Microb Health Dis 2020; 2: e326

European Helicobacter & Microbiota Study Group

THE INFLUENCE OF LUNG AND OROPHARYNGEAL MICROBIOTA-MYCOBIOTA ON VENTILATOR-ASSOCIATED PNEUMONIA OCCURRENCE IN CRITICALLY ILL PATIENTS: A SYSTEMATIC REVIEW

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Abstract – *Objective:* Ventilator-associated pneumonia (VAP) is a leading cause of infections in intensive care units. VAP is associated with prolonged length of invasive ventilation and increased mortality rate. Microbiota has been suggested to be involved in the development of numerous respiratory conditions, including acute bacterial infections. The aim of this study is to systematically review the evidence of microbiota and mycobiota influence on VAP occurrence.
Materials and Methods: Every article focusing on microbiota, mycobiota and ventilator-associated pneumonia available on the MEDLINE database was assessed. Studies were considered suitable for analysis if they included microbiota analysis and focused on patients receiving invasive mechanical ventilation.
Results: From 54 articles referenced on PubMed, 19 abstracts were selected for full-text assessment and 10 studies were included; only two reporting mycobiota analysis. Methods for DNA extraction, library preparation, sequencing and bioinformatics analysis were highly heterogeneous. A lower α diversity of lung microbiota seems to be associated with the occurrence of VAP and with an early colonization with *Enterobactericeae*. Lung microbiota composition co-evolves with oro-pharyngeal microbiota suggesting interconnections that could contribute to explain lung microbiota modification.
Conclusions: Lung and oro-pharynx microbiota are associated with the occurrence of VAP. Their dynamics is highly suggestive of transcolonization from the gut and occurrence of VAP is associated with a decreased α diversity. Surprisingly, gut microbiota influence on VAP occurrence has not been investigated. Data are also lacking on the fungal and viral compartments of microbiota. Further insights regarding these issues and a better understanding of the underlying mechanisms are needed before tailoring prevention of VAP based on microbiota composition.

Keywords: Microbiota, Mycobiota, Intensive care unit, Ventilator-associated pneumonia.

INTRODUCTION

Lower respiratory tract infections are the leading cause of nosocomial infections in intensive care unit (ICU)[1,2]. Pneumonia can be distinguished according to whether it occurs in the absence or in the presence of invasive mechanical ventilation. Ventilator-associated pneumonia (VAP) are defined by pneumonia occurring at least 48 hours after oro-tracheal intubation[3]. Despite

recent advances in the understanding of physio-pathological mechanisms responsible for VAP, few advances have been made in preventive measures. VAP affects 20-40% of patients receiving invasive mechanical ventilation and more than 70% of the most severe patients, such as those with acute respiratory distress syndrome. Its incidence is positively correlated with the duration of invasive mechanical ventilation (about 10 to 25‰ days of mechanical ventilation)[3-5]. The occurrence of VAP is associated with severe morbidity leading to increased duration of invasive mechanical ventilation and hospitalization and to a dramatic crude mortality rate ranging from 25 to 75% of patients[3,6,7]. Several risk factors for the development of VAP have been previously described; however, a role for oro-pharyngeal and lung microbiota has only recently been evoked. In fact, microbiota has been proven to be involved in numerous chronic respiratory diseases such as asthma, chronic obstructive pulmonary disease or cystic fibrosis but also in acute infectious diseases such as influenza or bacterial pneumonia[8]. The aim of this study is to systematically review the evidence of microbiota influence on VAP occurrence.

MATERIALS AND METHODS

Search Strategy

We searched the MEDLINE database for English language articles published from the inception of the database to May 11, 2020. A combination of MeSH/Emtree and title/abstract keywords was used. The search terms were "microbiota", "microbiome", "mycobiota", "mycobiome", "virobiota", "virobiome" and "ventilator-associated pneumonia".

Eligibility Criteria

Studies were considered suitable for inclusion in this systematic review if (1) they really investigated microbiota composition after next-generation sequencing and/or association of bacterial species identified by next-generation sequencing with clinical outcomes, (2) if they assess the occurrence of ventilator-associated pneumonia, (3) all the patients were adults, and (4) they were written in English and published in a journal with peer-reviewing. If the studies lacked outcome data or provided only flora investigation by routine culture, they were excluded. If the full text could not be retrieved or if the article was a commentary or a review or an erratum, it was excluded.

Selection of Studies and Data Extraction

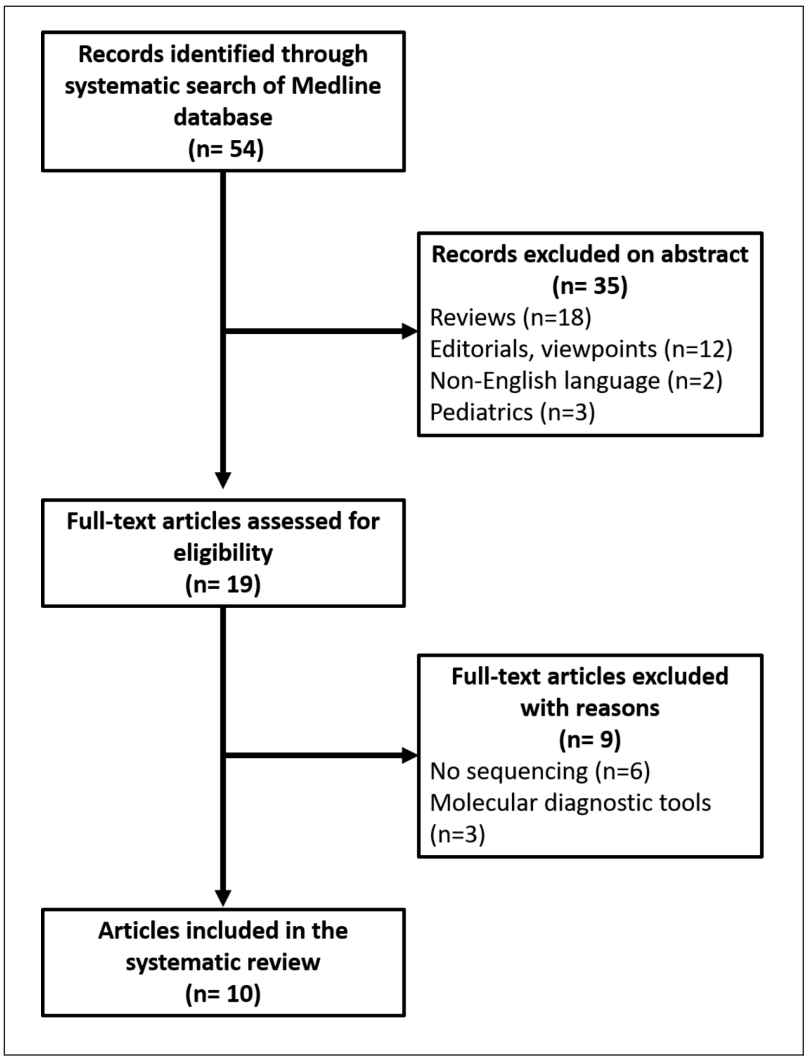
All the available data were extracted from each study by two investigators (RP and PB) independently according to the aforementioned inclusion criteria, and any differences were resolved by discussion with a third investigator (DG). The following data were collected from each study: the name of the first author, publication year, study design, number of patients, type of samples and of next-generation sequencing (NGS) primary outcome, and conclusions for the primary outcome.

RESULTS

STUDY SELECTIONS

From 54 articles referenced using the previously described request in MEDLINE database, 19 appeared to address issues relevant for this review and were selected for full-text assessment after reading the abstract. After full-text assessment, 10 studies were included in the systematic review (Fig. 1). Methods used to obtain amplicons and bioinformatics pipelines were highly heterogeneous between the different studies (Table 1). Four studies were relevant regarding the influence of lung microbiota-mycobiota on VAP occurrence (Table 2), 3 regarding the crosstalk between lung and oro-pharynx microbiota (Table 3) and 3 regarding both oro-pharynx and lung microbiota influence on VAP occurrence (Table 4).

Figure 1. Flow-chart.



The Role of Lung Microbiota in Ventilator Associated Pneumonia Occurrence

The first insight in the role of lung microbiota in VAP occurrence was the description of differences in the bacterial and fungal composition of lung microbiota of VAP patients *vs.* controls by Bousbia et al[9] in 2012. The authors identified a wide repertoire of 160 bacterial species of which 73 have not been previously reported in pneumonia and with 37 putative new species. They found significant differences between microbiota of patients with VAP and controls, with more bacteria belonging to *Bacilli* and *Gammaproteobacteria* in patients with VAP whereas anaerobic bacteria related to *Bacteroidia* and *Clostridia* were more represented in controls ($p < 0.01$). Regarding fungi, *Agaricomycetes* and an unclassified *Ascomycota* were only identified in the VAP cohort (Table 2). This study was the first to illustrate lung microbiota-mycobiota in ventilated patients and microbiota differences between patients with or without VAP.

A larger cohort including 35 patients receiving invasive ventilation (11 VAP) was described in 2017 by Zakharkina et al[10]. This study confirmed that duration of mechanical ventilation is associated with a decrease in lung microbiota α diversity (Shannon index) but that the administration of antibiotic therapy is not a major determinant of this decrease (fixed-effect regression coefficient (β): -0.03, 95CI: -0.05 to 0.005 and 0.06, 95CI: -0.17 to 0.30 respectively). Moreover, a significant difference in changes of β diversity was observed between patients who developed VAP and controls (Bray-Curtis distances: $p = 0.03$, Manhattan distances: $p = 0.04$) but no difference in terms of α diversity was observed. *Burkholderia*, *Bacillales*, and, to a lesser extent, *Pseudomonadales*, were positively correlated with these changes in ß diversity in patients who will subsequently develop VAP (Table 2).

TABLE 1. SEQUENCING PROCESS AND BIOINFORMATICS ANALYSIS FOR MICROBIOTA-MYCOBIOTA ANALYSIS.

Year	Authors	Extraction	Amplification	Sequencing	Bioinformatic analysis
2012	Bousbia et al[9]	Magna Pure DNA Isolation Kit II, Roche Diagnostics	**16S rDNA** Primers 536F/rp2 **18S rDNA** Primers P-ITS1/ITS2	ABI PRISM 3130xl genetic analyser, Applied Biosystems	Chimera removal: Black Box Chimera check program and analysis of each sequence BLAST profile Assembling: chromaspro software Assignment: GenBank database
2016	Hotterbeekx et al[14]	Masterpure complete DNA and RNA purification kit	**V3-V5 16S rRNA** Primers V345-341F/ V345-909R **ITS-II** Primers ITS-F3/ITS-R4	454 GS FLX sequencer, Roche	Chimera removal: UCHIME Assembling: Mothur Assignment: SILVA database Parallel analysis on the online server MetaGenome Rapid Annotation, Subsystem technology
2016	Sands et al[15]	Yeast/Bac DNA extraction kit	**16S rRNA** Primers 27f/1492r	MiSeq, Illumina	Chimera removal: Mothur Assembling: Mothur Assignment: RDP Multiclassifier tool
2016	Marino et al[16]	Gentra PureGene Yeast/Bacteria kit	**V4 16S rRNA** Primers 28F/388R	MiSeq, Illumina	Chimera removal: Mothur Assembling: Mothur Assignment: RDP Multiclassifier tool
2016	Kelly et al[17]	MoBio Power Soil DNA extraction kit	**V1-V2 16S rRNA** Primers 27F/338R	MiSeq, Illumina (454 GS FLX sequencer, Roche for healthy controls)	Chimera removal: QIIME 1.8.0 Assembling: QIIME 1.8.0 / PyNast Assignment: Greengenes and Living Tree Project database
2017	Zakharkina et al[10]	MoBio Power Soil DNA extraction kit	**16S rRNA** Primers F-16S-27/ R-16S-355	454 GS FLX sequencer, Roche	Chimera removal: ChimeraSlayer Assembling: QIIME, 1.9.0 Assignment: European Nucleotide Archive
2018	Huebinger et al[11]	MoBio Power Soil DNA extraction kit	**V4 16S rRNA** Primers 515F/806R	Ion Torrent Personal Genome Machine	Chimera removal: UCHIME Assembling: Mothur v1.3.6.1 Assignment: SILVA database
2018	Qi et al12	From freeze-dried powder	**V3-V4 16S rRNA** Primers F1/R2	MiSeq, Illumina	Chimera removal: USEARCH 8.0 Assembling: USEARCH 8.0 Assignment: SILVA database
2019	Sommerstein et al[18]	Qiagen DNA Minikit	**V4 16S rRNA** Primers F515/R806	MiSeq, Illumina	Chimera removal: DADA2 pipeline Assembling: DADA2 pipeline Assignment: European Nucleotide Archive
2019	Emonnet et al[19]	NucleoSpin Soil Kit, Macherey-Nagel	V3-V4 16S rRNA Primers 341F/785R Nested PCR after V1-V6 rDNA amplification Primers GM3/1061R	MiSeq, Illumina	Chimera removal: Mothur Assembling: USEARCH Assignment: RDP reference database

16S rRNA: gene coding for ribosomal 16S or 18S sub-unit RNA.

TABLE 2. INFLUENCE OF LUNG MICROBIOTA AND MYCOBIOTA ON VENTILATOR-ASSOCIATED PNEUMONIA OCCURRENCE.

Year	Authors	Design	Samples	Outcome	Brief results
2018	Huebinger et al[11]	Unicentric, prospective, case-control cohort	Broncho-alveolar lavage	Culture positive VAP (n: 7) Culture negative VAP (n: 7) Respiratory tract flora on culture (n: 8)	Culture positive VAP had the lowest levels of diversity (Shannon index): culture negative: 3.97 ± 0.65, respiratory tract flora: 2.06 ± 0.73, culture positive: 0.77 ± 0.36 Culture positive and respiratory tract flora samples showed increased levels of pro-inflammatory cytokines compared with culture negative ones.
2018	Qi et al[12]	Unicentric, prospective, case-control cohort study	Tracheal aspirate	*Pseudomonas aeruginosa* VAP (n: 36)	*Pa* VAP patients had lower levels of diversity (Shannon index) than controls (p: 0.003) *Pa* VAP patients microbiota was dissimilar to those of control patients (weighted UniFrac distance, R^2: 0.38, $p = 0.001$)
2017	Zakharkina et al[10]	Unicentric, prospective, case-control cohort study	Tracheal aspirate	VAP (n: 11)	Duration of mechanical ventilation was associated with a decrease in α diversity but not the administration of antibiotic therapy (Shannon index, fixed-effect regression coefficient (β): -0.03, 95CI: -0.05—0.005 and 0.06, 95CI: -0.17-0.30 respectively) A significant difference in change of diversity was observed between patients who developed VAP and controls (Bray-Curtis distances: $p = 0.03$, Manhattan distances: $p = 0.04$)
2012	Bousbia et al[9]	Unicentric, prospective, case-control cohort study	Broncho-alveolar lavage	Low-respiratory tract infections including VAP (n: 106)	Bacteria belonging to *Bacilli* and *Gammaproteobacteria* were dominant in patients whereas anaerobic bacteria related to *Bacteroidia* and *Clostridia* were dominant in controls (p: < 0.01). *Agaricomycetes* was only identified in VAP patients.

ICU: intensive care unit, Pa: *Pseudomonas aeruginosa*. VAP: ventilator-associated pneumonia.

TABLE 3. RELATION BETWEEN LUNG AND ORO-PHARYNX MICROBIOTA AND MYCOBIOTA.

Year	Authors	Design	Samples	Outcome	Brief results
2016	Hotterbeekx et al[14]	Unicentric, prospective, case-control cohort study	ETT	VAP (n: 44)	Presence of *Pseudomonas aeruginosa* negatively correlates with patient survival and with bacterial species diversity in endotracheal tube Patients with a relative abundance of *Pseudomonadaceae* < 4.6% and of *Staphylococcaceae* < 70.8% have the highest chance of survival *Candida spp.* were the most common fungi in the ETT mycobiota but no fungus was clearly identified as being associated with the occurrence of VAP
2016	Sands et al[15]	Unicentric, prospective, case-control cohort study	Dental plaque	Dynamics of dental plaque microbiota (n: 13)	A significant "microbial shift" in the dental plaque microbiota occurred during mechanical ventilation for 9/13 invasively ventilated patients. After extubation, relative abundance of potential respiratory pathogens decreased substituted by oral microbiota, mainly *Prevotella spp.* and streptococci
2016	Marino et al[16]	Unicentric, prospective, case-control cohort study	Dental plaque ETT BAL	Dynamics of dental plaque microbiota, ETT and lung microbiota (n: 12)	No significant differences in the microbial communities of these samples were evident Detected bacteria were primarily oral species with potential respiratory pathogens

BAL: broncho-alveolar lavage. ETT: endotracheal tube. VAP: ventilator-associated pneumonia.

TABLE 4. INFLUENCE OF ORO-PHARYNX MICROBIOTA ON VENTILATOR-ASSOCIATED PNEUMONIA OCCURRENCE.

Year	Authors	Design	Samples	Outcome	Brief results
2019	Emonnet et al[19]	Unicentric, prospective, case-control cohort study	Oropharyngeal swabs Tracheal aspirate	VAP (n: 18)	Low relative abundance of *Bacilli* at the time of intubation in the oropharyngeal microbiota is associated with the subsequent development of VAP (AUC: 0;85, $p < 0.0001$, sensitivity: 81.25%, specificity: 82.86%) No significant change in oro-pharyngeal microbiota was observed between patients who develop VAP and controls (PERMANOVA, $p > 0.05$) Molecular techniques are able to identify the causative pathogen identified by culture but also difficult-to-grow bacteria such as *Mycoplasma spp*. and anaerobes.
2019	Sommerstein et al[18]	Unicentric, prospective, case-control cohort study	Oropharyngeal swabs Tracheal aspirate	VAP (n: 5)	VAP patients have lower oropharyngeal microbiota α diversity than controls Detection of *Enterobacteriaceae* in oropharynx occurred early in the course of mechanical ventilation and consisted in a single OTU in 2/3 patients with enterobacterial VAP
2016	Kelly et al[17]	Unicentric, prospective, case-control cohort study	Oropharyngeal swabs Tracheal aspirate	Longitudinal analysis and correlation with VAP occurrence (15 patients, 4 VAP)	Critically ill patients had lower levels of α diversity of lung and oropharyngeal microbiota compared with controls and diversity further diminished over time of intubation Diagnosis of VAP correlated with low diversity and dominance of a single taxon and dominant taxa matched with clinical bacterial cultures when positive.

ICU: intensive care unit. OTU: operational taxonomic unit. VAP: ventilator-associated pneumonia.

A third study aimed to decipher the variations of lung microbiota according to the results of bacteriological cultures (culture positive, culture negative or respiratory tract flora on BAL) during the episode of VAP or systematic screening after 36h of invasive ventilation[11]. Twenty-two individual BAL were available: 7 BAL with positive culture, 7 with negative culture negative and 8 with a respiratory tract flora. Culture positive BAL had a lower α diversity than respiratory tract flora and culture negative BAL (Shannon index respectively 0.77 ± 0.36, 2.06 ± 0.73 and 3.97 ± 0.65). Moreover, culture positive BAL were dominated by single bacterial genera but not culture negative ones. Interestingly in this study, BAL classified as respiratory tract flora were more similar to the culture positive ones in the microbiome profile than the culture negative ones. Regarding immune responses, culture positive and respiratory tract flora samples showed increased levels of pro-inflammatory cytokines compared with culture negative ones (Table 2). The fact that those culture negative BAL had a greater number of bacterial species found by sequencing techniques but were associated with a lower pro-inflammatory cytokines production enhances the hypothesis of a resident non-pathogenic bacterial community as the normal lung environment.

Finally, Qi et al[12] specifically focused on *Pseudomonas aeruginosa* VAP using TA. Patients with *P. aeruginosa* VAP had significantly different lung microbiota composition compared with those of non-infected intubated patients (weighted UniFrac distance R^2: 0.38, $p = 0.001$). At baseline, independently of the subsequent occurrence of VAP, two clusters were identified depending on the primary diseases of the patients (Chi2-test, $p < 0.0001$). The first cluster, including patients with gastro-intestinal disorders, was dominated by *Proteobacteria* and the second, including patients with a respiratory disease, by *Firmicutes* and *Bacteroidetes*. Lung microbiota of *P. aeruginosa* VAP patients also had a lower α diversity than those of patients without VAP ($p < 0.01$). A negative correlation was found between presence of *Lactobacillus* in lung microbiota and clinical pulmonary infection score (CPIS) on the day of the diagnosis of VAP ($p = 0.01$) (Table 2).

The Link Between Lung and Oro-Pharynx Microbiota During Mechanical Ventilation

Since lungs and oropharynx are connected by the endotracheal tube (ETT) in patients receiving invasive mechanical ventilation, the first study focused on ETT microbiota-mycobiota and microbial consortium co-developing with the known pathogens *P. aeruginosa* and *Staphylococcus epidermidis*[13]. In this study including 203 patients, 44 of whom subsequently progressed to VAP, abundance of ETT biomass did not correlate with VAP etiology or patient survival ($p = 0.1$ for both). ETT colonization with *P. aeruginosa* was associated with a lower ETT microbiota α diversity than colonization with *S. epidermidis* ($p = 0.03$). Among the *S. epidermidis* colonized patients, presence of *Klebsiella pneumoniae* and *Serratia marcescens* were associated with occurrence of VAP (LDA > 4). *Candida spp*. were the most common fungi found in the ETT mycobiota, more frequently in ETT harboring *S. epidermidis* than *P. aeruginosa*, but no fungus was clearly identified as being associated with the occurrence of VAP (Table 3).

Sands et al[14] then focused on the changes of the dental plaque microbiota during mechanical ventilation. A microbial shift in the composition of the dental plaque was demonstrated during mechanical ventilation for 9 out of 13 patients with the acquisition of several potential respiratory pathogens including *Staphylococcus aureus*, *Streptococcus pseudopneumoniae* and *Escherichia coli*. Both the prevalence and abundance of potential respiratory pathogens were shown to decrease following extubation with a compositional change towards a more predominantly oral microbiota in terms of abundance, including *Prevotella spp.* and *Streptococci* (Table 3).

Assessing the link between the microbiota of dental plaque, ETT and lung microbiota, Marino et al[15] included 12 patients receiving invasive ventilation. No significant differences in the microbial communities of these samples were evident; detected bacteria were primarily oral species (e.g., *Fusobacterium nucleatum*, *Streptococcus salivarius*, *Prevotella melaninogenica*) with potential respiratory pathogens (*S. aureus*, *P. aeruginosa*, *Streptocuccus pneumoniae*

and *Haemophilus influenzae*) (Table 3). This high similarity between these microbiomes suggests major interconnections between the oropharyngeal and lung microbiota and a role for this axis in the development of VAP.

The role of Oropharynx-Lungs Axis in Ventilator-Associated Pneumonia Occurrence

In 2016, Kelly et al[16] further analyzed the dynamics of lung microbiota by repetitive tracheal TA of intubated patients, compared it to lower respiratory tract secretions obtained by bronchoscopy in healthy subjects and correlated the diversity with oro-pharyngeal microbiota and the occurrence of VAP. In this study, 15 intubated critically ill subjects had a lower initial α diversity of lung microbiota (Shannon index) than healthy controls, and this diversity lowered with the duration of mechanical ventilation but antibiotics exposure did not fully account for this decrease in lung microbiota diversity. Intubated subjects had distinct lower respiratory tract bacterial communities compared to healthy controls. Healthy subjects' lower respiratory tracts communities were dominated by the families *Prevotellaceae, Streptococcaceae* and *Veillonellaceae*, contrasting with intubated subjects who had lower respiratory tract communities dominated by specific taxa at most time points. Clinical diagnosis of VAP (n: 4) could correlate with a decreased α diversity of lung microbiota but the analysis did not reach statistical significance ($p = 0.08$). Nevertheless, patients with VAP had significantly lower lung microbiota α diversity compared with intubated patients without pneumonia ($p < 0.01$). Dominant taxa matched clinical bacterial cultures when cultures were positive. In case of suspicion of VAP with negative cultures, microbiota analysis allowed the detection of *Ureaplasma parvum* or *Enterococcus faecalis* in some patients (Table 4).

Another study[17] investigated the longitudinal dynamics of oropharyngeal microbiota and its relation with lung microbiota (TA). Five out of 10 patients developed VAP. Patients who developed *Enterobacteriaceae* VAP seemed to have lower oropharyngeal α diversity than controls despite the lack of statistical comparison due to the small size of the cohort. Dissimilarity (β diversity) was not different between *Enterobacteriaceae* VAP, *H. influenza* VAP and controls. Interestingly, detection of *Enterobacteriaceae* in oro-pharyngeal microbiota occurred mostly in patients who will subsequently develop VAP and early in the course of invasive ventilation. This colonization consisted in a single OTU in 2/3 patients suggesting the colonization of oropharyngeal microbiota by the pathogens before lung colonization (Table 4).

Finally, during the same time (2019), Emonet et al[18] performed a similar analysis on a larger cohort. This study included 18 late-onset "definite" VAP and 36 controls and suggests that a low relative abundance of *Bacilli* at the time of intubation in the oropharyngeal microbiota is associated with the subsequent development of VAP (AUC: 0;85, $p < 0.0001$, sensitivity: 81.25%, specificity: 82.86%). Even if lung and oropharyngeal microbiota were globally different at each point ($p < 0.05$), they showed a similar trend of changes over time with an increase in phyla *Proteobacteria* and *Tenericutes* and a decrease in the proportion of other major phyla, including *Firmicutes* with a similar decrease in bacterial diversity. Nevertheless, the variations between individuals were substantial; lung and oropharyngeal microbiotas sampled the same day from the same individual clustered well together. Besides these potential predictive properties, molecular techniques were also able to identify the causative pathogen which grows on culture but also difficult-to-grow bacteria such as *Mycoplasma spp*. and anaerobes (Table 4).

DISCUSSION

VAP occurrence is associated with a decrease in lung microbiota α diversity. As only 2 studies mention mycobiota analysis, no conclusion on the mycobiota role are permitted. In patients receiving invasive ventilation, bacterial species composing lung microbiota differ significantly between those developing or not VAP with an overrepresentation of *Enterobacteriaceae* in lung microbiota of patients who will subsequently develop VAP. Interestingly, this impairment of lung microbiota diversity is also associated with the duration of mechanical venti-

lation which could explain the increased frequency of VAP in patients receiving prolonged invasive mechanical ventilation.

Those results are consistent with the ability of lung microbiota to protect against respiratory infections with *S. pneumoniae* and *K. pneumoniae* by priming the pulmonary production of granulocyte-macrophage colony-stimulating factor via Nod2 and IL-17 stimulation19. Besides this immune impact, microbial interactions are of major importance. In fact, these microbiota analyses confirm the suggested interconnection between oropharynx and lung flora and its importance in the pathophysiology of VAP. This subject has been recently discussed by Soussan et al[20] under the scope of transcolonization. Evidence for a continuum of lung colonization from the oropharyngeal cavity has been extensively studied between the 1960s and the 1980s[21,22]. An increased isolation of Gram-negative bacteria in the oral cavity a few hours after oro-tracheal intubation suggests a communication between the oropharyngeal area and the digestive tract, that they usually colonize[23,24]. Oro-pharyngeal colonization can be a step to further colonization of the respiratory tract. Consistently with this suggestion, the presence of digestive fluid or pepsin has been demonstrated in human lungs[25-28] and migration of radiolabelled elements from the stomach to the lungs has been evidenced[29]. The sequence of an early oro-pharyngeal colonization by *Enterobacteriaceae*, as discussed above, followed by a later lung colonization is also in favour of transcolonization of the lungs from the gut through the stomach and the oropharyngeal cavity.

Numerous factors can participate in this transcolonization. In fact, critically ill patients experience numerous disturbances. Systemic perturbations are illustrated by immunological impairment after the response to severe infections as those occurring during the "immune-paralysis" phase after septic shock[30,31]. Regional modification as the patient's posture in supine position in combination with enteral nutrition, thoracic and abdominal pressure regimen, the presence of a gastric tube and treatments reducing lower oesophageal sphincter tonus or corticosteroids use all participate *via* modification of flora and increased reflux to the transcolonization from the gut to the oropharynx[20]. Intragastric proliferation itself is promoted by the reduction of digestive motility due to decreased peristalsis of the proximal small intestine and the increase in gastric pH due to continuous enteral nutrition, the presence of bilirubin in the gastric cavity or the use of proton pump inhibitor[25]. These modifications make the gastric environment more prone to *Enterobacteriaceae* proliferation and this increased inoculum has an enhanced ability of dissemination and oropharyngeal colonization. Those modifications surely explain part of lung colonization but cannot be the sole explanation as oropharyngeal flora disturbances sometimes appear without gastric colonization[25]. In fact, some Gram-negative bacteria are part of the resident oro-pharyngeal flora and can proliferate after salivary alterations associated with invasive ventilation.

After oropharyngeal alterations, transcolonization to the lungs can occur through several ways. Inhalation of bacteria is a first evident mechanism. Even if ETT avoid some macro-inhalation, the supine position of patients augments the leakage of fluid from supra-glottic space to the lungs. Furthermore, the presence of ETT counteract the natural system of prevention of micro-inhalation inhibiting airway closure, disrupting the mucociliary clearance and ultimately favouring the flow of secretions from the upper aerodigestive tract to the subglottic area with the lack of tightness of the ETT cuff impairing the blockade of those secretions[20].

These physiopathologic considerations are enhanced by some studies focusing on the link between extended-spectrum beta-lactamase (ESBL) gut colonization and subsequent ESBL-VAP. Several studies[32,33] demonstrated that ESBL VAP only occur in patients previously colonized in the gut by ESBL and that ESBL oropharyngeal colonization also precedes ESBL VAP. Interestingly, in case of ESBL-VAP, the infecting strain was indeed the gut colonizing strain as demonstrated by our team[34] and confirmed in a larger cohort by Denkel et al[35].

NGS development has allowed a more precise description of lung, oropharyngeal and gut floras with the identification of non-cultivable micro-organisms[36]. These investigations have led to the emergence of the "gut-lung axis" concept whose role in respiratory diseases, including acute bacterial infections, has been recently reviewed by our group[8]. This "gut-lung axis" concept is the application of the "transcolonization" concept emerged with the investi-

gation of flora modifications to the microbiota field. As described above, the events enhancing transcolonization are the same than those enhancing the microbial communication between gut and lung microbiotas within the gut lung axis. Gut microbiota also exerts a major influence on local lung immunity and immune response to infections[19,37,38]. Another argument in favour of a plausible role for gut microbiota in VAP occurrence is its known profound modification and decrease in diversity during the patients' stay in ICU[39]. However, the role of gut microbiota, *per se* or through the dynamics of lung microbiota, in the development of VAP has not been investigated and data are lacking.

Besides, inter-compartment cross-talks, inter-kingdom cross-talks between bacterial, fungal and viral microorganisms should not be underestimated. In fact, gut mycobiota seems to sum up most of gut bacterial microbiota[40]. Virobiota is more difficult to investigate because of technical issues but bacteriophages are key players in microbiota shaping and are probably of major importance[41,42].

These findings could pave the way to a new therapeutic approach by modulating the lung and/or gut microbiota but several pitfalls need to be resolved before a routine application.

In fact, results of manipulation of the microbiota are heterogeneous. Eight studies enrolled 1229 patients and found a relative risk for VAP according to probiotics administration between 0.30 and 1.41 and only 3 trials demonstrated a significant difference in favour of probiotics administration[43]. Apparent discrepancies can be explained by the fact that microorganisms administered as probiotics are not tailored by indication, or even better by patients, but are for now standard therapy without specific selection of the micro-organisms used. Identification of candidates through microbiota comparison between patients developing or not subsequent VAP could be part of the solution. Nevertheless, probiotics administration for the prevention of VAP seems to be safe, with only few side effects reassuring its use in critically ill patients after the worrisome PROPATRIA trial in severe acute pancreatitis[44]. Modulation of gut and/or lung microbiotas by probiotics administration is not as simple as what is usually believed since their effects do not only reside in their ability to colonize their environment but rather in their ability to share genes and metabolites and to interact with host epithelial and immune cells[45]. Before the implementation of such a therapeutic approach, a lot remains to be done with the identification of probiotics candidates tailored by indication or even by individuals.

In this context, an additional major limitation is the extreme heterogeneity in the methods used for microbiota analysis. As described in the Results sections, numerous different extraction kits, hypervariable regions with different primers and different sequencers were used and all of these parameters can dramatically modify the amplicons and so the sequenced reads obtained[46-49]. Different bioinformatics pipe-lines and databases were used and comparison of OTUs from a study to another is difficult. The development of DADA2 pipeline which determines exact sequence variants (or ASV) can enable comparison between studies but only if the same primers were used for the amplification of the region of interest[50,51]. It is therefore of major importance to obtain a consensus to establish a "standard method" allowing comparisons of the results obtained by different teams and rendering NGS results transposable into daily clinical management.

CONCLUSIONS

Lung and oro-phraynx microbiotas seem to play an excruciating role in the development of VAP and their modulation could so represent a new preventive approach. Nevertheless, data remain scarce about the determinants of their alterations and few studies lead to draw thorough conclusions about causality. Further insights in the role of gut microbiota and other microbial compartments, such as mycobiota and virobiota of both lung and gut are needed to better understand the microbial dynamics leading to lung dysbiosis and further development of VAP. Standardization of sequencing process and bioinformatics analysis is also highly warranted to allow comparison of results from different teams and to certify reproducibility. NGS guidance of preventive treatments regarding VAP occurrence in routine care should not be instigated before high-quality evidence resolving these pitfalls.

Authors' Declaration of Personal Interests

None of the authors have any conflict of interest to declare.

Declaration of Funding Interests

Not applicable

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