –0.255, 4.812 | –3.020, 6.192 | –2.980, 5.320 | –5.080, –0.490 | –3.303, 4.340 |
| Range | –11,460, 3.210 | –15,730, 20,480 | –16,780, 12,170 | –11,640, 8,440 | –16,780, 20,480 |
| Post-hoc | a | b | ab | a | b |
| IL-10 Nil (pg/mL) | 14.510 | 11.680 | 15.670 | 12.770 | 12.785 |
| Median | 10,595, 25,610 | 2,485, 15,125 | 12,040, 29,670 | 10,020, 17,900 | 9,950, 25,608 |
| Range | 1,510, 54,070 | 0.220, 49.750 | 1.230, 95.970 | 2.990, 85.300 | 0.220, 95.970 |
| Post-hoc | a | a | a | a | a |
| IL-10 Mitogen (pg/mL) | 194.795 | 142.410 | 40.860 | 244,800 | 157.945 |
| Median | 99,883, 337.895 | 75,490, 257.623 | 20,610, 111.370 | 157,559, 302.560 | 63,425, 274.325 |
| Range | 17,700, 572.650 | 0.370, 621,240 | 0.940, 451,030 | 85,420, 559,430 | 0.370, 621,240 |
| Post-hoc | a | a | a | a | a |
| IL-10 Mitogen-Nil (pg/mL) | 182.615 | 125.525 | 20.830 | 233,600 | 145,870 |
| Median | 88,688, 330.555 | 72,902, 246.447 | –6,130, 81.700 | 151,320, 289.850 | 49,815, 254,150 |
| Range | 11,150, 546.200 | –1,254, 613,970 | –97,300, 439,130 | 59,200, 546,660 | –90,730, 613,970 |
| Post-hoc | a | c | a | c | b |
| IL-17a Nil (pg/mL) | 17.830 | 18.725 | 17.670 | 21.060 | 18.230 |
| Median | 15,755, 19.310 | 16,807, 20.765 | 15,090, 20.660 | 15,680, 25.060 | 15,395, 21.067 |
| Range | 9,680, 25,720 | 13,430, 23,180 | 11,260, 24,060 | 10,900, 29,860 | 9,680, 29,860 |
| Post-hoc | a | a | a | a | a |
| IL-17a Mitogen (pg/mL) | 61.120 | 41.435 | 23.950 | 74.150 | 53.355 |
| Median | 39,913, 72,810 | 20,902, 78,292 | 20,210, 48,610 | 35,320, 85,720 | 25,835, 77,370 |
| Range | 19,570, 209,300 | 14,346, 99,440 | 12,270, 97,680 | 21,700, 98,780 | 12,270, 209,300 |
| Post-hoc | a | ac | c | b | b |
| IL-17a Mitogen-Nil (pg/mL) | 42.510 | 24.405 | 4.800 | 54.820 | 35.780 |
| Median | 22,962, 54.555 | 0.545, 58.362 | 2.470, 30.940 | 38,450, 64,260 | 5,830, 59.412 |
| Range | 0.380, 190,870 | –4,070, 85.690 | –9,710, 81,880 | –7,040, 85,450 | –9,710, 190,870 |
| Post-hoc | ab | ac | c | b | b |

NS: Not significant.
Post-hoc: If same letters p value is > 0.05, otherwise p < 0.05.

measurements.

Measurement methods of cytokines, OS parameters, and NM are given as a supplementary (s) (see sFile, Methods).

2.2. Data availability

All necessary raw data are available at Zenodo (https://doi.org/10.5281/zenodo.4771625).

2.3. Statistical analyses

The demographic variables, blood plasma cytokines/chemokines, and α-diversities of subjects in the study were summarized using the mean and standard deviation or median and interquartile range depending on their distribution. These variables were compared among groups using ANOVA or non-parametric factorial Kruskal-Wallis rank-sum test and were subsequently investigated using pairwise tests between sub-classes using the Wilcoxon rank-sum test. Differentially abundant bacterial taxa were identified using the discriminant analysis effect size (LEfSe). A logarithmic LDA score > 4 was considered statistically significant.

Principal coordinates analysis (PCoA) was used to explore and visualize the similarities or dissimilarities of samples based on their species-level compositional profiles.

Association between COVID enriched/depleted nasal microbial taxa, and plasma concentrations of cytokines/chemokines were evaluated using Spearman correlation to evaluate microbial network structures and coexistence patterns. Spearman correlations between nasal NM composition and plasma concentrations of cytokines/chemokines were determined using Mantel’s test based on the Bray Curtis distance matrix.

Associations between microbial community composition and patients’ parameters were assessed using permutational analysis of variance (PERMANOVA). PCoA and PERMANOVA were implemented in the vegan R package [23].
Fig. 1. Gen copies (A), and observed species (B) plots.

Fig. 2. Alpha diversity indices; Chao1 (A), Phylogenetic Diversity (PD) Whole Tree(WT) (B), and simpson (C).
3. Results

3.1. Characteristics of the participants

A total of 100 (F/M: 49/51) participants’ data were analysed. Their mean ± SD age was 53.09 ± 14.51 years. There was no significant difference in the mean age and F/M ratio between the 1st (recovered COVID-19) (n = 26), 2nd (mild COVID-19) (n = 24), 3rd (severe COVID-19) (n = 25), and 4th (healthy control) (n = 25) group participants (p > 0.05, all). Although the rate of presence of comorbidities (Hypertension, Diabetes Mellitus, coronary artery disease, chronic kidney disease, and cerebrovascular diseases) was not different between group 2 and 3, their comorbidity rates were both significantly higher than group 1 and 4 rates. Additionally, the rate of presence of comorbidities of group 4 was also significantly higher than group 1. In other words, the recovered COVID-19 patients’ group had the lowest rate of comorbidities. Although the alcohol use rate was not different between the groups (p > 0.05), the smoking rate was lowest in the recovered COVID-19 patients’ group. This lowest rate was significantly different from healthy controls and severe COVID-19 patients groups only (p < 0.05, both). Regarding body mass index (BMI), the healthy controls had the lowest median level and was only significantly different from severe COVID-19 patients’ BMI median level (See Table 1).

3.2. In vitro cytokines production profiles (Nil, Mitogen, and Mitogen-Nil tubes)

Comparison of in vitro cytokines production of Nil, Mitogen, and Mitogen-Nil tubes is shown in Table 2. The groups’ granulocyte-colony stimulating factor (GCSF) production in Nil and Mitogen tubes showed a different pattern. Regarding their subtraction (Mitogen-Nil) levels, it was lowest in the mild COVID-19 patients’ group. Although it was not significantly different from severe COVID-19 patients’ level, it was significantly lower from the other 2 study groups (p < 0.05, both). Although Mitogen and Mitogen-Nil the C-X-C motif chemokine ligand (CXCL)10 levels were not significantly different between the groups (p > 0.05, all), the Nil tube CXCL10 levels of the healthy control was significantly higher than the levels of the other 3 groups (P = 0.003). Nil, Mitogen, and Mitogen-Nil IL-1β production levels were also significantly different between the 4 study groups (P were 0.012, < 0.001, and < 0.001, respectively). The interesting point is that the recovered COVID-19 patients’ IL-1Mitogen and Mitogen-Nil concentrations were significantly higher than the other 3 study groups (P < 0.05, all). Although the IL-7 Nil levels of the healthy controls were significantly lower from the other 3 groups, the IL-7 Mitogen levels of the same groups were also the highest but only significantly different from the recovered COVID-19 patients’ group. As a result, their subtraction (i.e. Mitogen-Nil levels) was lowest in the healthy controls but only significantly different from the recovered COVID-19 patients. The IL-10 Nil levels were not different.
between the study groups. Regarding IL-10 Mitogen, and Mitogen-Nil levels, the mild COVID-19 median level was significantly lower from recovered COVID-19 patients and healthy controls groups. On the other hand, the severe COVID-19 patients’ Mitogen, and Mitogen-Nil IL-10 median levels were the lowest and significantly different from the other 3 study groups’ levels (see Table 2 for p values and post-hoc analysis). The IL-17a Mitogen and Mitogen-Nil levels of group 3 participants were significantly lower than group 1 and 4 (but not from group 2) participants’ levels. The other remaining cytokines production profiles were not significantly different between the 4 study groups (p > 0.05) (Table 2).

3.3. In vitro oxidative stress parameters

Although TOS and TAS levels were different in some tubes and/or study groups, OSI (TOS/TAS ratio) levels were not significantly different between the 4 study groups in Nil, Mitogen, and Mitogen-Nil tubes (p > 0.05, all) (Table 1).

3.4. Microbiota analysis results

Throughout the paper, k, p, c, o, f, g, and s are used to refer to the kingdom, phylum, class, order, family, genus, and species, respectively. As shown in Fig. 1A, the healthy control group has the lowest, and the severe COVID-19 group has the highest gene copies per microliter. As an α-diversity measure, a plot of Fig. 1B shows the observed species in each study group. This plot reveals that the observed species of the severe COVID-19 group is the lowest. The chao1 index is another α-diversity measure of the richness of the samples. The severe COVID-19 patients’ group had the lowest chao1 index levels, which was significantly different from the levels of the other 3 groups (Table 1, and Fig. 2A). In other words, severe COVID-19 led to the disappearance of some normally present species in the host’s nasopharynx. The Shannon diversity index is a count-based and Phylogenetic Diversity (PD) Whole Tree(WT) index is a tree-based diversity metric [24]. The Shannon diversity index was not significantly different between the groups, while the PD-WT index was lowest in the severe COVID-19 patients’ samples and significantly different from mild COVID-19 patients and healthy control groups. Another measure of richness is the Simpson diversity index. This index median level was also lowest in the severe COVID-19 patients’ group and was significantly lower from the recovered COVID-19 patients’ group. These all indicate decreased species richness of severe COVID-19 patients’ samples (Table 1, and Fig. 2).

Regarding β-diversity measures, PERMANOVA analysis revealed no significant difference between samples of the 4 study groups (p > 0.05). Further analysis by Bray-Curtis dissimilarity based principal coordinates analysis (PCoA) is shown in Fig. 3. On PCoA of weighted (quantitative) and unweighted (qualitative) UniFrac operational taxonomic units (OTUs) profile distances, some of the severe COVID-19 patients’ samples were separated from the main overlapped samples (more prominent in unweighted UniFrac metric distance (Fig. 3 B, and C) [25,26].

A comparison of NM at phylum level is shown in Fig. 4. At the phylum level, 3 of them were significantly different between the study groups (Kruskal-Wallis test). Fusobacteria were relatively more abundant in recovering patients and least in the severe COVID-19 patients’ group. The mean abundance (%) of this phylum in the severe COVID-19 patients’ group was significantly lower than the other 3 study groups. Saccharibacteria showed somewhat a similar pattern. The mean abundance of Spirochaetae was also low in severe COVID-19 patients’ group. But this difference reached a statistical significance only in comparison with the healthy controls group mean (See Table 3 for means and post-hoc analysis). Linear discriminant analysis Effect Size (LEfSe) analysis was used to identify taxa whose distributions are significantly and statistically different among study groups (Fig. 5). As in Yang XA, et al. study [27], 20 strains with an LDA effect size > 4 were identified by LEfSe analysis. It be noticed that none of these identified taxa was abundant in healthy controls. The distribution of the abundance of these strains in recovered, mild, and severe COVID-19 patients’ groups was 12, 5, and 3, respectively (See Table 4).
Fig. 5. Comparison of microbiota species by Linear discriminant analysis effect size (LEfSe) tool with a p-value < 0.05 and the effect size (LDA score) > 2.
Kines levels or microbiota - * Kruskal-Wallis test, Mann-Whitney

Table 3

| Phyla       | Groups                        | Recovered | Mild COVID-19 | Severe COVID-19 | Healthy Controls | P value* |
|-------------|-------------------------------|-----------|---------------|-----------------|-----------------|----------|
| Fusobacteria| Mean abundance(%)             | 0.109     | 0.091         | 0.049           | 0.071           | <0.05    |
|             | Post-hoc                      | b         | a             | c               | a               |          |
| Saccharibacteria| Mean abundance(%)             | 0.031     | 0.017         | 0.010           | 0.026           | <0.05    |
|             | Post-hoc                      | b         | a             | c               | b               |          |
| Spirochaetae| Mean abundance(%)             | 0.002     | 0.002         | 0.002           | 0.005           | <0.05    |
|             | Post-hoc                      | ab        | ab            | ab              | ab              |          |

a Kruskal-Wallis test, Mann-Whitney U test were applied for post-hoc pairwise comparisons and Bonferroni adjusted p values are given where equal letters indicate no significant differences (p > 0.05) and vice versa.

3.5. Correlation analysis

Correlation analysis showed no association between BMI and cytokine levels or microbiota α-diversity indices (i.e. the Shannon, chao1, observed species, PD whole tree, and simpson indices) (p > 0.05).). The Chol index showed a significant correlation with CXCL-10 Nil (R = 0.31, and p = 0.0016).

Correlation plots of each operational taxonomic unit (OTU) with *in vitro* cytokine production and/or oxidative stress parameters are performed. As in Yeoh YK, et study [28], only the significant correlations (R > 0.3 < -0.3) are given (Fig. 6 A–D).

IL-1β Mitogen-Nil and, and IL-1β Mitogen levels were both negatively correlated with p_Actinobacteria.c_Acetinobacteria.o_Micrococcales.f_Micrococcaceae.g_Rothia & both positively correlated with p_Fusobacteria.c_Fusobacteriia.o_Fusobacteriales (Fig. 6A, and B). On the other hand, IL-10 Mitogen, and IL-10 Mitogen-Nil levels were both positively correlated with p_Bacteroidetes.c_Bacteroidia.o_Bacteroidales.f_Porphromonadaceae (See (Fig. 6A-D for R and P values). The distribution of these 3 correlated microbiota taxa between the study groups is shown in eFigure 2A-C.

4. Discussion

More than one year is passed since the beginning of the COVID-19 pandemic. But still, we are trying to find our feet. According to our knowledge, this is the 1st study that evaluates *in vitro* oxidative stress parameters and cytokines production by PBMCs in COVID-19. Infections (including COVID-19) are expected to alter the oxidative stress status of the host [13,29]. Surprisingly, *in vitro* OSI levels, were not significantly different between the test tubes and/or study groups of this study (Table 1). Another striking finding of our study is the patterns of IL-10 in the 4 study groups and in the studied *in vitro* test tubes (i.e Nil, Mitogen, and their subtraction [Mitogen-Nil]) as well. Although basal (Nil tube) IL-10 production was not significantly different between the 4 study groups, it was highest in the severe COVID-19 patients’ group (p > 0.05). On the other hand, the antigens’ provoked (Mitogen) and Mitogen-Nil tubes IL-10 production pattern were opposite. In other words, Mitogen, and Mitogen-Nil IL-10 levels of the severe COVID-19 patients were significantly lower from the remainder 3 groups (p < 0.05). Additionally, Receiver operating characteristic (ROC) analysis yielded a cutoff point of ≤ 59.20 pg/ml to differentiate severe COVID-19 patients from the other remainder 3 group participants with a sensitivity and specificity of 86.67, and 72.00%, respectively. The area under the curve for this cutoff point was 0.78 [30]. Of course, this pilot cutoff point of IL-10 Mitogen-Nil needs to be validated by further large-scale studies. Interleukin-17a production showed a somewhat similar pattern as IL-10. The only difference between them is that the IL17a levels of the mild COVID-19 patients were also significantly lower from the recovered and control groups, but not from the severe COVID-19 patients (See Table 2). Interleukin-10 is an anti-inflammatory cytokine that helps regulate the immune response to pathogens, thereby preventing and/or minimizing damage to the host [31,32]. When this immunomodulatory effect of this cytokine is taken into consideration, one could not correlate the highness of serum IL-10 levels with the disease severity and/or the bad outcomes shown by the previous studies [4,33,34]. The lowest Antigens provoked IL-10 by the severe COVID-19 patients PBMCs is more illustrative of the beneficial immunomodulatory effect of IL-10 during infections. Some authors speculate that IL-10 influences the local microenvironment through tissue-specific regulatory mechanisms [35]. Experimental studies showed that orally administered probiotic *Bifidobacterium longum*51A has a beneficial protective effect against *Klebsiella pneumonia* induced lung injury. This beneficial effect is associated with the induction of IL-10 secretion in the lung [32,36]. More interestingly, intranasal administration of some probiotics had led to the modulation of T helper type 2 (Th2)-biased immune response and an increase in the IL-10 expression of the lung tissues [37]. Could these therapeutic approaches be used in the protection and/or decreasing the severity of COVID-19? Need to be evaluated. We should mention that our pilot study showed a significant correlation between *in vitro* IL-10 Mitogen and Mitogen-Nil levels and some NM species (see Fig. 6 C, and D). Some researchers have revealed that oral *Lactobacillus rhamnosus* GG administration has immunoregulatory properties. Administration of this probiotic has been associated with an increase of serum IL-10 levels. This increase was usually obvious after a 8 weeks of follow-up periods. Pessi T, et al, observed that the enhancement of IL-10 production in mitogen-induced cultures was preceded the in vivo rise [38]. This finding of the above study may explain why antigens provoked (but not basal) IL-10 levels correlate with our NM p_Bacteroidetes.c_Bacteroidia.o_Bacteroidales.f_Porphromonadaceae species.

The IL-17 cytokine family is not well understood. Although it is implicated in the pathogenesis of some auto-immune diseases, it is shown to have a protective and/or beneficial role in inflammation, infections, injury, and cancer [39]. Some studies showed elevated serum IL-17a in COVID-19. Therapies targeting this cytokine (anti-IL-17a inhibitor) are on the agenda of some researchers [40,41]. Our study results do not support this therapeutic approach. Another point that may support our study findings and our expectation of the ineffectivity of anti IL-17a inhibitors is the finding of lower levels of IL-17a in COVID-19 patients with prolonged SARS-CoV-2 RNA shedding during the acute phase of the disease [42]. So, our results show that *in vitro* provoked the production of these 2 cytokines (especially IL-10) may be candidate biomarkers in predicting the severity of COVID-19 disease [11]. Still, further detailed studies are needed in this field. In an open-label, Bayesian randomized clinical trial (CORIMUNO-ANA-1) [43], IL-1 in-hibitor drug “Anakirna” was found useless to improve the outcomes of mild-moderate COVID-19 pneumatic patients. In our study, these patients had the lowest in *in vitro* IL-1 β Nil, Mitogen, and Mitogen-Nil levels (Table 2). This finding of our studies is in line with the above CORIMUNO-ANA-1 trial results. So a such *in vitro* basal and evoked cytokine(s) production measures may guide future cytokine(s) based therapeutic trials. As we know from real-life practice, recently recovered COVID-19 patients are mostly protected from re-infection by the same virus (naturally acquired immunity). In our study, this group of patients had the highest Mitogen and Mitogen-Nil tubes IL-1β production levels.
### Table 4
Comparison of microbiota species with a LDA > 4.

| OTU | M – W U | The group in which taxa is more abundant | Effect size | p. value |
|------|---------|-----------------------------------------|------------|---------|
| k_Bacteria_p_Fusobacteria. c_Fusobacteriia. o_Fusobacteriales. f_Lepotrichiaceae. g_Lepotrichia. | 4,76 | Recovered COVID-19 | 3,32 | 0.0011 |
| k_Bacteria_p_Fusobacteria. c_α-Neisseria_prototropha. o_α-Neisseriales. | 4,99 | Recovered COVID-19 | 4,54 | 0.0004 |
| k_Bacteria_p_Proteobacteria. c_Alpha_protobacteria. o_Neisseriales. f_Neisseriaceae_g_α-Neisseria. | 5,00 | Recovered COVID-19 | 4,54 | 0.0006 |
| k_Bacteria_p_Bacteroidetes. c_Bacteroidia. o_Bacteroidales. g_Bacteroidaceae. | 4,65 | Recovered COVID-19 | 4,08 | 0.0080 |
| k_Bacteria_p_Bacteroidetes. c_Bacteroidia. o_Bacteroidales. g_Porphyrmonadaceae. | 4,61 | Recovered COVID-19 | 4,08 | 0.0023 |
| k_Bacteria_p_Bacteroidetes. c_Bacteroidia. o_Bacteroidales. g_Porphyrmonadaceae. g_Porphyrmonas. | 5,59 | Mild COVID-19 | 4,74 | 0.0130 |
| k_Bacteria_p_Fusobacteria. c_Fusobacteriia. o_Fusobacteriales. f_Fusobacteriaceae. g_Fusobacterium. | 4,79 | Mild COVID-19 | 4,57 | 0.0018 |
| k_Bacteria_p_Fusobacteria. c_Fusobacteriia. o_Fusobacteriales. f_Fusobacteriaceae. g_Fusobacterium. s_mucosa_perflava_subflava. | 4,07 | Recovered COVID-19 | 4,35 | 0.0021 |
| k_Bacteria_p_Phyllobacteriaceae. g_Phyllobacterium. s_periodonticum. | 4,55 | Severe COVID-19 | 4,07 | 0.0061 |
| k_Bacteria_p_Bacteroidetes. c_Betaproteobacteria. o_Neisseriales. | 5,00 | Recovered COVID-19 | 4,54 | 0.0006 |
| k_Bacteria_p_Pseudomonas. c_Pseudomonadaceae. | 4,69 | Mild COVID-19 | 4,35 | 0.0023 |
| k_Bacteria_p_Actinobacteria. c_Actinobacteriia. o_Actinomycetales. f_Micrococcales. | 4,55 | Severe COVID-19 | 4,07 | 0.0061 |
| k_Bacteria_p_Pseudomonas. c_Pseudomonadaceae. f_Pseudomonadaceae. g_Pseudomonas. | 5,05 | Recovered COVID-19 | 4,51 | 0.0006 |
| k_Bacteria_p_Actinobacteria. c_Actinobacteriia. o_Actinomycetales. | 4,55 | Severe COVID-19 | 4,07 | 0.0045 |
| k_Bacteria_p_Pseudomonas. c_Pseudomonadaceae. o_Pseudomonads. | 5,04 | Recovered COVID-19 | 4,58 | 0.0007 |
| k_Bacteria_p_Bacteroidetes. c_Betaproteobacteria. o_Bacteroidales. | 5,05 | Recovered COVID-19 | 4,51 | 0.0006 |
| k_Bacteria_p_Pseudomonas. c_Pseudomonadaceae. f_Pseudomonadaceae. g_Pseudomonas. | 4,79 | Mild COVID-19 | 4,37 | 0.0018 |
| k_Bacteria_p_Fusobacteria. c_Fusobacteriia. o_Fusobacteriales. f_Fusobacteriaceae. g_Fusobacterium. s_mucosa_perflava_subflava. | 5,39 | Mild COVID-19 | 4,77 | 0.0065 |

(p < 0.005) (see Table 2). This cytokine has a protective anti-viral effect. In some experimental studies, hyperactivation of the NLRP3 inflammation led to protection from influenza A virus infection via IL-1 β mediated neutrophil recruitment [44]. Studies with recombinant IL-1β showed its antiviral activities in several cell lines [45]. Could the protection of re-infection of this group be IL-1 β mediated? This issue may be clarified better by conducting such IL-1 β based evaluation before and after COVID-19 vaccination. By the way, IL-1β Mitogen and IL-1 β Mitogen-Nil were as negatively correlated with p_Bacteroidetes. c_Bacteroidia.o_Bacteroidiales.f_Porphyrmonadaceae species abundance (Figs. 6 A1-2, and B1-2). As shown in Table 4 and eFigure 2A, this taxon is more abundant in recovered COVID-19 patients. Other studies also showed that microbiota influences cytokine(s) (including IL-1β) production (and vice versa) [32,46].

Nonetheless, a study by Seo s, et al. showed that some species of gut microbiota stimulate newly recruited monocytes to induce NLRP3-dependent IL-1β realase [47]. Whether this is the case in our study too, needs to be furtherly studied. In the sight of future such studies, this cytokine and/or the mentioned microbiota species may be used as a prophylactic and/or preventive measure at the time of expected or real exposure to this virus. In their recent study, Nardelli C and colleagues found Fusobacteria a less abundant phylum in COVID-19 patients (in comparison to healthy controls) [21]. Our study results also showed the lowest abundance of this phylum in severe COVID-19 patients (in comparison to mild, recovered COVID-19 patients, and healthy controls (see Table 3).

Additionally, we identified the top 20 NM taxa candidates for biomarkers and/or intervention measures in the war against this challenging viral infection where its main transmission route is the respiratory system. The striking point is that none of these 20 microbiota taxa was abundant in the healthy controls. This finding also supports the potential role of these identified taxa in the acute phase and the convalescent period of COVID-19 infection.

This study has some limitations. The main limitation of this study is being a cross-sectional one. Still, we have included all types of COVID-19 patients (including the recovered one). Only critically ill COVID-19 patients were excluded. The reason of this exclusion is that postponing their urgent intervention(s) and treatment(s) for the purposes of a prophylactic and/or preventive measure at the time of expected or real exposure to this virus [48]. As we mentioned above, the rate of comorbidities and/or smoking was different between the study groups. The presence of comorbidities had even no or small effect on most of the parameters that showed significant differences between the study groups. Only IL-1β Mitogen levels showed a moderate association with the presence of the comorbidities (Omega squared (ω²) = 0.118, p < 0.001). On the other hand, Smoking did not show any significant relation with these study parameters. Nonetheless, these study parameters were significantly different between groups with no significant differences in these mentioned confounding factors (Table 1, and 2).

### 5. Conclusions
The findings of this pilot study in COVID-19 showed that in vitro cytokine production studies might be more beneficial than ordinary measuring blood levels. Additionally, the study identified some nasopharynx microbiota species that could be candidate biomarkers in managing this infection. There was a significant correlation between these microbiota species and in vitro cytokines production levels (especially IL-10 and IL-1β). Further large-scale studies pointing to these
findings are needed to help develop such biomarker(s) that can be used in the diagnosis and/or management of this challenging viral disease.

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**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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**Appendix A. Supplementary material**

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