Consistent Stool Metagenomic Biomarkers Associated with the Response To Melanoma Immunotherapy

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ABSTRACT The human gut microbiome plays an important role in both health and disease. Recent studies have demonstrated a strong influence of the gut microbiome composition on the efficacy of cancer immunotherapy. However, available studies have not yet succeeded in finding reliable and consistent metagenomic markers that are associated with the response to immunotherapy. Therefore, the reanalysis of the published data may improve our understanding of the association between the composition of the gut microbiome and the treatment response. In this study, we focused on melanoma-related metagenomic data, which are more abundant than are data from other tumor types. We analyzed the metagenomes of 680 stool samples from 7 studies that were published earlier. The taxonomic and functional biomarkers were selected after comparing the metagenomes of patients showing different treatment responses. The list of selected biomarkers was also validated on additional metagenomic data sets that were dedicated to the influence of fecal microbiota transplantation on the response to melanoma immunotherapy. According to our analysis, the resulting cross-study taxonomic biomarkers included three bacterial species: *Faecalibacterium prausnitzii*, *Bifidobacterium adolescentis*, and *Eubacterium rectale*. 101 groups of genes were identified to be functional biomarkers, including those potentially involved in the production of immune-stimulating molecules and metabolites. Moreover, we ranked the microbial species by the number of genes encoding functionally relevant biomarkers that they contained. Thus, we put together a list of potentially the most beneficial bacteria for immunotherapy success. *F. prausnitzii*, *E. rectale*, and three species of bifidobacteria stood out as the most beneficial species, even though some useful functions were also present in other bacterial species.

IMPORTANCE In this study, we put together a list of potentially the most beneficial bacteria that were associated with a responsiveness to melanoma immunotherapy. Another important result of this study is the list of functional biomarkers of responsiveness to immunotherapy, which are dispersed among different bacterial species. This result possibly explains the existing irregularities between studies regarding the bacterial species that are beneficial to melanoma immunotherapy. Overall, these findings can be utilized to issue recommendations for gut microbiome correction in cancer immunotherapy, and the resulting list of biomarkers might serve as a good stepping stone for the development of a diagnostic test that is aimed at predicting patients' responses to melanoma immunotherapy.

KEYWORDS gut microbiota, cancer immunotherapy, melanoma, metagenomics, fecal transplantation, compositional data analysis

Over the past decades, the main cancer treatment options were surgery, ionizing radiation, and chemotherapy. However, recently, novel approaches of treating melanoma and other cancerous types emerged. Since these approaches are based on

the activation of patients' own immunities, they collectively became known as immunotherapy. The therapeutic effects are achieved through the administration of immune checkpoint inhibitors (ICIs), which induce a T-lymphocyte-mediated immune response against tumors (1). However, responses to these treatments are notoriously heterogeneous and nondurable. A significant number of patients fail to benefit from ICIs (2), whereas others exhibit severe autoimmune side effects (3).

The gut microbiome takes part in metabolic reactions that are utterly important for human health. Human studies have reported differences in the gut microbiota compositions of cancer patients who exhibited different responses to immunotherapy (RI) (4–10). Moreover, the transplantation of feces from a responder patient may modulate the outcome of a nonresponder patient (11, 12). These experiments confirmed the crucial role of the gut microbiome in determining the efficacy of immunotherapy. The development of this line of research may lead not only to the creation of new diagnostic tools but also, possibly, to new medications and treatment strategies that are aimed toward increasing the effectiveness of immunotherapy. However, despite the large number of studies that have been published, researchers still have not reached a consensus on the gut microbial determinants of responsiveness to melanoma immunotherapy (RMI). Moreover, the published meta-analyses (13, 14) do not provide a clear answer to this question. While there is no doubt that the frequently cited objective factors, such as the complexity of metagenomic data, technical and/or biological variations, as well as specific individual factors, partially explain the lack of consistent results, some data analysis strategies may be better than others for revealing previously unidentified trends and dependencies.

In this study, we identified consistent biomarkers that are associated with positive ICIs therapy outcomes by applying compositional data analysis methods (15) to stool metagenomic data from earlier published studies. We also focused on melanoma-related data, as these are more abundant than are data related to other tumor types, and we improved the accuracy of our analysis by employing the approaches that we developed earlier, including MetaCherchant (16) and Recipient intestinE Colonization AnalysiS Tool (RECAST) (17). As a result, consistent stool metagenomic biomarkers that are associated with RMI were identified by comparing the stool samples of patients who displayed different treatment responses. We also validated our findings on available data sets that were dedicated to the impact of fecal microbiota transplantation on RMI as well as on other metagenomic data sets. Additionally, we established connections between taxonomic and functional biomarkers that allowed us to single out the potentially most beneficial bacteria that possibly contribute to RMI and to verify the results of previous analyses.

RESULTS

Discovery of consistent taxonomic biomarkers associated with RI. The aim of our study was to find consistent taxonomic biomarkers that are associated with RI. Thus, the metagenomic sequencing data of 358 stool samples from 5 studies that are available in the NCBI database were included in the analysis: the group comparison of melanoma patient stool samples with different RI outcomes (Frankel 2017, Gopalakrishnan 2018, and Matson 2018; here, called the group 1 data sets; 47 responders [R] versus 55 nonresponders [NR]; 102 metagenomes) as well as the data analysis results of the fecal microbiota transplantation influence on RI (Baruch 2021 and Davar 202; here, called the group 2 data sets; 9 donors; 6 responders; 19 nonresponders; 256 metagenomes). The sequencing statistics and other metadata that characterize the metagenomes are presented in Table S1.

The search for consistent taxonomic biomarkers that are associated with RMI was carried out in two steps. At the first stage, the taxa of the gut metagenomes that set R apart from NR were identified by applying the differential rankings approach via Songbird individually for the data sets in Group 1. The top 20 taxa displaying both positive and negative differential values were selected for further analysis. The log-ratio assessment of the selected taxa across metagenomes shows a clear, statistically
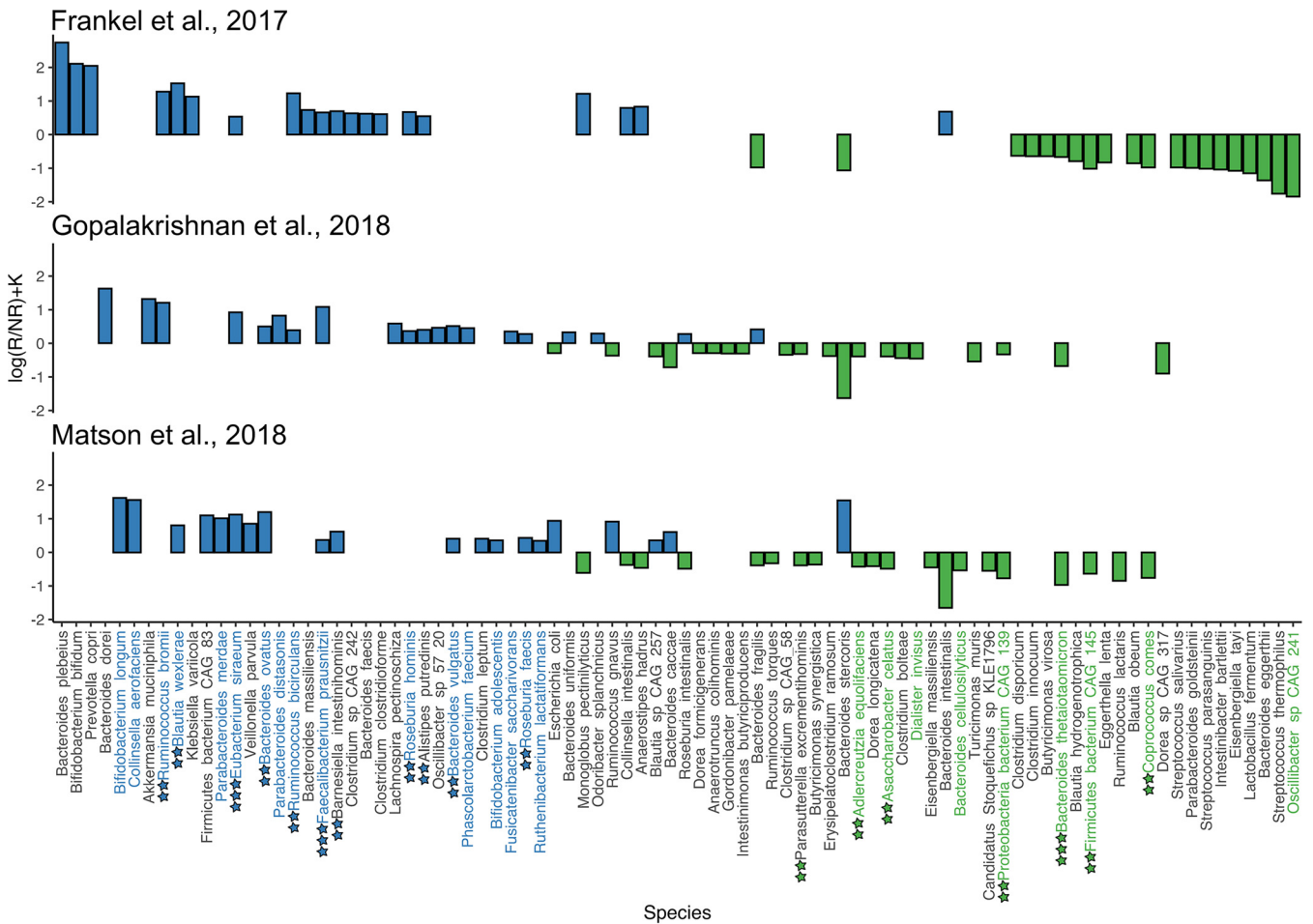
FIG 1 Species rankings and differentials generated by Songbird. Species are plotted on the x axis, and differentials describing the log-fold change in taxa associated with RI are plotted on the y axis. Positive coefficient levels correspond to RMI, and negative levels correspond to an unresponsiveness to melanoma immunotherapy (UMI). Two colored stars designate species associated with RMI and UMI in two data sets, and three stars designate species associated with RMI and UMI in three data sets. Species that contain consistent biomarkers are highlighted in blue and green.

significant difference between the R and NR groups (Fig. S1). The concatenated Songbird-derived rankings and differentials are illustrated in Fig. 1. According to our findings, nine bacterial species (*Faecalibacterium prausnitzii*, *Eubacterium siraeum*, *Ruminococcus bromii*, *Blautia wexlerae*, *Bacteroides ovatus*, *Ruminococcus bicirculans*, *Barnesiella intestinihominis*, *Roseburia hominis*, *Alistipes putredinis*, *Bacteroides vulgatus*, and *Roseburia faecis*) were predictors of RMI in at least two data sets of group 1. In contrast, seven bacterial species were identified to be predictors of UMI: *Bacteroides thetaiotaomicron*, *Parasutterella excrementihominis*, *Adlercreutzia equolifaciens*, *Asaccharobacter celatus*, Proteobacteria bacterium CAG 139, Firmicutes bacterium CAG 145, and *Coprococcus comes*.

It is noteworthy that the data set used in the study of Frankel 2017 encompassed patients with different treatment regimens. Nevertheless, the Songbird approach identified the species that marked the differences between the R and NR, groups regardless of the immunotherapy protocol (Fig. S2).

The aim of analyzing the group 2 data sets was to investigate the impact of fecal microbiota transplantation (FMT) on RI. It should be noted that the FMT-related data are more complex than the group comparison data (Group 1), as they usually consist of donor metagenomes and time series of recipient metagenomes. Therefore, the identification of biomarkers in group 2 was carried out in two steps: (i) the detection of donor-derived microbes in the samples of recipients and (ii) the identification of donor-derived microbial species that colonized the recipient and were associated with the improvement of symptoms of melanoma after immunotherapy.
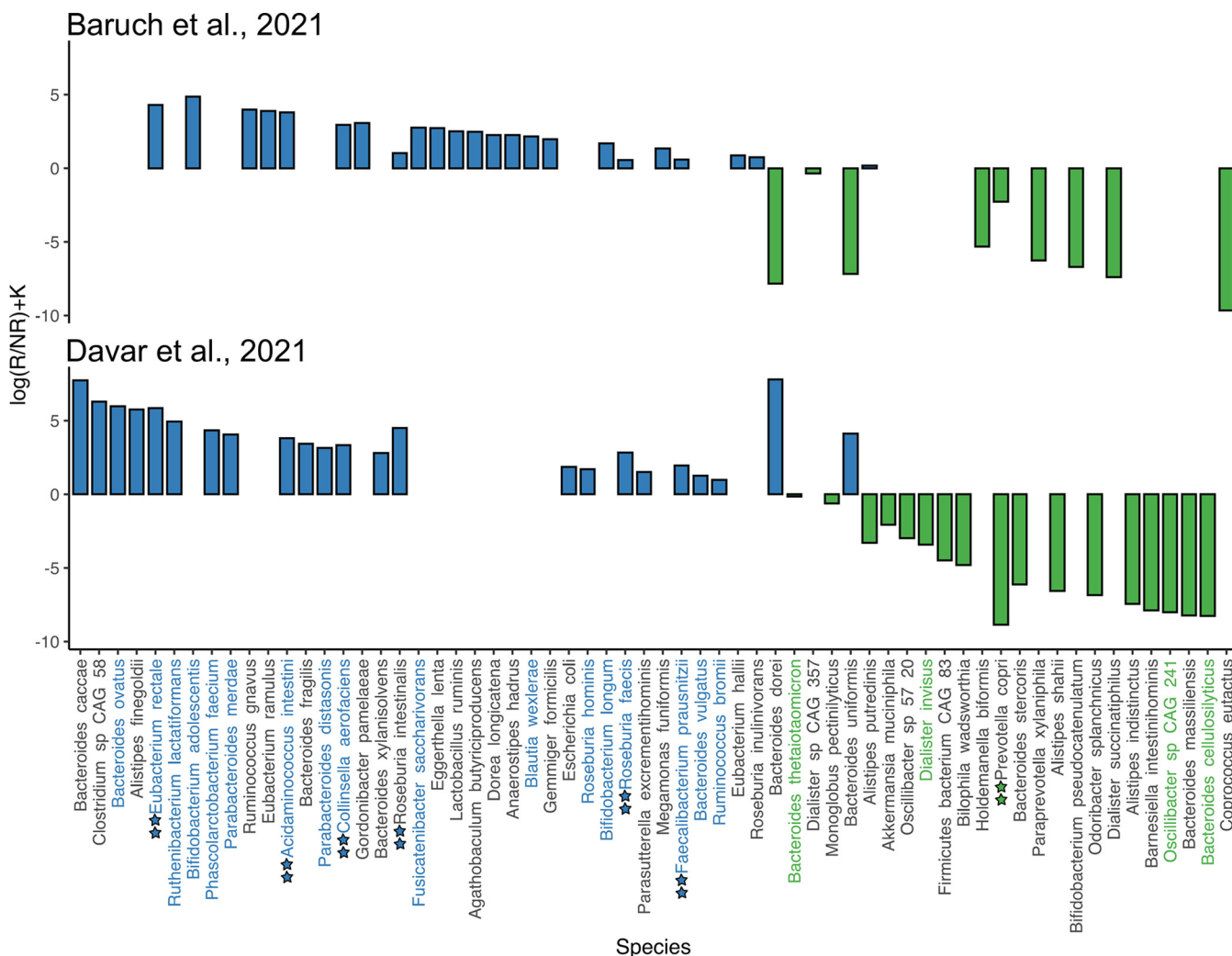
FIG 2 Donor-derived species rankings and differentials generated by Songbird. Microbial species are plotted on the *x* axis, and differentials describing the log-fold change in taxa associated with RI are plotted on the *y* axis. Positive coefficient levels correspond to an association with RMI and negative levels correspond to UMI. The colored stars designate species with positive or negative associations in both the Baruch 2021 and Davar 2021 data sets. Species that contain consistent biomarkers are highlighted in blue and green.

In the first step, the diversity of donor-derived microbes in the metagenomes of the recipients was determined using the RECAST approach. A total of at least 102 donor-derived bacterial species were identified in each recipient. The list of the top five bacteria that colonized the majority of recipients is: *Eubacterium rectale*, *F. prausnitzii*, *A. putredinis*, *R. faecis*, and *Bacteroides uniformis* (Fig. S3). According to variance analysis, the composition of donor-derived microbes was more dependent on the donor subject rather than on RMI in both of the FMT data sets (PERMANOVA, $R^2 = 0.20$, $P_{adj} < 0.05$; $R^2 = 0.07$, $P_{adj} < 0.05$, respectively, for Baruch 2021; $R^2 = 0.14$, $P_{adj} < 0.001$; $R^2 = 0.09$, $P_{adj} < 0.001$ for Davar 2021; Aitchison distance, 10,000 permutations).

In the second step, Songbird was used to perform multinomial regression for the detection of donor-derived microbes that were associated with RMI. The log-ratio assessment of the Songbird differential rankings of donor-derived microbial profiles showed a clear, statistically significant difference between the R and NR groups (Fig. S4). As the analysis of the group 1 data sets showed, the predictors of RMI included *E. reclate*, *Acidaminococcus intestini*, *Collinsella aerofaciens*, *Roseburia intestinalis*, *R. faecis*, and *F. prausnitzii*. Only *Prevotella copri* was consistently associated with UMI (Fig. 2). Interestingly, *E. rectale* was a stronger predictor of RMI for the FMT-related data sets, in comparison to *F. prausnitzii*. Firmicutes appeared to be beneficial in both data sets (maintaining a difference in the species

TABLE 1 Consistent taxonomic biomarkers associated with RMI, resulting from the analysis of the data sets for groups 1 and 2

Species (% of samples with nonzero relative abundance)	Phyla	Studies included in our analysis	Other studies
Faecalibacterium prausnitzii (94)	Firmicutes	Frankel 2017, Gopalakrishnan 2018, Matson 2018, Baruch 2021, Davar 2021	4, 5, 7–10, 13, 55, 56
Roseburia faecis (70)	Firmicutes	Gopalakrishnan 2018, Matson 2018, Baruch 2021, Davar 2021	14
Bacteroides ovatus (96)	Bacteroidetes	Gopalakrishnan 2018, Matson 2018, Davar 2021	57
Bacteroides vulgatus (99)	Bacteroidetes	Gopalakrishnan 2018, Matson 2018, Davar 2021	24, 58
Blautia wexlerae (80)	Firmicutes	Frankel 2017; Matson 2019, Baruch 2021	
Collinsella aerofaciens (79)	Actinobacteria	Matson 2018, Baruch 2021, Davar 2021	7
Eubacterium siraeum (52)	Firmicutes	Frankel 2017, Gopalakrishnan 2018, Matson 2018	56, 59
Roseburia hominis (71)	Firmicutes	Frankel 2017, Gopalakrishnan 2018, Davar 2021	14
Ruminococcus bromii (47)	Firmicutes	Frankel 2017; Gopalakrishnan 2018, Davar 2021	8, 60, 61
Acidaminococcus intestini (36)	Firmicutes	Baruch 2021, Davar 2021	
Bifidobacterium adolescentis (47)	Actinobacteria	Matson 2019, Baruch 2021	7, 61
Bifidobacterium longum (53)	Actinobacteria	Matson 2019, Baruch 2021	7, 19, 62, 63
Eubacterium rectale (87)	Firmicutes	Baruch 2021, Davar 2021	
Fusicatenibacter saccharivorans (79)	Firmicutes	Gopalakrishnan 2018, Baruch 2021	
Parabacteroides distasonis (96)	Bacteroidetes	Gopalakrishnan 2018, Davar 2021	24, 64
Parabacteroides merdae (89)	Bacteroidetes	Matson 2018, Davar 2021	7, 61
Phascolarctobacterium faecium (59)	Firmicutes	Gopalakrishnan 2018, Davar 2021	
Ruminococcus bicirculans (46)	Firmicutes	Frankel 2017, Gopalakrishnan 2018	
Ruthenibacterium lactatiformans (95)	Firmicutes	Matson 2018, Davar 2021	64

content), whereas Bacteroidetes and Actinobacteria demonstrated conflicting results in terms of their impacts on immunotherapy efficacy. For example, *Bacteroides dorei* and *B. uniformis* were strongly associated with RMI in the Davar 2021 data set but not in the Baruch 2021 data set. In contrast, *A. putredinis* was associated with a beneficial impact on immunotherapy in the Baruch 2021 data set but not in the Davar 2021 data set. Moreover, Actinobacteria emerged as a significant predictor of immunotherapy outcome only in the Baruch 2021 data set, whereas a major part of Bacteroidetes emerged as a significant predictor of RI only in the Davar 2021 data set. However, these differences may be due to technical biases that are linked to differences in DNA extraction procedures (18) but not with biological phenomena.

The second stage of the analysis was to put together a list of consistent biomarkers following this scheme: (i) microbial species that were associated with RMI, according to the Songbird results in more than one data set, were added to the list; (ii) microbial species that were associated with UMI in at least one data set were excluded from the list of biomarkers, regardless of the number of data sets in which they were associated with RMI. The identified taxonomic biomarkers have been associated with RMI in other studies, which we mention in Table 1.

The resulting list of consistent taxonomic biomarkers that are associated with RMI included 19 bacterial species: 12 Firmicutes, 4 Bacteroides, and 3 Actinobacteria. Many major short-chain fatty acid producers, such as *F. prausnitzii*, *R. faecis*, *R. hominis*, *R. bromii*, and *E. rectale*, were included in the list. Interestingly, *F. prausnitzii* was identified to be a significant predictor of RMI in all of the data sets that were included in the analysis. In addition, we found nine studies in the scientific literature that also reported the beneficial role of *F. prausnitzii* in immunotherapy. All of the identified consistent taxonomic biomarkers coincided with the colonizers that were identified in the FMT data sets (Fig. S3). However, only 17 out of 19 (excluding *Ruminococcus bicirculans* and *E. siraeum*) were associated with RMI in the FMT-related data sets. Moreover, *E. reclate* and *A. intestini* were found to be predictive of RMI only in the FMT-related data sets. It should also be noted that *F. prausnitzii* and *Roseburia* spp. have been identified as cross-cohort biomarkers of a response to ICIs therapy in previously published meta-analyses (13, 14), which further confirms the roles of these bacteria in producing a positive response to ICIs therapy.

Consistent biomarkers associated with UMI were identified by applying the same methodology. The resulting list included 4 Firmicutes, 2 Bacteroides, 2 Actinobacteria, and 1 Proteobacterium; the species were *Bacteroides thetaiotaomicron*, *Adlercreutzia equolifaciens*, *Asaccharobacter celatus*, *Bacteroides cellulosilyticus*, *Coprococcus comes*, *Dialister invisus*, Firmicutes bacterium CAG 145, *Oscillibacter* sp CAG 241, and Proteobacteria bacterium CAG 139. However, only *B. thetaiotaomicron* was predictive of UMI in 4 data sets (Frankel 2017, Gopalakrishnan 2018, Matson 2018, Davar 2021). The rest of the bacteria from this list showed consistent results in only two data sets. Concurrently, in the group 2 data sets, there were no consistent biomarkers of UMI among the analyzed studies. Only *P. copri* displayed predictive power of UMI in both Baruch 2021 and Davar 2021. However, as it also displayed predictive power of RMI in Frankel 2017, it was excluded from the list.

Validation of consistent taxonomic biomarkers using group comparison of metagenomics data sets. To test the identified consistent taxonomic biomarkers, we applied group comparison to the metagenomic data sets that were published by Spencer 2021 and Lee 2022. There were a total of 322 metagenomes that were divided into 164 responders and 158 nonresponders. We compared the list of consistent biomarkers by using the Songbird differential values in Spencer 2021 and Lee 2022. Microbial species that exhibited contradictory results in at least one of the additional data sets were excluded from further analysis. As a result, only four bacterial species, namely, *B. adolescentis*, *F. prausnitzii*, *E. rectale*, and *P. merdae*, were found to be consistent biomarkers of RMI in additional data sets. One species, namely, *A. equolifaciens*, was found to be a biomarker of UMI. After further verification, *P. merdae* and *A. equolifaciens* were removed from this list, as their differential values at least in one of the additional data sets were <0.1, and they did not exhibit differences between the R and NR groups in the log-ratio analysis. Thus, from the list of early identified cross-study taxonomic biomarkers, only *B. adolescentis*, *F. prausnitzii*, and *E. rectale* were reproducible in the Spencer 2021 and Lee 2022 data sets. It is noteworthy that only *F. prausnitzii* was found to be a predictor of RMI in all seven of the data sets that were included in the analysis and that *E. rectale* and *B. adolescentis* were consistent in four of the data sets. Next, we carried out a log-ratio assessment of *B. adolescentis*, *F. prausnitzii*, and *E. rectale* across metagenomes in additional data sets. Since representative biomarkers of UMI were not identified, only taxa with differential values of <−0.5 were selected as a set of denominators.

Today, we know that many species of bifidobacteria, by stimulating the immune system, can improve the response to ICIs therapy (19, 20). However, in our study, only *B. adolescentis* displayed strong predictive power of a RMI, whereas another bifidobacteria species was associated with UMI. To demonstrate these effects, we added *B. longum* to the log-ratio assessment, and it was found to be a reproducible predictor of RMI in the group 1 and 2 data sets but not in the additional data sets. As a result, the log-ratio assessment of the selected taxa across metagenomes showed a clear, statistically significant difference between the R and NR groups in both the Spencer 2021 and Lee 2022 data sets. The results are presented in Fig. 3. The Songbird differential values across the identified taxonomic biomarkers and data sets are presented in Table S2.

Discovery of consistent functional biomarkers associated with RI. The discovery of consistent functional biomarkers that are associated with RMI was achieved by employing the same approach as that used in the taxonomic analysis. The top 200 functional features displaying positive or negative differential values were selected for further analysis. The log-ratio assessment of the selected KEGG orthology groups (KOG) of the analyzed metagenomes or donor-derived microbial profiles for the additional data sets demonstrated a clear, statistically significant difference between the R and NR groups (Wilcoxon rank-sum test, $P < 0.001$). A total of 140 KOGs that were associated with RMI in more than one data set were identified as a result of analyzing the group 1 and 2 data sets, whereas 57 KOGs were associated with poor RMI. After the validation of the results using the Spencer 2021 and Lee 2022 data sets, 101 KOGs were associated with RMI, and 20 KOGs were associated with poor RMI (Table S3). Among the list of consistent functional biomarkers of RMI were KOGs that are involved in polysaccharide metabolism (K16148, K16147, K01210, K01218, K01136) as well as in peptidoglycan (K07284, K05364, K12554)
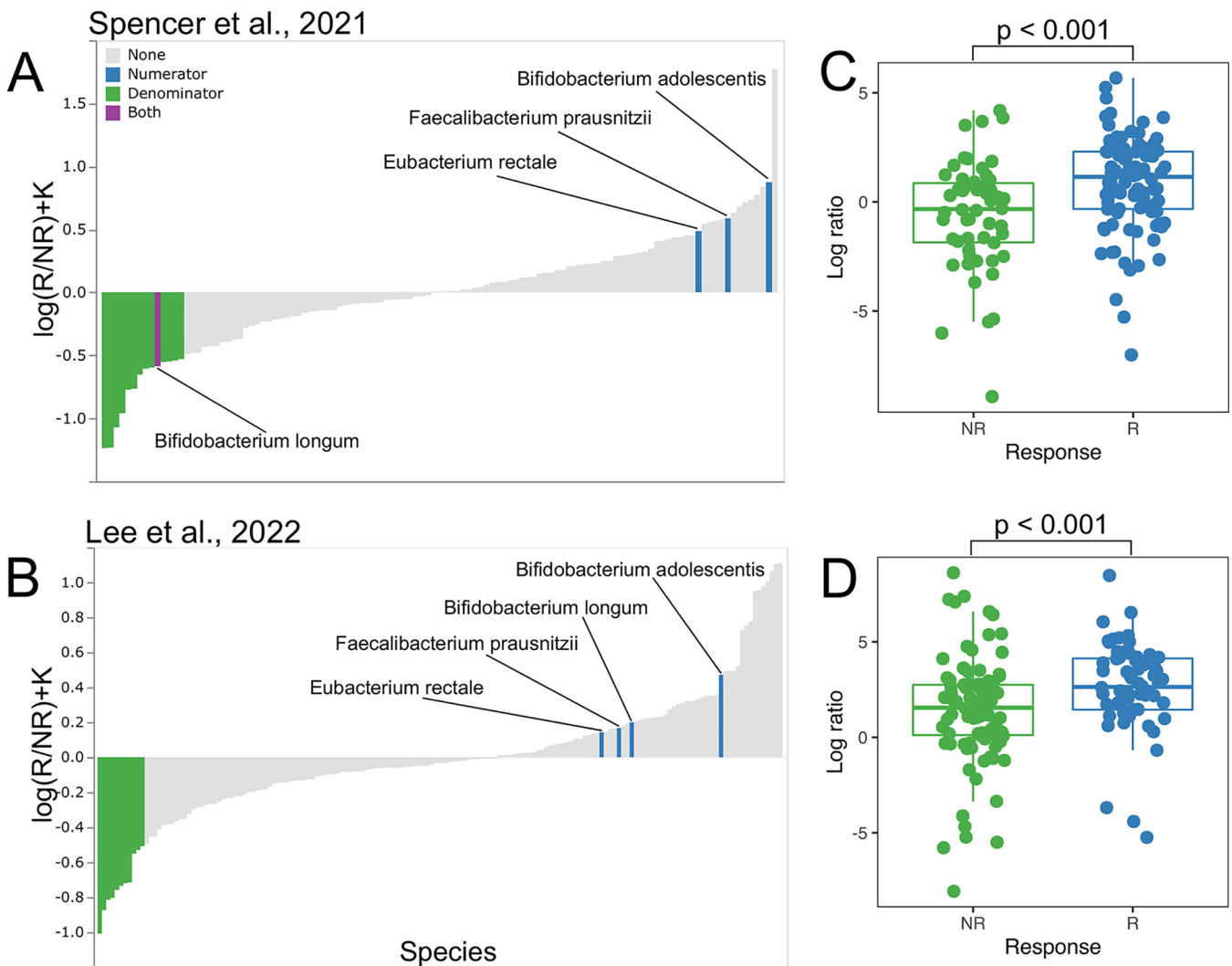
FIG 3 Validation of the consistent taxonomic biomarkers associated with RMI or UMI using additional metagenomic data sets. (A and B) Feature rankings and differentials generated by Songbird. Microbial species are plotted on the x axis, and differentials describing the log-fold change in features with regard to RI are plotted on the y axis. The validated consistent taxonomic biomarkers of RMI are highlighted in blue. Taxonomic features with differential values of <-0.5 that were selected as a set of denominators are highlighted in green. (C and D) Boxplots of the log ratios of the selected features are highlighted in color in panels A and B. The log-ratios statistical assessment, which was performed using the Wilcoxon rank-sum test, shows a clear significant difference between the R (responders) and NR (nonresponders) groups. P values are shown in the figure. The plots in panels A and C correspond to the Spencer 2018 data set, and the plots in panels B and D correspond to the Lee 2022 data set.

and fatty acid biosynthesis (K11533, K11263). It should be noted that the gluABCD gluta-mate uptake system (K10005, K10006, K10007, K10008) and the phosphotransferase (PTS) encoded by the gfrABCD operon (K19506, K19507, K19508, K19509), which leads to the utilization of Maillard reaction products (MRPs), such as fructoselysine/glucoselysine, are also predictors of RMI. Among other significant predictors of RMI were gene groups that are involved in the biosynthesis of cofactors, such as cobalamin (K13542) and ubiquinone (K03688). Moreover, the KOGs corresponding to sporulation (K18955, K06383, K06297, K06313, K06330, K06413) and motility/secretion systems (K02653, K02398, K02417, K02278) were also associated with RMI. Concurrently, among other functional biomarkers of UMI were KOGs derived from pathogenic bacteria, such as KOGs that are involved in aerobactin biosynthesis (K03894, K03895, K03896, K03897).

Identification of the relation between taxonomic and functional biomarkers of RI. Using the MetaCharchant and Kraken2 tools, we studied the relationship between the taxonomic results and the consistent functional biomarkers described above (Table S4). According to the results presented in Fig. 4A, 45 Firmicutes, 18 Actinobacteria, 17
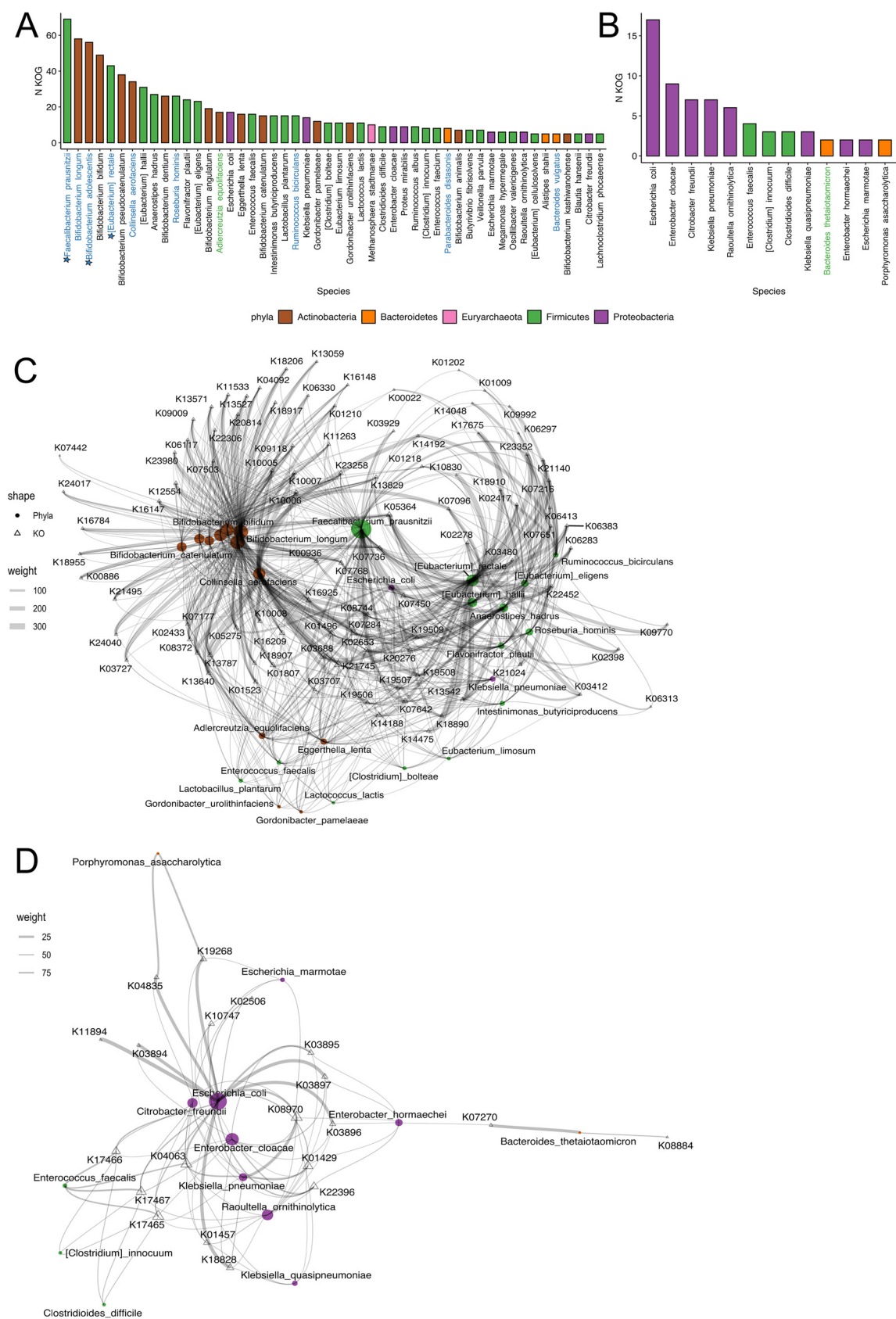
FIG 4 Taxonomic affiliation of consistent functional biomarkers of RI. (A and B) The number of KOGs linked to each bacterial species, as identified by Kraken 2. The bacterial species are plotted on the x axis, and the numbers of KOGs are plotted on the

Proteobacteria, 11 Bacteroidetes, 3 Euryarchaeota, 1 Synergistetes, and 1 Verrucomicrobia bacteria were linked to functional biomarkers that are associated with RMI. It is noteworthy that 9 out of 19 phyla (approximately 47%) have been included in the group 1 and 2 data sets taxonomic biomarkers that are associated with RMI, which are presented in Table 1. According to a variance analysis, different bacterial phyla were linked to different bio-markers (PERMANOVA, $R^2 = 0.12$, $P < 0.001$; Bray-Curtis dissimilarity, 10,000 permutations). The number of KOGs associated with RMI was highest in the species belonging to Actinobacteria (Wilcoxon rank-sum test, $P_{adj} < 0.05$). Firmicutes, Bacteroidetes, and Proteobacteria were least linked to the selected gene groups, even though the results were not statistically significant (Wilcoxon rank-sum test, $P_{adj} > 0.05$). The top five bacteria that contained KOGs that were most strongly linked to RMI were: *F. prausnitzii*, *B. longum*, *B. adolescentis*, *Bifidobacterium bifidum*, and *E. rectale*. It is noteworthy that this list is par-tially similar to the list of consistent taxonomic biomarkers that resulted from the analysis of the Spencer 2021 and Lee 2022 data sets (*F. prausnitzii*, *B. adolescentis*, and *E. rectale*) (Fig. 3). Interestingly, a combination of *F. prausnitzii*-linked KOGs and any of the bifidobac-teria *B. longum*, *B. adolescentis* and *B. bifidum* was seen in approximately 85% of the cases of consistent functional biomarkers of RMI.

In contrast, 8 Proteobacteria, 3 Firmicutes, and 2 Bacteroidetes bacteria were associated with functional biomarkers of UMI. The top five bacterial species that contained the high-est numbers of functions associated with biomarkers of UMI were the opportunistic and potential opportunistic species *Escherichia coli*, *Enterobacter cloacae*, *Citrobacter freundii*, *Klebsiella pneumoniae*, and *Raoultella ornithinolytica* (Fig. 4B). All of the identified aerobac-tin biosynthesis KOGs belonged to *E. coli* and *E. cloacae*. It is noteworthy that we identified bacterial species, including *Escherichia coli*, *E. cloacae*, *B. thetaiotaomicron*, and others, that are linked to functional biomarkers of both RMI and UMI.

We also carried out a network analysis of the relationship between the taxonomic results and the consistent functional biomarkers. The bacterial phyla that differed in their content of functional biomarkers of RMI were also identified (Fig. 4C). Interestingly, *F. prausnitzii* occu-pied an intermediate position between the Firmicutes and Actinobacteria clusters, and it was linked to the specific KOGs of both of the bacterial phyla. The functional biomarkers of UMI were predominantly linked to Proteobacteria and did not form distinct clusters (Fig. 4D).

DISCUSSION

The international scientific community is being actively engaged in studying the influence of the human intestinal microbiota on the efficacy of cancer immunotherapy. However, despite the large number of published studies, researchers still cannot find reliable and consistent gut microbial determinants of RMI. Thus, the development and validation of new tools of metagenomic analysis and reanalysis of the published data may help us reveal new links between the composition of the gut microbiome and the efficacy of immunotherapy. In this study, we focused on melanoma-related metage-nomic data due to its abundance, in comparison with data related to other tumor types. Using the Songbird tool for compositional data analysis (21), we analyzed the gut metagenomes from seven published studies and identified three bacterial species as cross-study taxonomic biomarkers that are associated with RI: *F. prausnitzii*, *B. ado-lescentis*, and *E. rectale*. Nevertheless, only *F. prausnitzii* was consistent across the data sets that were included in the analysis. It is noteworthy that the identified biomarkers were congruent with the results of other published studies (Table 1). Moreover, despite the fact that the Frankel 2017 and Lee 2022 data sets included patients who were

FIG 4 Legend (Continued)

y axis. The color bars indicate the phyla of the bacteria. The species that have been selected as consistent taxonomic biomarkers in the group 1 and group 2 data sets are highlighted in blue and green. The taxonomic biomarkers associated with RMI and validated on the Spencer 2021 and Lee 2022 data sets are marked by blue stars. (C and D) The networks show taxonomic affiliations belonging to validated positive functional biomarkers. Triangles correspond to bacterial phyla, and circles correspond to KOGs. The widths of the circles correspond to the numbers of related KOGs. The plots in panels A and C correspond to the validated consistent functional biomarkers that are associated with RMI, whereas the plots in panels B and D correspond to UMI.

exposed to different treatment regimens (anti-PD1, anti-CTLA4, or a combination of both), the identified biomarkers still described the difference between the R and NR patient groups well. Many have suggested that *F. prausnitzii*, *E. rectale*, and bifidobacteria exhibit immunomodulatory potential in relation to both healthy people and COVID-19 patients (22, 23). We assume that there are universal stool metagenomic biomarkers that are reflective of the state of the immune system. Nonetheless, extensive clinical studies are needed to confirm these hypotheses.

Furthermore, we identified consistent functional biomarkers of RMI that are related to polysaccharide metabolism, peptidoglycan and fatty acid biosynthesis, the gluABCD glutamate uptake system, fructoselysine/glucoselysine utilization by gfrABCD PTS, as well as groups of genes that are involved in the biosynthesis of cofactors, such as cobalamin and ubiquinone. Interestingly, the groups of genes related to sporulation and motility/secretion systems were predictive of RMI. Today, we know that the gut microbial community promotes a variety of digestive metabolic functions, some of which may be associated with the improved functioning of the immune system and RMI. The identified biomarkers are corroborated by the results of other published findings regarding the molecular mechanisms involved in RI. For example, the increased intake of plant polysaccharides improves RMI (10, 24). Moreover, *F. prausnitzii*, *E. rectale*, and *B. adolescentis*, which were pinpointed as taxonomic biomarkers, are major producers of butyrate and other short-chain fatty acids. Gut microbiome-derived SCFAs are necessary for fine-tuning the body's metabolism and cytotoxic immunity, both of which improve the efficacy of immunotherapy (25–27). Moreover, gene groups that are involved in sporulation and motility can be characterized as pattern recognition receptor (PRR) ligands, and they are involved in the modulation of host immunity and in the efficacy of immunotherapy (20, 28–31). Another finding of this study that may be relevant to the modulation of RMI by gut microbes is that the gut bacteria may affect food safety by tweaking the generation of Maillard reaction products (32). We determined that the gfrABCD PTS system, which is used by bacteria as a fructoselysine/glucosolysine disposal system, can be utilized for the prediction of immunotherapy success. This discovery fits well in our paradigm, as fructoselysine may be used by intestinal bacteria as an additional substrate for butyrate production (33), which thereby boosts the probiotic component of the microbiome and increases the body's receptivity of cancer immunotherapy.

Using our previously developed computational pipeline (16), we ranked the bacterial species by the numbers of consistent functional biomarkers contained in them. In total, 96 bacterial species were linked, to some extent, to KOG predictors of RMI. Based on these results, we hypothesized that the irregularities between previous studies might be explained by the fact that beneficial functions are distributed among different bacterial species. Only *F. prausnitzii* contained approximately 70% of the identified functions that are beneficial to immunotherapy success. Moreover, multiple studies demonstrated the crucial role of *F. prausnitzii* in determining the body's RI (Table 1). Thus, *F. prausnitzii* appears to be a key determinant of immunotherapy success. Interestingly, the combination of *F. prausnitzii*-linked KOGs with any of the bifidobacteria *B. longum*, *B. adolescentis*, or *B. bifidum* covered approximately 85% of the identified consistent biomarkers of successful immunotherapy. It is possible that rational design of probiotics must take into account the symbiotic bonds between beneficial bacteria, as they can lead to synergetic beneficial effects, overall. Studies have already shown that the combination of *F. prausnitzii* and bifidobacteria improves butyrate production, growth, and gut colonization (34, 35). Although there is no doubt that bifidobacteria exert a wide range of positive properties related to human immunity, according to our results, only *B. adolescentis* was a predictor of successful immunotherapy. Concurrently, despite the large number of immunotherapy-related beneficial KOGs that were harbored by *B. longum*, the results of the validation of the taxonomic biomarkers for this species were not reproducible. Moreover, *B. bifidum* was not identified to be a consistent taxonomic predictor, despite being associated with a large number of beneficial KOGs. We conjecture that these results might be explained by

the fact that the beneficial properties exhibited by bifidobacteria toward immunotherapy are strain-specific (20) or can be manifested via interactions with other intestinal microorganisms.

In addition to searching for biomarkers of RMI, we set out to establish which distinctive features, other than a low abundance of beneficial microbes, are unique to NRs. However, we did not find any representative taxonomic biomarkers in NRs. Interestingly, the "Anna Karenina principle" seems to be applicable to animal microbiomes in the sense that, unlike balanced microbiomes, which seem to have common features, dysbiotic microbiomes have little to none, paralleling Leo Tolstoy's dictum that "All happy families look alike; each unhappy family is unhappy in its own way." (36). Nevertheless, we identified functional biomarkers of UMI, such as the KOGs that are involved in aerobactin biosynthesis. As reported by many studies, the production of aerobactin is a virulence factor that has been found in intestinal pathogens. We identified some links between aerobactin biosynthesis and both *E. coli* and *E. cloacae*. It is noteworthy that *E. coli* has also been linked to a large number of functional biomarkers that are characteristic of RMI. We conjecture that *E. coli* strains can be both beneficial or opportunistic in regard to melanoma immunotherapy. In addition, we could not conclude which group *B. thetaiotaomicron* belongs to, as it did not display any distinctive pattern between the compared data sets. This is congruent with the literature, which states that the role of *B. thetaiotaomicron* in modulating immunotherapy outcome is ambiguous. On the one hand, the administration of *B. thetaiotaomicron* improved RMI in mice (28). On the other hand, *B. thetaiotaomicron* was suggested as a determinant of UMI (8). *B. thetaiotaomicron* has also been blamed for exacerbating enteric infections (37, 38). Thus, it is feasible that ascribing a concrete status to *B. thetaiotaomicron* depends to a large extent on the presence of pathogenic bacteria in the metagenome.

The impact of fecal microbiota transplantation on RI was strongly dependent on the donor's microbiome. However, the most beneficial donated samples did not work alike for all of the patients. In other words, the FMT immunomodulatory success depends on both the characteristics of the donor microbiome and the recipient's intestinal environment (or other unknown factors). Moreover, species that have been linked to successful therapy have also colonized NRs. It is possible that the engraftment of a broad array of donor-derived species is a side effect of FMT (39). At the same time, unique, hidden aspects of the donor microbiome, such as molecular structures that are secreted by specific strains or certain bacteriophages, can account for RMI (20, 40). Also, other factors, such as the microbial load (which cannot be assessed using metagenomic data) in the donor stool samples and/or in the recipient's mucosa (41), may be a strong determinant of the success of FMT. Interestingly, the most efficient microbial colonizers, namely, *F. prausnitzii* and *E. rectale*, were also associated the most with beneficial KOGs, and they were included in the list of validated consistent taxonomic biomarkers. One possible explanation is that the adaptive evolutionary processes that led to the selection of the most beneficial species for the host also led to the selection of the most competitive microbial symbionts.

Conclusions. Today, it is widely known that the gut microbiome exerts many positive effects on the functioning of the host organism, ranging from metabolic functions to the influence of microbial-derived molecular structures on cell-specific receptors. Since metagenomic sequencing is not suitable for quantifying the fecal microbial load, we can assume that the general microbiome signature detected by human cells is degraded in nonresponders, in comparison to responders, due to infection or some other reason, and that the identified biomarkers are indicators of the microbiome structure degradation. Moreover, the specific characteristics of the Rs' intestinal communities, which are transferable from one person to another, may determine the effectiveness of immunotherapy. To put it briefly, the true biological nature of the observed phenomena has not yet been established. Nevertheless, some useful conclusions can be drawn. For example, the inclusion of digestive fibers and probiotic strains of bifidobacteria into treatment regimens will definitely improve the chances of successful immunotherapy. However, the question of which, among commercial probiotics, is the most effective remains open. In addition, it is likely that taking steps toward enhancing intestinal infection control will also improve the efficacy of ICIs.

TABLE 2 Characteristics of the metagenomic data sets that were used in the study

Dataset	Immunotherapy type	Individuals/Samples	Responders/Nonresponders	Reads per metagenome (mean ± SD), mln	Sequencing platform (read length, bp)
Frankel 2017	anti-PD1/anti-CTLA4	39/39	19/20	36.8 ± 10.6	Illumina (100)
Gopalakrishnan 2018	anti-PD1	25/25	14/11	15.9 ± 4.0	Illumina (100)
Matson 2018	anti-PD1	38/38	14/24	39.7 ± 16.9	Illumina (150)
Baruch 2021	anti-PD1	10/42	3/7	15.8 ± 5.5	Illumina (150)
Davar 2021	anti-PD1	15/214	3/12	11.1 ± 3.8	Illumina (150)
Spencer 2021	anti-PD1	158/158	100/58	12.5 ± 7.8	Illumina (100 to 150)
Lee 2022	anti-PD1/anti-CTLA4	164/164	100/64	24.7 ± 18.3	Illumina (150)

MATERIALS AND METHODS

Metagenomic data. Sequencing data from samples of gut metagenomes of melanoma patients who displayed different responses to ICIs were collected before any interventions from three published studies (5, 7, 8). These data were used to analyze the associations between taxonomic or functional metagenomic profiles and the responsiveness of patients to immunotherapy. Additional data that showed the impact of FMT on immunotherapy (11, 12) were used to validate the identified biomarkers. To validate the selected biomarkers, two additional metagenomic data sets were used (10, 14). In total, 680 metagenomic stool samples were used in the study, of which 374 metagenomes were from melanoma patients with RMI (responders group, R) and 306 metagenomes were from melanoma patients who were unresponsive to immunotherapy (nonresponders group, NR). The characteristics of the metagenomic data sets that were used in the study are presented in Table 2.

Data analysis strategy. Raw metagenomic data were downloaded from the NCBI/EBI public data repositories, using the SRA-Toolkit's fastq-dump tool (42). The assessment of read quality and the filtering out of poor quality reads were carried out using the FastQC software package (https://github.com/s-andrews/FastQC). Technical sequences and bases with a quality of lower than a 30 Phred score were trimmed using Trimmomatic (43). Human sequences were removed from the metagenomes using bbmap (44) and the GRCh37 human genome version. The described preprocessing computational steps that were applied to the metagenomics reads were implemented in the Assnake metagenomics pipeline (https://github.com/ASSNAKE). The donor-derived reads of post-FMT stool metagenomes in FMT-related data sets were identified using the RECAST algorithm (17). The taxonomic and functional profiles of the processed stool metagenomes or the donor-derived metagenomic reads were obtained using the MetaPhlAn3 (45) and HUMAnN2 approaches (46) along with the KEGG database (47).

The identification of consistent taxonomic and functional biomarkers that are associated with RMI was performed in two steps. For the first step, using the taxonomic and functional profiles of stool metagenomes or donor-derived reads (for the FMT-related data), microbial species with increased or decreased abundance were identified in patients who were responsive to immunotherapy using the Songbird (21) and Qurro (48) tools that are implemented in the QIIME2 framework (49). The second step was the creation of a list of consistent biomarkers, which was done by following this methodology: (i) microbial species that were associated with RMI in more than one data set were added to the list; (ii) microbial species that were associated with UMI in at least one data set were excluded from the list of biomarkers, regardless of the numbers of data sets in which they were associated with a positive outcome. The specific UMI biomarkers were identified in a similar way.

The search for links between taxonomic and functional biomarkers was carried out as follows. The identification of taxonomic affiliations and the reconstruction of the metagenomic context of the functional biomarkers was performed using the MetaCherchant tool (16). Unitigs with a length of >1,000 bp were selected for subsequent taxonomic annotation using Kraken 2 (50) and a precomputed database (https://lomanlab.github.io/mockcommunity/mc_databases.html). The resulting data were processed using the ggraph package for GNU/R (51).

Additional statistical analyses and visualizations were carried out using the vegan package (52), ggplot2 library, and standard statistical techniques that are implemented in GNU/R. The PERMANOVA (adonis function of the vegan package), Bray-Curtis dissimilarity (52), and Aitchison distance (53, 54) were used for measuring the distances and dissimilarities in the comparisons of the taxonomy profiles of the read categories and functional biomarkers content. The Benjamini-Hochberg procedure was used to correct for multiple hypothesis testing.

Data availability. In this study, we used data from open sources, which are available at the NCBI and EBI Sequence Read Archives under the BioProject accession numbers PRJNA397906, PRJEB22893, PRJNA399742, PRJNA678737, PRJNA672867, PRJNA770295, and PRJEB43119. All of the results of the project are presented in the article text and in the additional materials.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, TIF file, 0.2 MB.

FIG S2, TIF file, 0.1 MB.

FIG S3, TIF file, 1.7 MB.

FIG S4, TIF file, 0.1 MB.
TABLE S1, XLSX file, 0.02 MB.
TABLE S2, XLSX file, 0.01 MB.
TABLE S3, XLSX file, 0.02 MB.
TABLE S4, XLSX file, 0.8 MB.

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