

Supplemental Online Content

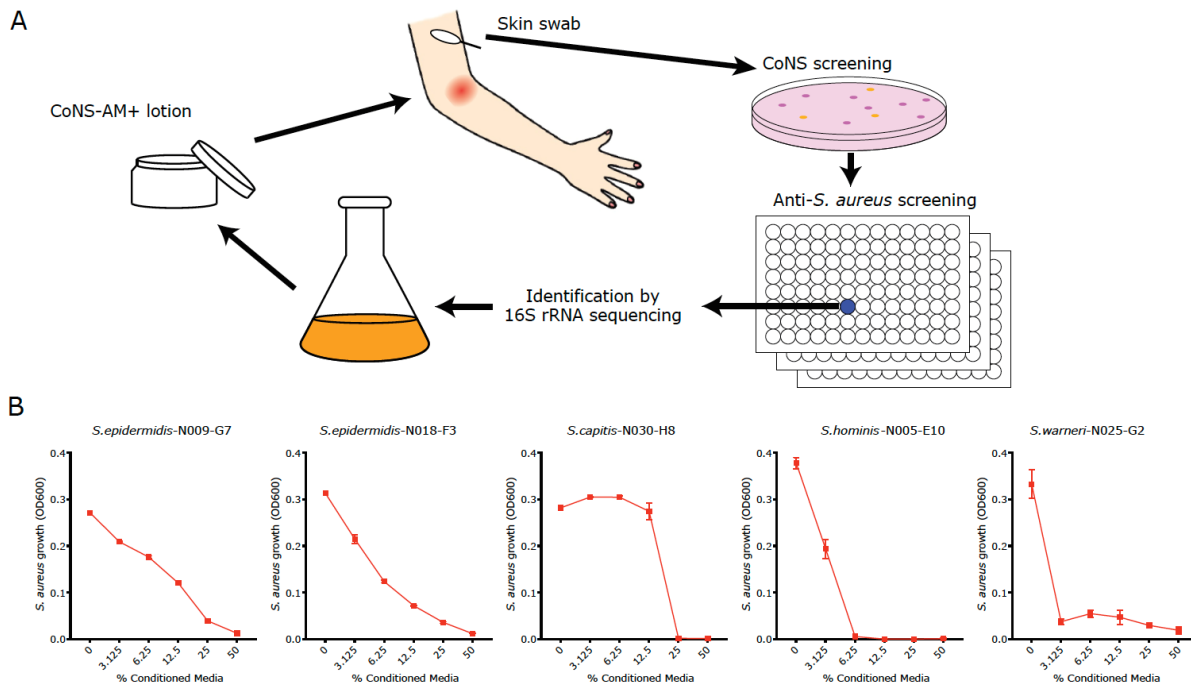
Nakatsuji T, Gallo RL, Shafiq F, et al. Use of autologous bacteriotherapy to treat *Staphylococcus aureus* in patients with atopic dermatitis. *JAMA Oncol*. Published online June 16, 2021. doi:10.1001/jamaoncol.2021.1311

eFigure. Screening for autologous CoNS-AM+ with capacity to inhibit *S. aureus*

eTable. Clinical characteristics of patients with AD treated with CoNS-AM+ or the vehicle

eMethods.

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



eFigure. Screening for autologous CoNS-AM+ with capacity to inhibit *S. aureus*

A, Study design of rational bacteriotherapy using autologous strain of CoNS-AM+ with capacity to inhibit *S. aureus* for patients with AD.

B, Growth inhibition curve conditioned media from each CoNS-AM+ against *S. aureus* strain isolated from lesional skin of each AD patient. Data represent Mean \pm SEM of 4 individual replicates.

eTable. Clinical Characteristics of Patients With AD Treated With CoNS-AM+ or the Vehicle

Patient demographic characteristics	Mean (SD)	
	Patients with AD treated with CoNS-AM+	Patients with AD treated with the vehicle
Age, y	28.2 (16.3)	25.5 (12.2)
Sex, No. (%)		
Male	2 (20.0)	2 (33.3)
Female	3 (80.0)	4 (66.6)
Race, No. (%)		
White	1 (20.0)	2 (33.3)
Asian	4 (80.0)	4 (66.7)
BMI	25.1 (6.3)	24.6 (5.2)
Baseline EASI	2.48 (0.99)	2.83 (1.02)

Abbreviations: AD, atopic dermatitis; BMI, body mass index (calculated as weight in kilograms divided by height in meters squared); CoNS-AM+, antimicrobial-producing coagulase-negative staphylococcus.

eMethods:

Human Subjects

This clinical trial was conducted under a contract with KAI Research Inc. (Rockville, MD) and the National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS). The approach of bacteriotherapy with autologous strain of CoNS for subjects with AD has been officially approved by US Food and Drug Administration (FDA), and this protocol has been filed as an investigational new drug application (IND) (UCSD Approval #15786). This trial is registered at ClinicalTrials.gov (NCT03158012). All experiments involving human subjects were carried out according to UCSD IRB-approved protocols (Project#071032). Informed consent was obtained from all subjects.

Adults with AD were recruited to the University of California, San Diego (UCSD), San Diego, CA (demographic data are shown in eTable 1). All subjects refrained any treatments or behaviors that could potentially affect the skin microbiome prior to sample collection (refer to Supplementary information for sampling collection criteria).

At the screening visit, live bacteria samples were obtained from lesional skin (antecubital fossa) and nonlesional skin (forearm) by swabbing (See below). Sample from lesional skin was used for measurement of *S. aureus* survival. *S. aureus*-culture positive was defined as >1 CFU/cm². If AD subject was *S. aureus*-culture positive, CoNS-AM+ strains were screened against *S. aureus* strain from the same subject.

41 individuals with active AD were screened for *S. aureus* colonization, and 15 subjects were *S. aureus*-culture positive (eFigure 1). Nine subjects were randomly assigned to active drug, while 6 subjects were assigned to vehicle treatment using an online randomizer (www.randomization.com). Three subjects with low frequency of CoNS-AM+ upon screening

and one subject who was *S. aureus* negative at baseline visit were excluded. Thus, we reported data from 5 subjects who received active treatment and 6 subjects who received placebo treatment in this study.

Sample collection criteria

Skin swab sample collection was performed on a subset of participants at the University of California, San Diego, San Diego, CA, under the research plan approved by IND (approval #: 15786). Participants were screened based on following criteria.

AD patient inclusion criteria

1. Male or female subjects who are not pregnant or lactating. Female subjects of child-bearing potential must have a negative urine pregnancy test on the day of the screening visit in order to be eligible for the study.
2. 18-80 years of age
3. Diagnosis of atopic dermatitis for at least 6 months using the Hanifin and Rajka Diagnostic Criteria for atopic dermatitis
4. Presence of lesional atopic dermatitis skin in both antecubital fossae
5. Positive *S. aureus* colonization based on results of a skin culture taken from one of their AD-affected antecubital fossae
6. Positive for antimicrobial CoNS species from non-lesional AD skin

AD exclusion criteria

1. Use of any topical AD treatments (including topical steroids, topical calcineurin inhibitors) to either arm within one week of the Treatment visit
2. Use of any antihistamines 7 days within one week of the Treatment visit

3. Use of any oral/systemic AD therapies (steroids) within 28 days of the Treatment visit
4. Severe AD that would worsen significantly from holding a participant's usual topical/oral AD medications for the time periods required in the inclusion/exclusion criteria (one week prior to the Treatment visit for topical medications and antihistamines and 28 days prior to Treatment visits for oral medications)
5. Subjects who have taken a bleach bath within a week prior to the Treatment visit, or who take bleach baths during the study
6. Patients with severe medical condition(s) that in the view of the investigator prohibits participation in the study
7. Subjects with Netherton's syndrome or other genodermatoses that result in a defective epidermal barrier
8. Any subject who is immunocompromised (e.g. provides researchers with a history lymphoma, HIV/AIDS, Wiskott-Aldrich Syndrome) or has a history of malignant disease (with the exception of non-melanomatous skin cancer). This information will be gathered verbally from the patient while taking a medical history from the patient, and will not involve further testing such as an HIV test.
9. Subjects with a history of psychiatric disease or history of alcohol or drug abuse that would interfere with the ability to comply with the study protocol
10. Active bacterial, viral or fungal skin infections
11. Any noticeable breaks or cracks in the skin on either arm, including severely excoriated skin or skin with open or weeping wounds suggestive of an active infection or increased susceptibility to infection.
12. Ongoing participation in another investigational trial

13. Use of any oral or topical antibiotic for up to four weeks prior to the Treatment visit
14. Use of any systemic immunosuppressive therapy (e.g. CsA, MTX, etc.) within four weeks of the Treatment visit.
15. Sensitivity to or difficulty tolerating Dove fragrance-free bar soap or Cetaphil lotion
16. Subjects with prosthetic heart valves, pacemakers, intravascular catheters, or other foreign or prosthetic devices.
17. Allergy or intolerability to soy or macadamia nuts.
18. Participant who has a condition or is in a situation that, in the investigator's opinion, may put the patient at significant risk, or may significantly interfere with the patient's participation in the study.

Collection of live bacteria from skin

Live bacterial samples were obtained by swabbing lesional skin (antecubital fossa) or nonlesional skin (upper arm) and storing swabs in 1mL of 85% TSB/15% glycerol at -80 °C. Bacterial suspensions were inoculated on a mannitol salt agar with egg yolk for selective growth of *Staphylococcus* species. CoNS was distinguished from *S. aureus* according to mannitol metabolism (*S. aureus*: yellow color colony, CoNS: pink color colony), and the egg yolk reaction (yellow halo around a *S. aureus* colony). To quantify abundance of live *S. aureus*, bacterial suspension was inoculated on a Baird-Parker agar plate, *S. aureus*-selective media.

Screening for antimicrobial activity

CoNS-AM+ were isolated from nonlesional sites with unbiased high-throughput screening according to our previous study (8). Briefly, up to 700 individual colonies of CoNS

from nonlesional skin were randomly picked from mannitol salt agar plates and cultured in TSB overnight. Sterile conditioned media was prepared by filtration through 0.22 μ m PVDF membrane. The antimicrobial activity in each sterile filtered media (100 μ L) was evaluated by mixing with fresh TSB (10 μ L) containing 1×10^4 colony-forming units (CFU) of *S. aureus* clone isolated from the lesional skin of the same AD subject. Potent antimicrobial CoNS (CoNS-AM+) was defined as those that completely suppressed *S. aureus* growth after 22 hrs. Potency of antimicrobial activity was further characterized by serial dilution antimicrobial assay. CoNS-AM+ with potent antimicrobial activity against *S. aureus* strain from each subject was sub-cloned more than 3 times to obtain a pure single strain. Purified strain was stored as a master glycerol stock at -80°C.

All CoNS isolates were stored frozen for species identification by sequencing full-length 16S rRNA gene. Full-length 16S rRNA genes were amplified from 48 representative colonies with universal 16S primers, 27-F and 1525-R. Amplicons were sequenced from both ends by Sanger method. Obtained 16S rRNA gene sequence was searched against Ribosomal Database Project (<http://rdp.cme.msu.edu/>) to assign operational taxonomic units at species level.

Preparation of skin lotion

Master glycerol stock was streaked on a TSB agar plate and incubated at 37°C for 18 hours. A single colony was cultured in 5 mL of animal-free TSB (Corning 61-411-RO) at 37°C for 18 hours with shaking at 200 rpm. Bacterial cells were washed with saline 3 times and re-suspended in 5 mL saline. Bacterial density was estimated by measuring OD₆₀₀. Each CoNS strain was formulated at 1×10^7 CFU/gram in Cetaphil™ lotion (Galderma), which was confirmed not to

affect bacterial viability. Vehicle cream was formulated with equal amount of saline only. For quality control, microbial DNA was extracted from 100 mg of formulated lotion with UltraClean Microbial DNA isolation kit (MolBio Inc.). Potential contamination of active or vehicle formulation was evaluated by qPCR using Microbial DNA qPCR kit (Qiagen) for *Escherichia coli*, *Clostridia* spp, *Salmonella enterica*, *Streptococcus pyogenes*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*. To detect possible contamination by *Clostridium sporogenes* and *S. aureus*, Techne Pro qPCR kit (Techne, Inc) was used. To detect possible contamination by fungus, pre-developed primer/probe (FungiQuant) was used. FungiQuant primer/probe quantitatively detects a broad range of fungus species, including *Trichophyton rubrum*. When formulation contains less than 1 copy of target DNA (i.e., Ct value is 6 cycles greater than 100 copies of target DNA fragment used as positive control), we used the formulation in the AD subject.

Single-use aliquot of formulated lotion (2 grams) was added to a sterile container. A four-day supply (18 single-use aliquots) was provided to each participant at day 0 and day 4 visits.

Interim analysis

An interim analysis was performed because of difficulties in enrollment due to a lower rate of staphylococcus positivity than expected. We requested approval from our sponsor National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS) for interim analysis once we had randomized 15 subjects. In total 41 subjects were screened in order to randomize 15 subjects (eFigure 2 in Supplementary Information). The presented data are from the interim analysis officially conducted.

Full-length 16S rRNA sequencing of skin microbiome

Bacterial DNA was collected from pre-measured areas similar to those used for bacterial culture collection using swabs pre-moistened with Tris-EDTA buffer containing 0.1% TritonX-100 and 0.05% Tween-20 (w/v). Microbial DNA was extracted with PureLink Microbiome DNA purification kit and eluted with elution buffer. Near full-length fragment of 16S rRNA gene was amplified with 27F and 1492R primers with barcode on the forward primer by a 35-cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 minutes, followed by 35 cycles of 94°C for 30 seconds, 53°C for 40 seconds, and 72°C for 90 seconds, after which a final elongation step at 72°C for 5 minutes was performed. After amplification, PCR products were checked in 2% agarose gel to determine the success of amplification and the relative intensity of bands. All samples were pooled together in equal proportions based on their molecular weight and DNA concentrations. The PCR pool was then purified using Ampure PB beads (Pacific Biosciences).

The SMRTbell libraries (Pacific Biosciences) were prepared following the manufacturer's user guide, and sequencing performed at MR DNA (www.mrdnlab.com, Shallowater, TX, USA) on the PacBio Sequel following the manufacturer's guidelines. After completion of initial DNA sequencing, each library underwent a secondary analysis, Circular Consensus Sequencing, using PacBio's CCS algorithm. The CCS algorithm aligned the forward 'FWD' and reverse 'REV' subreads from each template to generate consensus sequences, thereby correcting the stochastic errors generated in the initial analysis. Sequence data was then processed using the MR DNA analysis pipeline (MR DNA, Shallowater, TX, USA). In summary, the CCS sequencing data was depleted of barcodes, oriented 5' to 3', sequences <150bp removed, and sequences with ambiguous base calls removed. Sequences were denoised, OTUs generated, and chimeras

removed. Operational taxonomic units (OTUs) were defined by clustering at 3% divergence (97% similarity). To analyze the microbial composition at species level, 13,000 reads/ sample (Average) were analyzed. Final OTUs were taxonomically classified using BLASTn against a curated database derived from RDPII and NCBI (www.ncbi.nlm.nih.gov, <http://rdp.cme.msu.edu>). Desimilarity of each sequence data was assessed by calculating Jaccard Distance with with QIIME (<http://qiime.org>).

Statistical analyses

For non-normally distributed variables such as abundance of 16S rRNA gene and CFU of *S. aureus*, data were normalized by log 10 transformation and two-tailed parametric t-test was used to compare data from subjects with active or vehicle treatment at each time point. Unpaired t-tests (two-tailed) were used to compare EASI scores from subjects with active or vehicle treatment at each time point. Statistical analyses were performed using Prism (Version 8.0, Graph Pad).

The sample size was calculated using G-Power Data Analysis software for a two-tailed t-test for independent samples, type II error rate of 0.2, and type I error rate of 0.05. The values used in the analysis were determined from a pilot study in which we quantified the reduction in *S. aureus* on the forearms of 5 AD patients twenty-four hours after treatment with a live microbiome transplant.⁸