Bioscience of Microbiota, Food and Health Vol. 41 (3), 94–102, 2022

Review

The role of respiratory microbiota in the protection against viral diseases: respiratory commensal bacteria as next-generation probiotics for COVID-19

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Received February 5, 2022; Accepted March 5, 2022; Published online in J-STAGE March 29, 2022

On March 11, 2020, the World Health Organization declared a pandemic of coronavirus infectious disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and imposed the biggest public health challenge for our civilization, with unforeseen impacts in the subsequent years. Similar to other respiratory infections, COVID-19 is associated with significant changes in the composition of the upper respiratory tract microbiome. Studies have pointed to a significant reduction of diversity and richness of the respiratory microbiota in COVID-19 patients. Furthermore, it has been suggested that *Prevotella*, *Staphylococcus*, and *Streptococcus* are associated with severe COVID-19 cases, while *Dolosigranulum* and *Corynebacterium* are significantly more abundant in asymptomatic subjects or with mild disease. These results have stimulated the search for new microorganisms from the respiratory microbiota with probiotic properties that could alleviate symptoms and even help in the fight against COVID-19. To date, the potential positive effects of probiotics in the context of SARS-CoV-2 infection and COVID-19 pandemics have been extrapolated from studies carried out with other viral pathogens, such as influenza virus and respiratory syncytial virus. However, scientific evidence has started to emerge demonstrating the capacity of immunomodulatory bacteria to beneficially influence the resistance against SARS-CoV-2 infection. Here we review the scientific knowledge regarding the role of the respiratory microbiota in viral infections in general and in the infection caused by SARS-CoV-2 in particular. In addition, the scientific work that supports the use of immunomodulatory probiotic microorganisms as beneficial tools to reduce the severity of respiratory viral infections is also reviewed. In particular, our recent studies that evaluated the role of immunomodulatory *Dolosigranulum pigrum* strains in the context of SARS-CoV-2 infection are highlighted.

Key words: probiotics, respiratory viral infections, respiratory microbiota, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), coronavirus infectious disease 2019 (COVID-19), *Dolosigranulum pigrum*

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), one of seven known coronaviruses able to infect humans, is the etiological agent of coronavirus infectious disease 2019 (COVID-19). This virus, first reported in 2019 in Wuhan (China) and now known as the Wuhan variant, possessed significant genomic similarity to the SARS-CoV-1 virus (up to 70%) [1, 2]. COVID-19 was declared a pandemic by the World Health Organization (WHO) on March 11, 2020, and imposed

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doi: 10.12938/bmfh.2022-009

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COVID-19 can induce a myriad of generic cold-like symptoms, such as high fever, cough, shortness of breath, fatigue, sore throat, nausea, and loss of smell and taste, being a mild disease for up to 80% of cases; however, up to 20% of infected subjects require some level of medical care, and in about 5% of cases, intensive care is required due to extensive lung damage caused by inflammation, pneumonia, and acute respiratory distress syndrome (ARDS). In addition, although this virus is mainly identified in the respiratory tract, it can also infect different human tissues and organs. This lack of specificity can lead to infections in the gut [3], liver [4], and even the nervous system [5], causing life-threatening diseases like hepatitis and encephalitis.

The possible routes of infection by the SARS-CoV-2 virus include respiratory droplets, airborne particles, and contaminated surfaces through the upper respiratory tract (URT); however, although the exact method of transmission is still up to debate [6], there is enough evidence to consider this virus airborne [7]. Besides, the human URT is not a sterile environment, as it is exposed to a constant inflow of microorganisms and viruses [8] and is colonized by a diverse community of microorganisms. This community, also known as a microbiota, is composed of those microorganisms that successfully colonize these microenvironments, including microorganisms that live in a symbiotic state, transient microorganisms, pathobionts [9], and microbes with pathogenic potential.

The complex interactions between the microbiota and its hosts have been described as important for human health and development [10], playing relevant roles in the susceptibility and severity of infectious diseases of the respiratory tract [11–13] (Fig. 1). Herein, we review the scientific knowledge regarding the role of the respiratory microbiota in viral infections in general and in the infection caused by SARS-CoV-2 in particular. In addition, the scientific work that supports the use of immunomodulatory probiotic microorganisms as beneficial tools to reduce the severity of respiratory viral infections is also reviewed. In particular, the recent studies that evaluated the role of immunomodulatory probiotic microorganisms in the context of SARS-CoV-2 infection are highlighted.

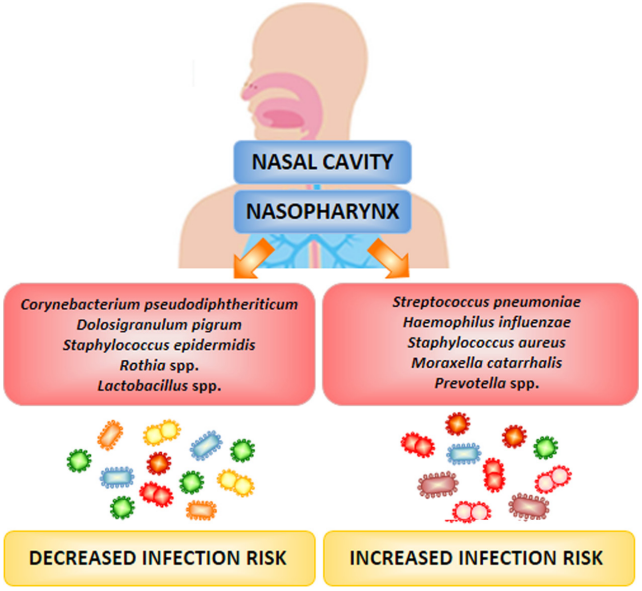

Fig. 1. Members of the respiratory microbiota with beneficial or detrimental effects on the infection risk. The composition of the nasal cavity and nasopharynx microbial populations influence the susceptibility to respiratory tract infections. A higher burden of opportunistic pathogens, including *Streptococcus pneumoniae*, *Moraxella catarrhalis*, *Staphylococcus aureus*, *Haemophilus influenzae*, and *Prevotella* spp., is positively correlated with an increased risk of respiratory tract infections. On the other hand, the respiratory tract commensal bacteria that are predominant in healthy non-infected hosts include *Dolosigranulum pigrum*, *Staphylococcus epidermidis*, *Corynebacterium* spp., *Rothia* spp., and *Lactobacillus* spp. The abundances of these bacteria in the upper respiratory tract are negatively correlated with lower risks of respiratory tract infections [11–13].

ASSOCIATIONS OF RESPIRATORY MICROBIOTA COMPOSITION WITH RESPIRATORY TRACT INFECTION SEVERITY

Respiratory tract infections (RTIs) are seasonal and common; an average person will experience various episodes over a year. RTIs also have a higher incidence in cold countries than in tropical countries [14], as cold temperatures and low humidity are associated with an increase in their frequency [15]. RTIs caused by the prevalent respiratory viruses, such as influenza virus (IFV), respiratory syncytial virus (RSV), and rhinovirus (RV), are normally self-limited in healthy individuals, receding in a couple of weeks. However, these infections are also responsible for significant morbidity and mortality in susceptible groups, including children, immunosuppressed patients, and the elderly [16], who suffer from a natural reduction of immune system potency, known as senescence [17]. RTIs are both a public health concern, with 4.25 million deaths/year globally [18], and an economic burden, with costs exceeding $130 billion/year for viral RTIs [16, 19] in the USA alone.

The microbiotas of the upper and lower respiratory tracts (URT and LRT, respectively) have been described as offering mechanisms mediated by commensal microorganisms to prevent the growth and colonization of pathobionts and viral infections. For instance, common bacterial pathogens, including *Streptococcus pneumoniae* and *Haemophilus influenzae*, are common inhabitants of a healthy URT, but their abundances are kept under control by other members of the microbial community. However, reduced microbial diversity and/or the loss of beneficial and protective bacteria caused by changes in the host's health state [20], known as dysbiosis, can hinder this protective layer and facilitate the enrichment and dominance of pathogens, which could lead to life-threatening respiratory infections, such as pneumonia and meningitis [21, 22]. Studies have shown that birth and feeding modes have a strong influence on the formation of the respiratory microbiota [23, 24]. Natural birth and breastfeeding promote a healthier microbiota and protection against infections and allergies [23, 25, 26]. Those studies with infants are important to show the causal direction of the influence of the microbiota (already present in individuals) on the susceptibility to infections and diseases. On the other hand, the usage of antibiotics has been demonstrated to negatively affect the respiratory microbiota,

doi: 10.12938/bmfh.2022-009

increasing the risk of severe respiratory diseases or recurrent infections [27].

Acute RTI caused by the IFV, a highly infectious virus and the etiological agent of the flu (influenza), was recognized as a major public health concern due to its annual cycle of infection. Its frequent nucleotide substitutions and genomic rearrangement lead to an arms race between the development of vaccines and viral mutations, contributing to the seasonality of influenza epidemics worldwide [28], and even pandemic strains. The most notorious flu pandemic, the Spanish flu, resulted in up to 20–50 million deaths worldwide [29] more than a century ago, being the last great global pandemic before the ongoing pandemic caused by the SARS-CoV-2 virus. Interestingly, autopsy analysis of deceased patients from 1918 revealed a high frequency of secondary superinfection caused by URT bacteria [30], which could have contributed to this extremely high death rate. Indeed, IFV infections have been shown to disturb the microbial composition in the URT [31], affecting the relative abundances of bacteria from the genera *Staphylococcus*, *Fusobacterium*, *Bacteroides,* and others [32]. This temporary dysbiosis caused by viral infections was described as a predisposing factor due to environmental changes (such as permeability and mucus production) and the loss of the protective layer mediated by interactions between the host and a healthy microbiota [33, 34].

Other important respiratory infections include severe cases of bronchiolitis, the most common respiratory infection in infants [35], which is a public health problem worldwide, as it accounts for 18% of all infant hospitalizations in the USA alone [36]. This pathology is most commonly associated with infections by RSV [37] and RV, including its 3 subspecies (RV-A, RV-B, and RV-C). In a 2016 study, researchers were able to identify 4 different nasopharyngeal microbiome types when analyzing samples from 1,600 infants. Those microbiomes mainly differed with regard to the dominant bacterial species, such as *Haemophilus*-dominant (19.2% of samples), which is linked to an increased rate of intensive care use [35], *Moraxella*-dominant (21.9% of samples), *Streptococcus*-dominant (28.2%), and mixed profiles. In a subsequent study, they identified an association between the *Haemophilus*-dominated microbiota and delayed clearance of RSV in infants [38], suggesting its role in the severity of this disease.

In a study published in 2019, researchers compared the nasopharynx microbiota of 774 infants using a multinomial logistic regression adjusted for 8 covariates and concluded that the microbiota composition differed with regard to dominant bacterial species among patients infected by different viruses (RSV, RV-A, RV-B, and RV-C). They reproduced the four different nasopharyngeal microbiome types: *Haemophilus*-dominant, *Moraxella*-dominant, *Streptococcus*-dominant, and mixed profiles. However, their results suggested that patients with a nasopharyngeal microbiota dominated by the *Haemophilus* genus were more likely to develop bronchiolitis caused by RV-A, patients with a mixed microbiota were more likely to have RV-B, and those with a microbiota dominated by *Moraxella* genus were more likely to have RV-C when compared with RSV-only samples [39].

These works clearly indicate the need for deeper and larger-scale clinical works to determine associations between the presence/absence of microorganisms in the respiratory microbiota with the susceptibility to respiratory viral infections.

NASAL ADMINISTRATION OF BENEFICIAL MICROBES FOR IMPROVING THE PROTECTION AGAINST VIRAL INFECTIONS

Research from the last two decades has shown that the nasal administration of beneficial immunomodulatory microbes, particularly lactobacilli, is an interesting alternative to improve the protection against respiratory viral infections [40–42]. Early studies focused on the ability of immunomodulatory lactobacilli to beneficially modify the outcome of IFV infection. Hori *et al.* [41] reported that the treatment of BALB/c mice with *Lacticaseibacillus casei* Shirota by the nasal route stimulated cellular respiratory immunity and increased the protection against IFV challenge. The Shirota strain enhanced protection by increasing systemic and respiratory NK cell activity and the production of IFN-γ and TNF-α by respiratory lymphocytes. Similar observations were reported with *Lacticaseibacillus rhamnosus* GG [40], *Lactobacillus pentosus* S-PT84 [42], and *L. rhamnosus* CRL1505 [43] administered by the nasal route.

The exposure of the respiratory mucosa to viruses also results in a pathogen-specific IgA response in the airways and IgG production in the deep lung. The ability of IgA to mask adhesion epitopes and induce viral agglutination together with the neutralization capacity of IgG are key factors in the defense against IFV infection. It was shown that nasal priming of mice with *L. rhamnosus* CRL1505 enhanced the production of respiratory and systemic specific antibodies after IFV challenge [43]. The improved humoral response induced by *L. rhamnosus* CRL1505 was associated with a significant reduction of viral titers and body weight loss.

It was also shown that immunomodulatory lactobacilli are capable of improving innate antiviral defenses in the respiratory tract. We observed that nasal priming with *L. rhamnosus* CRL1505 prior to an IFV challenge modified the levels and kinetics of cytokines and inflammatory cells in mice [44]. We also observed that mice treated with the CRL1505 strain had increased levels TNF-α, IL-6, neutrophils, and macrophages in the respiratory tract in the early stages of infection, while the pro-inflammatory factors and cells decreased later during the course of IFV infection. Of note, in control mice, those parameters continued increasing. The different kinetics of the innate antiviral response induced by *L. rhamnosus* CRL1505 correlated with a reduced severity of pulmonary damage when compared with control mice [44].

Other recent studies have focused on the ability of immunomodulatory lactobacilli to protect against RSV infection. In this regard, to simulate the pro-inflammatory and physiopathological alterations produced by RSV in the lung, we used an experimental model of lung inflammation based on the administration of the dsRNA analog poly(I:C) [45, 46]. The activation of Toll-like receptor 3 (TLR3) signaling by poly(I:C) administration induces the production of pro-inflammatory cytokines and chemokines and the recruitment of inflammatory cells into the respiratory tract, which leads to marked tissue damage and impairment of lung function [46]. In this experimental model, we demonstrated that nasal priming with *L. rhamnosus* CRL1505 or *Lactiplantibacillus plantarum* CRL1506 reduced the production of TNF-α, IL-6, IL-8, and monocyte chemoattractant protein-1 (MCP-1) and increased the production of IL-10 in the respiratory tract after the challenge with poly(I:C) [46]. In

addition, we showed that the nasal treatment with the CRL1505 or CRL1506 strains also increased the levels of type I interferons (IFNs) (IFNα/β) and IFN-γ in the respiratory tract after poly(I:C) administration [46]. The higher IFN-γ levels in the respiratory tract of lactobacilli-treated mice were associated with augmented numbers of $CD3^+CD4^+IFN\text{-}\gamma^+$ T cells [46], while the improved levels of type I IFNs correlated with enhanced numbers of $CD11c^+SiglecF^+IFN\text{-}\beta^+$ alveolar macrophages [47].

Later, we examined whether *L. rhamnosus* CRL1505 or *L. plantarum* CRL1506 was capable of enhancing the resistance of infant mice to RSV. Nasally administered CRL1505 or CRL1506 strains significantly diminished lung RSV loads and tissue injuries [45]. As observed in the poly(I:C) challenge experiments, lactobacilli-treated mice were able to differentially modulate the respiratory antiviral immune response by decreasing TNF-α and IL-6 and increasing IL-10, $CD3^+CD4^+IFN\text{-}\gamma^+$ T cells [45], and $CD11c^+SiglecF^+IFN\text{-}\beta^+$ alveolar macrophages [47]. In fact, we demonstrated that a different cytokine profile, in particular, the levels of IFN-γ, IFN-β, and IL-10, is necessary to achieve full protection against RSV in infant mice. To our knowledge, there are no other reports in which the effects of nasally administered immunomodulatory lactobacilli on protection against RSV were assessed. However, by using the pneumonia virus of mice (PVM), which is also a virus from the *Paramyxoviridae* family, it was shown that immunomodulatory lactobacilli are able to enhance the resistance against pneumoviruses. It was shown that nasal priming with *L. plantarum* NCIMB 8826 improved the survival of acute PVM infection through the suppression of virus replication and efficient regulation of the virus-induced pro-inflammatory response [48–50].

More recent studies have shown that certain strains of respiratory commensal bacteria with immunomodulatory abilities could exert a beneficial effect in the context of viral respiratory infections similar to those described for probiotic lactobacilli. Research from the last decade demonstrates that some species of respiratory commensal bacteria, including *Dolosigranulum* spp. and *Corynebacterium* spp., have a protective role in the respiratory tract [51], considering that the levels of these microorganisms have been correlated with improved resistance against bacterial [52, 53] and viral infections [54–56]. In line with these reports and using the murine models of poly(I:C)-mediated inflammation and RSV infection, we demonstrated that nasal administration of *Dolosigranulum pigrum* 040417 or *Corynebacterium pseudodiphtheriticum* 090104 beneficially regulated the respiratory antiviral innate immune response [55, 57]. Administration of the 040417 or 090104 strain to mice enhanced the resistance to RSV infection and diminished the inflammatory-mediated lung damage. Furthermore, we showed that the improved antiviral immunity induced by *D. pigrum* 040417 or *C. pseudodiphtheriticum* 090104 was related to their capacities to stimulate $CD11c^+CD11b^{high}MHCII^+$ and $CD11c^+CD103^+MHCII^+$ lung dendritic cells (DCs) and enhance the response of respiratory $CD3^+CD4^+IFN\text{-}\gamma^+$ T cells [55, 57]. We recently extended and complemented those previous findings by showing for the first time that *D. pigrum* 040417 regulates the response of respiratory epithelial cells to TLR3 activation [58]. In our experiments, higher levels of IFN-β and IL-6 and lower concentrations of CXCL8, CCL5, and CXCL10 were found in Calu-3 cells pretreated with *D. pigrum* 040417 and then challenged with poly(I:C) in comparison with respiratory epithelial cells stimulated only with the TLR3 agonist. These findings were in line with the *in vivo* experiments showing that mice nasally treated with the 040417 strain had improved levels of IFN-β, reduced concentrations of inflammatory cytokines and chemokines, and diminished numbers of neutrophils in the respiratory tract after poly(I:C) or RSV challenge [57]. So, the results allow us to speculate that *D. pigrum* 040417 would establish complex molecular interactions with respiratory epithelial cells modifying their immunobiology and increasing their defense functions, as has described for beneficial microorganisms that interact with the epithelial cells of the intestinal mucosa [59–61].

The detailed studies of the respiratory immune response in all the works mentioned above allow us to conclude that the modulation of innate immunity by beneficial microorganisms would be of fundamental importance for their ability to increase protection against viral infections (Fig. 2): a) the stimulation of innate immunity could be sufficient to limit viral replication, and if this response is also efficiently regulated, to avoid lung damage produced by the viral inflammatory process. b) The beneficial regulation of innate immunity can subsequently modulate the adaptive immune response, both cellular and antibody-mediated adaptive immune responses. In this regard, it was shown that DCs

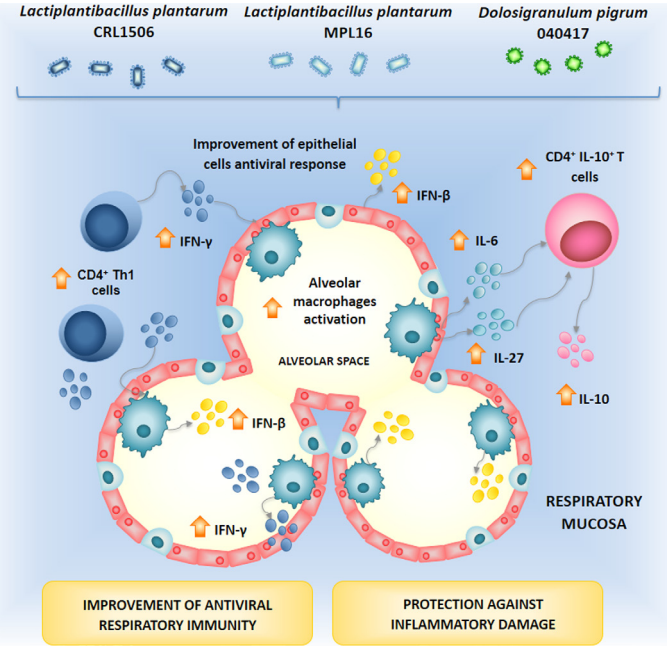

Fig. 2. Immune mechanisms used by beneficial microorganisms to improve protection against respiratory viral infections. When administered nasally, the immunomodulatory microorganisms *Lactiplantibacillus plantarum* MPL16, *L. plantarum* CRL1506, and *Dolosigranulum pigrum* 040417 reach the pulmonary alveoli by inhalation or mucosal dispersion. The beneficial microbes interact with alveolar macrophages and respiratory epithelial cells, increasing their ability to produce type I IFNs (IFN-β) in response to viral challenges. Type I IFNs also favor the activation of Th1 lymphocytes that produce IFN-γ, enhancing the respiratory antiviral immunity. In addition, alveolar macrophages stimulated by beneficial microbes are capable of producing IL-6 and IL-27, which stimulate regulatory T cells, enhancing their production of IL-10. Through these mechanisms, the MPL16, CRL1506, and 040417 strains enhance the protection against inflammatory damage during the course of viral infections.

doi: 10.12938/bmfh.2022-009

undergo maturation in response to type I IFNs *in vitro* and that IFN-α/β potently enhances adaptive immune responses *in vivo* through the stimulation of dendritic cells (DCs) [62]. Type I IFNs increase the expression of MHC-II, CD40, and CD86 in DCs, which then acquire a higher ability to stimulate $CD4^{+}IFN\text{-}\gamma^{+}$ and $CD8^{+}IFN\text{-}\gamma^{+}$ T cells [63, 64]. In addition, IFN-α/β promotes antibody responses *in vivo* by stimulating DCs [65]. c) Most importantly, the modulation of the innate respiratory immunity is not specific for a particular virus but rather promotes mechanisms that can help to protect against different viruses reaching the respiratory tract. Thus, despite the fact that studies of the beneficial effects of nasally administered microorganisms have focused on IFV and RSV infections, it is possible to speculate that they could also protect against other respiratory viruses, such as the new coronavirus SARS-CoV-2.

CHANGES IN RESPIRATORY MICROBIOTA COMPOSITION IN COVID-19

Similar to other respiratory infections, COVID-19 is associated with significant changes in the composition of the URT microbiome, which could potentially lead to dysbiosis [66]. An early study pointed to a significant reduction of diversity and richness as measured by two alpha-diversity metrics, the Shannon and Chao indexes, and to an unexpected increase of the Propionibacteriaceae family compared with other taxa [67]. This reduction of microbial diversity linked to COVID-19 was later confirmed by an independent study, which reported an increase of *Staphylococcus* abundance in critical patients [68]. Bacteria from the genera *Prevotella*, *Campylobacter,* and *Streptococcus*, are common inhabitants of the URT microbiota; however, they have been identified as enriched in COVID-19 patients, and among them, two *Streptococcus* strains have been observed to stimulate the expression of the ACE2 surface protein in Vero cells *in vitro* [69]. Such host-microorganism interactions could result in an augmentation of infected cells, as this protein is the receptor used by the SARS-CoV-2 virus to invade epithelial cells.

The role of the microbiome in the host's biological processes, such as homeostasis, metabolism, and immunity, is being actively investigated, as it could help aggravate or alleviate the host's health conditions [70]. In this sense, researchers have been trying to identify potential biomarkers in the microbiotas of patients that could explain differences in COVID-19 severity, with one of the first studies investigating the impact of SARS-CoV-2 infection and the URT microbiota published by us [71]. We identified operational taxonomic units (OTUs) linked to severity groups, with those from the genus *Prevotella* being the most abundant in patients belonging to the most critically ill patients and this genus of bacteria also being identified as the most abundant in patients with severe COVID-19 cases by independent studies [72, 73]. The genus *Prevotella* is linked to an increase of Th17-mediated mucosal inflammation [74], and there is growing evidence of the role of this inflammatory response in severe lung pathology caused by coronaviruses [75].

Other bacterial genera, such as *Staphylococcus, Lawsonella*, *Alloprevotella*, *Treponema,* and *Finegoldia* were identified as having increased relative abundances in severe cases of COVID-19 [71, 73]. Although the roles of most of these microorganisms in COVID-19 severity are unknown, co-infections caused by the bacteria *Staphylococcus aureus* in COVID-19 patients are responsible for an increased patient mortality rate when compared with patients only infected with SARS-CoV-2, as demonstrated by recent studies [76, 77]. These co-infection cases, including methicillin-sensitive and methicillin-resistant strains, were described as hospital-acquired, since they are facilitated by interventions for critical COVID-19 patients in intensive care units (ICUs), such as mechanical ventilation. *S. aureus* co-infection is a known complicating factor for respiratory viral epidemics and pandemics [78], including the historical Spanish flu pandemics, and also helps increase the mortality and severity of seasonal influenza cases [79].

Besides the health state of the patient, the nasopharynx microbiota is also greatly affected by the age of the subject, being less diverse and richer in young children, with a higher abundance of bacteria classified as *Dolosigranulum*, than in adults and the elderly [80]. This natural changing or maturing of the microbiota is speculated to be one of the reasons why children are less affected by COVID-19 than other age groups [80]. Indeed, a negative association between beneficial bacterial genera, such as *D. pigrum* and *Corynebacterium* spp., and the increase of COVID-19 severity was observed by two independent studies. In the first, the authors identified through a network analysis that an OTU classified as *Dolosigranulum* was positively associated with *Corynebacterium* and negatively associated with *Prevotella* [71]. The second study applied a nonmetric multidimensional scaling (NMDS) using the Bray-Curtis distance and partial least-squares discriminant analysis and also identified *Dolosigranulum* and *Corynebacterium* as significantly more abundant in healthy subjects [68].

The development of new methods for DNA extraction, sequencing, and cultivation, as well as new analytical methods, is enabling the search for new microorganisms with probiotic properties that could alleviate symptoms and even help in the fight against infectious diseases, which was not possible in the past. Nonetheless, the potential of URT microorganisms to improve the protection against common respiratory infections, including flu, bronchiolitis, and COVID-19, is exciting and could be responsible for increasing the survivability of patients and reducing the yearly economic impact caused by respiratory infections.

BENEFICIAL MICROBES FOR IMPROVING THE PROTECTION AGAINST COVID-19

The immune response plays a critical role in the outcome of respiratory viral infections like those produced by SARS-CoV-2. In fact, it was demonstrated that an exacerbated disease due to immune-mediated pulmonary injury leads to higher morbidity and mortality in severe COVID-19 patients [81, 82]. Thus, approaches capable of regulating the viral-induced immunopathology are being actively researched to prevent lethal SARS-CoV-2 infections. In this regard, as we described above, nasally administered immunomodulatory microorganisms have the potential to beneficially influence the respiratory antiviral immune response. We observed that the treatment of Calu-3 cells with immunomodulatory *L. plantarum* or *D. pigrum* strains differentially modulated the production of type I IFNs, antiviral factors, and inflammatory cytokines and chemokines in response to TLR3 activation. Thus, we speculated that immunomodulatory *L. plantarum* or *D. pigrum* strains would be capable of interacting

with epithelial cells, inducing molecular changes that enhance their antiviral defenses. Our recent experiments showed that our hypothesis was correct [58, 83]. We showed that the pretreatment of Calu-3 cells with immunomodulatory *L. plantarum* (strains MPL16 or CRL1506) modulated the innate antiviral immune response of the respiratory epithelial cells triggered by SARS-CoV-2 infection, reducing the virus replication [58]. We also reported for the first time that an immunomodulatory respiratory commensal bacterium, *D. pigrum* 040417, was capable of regulating the innate antiviral immune response of respiratory epithelial cells, conferring improved protection against SARS-CoV-2 [83]. Our results were in agreement with published works that evaluated the potential implication of the respiratory microbiota on the severity and outcome of SARS-CoV-2 infection, as we described above [71, 80, 84].

Our results also suggest that *L. plantarum* MPL16, *L. plantarum* CRL1506, and *D. pigrum* 040417 enhanced the resistance of Calu-3 cells to SARS-CoV-2 by modulating the immune response activated by the viral dsRNA recognizing system. The detection of dsRNA by the pattern recognition receptors expressed in respiratory epithelial cells leads to the production of type I and type III IFNs and the expression of IFN-stimulated genes (ISGs) [85]. In this regard, it was shown that the challenge of Calu-3 cells with SARS-CoV-2 stimulated the JAK/STAT signaling pathway leading to IFN-α/β and IFN-λ production [86]. Furthermore, it was shown that SARS-CoV-2 stimulates the expression of hundreds of antiviral genes in respiratory epithelial cells [87]. In fact, transcriptional studies in Calu-3 cells described the upregulation of *TLR3, DDX58*, *IRF7, IRF9, OAS, Mx1, STAT1*, and *STAT2* after infection with SARS-CoV-2 [87]. Interestingly, it was reported that SARS-CoV-2 is much more sensitive to type I IFNs than SARS-CoV in Calu-3 cells [88]. In a study using a Calu-3 cell *in vitro* system, it was also reported that SARS-CoV-2 viral replication is inhibited by treatment with type I IFNs in a dose-dependent manner and that inhibition of the antiviral JAK/STAT signaling pathway significantly increases SARS-CoV-2 multiplication [86]. So, the early production of type I IFNs in the respiratory epithelium or exogenous therapeutic administration of them is thought to efficiently counteract SARS-CoV-2 replication [89]. Furthermore, the early improved production of IFN-β and subsequently enhanced expression of the ISGs *TLR3, DDX58, Mx1*, and *OAS1* induced by *D. pigrum* 040417, *L. plantarum* MPL16, or *L. plantarum* CRL1506 in Calu-3 cells would be associated with the lower SARS-CoV-2 replication observed in our experiments [58, 83].

Our experimental data also suggested that the modifications of the cytokine profile in SARS-CoV-2-challenged respiratory epithelial cells induced by *L. plantarum* MPL16, *L. plantarum* CRL1506, or *D. pigrum* 040417 would not only help to reduce the replication of the virus but could also additionally assist in diminishing the inflammatory damage caused by the unregulated immune response. Delayed IFN production has been associated with the induction of a compensatory strong inflammatory response, which leads to immunopathology in critically ill COVID-19 patients [90]. In this regard, strong activation of the nuclear factor kappa B (NF-kB) signaling pathway was detected in the respiratory epithelium after SARS-CoV-2 challenge. The activation of this signaling pathway leads to the increased expression of inflammatory cytokines/chemokines [87]. Transcriptomic studies demonstrated remarkably increased upregulation of *CCL2, CXCL8, CSF3, CSF2,* and *CXCL10* in SARS-CoV-2-challenged Calu-3 cells [87], and these inflammatory cytokines/chemokines were shown to be highly elevated in the blood of severe COVID-19 patients [91, 92]. In line with these previous findings, we detected that SARS-CoV-2 infection increased the production of CXCL8, CCL5, and CXCL10 in Calu-3 cells [58, 83]. Interestingly, *L. plantarum* MPL16 and CRL1506 [58] and *D. pigrum* 040717 [83] significantly diminished the production of these chemoattractants for neutrophils and T cells in respiratory epithelial cells infected with SARS-CoV-2. Considering that epithelial cells contribute to regulation of the recruitment and activation of immune cells in the respiratory tract [93], it could be speculated that the enhancement of IFN-β and the reduction of inflammatory cytokines/chemokines induced by beneficial immunomodulatory microorganisms would contribute *in vivo* to the avoidance of inflammatory damage. Furthermore, we have previously reported that nasal priming of mice with *L. plantarum* CRL1506 [45] or *D. pigrum* 040417 [57] increased lung $CD3^{+}CD4^{+}IL\text{-}10^{+}$ T cells after poly(I:C) administration or RSV infection, contributing to the control of inflammatory-mediated lung tissue damage. Thus, a deeper evaluation of the anti-inflammatory protective effects of the MPL16, CRL1506, and 040417 strains in the context of SARS-CoV-2 infection, through modulation of the respiratory epithelium and/or regulatory T cells responses, is an interesting topic for future research.

Our previous studies revealed a strain-dependent capacity of *Lactobacillus* spp. to regulate the antiviral immune response in the intestinal [59, 94] and respiratory [45, 47, 95] mucosa of mice. In line with those *in vivo* findings, our *in vitro* transcriptional experiments performed with intestinal epithelial cells demonstrated a strain-dependent ability of lactobacilli to modulate the TLR3-mediated antiviral defenses [59, 60, 96]. The results obtained in Calu-3 cell cultures were in line with those previous findings. While *L. plantarum* MPL16 and CRL1506 differentially modulated the response of Calu-3 cells to poly(I:C) challenge or SARS-CoV-2 infection, the strains *L. plantarum* MPL18 and CRL1905 were not capable of inducing those beneficial effects [58]. Furthermore, although both MPL16 and CRL1506 strains modulated the antiviral response of Calu-3 cells, *L. plantarum* MPL16 was more efficient in improving protective immunity against SARS-CoV-2. Moreover, we were the first to demonstrate a similar strain-dependent ability of respiratory commensal bacteria of the species *D. pigrum* and *C. pseudodiphtheriticum* to enhance antiviral immunity in the lungs [57]. Our studies in Calu-3 cells were in line with these *in vivo* findings, as *D. pigrum* 040417 increased the protective immunity against SARS-CoV-2 while *D. pigrum* 030918 induced no effect [83]. Thus, the data obtained from our comparative studies using different *L. plantarum* and *D. pigrum* strains emphasize the need for exhaustive studies of the specific strains that will be used to protect against SARS-CoV-2.

Of note, although *L. plantarum* MPL16, *L. plantarum* CRL1506, and *D. pigrum* 040417 each reduced the susceptibility of Calu-3 cells to SARS-CoV-2, these beneficial microorganisms were not capable of completely avoiding the viral infection. A possible alternative to achieve a greater protective effect could be the use of two or more combined strains to form an "immunobiotic consortium" that enhances the beneficial effects on innate antiviral immunity. Interestingly, it was reported that

Corynebacterium was positively associated with *Dolosigranulum* in a co-abundance network analysis in mild COVID-19 patients, while this co-abundance connection was lost in severe cases [71]. Thus, considering the frequent co-occurrence of *Dolosigranulum* spp. and *Corynebacterium* spp. because of the ability of the former to facilitate the expansion of corynebacteria [52], an immunobiotic consortium of immunomodulatory strains of these species of respiratory commensal bacteria could be an attractive alternative to improve protection against SARS-CoV-2. Another interesting question to answer with future research is if lactobacilli isolated from the human nasopharynx have a greater effect than that observed with food-derived lactobacilli, such as MPL16 and CRL1506 strains, and/or if they can create better synergism with respiratory commensal bacteria such as *D. pigrum* 040417.

CONCLUSIONS

To date, the potential positive effects of probiotics in the context of SARS-CoV-2 infection and COVID-19 pandemics have been extrapolated from studies carried out with other viral pathogens, such as IFV, RSV, and PVM [97, 98]. However, scientific evidence has started to emerge that demonstrates the capacity of immunomodulatory bacteria to beneficially influence the resistance against SARS-CoV-2 infection. Our own *in vitro* studies have provided evidence that immunomodulatory *L. plantarum* differentially modulates the innate antiviral immune response in respiratory epithelial cells and favorably influences their resistance to SARS-CoV-2 infection. In addition, some studies have provided evidence that the respiratory microbiota influence SARS-CoV-2 infection and COVID-19 severity [71, 80, 84]. Our results support these findings, as immunomodulatory *D. pigrum* strains enhance the resistance of respiratory epithelial cells to SARS-CoV-2 infection by improving the innate antiviral response. Although further mechanistic and *in vivo* studies are required to propose specific strains of immunomodulatory bacteria, like *L. plantarum* MPL16, *L. plantarum* CRL1506, or *D. pigrum* 040417, for the prevention or treatment of respiratory infections caused by SARS-CoV-2, the scientific evidence summarized in this review indicates that there is a high probability that immunomodulatory probiotics targeting the respiratory tract can contribute significantly to combat the COVID-19 pandemic.

ACKNOWLEDGMENTS

This study was supported by grants from a project of the NARO Bio-oriented Technology Research Advancement Institution (research program on the development of innovative technology, No. 01002A), a Grant-in-Aid for Scientific Research (A; 19H00965) from the Japan Society for the Promotion of Science (JSPS), the Japan Racing Association (JRA) Livestock Industry Promotion Project, and the Association for Research on Lactic Acid Bacteria (to HK). The research in this review was also supported by ANPCyT–FONCyT Grant PICT-2016-0410 (to JV), the JSPS Core-to-Core Program (A. Advanced Research Networks project: Establishment of international agricultural immunology research-core for a quantum improvement in food safety), and AMED Grant Number JP21zf0127001. Research in this review has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska–Curie grant agreement No. 801522 and the Science Foundation Ireland and was co-funded by the European Regional Development Fund through ADAPT Centre for Digital Content Technology grant number 13/RC/2106.

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