Supplementary Material

Double-Blind Randomized Placebo Controlled Trial of a *Lactobacillus* Probiotic Blend in Chronic Obstructive Pulmonary Disease

Authors: Teodora Nicola[1], Nancy M. Wenger[1], Kara Seidman[1], Michael Evans[1], Youfeng Yang[1], Dongquan Chen[2], William J. Van Der Pol[3], Amar Walia[1], Elliot J. Lefkowitz[4], Jun Wang[5], Ashley LeMoire[5], Lois Lin[5], Casey Morrow[6], Namasivayam Ambalavanan[1], Amit Gaggar[7], Charitharth Vivek Lal[1]

Affiliations:

1. Department of Pediatrics, University of Alabama at Birmingham, Birmingham, AL, United States.

2. Department of Preventive Medicine, University of Alabama at Birmingham, Birmingham, AL, United States.

3. Center for Clinical and Translational Science, University of Alabama at Birmingham, Birmingham, AL, United States.

4. Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL, United States.

5. Nutrasource Pharmaceutical and Nutraceutical Services, Inc., Guelph, ON

6. Department of Cell, Developmental and Integrative Biology, University of Alabama at Birmingham, Birmingham, AL, United States.

7. Division of Pulmonary, Allergy and Critical Care Medicine, Department of Medicine, University of Alabama at Birmingham, AL, United States.

This work has not been previously presented or published

Contact Information:

Charitharth Vivek Lal, MD[1]

Suite 9380, Women and Infants Center, University of Alabama at Birmingham

1700 6th Ave South, Birmingham, AL 35233

Tel 205-934-4680

Fax 205-934-3100

Email: clal@uabmc.edu

Supplemental Methods

Inclusion and Exclusion Criteria

Inclusion Criteria

1. Adult participants who are 18-80 years of age (inclusive).
2. Have been diagnosed with COPD or NCFBE and have been on a stable treatment regimen for ≥ 6 months at screening.
3. Have a body mass index (BMI) between 18.0-34.9 kg/m2 (inclusive).
4. Have normal or acceptable to the investigator vital signs (blood pressure, respiratory rate, heart rate) and normal or acceptable to the investigator physical exam findings (if applicable) at screening.
5. Individuals of childbearing potential must agree to practice a medically acceptable form of birth control for a certain time frame prior to the first dose of study product and throughout the study, including:
    a. use for at least 3 months prior to the first dose of study product: hormonal contraceptives including oral contraceptives, hormone birth control patch (e.g., Ortho Evra), vaginal contraceptive ring (e.g., NuvaRing), injectable contraceptives (e.g., Depo-Provera, Lunelle), or hormone implant (e.g., Norplant System)
    b. use for at least 1 month prior to the first dose of study product: double-barrier method, intrauterine devices, or complete abstinence from sexual intercourse that can result in pregnancy
    c. vasectomy of partner at least 6 months prior to the first dose of study product

6. Individuals with potential to impregnate others must agree to use condom or other medically acceptable methods to prevent pregnancy throughout the study. Complete abstinence from sexual intercourse that can result in pregnancy is also acceptable.
7. Agree to refrain from treatments listed in Section 6.5 in the defined timeframe.
8. Willing and able to agree to the requirements and restrictions of this study, be willing to give voluntary consent, be able to understand and read the questionnaires, and carry out all study-related procedures.

Exclusion Criteria

1. Participant has a history of heart disease, uncontrolled high blood pressure (i.e., ≥160 mmHg systolic or ≥100 mmHg diastolic), renal or hepatic impairment/disease, or uncontrolled diabetes (Type I or Type II) defined as not taking a stable dose of diabetes mellitus medication on the current regimen for a minimum of three months.
2. Participants on oxygen therapy.
3. Received a vaccine for COVID-19 in the 2 weeks prior to screening or during the study period, current COVID-19 infections, or currently have the post COVID-19 condition as defined by World Health Organization (WHO) (i.e., individuals with a history of probable or confirmed SARS-CoV-2 infection, usually 3 months from the onset of COVID-19 with symptoms that last for at least 2 months and cannot be explained by an alternative diagnosis).
4. Participant has a history of unstable thyroid disease, immune disorders and/or immunocompromised (e.g. HIV/AIDS), a history of cancer (except localized skin cancer without metastases or in situ cervical cancer) within 5 years prior to screening visit.

5. Major surgery in 3 months prior to screening or planned major surgery during the course of the study.
6. Participant has consumed probiotic supplements and is unwilling to stop at least one week prior to screening and throughout the study. Supplemental probiotics may include standalone probiotic supplements, vitamins with probiotics, and any foods supplemented with probiotics.
7. Participant is currently being prescribed antibiotics or states that they have been prescribed antibiotics within the 3 months prior to screening.
8. Participant has consumed supplemental enzymes and are unwilling to stop at least one week prior to screening and throughout the study. Supplemental enzymes may include standalone enzyme supplements, probiotic supplements with enzymes, and any medications containing enzymes.
9. Participant has an abnormality or obstruction of the gastrointestinal tract precluding swallowing (e.g. dysphagia) and digestion (e.g. known intestinal malabsorption, celiac disease, inflammatory bowel disease, chronic pancreatitis).
10. Participants who are lactating, pregnant or planning to become pregnant during the study.
11. Have a known sensitivity, intolerability, or allergy to any of the study products or their excipients, or any of the rescue medications.
12. Receipt or use of an investigational product in another research study within 28 days prior to baseline/Visit 2.

Restricted Concomitant Treatments

1. Systemic Antibiotics

    a. Prohibited within 3 months prior to the first dose of study product and for the duration of the study; usage during this timeframe will lead to screen failure or early termination

2. COPD and NCFBE therapies
    a. Permitted if a stable dose has been used for 90 days and is maintained for the duration of the study; start or stop of usage or changing dosage during the study will lead to early termination
3. Supplements
    a. Prohibited within 1 week prior to the first dose of study product and for the duration of the study; usage during this timeframe will lead to screen failure or early termination
    b. Standalone probiotic supplements, vitamins with probiotics, and any foods supplemented with probiotics
    c. Standalone enzyme supplements, probiotic supplements with enzymes, and any medications containing enzymes
    d. Supplements with holy basil leaf extract, turmeric root extract or vasaka leaf extract as the main ingredient
4. Any other medication or vaccine (including over the counter or prescription medicines, vitamins, and/or supplements) that the participant is receiving at the time of enrollment or receives during the study must be recorded along with:
    a. Reason for use
    b. Dates of administration including start date and if applicable, end date
    c. Dosage information including dose and frequency

Safety and Quality of Life Analyses

All safety analyses were conducted on the safety population. Treatment-emergent adverse events (TEAEs) were summarized by AE term, severity and relationship for each group. All serious adverse events were presented separately. Vital signs and laboratory tests data were summarized by product and visit. Change from baseline was calculated and summarized. Summary statistics include mean, standard deviation, minimum, median, and maximum. No inferential statistics were presented. For laboratory data, shift tables were provided to reflect the change of clinical evaluation from baseline to post-dose or end of study.

Weighted scores were calculated for the SGRQ total and component scores as described in the SGRQ Manual (March 2022) (1). An analysis of covariance (ANCOVA) model was used to analyze the change in weighted SGRQ scores, with study product as the fixed effect and baseline score as the covariate. The least square (LS) means for each product and difference in LS mean between the two products, together with the 2-side 95% confidence interval (CI) was used to assess the effects of the study products. Significance was set at 0.05. Frequency tables, including count and percentage, were provided by study product for the 17 questions and sub-items of the questions.

Biomarker Analysis

Serum and sputum pro-inflammatory biomarkers were measured by ELISA. MMP-9 was measured by Human MMP9 ELISA – DuoSet DY911-05 (R&D Systems), CRP by Human CRP ELISA – Duoset DY1707 (R&D Systems), and IL-6 by Human IL-6 DY206 (R&D Systems). Between-group and within-group analysis was conducted using a Mann-Whitney test and Kruskal-Wallis test (MMP-9 within-group comparison only). Median values with interquartile range for each group are presented. Significance was set at $P^{*}<0.05$.

Quantitative PCR

DNA was extracted from stool and sputum samples of participants randomized to resB® using Zymobiomics DNA Mini-Prep kit (Zymo Research). PCR (Qiagen Fast Cycling PCR kit) was performed on samples with at least 3 ng/μL total DNA by pico green quantification (Quant-It dsDNA Assay Kit, ThermoFisher). As a post-hoc analysis, qPCR was conducted to detect and quantify the presence of the specific *Lactobacillus* strains from the supplement in participants' stool samples. A common 16S gene-based forward primer was utilized with strain-specific reverse primers at a similar site (0.4uM), in a 25 μL reaction with 30 ng DNA template, using SYBR Green dye (LTi) to monitor in real time. Analysis of PCR reactions was performed on 2% agarose gels. Densitometry (% and ng values) was calculated from the imaged PCR gels using a GS-900 densitometer and Image Lab software (Version 6.1, Bio-Rad). Bands were gel extracted (Qiagen Qiaquick Gel Extraction Kit) and sequenced to confirm species identity. ZymoBiomics Gut Microbiome Standard (negative control) was used to demonstrate lack of nonspecific amplification. The absolute abundance of these species was compared between baseline and Week 12 using t-test within the resB® group.

Microbiome Amplicon Sequencing and Analysis

16S amplicon sequencing was performed using the Illumina MiSeq platform at the University of Alabama at Birmingham Microbiome Resource Core Facility under the direction of Dr. Casey Morrow as previously described (2-4). Sequencing data quality control, alignment and demultiplexing were performed by using a custom script built using QIIME 2 with amplicon sequence variants (ASVs) identified using SILVA (v138) (5, 6). Processed ASV tables were imported into MicrobiomeAnalyst for further analysis. The top 50 ASV species were categorized into taxonomy and filtered data. ASVs that appeared in less than two samples, were prevalent in

10% of samples and less than 5% of the inter quartile range were removed, leading to the removal of 504 low abundance features. Cumulative sum scaling was performed but not rarefication or transformation. Alpha diversity was quantified with the Shannon and Chao1 Indices. We visualized beta diversity with principal coordinates analysis of Bray–Curtis dissimilarity matrices and performed significance testing using permutational multivariate analysis of variance (PERMANOVA) and permutational multivariate analysis of dispersion (PERMDISP). Feature selection was performed using MetagenomeSeq and DESeq2.

Supplemental Tables

Supplemental Table 1. Treatment-emergent adverse events (TEAEs)

		Test Product			Placebo			Total	
		Participants (N=19)	Events (N=3)		Participants (N=18)	Events (N=9)		Participants (N=37)	Events (N=12)
Overall		2 (10.5%)	3 (100%)		4 (22.2%)	9 (100%)		6 (16.2%)	12 (100%)
Relation	Related	0 (0%)	0 (0%)		0 (0%)	0 (0%)		0 (0%)	0 (0%)
	Suspected	0 (0%)	0 (0%)		0 (0%)	0 (0%)		0 (0%)	0 (0%)
	Not related	2 (10.5%)	3 (100%)		4 (22.2%)	9 (100%)		6 (16.2%)	12 (100%)
Severity	Severe	0 (0%)	0 (0%)		0 (0%)	0 (0%)		0 (0%)	0 (0%)
	Moderate	0 (0%)	0 (0%)		0 (0%)	0 (0%)		0 (0%)	0 (0%)
	Mild	2 (10.5%)	3 (100%)		4 (22.2%)	9 (100%)		6 (16.2%)	12 (100%)
Serious		0 (0%)	0 (0%)		0 (0%)	0 (0%)		0 (0%)	0 (0%)
Leading to Discontinuation		0 (0%)	0 (0%)		0 (0%)	0 (0%)		0 (0%)	0 (0%)

Data is expressed as number of participants (percent of participants).

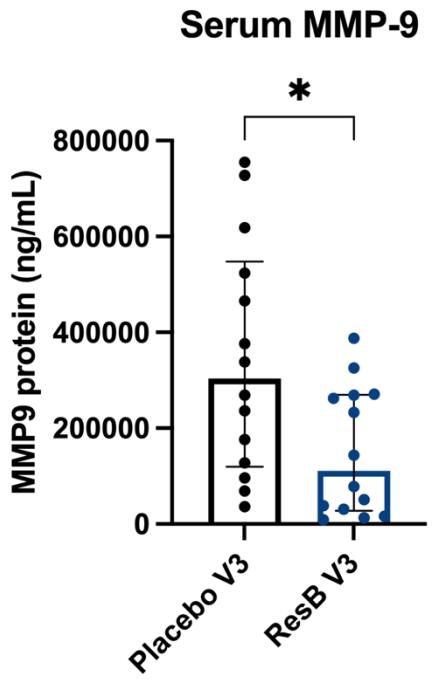
Table 2. Statistical results for alpha diversity measurements.

	statistic	pval	p.adj
ResB-baseline vs Placebo-baseline	-1.0138	0.31787	0.38144
ResB-baseline vs Placebo-_end-of-study	-2.4251	0.020962	0.069517
ResB-baseline vs ResB-_end-of-study	-2.3834	0.023172	0.069517
Placebo-baseline vs Placebo-_end-of-study	-1.3292	0.19291	0.28936
Placebo-baseline vs ResB-_end-of-study	-1.3319	0.19206	0.28936
Placebo-_end-of-study vs ResB-_end-of-study	-0.051646	0.95913	0.95913

Table 3. Statistical results for beta diversity measurements.

	F.Model	R2	pval	p.adj
ResB-baseline vs Placebo-baseline	0.99479	0.028427	0.368	0.5835
ResB-baseline vs Placebo-_end-of-study	0.98741	0.029052	0.389	0.5835
ResB-baseline vs ResB-_end-of-study	0.25517	0.0076732	0.906	0.964
Placebo-baseline vs Placebo-_end-of-study	0.24777	0.0074523	0.964	0.964
Placebo-baseline vs ResB-_end-of-study	1.5944	0.046088	0.157	0.5835
Placebo-_end-of-study vs ResB-_end-of-study	1.1204	0.033828	0.307	0.5835

Supplemental Figure 1. Serum MMP-9 was significantly lower in the resB®-treated group compared to placebo at Visit 3.



REFERENCES:

1. Jones PW, Quirk FH, Baveystock CM, Littlejohns P. A self-complete measure of health status for chronic airflow limitation. The St. George's Respiratory Questionnaire. Am Rev Respir Dis. 1992;145(6):1321-7.
2. Wenger NM, Qiao L, Nicola T, Nizami Z, Martin I, Halloran BA, et al. Clinical trial of a probiotic and herbal supplement for lung health. Frontiers in Nutrition. 2023;10.
3. Kumar R, Eipers P, Little RB, Crowley M, Crossman DK, Lefkowitz EJ, et al. Getting started with microbiome analysis: sample acquisition to bioinformatics. Curr Protoc Hum Genet. 2014;82:18.8.1-.8.29.
4. Van Der Pol WJ, Kumar R, Morrow CD, Blanchard EE, Taylor CM, Martin DH, et al. In Silico and Experimental Evaluation of Primer Sets for Species-Level Resolution of the Vaginal Microbiota Using 16S Ribosomal RNA Gene Sequencing. J Infect Dis. 2019;219(2):305-14.
5. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nature Biotechnology. 2019;37(8):852-7.
6. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 2013;41(Database issue):D590-6.