Supplemental Online Content

Johnstone J, Meade M, Lauzier F, et al. Effects of probiotics on incident ventilator-associated pneumonia in critically ill patients: a randomized clinical trial systematic review. *JAMA*. doi:10.1001/jama.2021.13355

This supplemental material has been provided by the authors to give readers additional information about their work.

eText: Additional Microbiology Materials and Methods

Capsule Quality Assurance

Probiotic Procurement:

Study product (probiotic and placebo) were in blister packs of 10 capsules per card. From each site participating in PROSPECT, 1 capsule from every 10^{th} card (e.g., 1 of every 100 capsules) of both the probiotic and placebo scheduled for administration to randomized patients were sent via regular post to the Laboratory for Interdisciplinary Microbiome Research at McMaster University (Hamilton, Ontario, Canada). Shipping and handling procedures followed established protocols ensuring the integrity of the study product during transportation.

Assessment of the Bacterial Content of the Study Product:

Viable bacteria in the capsule was carried out by standard quantitative culture methods [1]. Bacterial content of the capsules was assessed by aseptically opening the capsule and diluting the content in sterile H_2O [2]. Further dilutions were carried out in culture medium as required. Colony-forming units (CFUs) were enumerated on Man, Rogosa and Sharpe (MRS; BD) and Brain Heart Infusion (BHI; BD) agar media with the appropriate dilution. The presence of microorganisms on the surface of the placebo capsules was investigated prior to opening the capsules to test the content by rolling over the capsule on a BHI agar plate. Agar plates were incubated at 37°C for 48 hours aerobically with 5% CO_2 and an additional incubation at room temperature for >24 hours for fungi detection and enumeration.

Statistical Analysis:

We conducted a one-sided t-test with a mu_0 of 10^{10} to ensure that the capsule counts remained above the 10^{10} CFUs/capsule threshold. A *p* value < 0.05 was considered significant.

Strain Typing of *Lactobacillus* spp. Clinical Isolates Recovered From Trial Patients

Definitions of adverse events and serious adverse events in PROSPECT were defined *a priori* [3]. Clinical isolates of *Lactobacillus* spp. recovered from patients with potential adverse events at each participating site involved in PROSPECT were shipped to the Laboratory for Interdisciplinary Microbiome Research at McMaster University (Hamilton, Ontario, Canada) for strain typing when available.

Genomic DNA was obtained by adding a colony in 50µL of 5% Chelex and incubating the mixture at 95 °C for 15 minutes. Supernatant was recovered and used as template for PCR amplification. The *Lactobacillus* spp. isolates were tested by PCR in order to determine if they were *Lactobacillus rhamnosus* strain GG by using primers targeting the prophage Lc-Nu [4]. Colony PCR was performed using Lc-Nu_f (5'- TATCTTGACCAAACTTGACG-3) and Lc-Nu_r (5'- CAATCTGAATGAACAGTTGTC-3'). The PCR conditions included an initial denaturation at 94˚C for 2 min followed by 35 cycles of 94˚C for 30 seconds, 54˚C for 30 seconds, 72˚C for 45 seconds, followed by a final extension at 72˚C for 10 minutes.

Lactobacillus spp. isolates that were PCR negative for the GG- specific Lc-Nu prophage were further investigated using Random Amplification of Polymorphic DNA (RAPD) PCR. The PCR reaction was completed in 30µL and included 1µL of template, 20 pmol of RAPD-1254 primer (5′-CCG CAG CCA A-3′), 250 µM of each dNTPs, 3 mM $MgCl_2$ 1 U of Taq polymerase (Invitrogen) The PCR conditions were followed as described previously [5] and the RAPD amplicon profiles compared to those of *Lactobacillus rhamnosus* strain GG and other *Lactobacillus* spp. by agarose gel electrophoresis.

eReferences

1. Miller JH. A Short Course In Bacterial Genetics: a Laboratory Manual and Handbook for Escherichia Coli and Related Bacteria. Plainview, N.Y.: Cold Spring Harbor Laboratory Press; 1992.

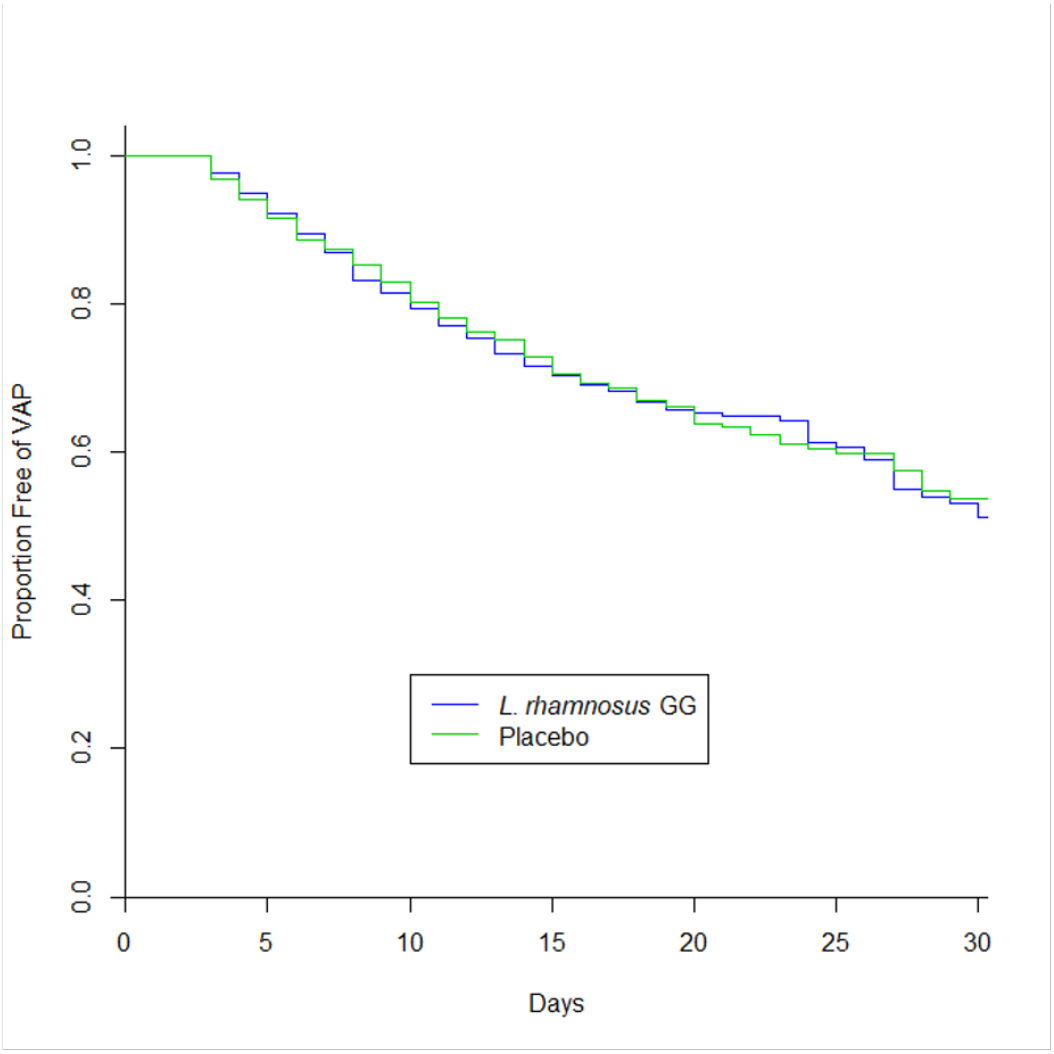
2. Lamarche D, Rossi L, Shah Mi, et al. Quality control in the conduct of a probiotic randomized trial. Can Crit Care forum. Toronto, ON, October, 28, 2014.

3. Johnstone J, Heels-Ansdell D, Thabane L, et al. Evaluating probiotics for the prevention of ventilator-associated pneumonia: a randomised placebo-controlled multicentre trial protocol and statistical analysis plan for PROSPECT. BMJ Open 2019;9(6):e025228.

4. Brandt K, Alatossava T. Specific identification of certain probiotic Lactobacillus rhamnosus strains with PCR primers based on phage-related sequences. Int J Food Microbiol 2003;84(2):189–96.

5. Pacheco ABF, Guth BEC, Soares KCC, Nishimura L, Almeida DFD, Ferreira LCS. Random Amplification of Polymorphic DNA Reveals Serotype- Specific Clonal Clusters among Enterotoxigenic Escherichia coli Strains Isolated from Humans. J Clin Microbiol 1997;35:5.

eFigure. Kaplan-Meier Curves for the Primary Outcome, Ventilator-Associated Pneumonia



No. at risk							
L. rhamnosus GG	1318	1065	516	274	139	84	56
Placebo	1332	1057	529	260	146	88	52

Legend for eFigure 1: In this figure we show Kaplan-Meier time-to-event curves for patients randomized to twice daily enteral *Lactobacillus rhamnosus* GG or placebo for the primary outcome of centrally adjudicated ventilator-associated pneumonia.

eTable 1: Alternative Pneumonia Definition Outcomes

	L. rhamnosus GG n=1318	Placebo n=1332	Absolute Difference [f], % (95% CI)	Hazard Ratio (95% CI)	P-value
Alternative Pneumonia Definitions, number (%):					
Clinical Pulmonary Infection Score ≥6 [a] [24]	329 (25.0)	331 (24.8)	0.1 (-3.2, 3.4)	0.97 (0.82, 1.13)	0.66
Center for Disease Control [b] [25]					
Pneumonia	100 (7.6)	107 (8.0)	-0.4 (-2.5, 1.6)	0.93 (0.70, 1.24)	0.63
Pneumonia, microbiologic confirmation	5 (0.4)	10 (0.8)	-0.4 (-0.9, 0.2)	0.52 (0.18, 1.52)	0.23
Calandra Definition [c] [23]					
Microbiologically confirmed	234 (17.8)	224 (16.8)	-	-	
Probable pneumonia	86 (6.5)	103 (7.7)	-	-	
Possible pneumonia	4 (0.3)	2 (0.2)	-	-	
Any pneumonia	324 (24.6)	329 (24.7)	-0.1 (-3.4, 3.2)	0.98 (0.83, 1.14)	0.76
American College of Chest Physicians [d] [20]	327 (24.8)	336 (25.2)	-0.4 (-3.7, 2.9)	0.94 (0.81, 1.11)	0.47
REDOXS Trial Definition [e] [22]					
Definite pneumonia	0 (0.0)	0 (0.0)	-	-	
Probable pneumonia	240 (18.2)	226 (17.0)	-	-	
Possible pneumonia	84 (6.4)	103 (7.7)	-	-	
Any pneumonia	324 (24.6)	329 (24.7)	-0.1 (-3.4, 3.2)	0.97 (0.83, 1.14)	0.74
Pneumonia confirmed by Invasive Quantitative Testing (e.g., BAL, PSB)	32 (2.4)	19 (1.4)	1.0 (-0.1, 2.1)	1.53 (0.85, 2.76)	0.16

[a] Clinical pulmonary infection score (CPIS), is a score that grades 6 domains on a scale from 0 to 2; any score ≥6 correlates with pneumonia. The score incorporates: 1) quantity and character of tracheal secretions (rare, moderate/large or mucropurulent, regardless of the amount); 2) radiographic infiltrates; 3) body temperature; 4) blood leukocyte count and number of band forms; 5) arterial oxygen tension/ inspiratory fraction of oxygen (PaO_2/FiO_2) and 6) presence of pathogenic bacteria [24].

[b] The Center for Disease Control definition of pneumonia required: 1) Two or more serial chest radiographs with at least 1 of the following: New or progressive or persistent infiltrate; consolidation; or cavitation and 2) at least 1 of the following: Fever; leukopenia or leukocytosis; and for adults >70 years old, altered mental status with no other recognized cause and 3) at least 2 of the following: New onset of purulent sputum or change in character of sputum ; new onset or worsening cough, or dyspnea, or tachypnea; rales or bronchial breath sounds; worsening gas exchange or increased oxygen requirements or increased ventilator demand [25]. Microbiologically confirmed pneumonia required the preceding criteria and at least 1 of the following: Positive growth in blood culture not related to another source of infection; positive growth in culture of pleural fluid; positive quantitative culture from bronchoscopy; ≥5% BAL-obtained cells containing intracellular bacteria on direct microscopic exam; histopathological evidence (biopsy or autopsy) of pneumonia.

[c] Calandra Definitions included the following categories: 1) Microbiologically confirmed pneumonia: New or progressive radiographic infiltrate, along with a high clinical suspicion of pneumonia plus a definite cause established by the recovery of a probable etiologic agent from a) an uncontaminated specimen (e.g. blood, pleural fluid, transtracheal aspirate, or transthoracic aspirate); b) the recovery from respiratory secretions of a likely pathogen that does not colonize the upper airways (e.g., *Mycobacterium tuberculosis*, *Legionella* species, influenza virus, or *Pneumocystis jiroveci* (*carinii*); c) recovery of a likely/possible respiratory pathogen in high concentrations using quantitative cultures of a lower respiratory tract sample (endotracheal aspirate, BAL, or protected specimen brush); or d) positive serology. 2) Probable pneumonia: New or progressive radiographic infiltrate along with a high clinical

suspicion of pneumonia plus a) detection of a likely pulmonary pathogen in respiratory secretions (expectorated sputum, endotracheal or bronchoscopic aspirate, or quantitatively cultured bronchoscopic BAL fluid or brush catheter specimen), but in concentrations below the diagnostic threshold, or b) the presence of a negative lower respiratory tract culture if collected within 72 hrs after starting a new antibiotic regimen. 3) Possible pneumonia: Abnormal chest radiograph of uncertain cause, in a patient with a low or moderate clinical suspicion of pneumonia, but with microbiological or serological evidence of definite or probable pneumonia (as defined above) [23].

[d] American College of Chest Physicians – Ventilator-associated pneumonia criteria defined as 2 of the following: 1) temperature >38°C or <36°C; 2) leukocytosis, 3) purulent tracheal secretions, 4) decreased PaO_2. If 2 or more criteria present are present, chest radiograph should be evaluated for evidence of alveolar infiltrates, air bronchograms, and new or worsened [20].

[e] REDOXS Trial Pneumonia Classification: Requires clinical suspicion of pneumonia, defined as new, progressive or persistent radiographic infiltrates and signs and/or symptoms of infection (fever, leukocytosis, worsening oxygenation, purulent secretions, etc.) and the following classification: 1) Definite pneumonia: radiographic finding of abscess and positive needle aspirate or histologic proof by open lung biopsy or at post mortem. 2) Probable pneumonia: must be associated with positive culture of pathogen known to cause pneumonia. 3) Possible pneumonia: no microbiological confirmation in the setting of a clinical suspicion of pneumonia as described above, and a clinical course comparable with pneumonia including institution of appropriate antimicrobial therapy [22].

[f] Unadjusted absolute difference.

REDOXS=Reducing Oxidative Stress Study; BAL=bronchoalveolar lavage; PSB=protected specimen brush catheter

eTable 2: Sensitivity Analyses: Ventilator-Associated Pneumonia

	L. rhamnosus GG	Placebo	Effect Estimate	P-value
Proportions Analysis n=2650, number (%)	**n=1318**	**n=1332**	**Odds Ratio (95% CI)**	
Ventilator-associated pneumonia [a]	289 (21.9)	284 (21.3)	1.03 (0.85, 1.25)	0.74
Competing Risk Analysis n=2650, number (%)	**n=1318**	**n=1332**	**Hazard Ratio (95% CI)**	
Ventilator-associated pneumonia [b]	289 (21.9)	284 (21.3)	1.02 (0.87, 1.19)	0.82
Per Protocol Analysis n=2089, number %)	**n=1040**	**n=1049**	**Hazard Ratio (95% CI)**	
Ventilator-associated pneumonia [c]	222 (21.3)	214 (20.4)	1.03 (0.85, 1.25)	0.76
Ventilator-Associated Pneumonia diagnosed on day 2 or later n=2650, number (%)	**n=1318**	**n=1332**	**Hazard Ratio (95% CI)**	
Ventilator-associated pneumonia [d]	315 (23.9)	308 (23.1)	1.04 (0.88, 1.22)	0.64

Legend for eTable 2: In this table we show 4 pre-specified sensitivity analyses related to the effect of probiotics on the primary outcome of ventilator-associated pneumonia. For definitions, please see statistical analysis plan [18] and associated references.

[a] Analysis stratified by medical/surgical/trauma admission category and center.

[b] In this sensitivity analysis, death is considered a competing risk. Overall, 575 patients died in ICU, but 138 of these patients developed ventilator-associated pneumonia antemortem; therefore, 437 deaths are considered competing events.

[c] Including only patients who received at least 1 dose of study product on ≥90% of study days.

[d] Including patients in the pneumonia group if pneumonia developed the first day after randomization.

eTable 3: Per-Protocol Analyses for Other Infectious Outcomes

	***L. rhamnosus* GG n=1040**	**Placebo n=1049**	**Hazard Ratio (95% CI)**	**P-value**
Any *Clostridioides difficile* Infection, number (%)	24 (2.3)	24 (2.3)	0.85 (0.47, 1.53)	0.58
In ICU	14 (1.3)	18 (1.7)		
Post ICU discharge in hospital	10 (1.0)	6 (0.6)		
Any Bacteremia, number (%)	77 (7.4)	72 (6.9)	1.06 (0.76, 1.48)	0.73
Intra-abdominal infection, number (%)	16 (1.5)	13 (1.2)	1.12 (0.51, 2.46)	0.78
Positive urine culture, number (%)	129 (12.4)	137 (13.1)	0.93 (0.72, 1.21)	0.60
Upper urinary tract infection [a], number (%)	2 (0.2)	3 (0.3)	1.06 (0.15, 7.69)	0.95
Skin/ soft tissue infection, surgical site, number (%)	20 (1.9)	24 (2.3)	0.84 (0.44, 1.63)	0.61
Skin/ soft tissue infection, nonsurgical, number (%)	30 (2.9)	21 (2.0)	1.20 (0.67, 2.13)	0.55
Other infections [b], number (%)	23 (2.2)	25 (2.4)	0.97 (0.54, 1.73)	0.91
Any infection [c], number (%)	320 (30.8)	318 (30.3)	0.97 (0.83, 1.14)	0.72

Legend for eTable 3: In this table we include 2098 patients who received at least 1 dose of study product on at least 90% of study days. We present all non-VAP ICU-acquired infections adjudicated by independent review blinded to center and allocation. Incident infections occurred 2 days or more following ICU admission. All definitions detailed in [18].

[a] Upper urinary tract infection = microbiologically confirmed abscess or other radiographic or surgical evidence of upper urinary tract infection with or without positive urine culture (positive urine culture alone not included)

[b] Other infections (e.g., meningitis, encephalitis, osteomyelitis, septic arthritis, sinusitis, mediastinitis, etc)

[c] Any infection (any of the foregoing infections, not including positive urine cultures alone; considering only the adjudicated pneumonia outcome)

eTable 4: Antimicrobial Administration

	***L. rhamnosus* GG n=1177**	**Placebo n=1185**	**P-value**
Days of Therapy (DOT) per 1000 patient-days			
Antibiotics	1159	1205	0.68
Antifungals	70	74	0.75
Antivirals	44	44	0.62
Defined Daily Dose (DDD) per 1000 patient-days			
Antibiotics	1524	1630	0.66
Antifungals	100	106	0.77
Antivirals	108	98	0.62
Antimicrobial-Free Days (AFD) per 1000 patient-days			
Antibiotics	310	293	0.80
Antifungals	938	932	0.77
Antivirals	959	960	0.61

Legend for eTable 4: In this table we show antibiotic, antiviral and antifungal administration reflecting 32,162 ICU-days (16,246 in probiotics and 15,916 in placebo groups). Antibiotics administered in the ICU were collected during the pilot trial but not the dose or frequency; therefore, data from 2362 patients are represented in this analysis. For definitions, please see statistical analysis plan [18]. P-values were generated using Wilcoxon rank sum test.

eTable 5: Characteristics of Patients With Lactobacillus Isolates: Serious Adverse Events and Adverse Events

Number	Age, Sex, Admission Diagnosis	APACHE II Score	Days Post Enrolment	Source of Isolate	Study Center Genus, Species	Centrally Confirmed Genus, Species, Strain	Outcome Severity	Health Status in Relation to Isolate	Final Status
SAE #1	75yo male Post-operative lobectomy	11	44	Blood	*L. casei/ paracasei/ rhamnosus*	*L. rhamnosus* GG	Serious Adverse Event (Prolongation of ICU admission)	Died 22 days later Treating team judged as unrelated to *L. rhamnosus* GG infection	Discharged from ICU Died in hospital
SAE #2	52yo female Hemorrhagic shock	26	94	Blood and liver abscess	*Lactobacillus* spp.	*L. rhamnosus* GG	Serious Adverse Event (Prolongation of ICU admission))	Died 72 days later Treating team judged as possibly related to *L. rhamnosus* GG infection	Discharged from ICU Readmitted to ICU and died
AE #1	60 yo male Overdose	25	3	Blood	*Lactobacillus* spp.	*L. rhamnosus* GG	Adverse Event	No associated sequelae Died 7 days later	Died in ICU
AE #2	68 yo male Septic shock (pneumonia)	22	2	Blood	*Lactobacillus* spp.	L. *rhamnosus* GG	Adverse Event	No associated sequelae Died on same day	Died in ICU
AE #3	80 yo female Traumatic brain injury	22	4	Blood	*Lactobacillus* spp.	*L. rhamnosus* GG	Adverse Event	No associated sequelae Died 3 days later	Died in ICU
AE #4	69 yo male Lower gastrointestinal bleed	24	8	Pleural fluid	*L. casei*	Unknown, isolate not sent to Surette laboratory	Adverse Event	No associated sequelae Died 4 days later	Died in ICU
AE #5	46 yo female Septic shock (necrotizing fasciitis)	23	40	Urine	*Lactobacillus* spp.	*L. rhamnosus* GG	Adverse Event	No associated sequelae Died 73 days later	Discharged from ICU Readmitted to ICU and died
AE #6	63 yo female Hepatic encephalopathy	18	49	Peritoneal fluid	*Lactobacillus rhamnosus*	Unknown, isolate not sent to Surette laboratory	Adverse Event	No associated sequelae Died 103 days later	Discharged from ICU Died in hospital

AE #7	59 yo male Septic and cardiogenic shock	34	13	Blood	*L. casei*	*L. rhamnosus* GG	Adverse Event	No associated sequelae Died 55 days later	Died in ICU
AE #8	75 yo male Pneumonia	25	6	Blood	*Lactobacillus* spp.	*L. rhamnosus* GG	Adverse Event	Recovered with no associated sequelae	Discharged from ICU Discharged from hospital
AE #9	51 yo male Intra-abdominal sepsis *	25	2	Blood	*L. rhamnosus*	Unknown, isolate not sent to Surette laboratory	Adverse Event	Recovered with no associated sequelae	Discharged from ICU Discharged from hospital
AE #10	69 yo female Hemolytic uremic syndrome	17	9	Blood	*Lactobacillus* spp.	*L. rhamnosus GG*	Adverse Event	Recovered with no associated sequelae	Discharged from ICU Discharged from hospital
AE #11	46 yo male Pneumonia	20	12	Blood	*Lactobacillus* spp.	*L. rhamnosus* GG	Adverse Event	Recovered with no associated sequelae	Transferred from ICU to other hospital
AE #12	56 yo male Septic shock (Fournier's gangrene)	28	2	Blood	*Lactobacillus* spp.	L. *rhamnosus* GG	Adverse Event	Recovered with no associated sequelae	Discharged from ICU, Transferred to other hospital
AE #13	68 yo female Gastrointestinal obstruction	25	21	Intra-abdominal abscess	*Lactobacillus* spp.	*L. rhamnosus* GG	Adverse Event	Recovered with no associated sequelae	Discharged from ICU Discharged from hospital
AE #14	28 yo male Meningo-encephalitis	13	27 On ward after ICU discharge	Urine	*Lactobacillus* spp.	Unknown, isolate not sent to Surette laboratory	Adverse Event	Recovered with no associated sequelae	Discharged from ICU Discharged from hospital