Probiotic for pathogen-specific *Staphylococcus aureus* decolonisation in Thailand: a phase 2, double-blind, randomised, placebo-controlled trial

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Summary

Background Decolonisation is considered a valuable means to reduce *Staphylococcus aureus* infection rates. However, previous topical strategies targeting the nose or skin had little success, and oral antibiotic-based decolonisation is ill advised because of eradication of the microbiota and development of antibiotic resistance. We previously showed that the probiotic *Bacillus subtilis* significantly diminished *S aureus* at the main intestinal colonisation site via specific bacterial interaction in mice; in this study, we tested this probiotic approach to control *S aureus* colonisation in humans.

Methods We did a single-centre, phase 2, double-blind, randomised, placebo-controlled trial in adults from the Songkhla region of Thailand who were colonised by *S aureus*. Eligible participants were adults (aged ≥18 years) without history of intestinal disease, antibiotic treatment, or hospital admission within the previous 90 days. Participants were excluded if they were pregnant, breastfeeding, taking probiotics, or had diarrhoea. Participants were allocated (1:1) to groups by computer randomisation in blocks of four, and research coordinators were masked to group allocation. Participants received 250 mg of probiotic *B subtilis* MB40 or placebo once per day for 30 days and *S aureus* colonisation was determined after the last dose was received. The primary outcome was colonisation by *S aureus* (continuous, mean decrease in colony-forming-unit count) in the intestine (by faecal counts) and nares (by nasal swabs) after intervention (30-day regimen of *B subtilis* probiotic). This trial is registered with the Thai Clinical Trials Registry, TCTR20210128003.

Findings The trial was done between Jan 29 and June 30, 2021, with enrolment taking place from Jan 29 to April 6, 2021. 115 participants were colonised by *S aureus*, either in the intestine (n=84), nose (n=50), or both (n=19), and were randomly assigned to treatment (n=55) and placebo groups (n=60). Oral probiotic *B subtilis* resulted in significant reduction of *S aureus* in stool (96·8%; p<0·0001) and nose (65·4%; p=0·0002). There were no differences in adverse effects or significant microbiome changes between the intervention and placebo groups.

Interpretation *B subtilis* probiotic eliminated more than 95% of the total *S aureus* colonising the human body without altering the microbiota. This probiotic strategy offers several key advantages over presently used decolonisation strategies for potential use in people with chronic or long-term risk of *S aureus* infection. Furthermore, by establishing a defining role of the intestinal colonisation site, our findings call for revisiting fundamental notions about *S aureus* colonisation.

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Introduction

Staphylococcus aureus is a human pathogen that can cause several serious and often fatal infections. Treatment is complicated by widespread antibiotic resistance, such as in methicillin-resistant *S aureus* (MRSA)[1]. In the USA, *S aureus* kills more people than any other antibiotic-resistant pathogen, with an annual death toll of 20 000 caused by bloodstream infections alone.[2] In most studies, between a quarter to a third of the studied population is reported to be permanent asymptomatic carriers of *S aureus*.[3,4] Because *S aureus* infections usually originate from asymptomatic colonisation,[5,6] decolonisation has frequently been suggested to reduce the prevalence of *S aureus* infections.[7–9] *S aureus* decolonisation strategies have generally used antibiotics, which are inherently problematic because of the dangers associated with the destruction of the natural microbiota and the spread of antimicrobial resistance.[10,11] Most strategies have targeted the nares,[7,9] which are considered the most important *S aureus* colonisation site,[3] and some also included skin decolonisation with antiseptics.[7] However, *S aureus* isncreasingly shown to also colonise the intestine,[4] and there are several reports demonstrating that, similar to nasal carriage, intestinal carriage is a

Research in context

Evidence before this study

Staphylococcus aureus decolonisation within the human body is generally considered a valuable approach to prevent infection with this pathogen. There have been several studies in which topical antibiotics or antiseptics were used to rid specific parts of the human body of *S aureus*, usually the nose and more rarely, the skin. Such measures only had limited and temporary success. This is believed to be at least in part because of recolonisation from the intestine, a site where large numbers of *S aureus* colonies are present. However, intestinal *S aureus* colonisation can only be targeted by oral antibiotic treatment, which propagates antibiotic resistance and increases the risk of infection, and is therefore advised against by the Infectious Diseases Society of America.

Added value of this study

In this study, we show that orally administered probiotic *Bacillus subtilis* strongly diminishes *S aureus* colonisation of the human intestine without a substantial effect on the microbiome, and even affects *S aureus* numbers in the nose as a colonisation site distal to the site of intervention. Although previously employed topical approaches only affect a minor portion of the total *S aureus* colonising humans, this method achieves that which was previously impossible, a reduction of a large portion (>95%) of the total number of *S aureus* colonies in humans, without adverse side-effects.

Implications of all the available evidence

The probiotic method of decolonisation that we propose could be of great value in settings with frequent *S aureus* infections, such as nursing homes, long-term care hospitals, or surgical wards. Furthermore, our findings indicate a pivotal role of the intestinal *S aureus* colonisation site and call for a categorical rethinking of *S aureus* colonisation dynamics and the setup of *S aureus* decolonisation strategies.

source for infection.[6,12,13] For example, in 2010, one study showed that intestinal but not nasal carriage is associated with skin and soft-tissue infections in children.[14] Notably, reinoculation from intestinal carriage may explain previously reported rapid recolonisation and unsuccessful *S aureus* decolonisation treatment that was directed at the nose or skin.[4] Rarely, oral antibiotics have been given to achieve comprehensive systemic decolonisation including that of the intestine, but given what we know now about the role of the natural intestinal microbiome in preventing overgrowth of pathogens, this use of antibiotics is hardly considered an appropriate strategy and not recommended by the Infectious Diseases Society of America.[15]

Probiotics are live microorganisms, which confer a health benefit on the host when administered in adequate amounts.[16] In contrast to antibiotics, probiotics do not have deleterious influences on the microbiota and do not lead to resistance in most instances.[17] Several strains of *Bacillus subtilis* are classified as probiotics that are commercially available in monospecies form or as a component of mixed probiotic formulae. *B subtilis* probiotic is taken as spores that germinate in the intestine[18] that, in comparison with other probiotic microorganisms, has the advantage of surviving passage through the stomach. We previously showed that most strains of *Bacillus* spp, including most strains of *B subtilis*, secrete molecules that specifically inhibit *S aureus* quorum sensing, a mechanism we demonstrated is essential for *S aureus* intestinal colonisation,[19] and orally administered *B subtilis* significantly diminished *S aureus* intestinal colonisation in mice.[19]

Prompted by our mechanistic findings, we here analysed whether a regimen of *B subtilis* (strain MB40) can decrease *S aureus* colonisation in humans and thereby overcome the problems related to topical decolonisation efforts and the use of antibiotics. Our study presents a strategy for *S aureus* colonisation that is safe, without harm to the existing microbiota, and efficacious. Furthermore, our data call for a categorical rethinking of *S aureus* colonisation dynamics and decolonisation strategies.

Methods

Study design

We did a single-centre, phase 2, double-blind, randomised, placebo-controlled trial at Prince of Songkla University (Hat Yai, Thailand) with participants from the Songkhla region of Thailand to assess the efficacy of *B subtilis* (strain MB40) at reducing intestinal and nasal colonisation in healthy individuals colonised with *S aureus*. Ethics approval was obtained by the Human Research Ethics Committee, Faculty of Medicine, Prince of Songkla University (reference number Zhs5-qWmp-qUCq-1xnX).

Participants

Eligible participants were adults aged 18 years or older who had no history of intestinal disease, antibiotic treatment, or hospital admission within the previous 90 days. Participants were excluded if they were pregnant, breastfeeding, taking probiotics, or had diarrhoea. Written informed consent was obtained from all participants. No effort was made to balance the groups on the basis of age, race or ethnic group, or sex.

We did a pretrial screen to assess who had a permanent colony of either *S aureus* or *Bacillus*, whereby we collected nasal swabs and faecal samples from every individual at two timepoints within a 4-week interval (figure 1). We screened for *S aureus* and *Bacillus* spp by plating on mannitol salt agar, which is selective for staphylococci and bacilli and on which *S aureus* and *Bacillus* spp can easily be differentiated from each other and other

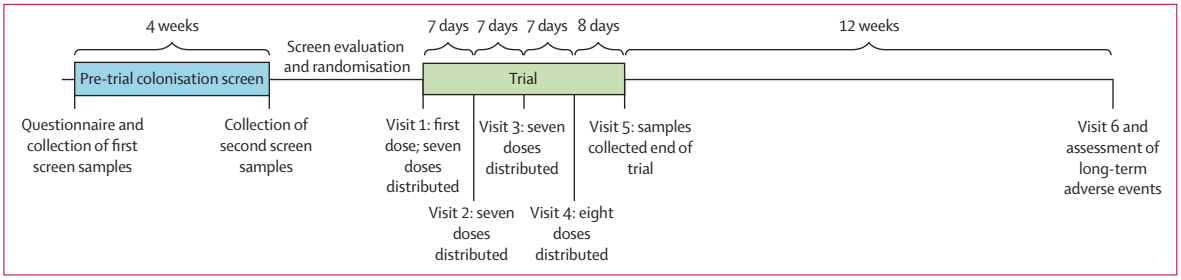

Figure 1: **Timeline of pretrial and trial procedures**

microorganisms by their morphology. To that end, dilutions of nasal swabs (from both nares) or of 1 g faecal matter suspended in 1 mL phosphate-buffered saline (PBS) were plated and grown overnight at 37°C on mannitol salt agar plates and entire plates of countable dilutions were counted. Representative colonies were confirmed for species identity using MALDI-TOF mass spectrometry and 16S rRNA sequencing. Participants were considered to have a permanent colony at a specific site (intestinal or nasal) of either *S aureus* or *Bacillus* spp if two positive samples (with at least one species-confirmed colony) were obtained at both timepoints.

Eligible participants who had a permanent colony of either *S aureus* or *Bacillus* spp were also interviewed by a research assistant using a structured questionnaire that included the collection of demographic and socio-economic data.

Randomisation and masking

Individuals with *S aureus* colonisation were randomly assigned (1:1; figure 2) to the intervention or control group. The randomisation code was computer-generated using Microsoft Excel, and randomisation was done in blocks of four.

Participants received probiotic or placebo, which were indistinguishable in appearance and texture, in sealed, non-transparent medical zip bags. The bags were coded by numbers by a research assistant and handed to participants by another research assistant who did not have information on group allocation or contents of the bags. The research assistants who generated the sequence, enrolled participants, and assigned them to the trial did not have any involvement in trial analyses. The nurses in the research clinic assessing adverse effects and the individuals analysing the data were masked to group allocation.

Procedures

Participants in the intervention group received a Good Manufacturing Practice and Good Hygiene Practice-certified capsule (MySkinRecipes, Bangkok, Thailand) that had been filled with 250 mg *B subtilis* strain spores, previously tested for safety and general probiotic effects on gastrointestinal health in humans (OPTI-BIOME *B subtilis* strain MB40; BIO-CAT Microbials, Shakopee, MN, USA; American Type Culture Collection, PTA-122264),[20] corresponding to a dose of 10×10^9 colony-forming units (CFU) once per day for 30 days, or 250 mg maltodextrin (Chemipan, Bangkok, Thailand) filled in the same type of capsules as a placebo. The OPTI-BIOME probiotic formula (with strain *B subtilis* MB40) was selected among several *B subtilis* strains frequently used in commercially available *B subtilis* probiotic or potentially probiotic formulae (Natto, R0179) on the basis of its considerably higher production of fengycins, which are the active molecules in *B subtilis* that inhibit the *S aureus* quorum-sensing system we had found to be essential for *S aureus* intestinal colonisation[19] (appendix p 3). The microbiological purity of the OPTI-BIOME formula was confirmed directly before the start of the intervention.

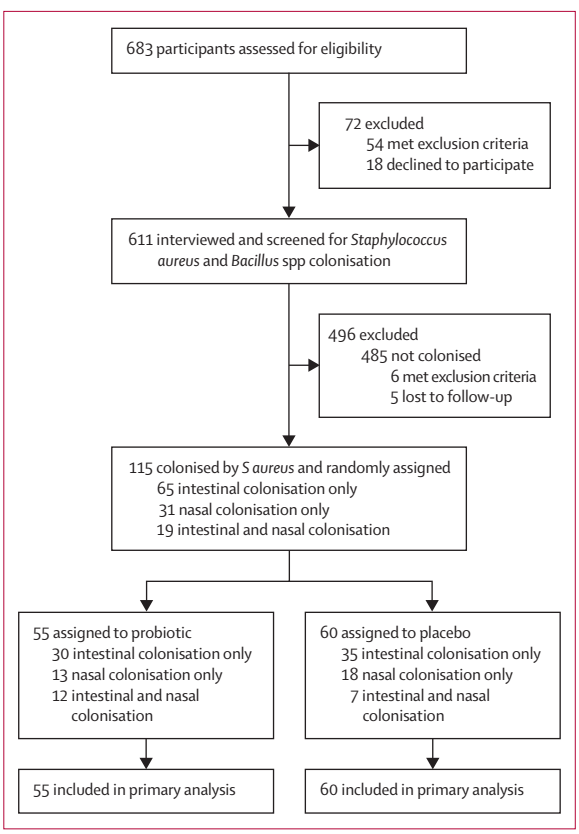

Figure 2: **Trial profile**

See **Online** for appendix

	Intestinal colonisation			Nasal colonisation		
	Total (n=84)	Probiotic (n=42)	Placebo (n=42)	Total (n=50)	Probiotic (n=25)	Placebo (n=25)
Sex						
Male	39	18 (46%)	21 (54%)	23	10 (43%)	13 (57%)
Female	45	24 (53%)	21 (47%)	27	15 (56%)	12 (44%)
Age, mean (SD)	36·20 (12·95)	36·19 (4·03)	36·21 (11·94)	34·2 (11·07)	34·32 (12·31)	34·08 (9·94)
Occupation						
General employee	13	7 (54%)	6 (46%)	20	7 (35%)	13 (65%)
Farmer	17	5 (29%)	12 (71%)	2	1 (50%)	1 (50%)
Federal employee	5	1 (20%)	4 (80%)	2	2 (100%)	0
Grocer	3	0	3 (100%)	1	1 (100%)	0
Health-care worker	5	2 (40%)	3 (60%)	1	0	1 (100%)
Student	27	18 (67%)	9 (33%)	14	8 (57%)	6 (43%)
Unemployed	9	6 (67%)	3 (33%)	7	5 (71%)	2 (29%)
Veterinarian	3	2 (67%)	1 (33%)	2	1 (50%)	1 (50%)
Business owner	2	1 (50%)	1 (50%)	1	0	1 (100%)
Smoking						
≥3 times per week	39	25 (64%)	14 (36%)	15	9 (60%)	6 (40%)
1–2 times per week	0	0	0	0	0	0
Never	45	17 (38%)	28 (62%)	35	16 (46%)	19 (54%)
Alcohol consumption						
≥3 times per week	3	2 (67%)	1 (33%)	3	2 (67%)	1 (33%)
1–2 times per week	34	16 (47%)	18 (53%)	14	9 (64%)	5 (36%)
Never	47	24 (51%)	23 (49%)	33	14 (42%)	19 (58%)
Underlying condition						
Allergic rhinitis	2	2 (100%)	0	18	9 (50%)	9 (50%)
Asthma	3	1 (33%)	2 (67%)	4	2 (50%)	2 (50%)
Diabetes	2	2 (100%)	0	0	0	0
Hypertension	1	0	1 (100%)	1	1 (100%)	0

All data are n (%) unless otherwise indicated.

Table 1: **Participant baseline data**

To that end, absence of contaminating micro-organisms was ascertained by bacterial contamination screening (Enterobacteriaceae, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Enterococcus* spp, and *S aureus*). Briefly, 1 g of each probiotic formula was suspended in 1 mL PBS and diluted. For *Enterobacteriaceae*, *P aeruginosa*, and *A baumannii*, the suspension was cultured on MacConkey agar and incubated at 37°C for 18 h. For *Enterococcus* spp, the suspension was cultured on bile esculin agar both with and without vancomycin (6 mg/L) and incubated at 37°C for 24 h. For *S aureus*, the suspension was cultured on mannitol salt agar and incubated at 37°C for 24 h.

After assignment to a trial group, participants received the first dose of *B subtilis* or placebo orally at a research clinic at Prince of Songkla University. On visit 1, participants were given doses for 7 days to take at home and returned to the research clinic weekly for three consecutive weeks (visits 2–4; figure 1) to receive further doses once per day. On visit 4, participants received eight doses, all together resulting in a total of 30 treatment days. After completing the 30-day treatment, nasal swab and faecal samples were analysed for *S aureus* and *Bacillus* the next day (figure 1).

At each weekly visit participants were asked to report any adverse events. Participants were also asked to visit the research clinic again (visit 6) 12 weeks after completing the study to report any long-term adverse events (figure 1).

For microbiome analysis, genomic DNA from each faecal sample was extracted using a QIAamp DNA stool Minikit (Qiagen; Germantown, MA, USA) according to the manufacturer's instructions. DNA was quantified using a Nanodrop spectrophotometer and paired-end sequencing of the 16S rRNA V3-V4 region was done by PSOMAGEN (Rockville, MA, USA) using an Illumina MiSeq system. All obtained paired-end sequences were identified and quantified for the abundance of Operational Taxonomic Units (OTUs) using Quantitative Insights Into Microbial Ecology (QIIME 1.9.1). This study used the Nephele (release 1.6) platform. The sequences were assigned to OTUs with the uclust-based open-reference OTU picking protocol from QIIME and the Greengenes 13_8 reference sequence set at 99% similarity.

	Stool CFU before intervention (95% CI)	Stool CFU after intervention (95% CI)	Reduction percentage (p value before vs after intervention)*	OPTI-BIOME vs placebo, p value†	Nose CFU before intervention (95% CI)	Nose CFU after intervention (95% CI)	Reduction percentage (p value before vs after intervention)	OPTI-BIOME vs placebo (p value)†
All participants with OPTI-BIOME	20 213 (12026 to 28401)	646 (261 to 1031)	96·8 (<0·0001)	0·0001	1576 (1130 to 2022)	564 (265 to 863)	65·4 (<0·0001)	0·0002
All participants with placebo	15 350 (9729 to 20971)	12 532 (7547 to 17571)	19·4 (0·12)	NA	1306 (911 to 1702)	1116 (756 to 1476)	14·6 (0·084)	NA
Participants only colonised in the intestine with OPTI-BIOME	18 027 (8757 to 27297)	659 (150 to 1168)	96·3 (<0·0001)	0·0007	ND	ND	ND	ND
Participants only colonised in the intestine with placebo	14 581 (8673 to 20490)	12 499 (6952 to 18046)	14·3 (0·33)	NA	ND	ND	ND	NA
Participants only colonised in the nose with OPTI-BIOME	ND	ND	ND	ND	1508 (1059 to 1956)	400 (176 to 624)	73·5 (0·0005)	0·0035
Participants only colonised in the nose with placebo	ND	ND	ND	NA	1288 (898 to 1677)	1048 (673 to 1423)	18·6 (0·13)	NA
Participants colonised in nose and intestine with OPTI-BIOME	25 680 (6591 to 44769	612 (48 to 1175)	97·6 (0·0005)	0·083	1650 (767 to 2533)	742 (131 to 1353)	55·0 (0·0005)	0·021
Participants colonised in nose and intestine with placebo	19 194 (–2017 to 40406)	12 697 (–2537 to 27931)	34·0 (0·16)	NA	1354 (71 to 2638)	1291 (207 to 2376)	4·7 (0·61)	NA

CFU=colony-forming units. NA=not applicable. ND=not determined. *Wilcoxon matched-pairs signed-rank test. †Mann-Whitney test of CFU differences in individuals at a given site.

Table 2: **Trial results**

Outcomes

The primary outcome was colonisation by *S aureus* (continuous, mean decrease in CFU count) in the intestine (by faecal counts) and nares (by nasal swabs) after intervention (30-day regimen of *B subtilis* probiotic). Secondary outcomes were intestinal and nasal colonisation by *B subtilis* after intervention, and characterisation of the intestinal microbiome.

Statistical analysis

The power analysis was based on a previous study that had shown that the mean density of *S aureus* in faecal human samples was 5·1 (SD 1·5 $\log_{10}$ CFU/g.[21] We estimated that probiotic *B subtilis* would reduce the amount of *S aureus* by 25% (to 3.82, 1·5 $\log_{10}$ CFU/g). The power calculation was done using ClinCalc by a continuous endpoint method (mean CFU) and two independent samples at 0·01 probability of a type 1 error and 0·1 probability of a type 2 error. Based on the power calculation, the required sample size was 41 participants per group. All enrolled participants were included in primary and safety analyses.

To estimate how many individuals had to be screened for *S aureus* colonisation in the pretrial selection, we assumed a colonisation rate in the Thai rural community where our study was done of about 12·5% to 13% based on our previous study in the same community.[19] At a 95% CI with an incidence rate of 12·75% and an allowable error of 2·5%, the required sample size for our initial screen was 684 participants.

Prism 8 (version 8.2.1) for Mac OS was used for statistical analyses.

The primary outcome (efficacy of decolonisation) was analysed by two-tailed Wilcoxon matched-pairs signed-rank tests within treatment and placebo groups, and by two-tailed unpaired Mann-Whitney tests comparing differences before and after treatment in CFU between treatment and placebo groups. These non-parametric tests were used because groups did not show normal distribution by Anderson-Darling, D'Agostino and Pearson, Shapiro-Wilk, and Kolmogorov-Smirnov tests. Statistical analysis comparing adverse effects between treatment and placebo groups was by Fisher's exact test. All error bars show the SD of the mean for non-logarithmic and the standard deviation of the geometric mean for logarithmic scales.

This trial was registered with the Thai Clinical Trials Registry, TCTR20210128003.

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

For the **ClinCalc** power calculation tool see https://clincalc.com/stats/samplesize.aspx

Results

Between Jan 29 and April 6, 2021, 611 participants were enrolled and screened for *S aureus* and *Bacillus* spp intestinal and nasal colonisation. 115 (19%) of these 611 screened participants were colonised by *S aureus*, either only in the intestine (n=65), only in the nose (n=31), or in both locations (n=19), and randomly assigned to a trial group (figure 2). Following randomisation, 55 participants (30 with colonies in the intestine only, 13 with colonies in the nose only, and 12 with colonies in both locations) were assigned to the treatment group, and 60 participants (35 with colonies in the intestine only, 18 with colonies in the nose only, and 7 with colonies in both locations) were assigned to the control group (figure 2). Baseline characteristics of

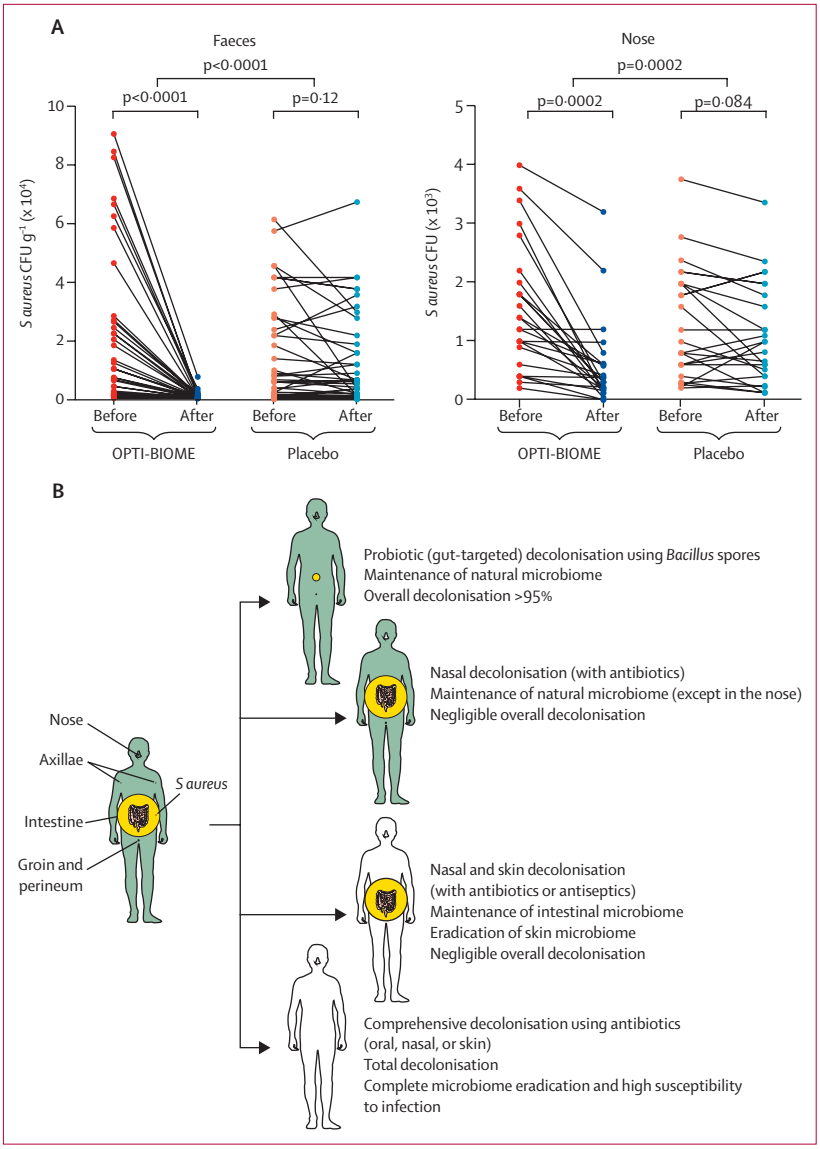

Figure 3: **Trial results with colonisation levels by individual and study interpretation**
(A) Pairwise comparison of colonisation before and after intervention for specific individuals, and statistical analysis of the effect difference between treatment and placebo by comparing differences between colonisation before and after intervention using Mann-Whitney tests. Statistical analysis of difference between data before and after intervention within a group was done by Wilcoxon matched-pairs signed-rank test. All error bars show the mean (SD). (B) *S aureus* distribution in the human body and comparison of decolonisation strategies. The average abundance of *S aureus* in individuals with *S aureus* colonies in the intestine, nares, and predominant further skin colonisation sites (axillae and the groin and perineum) was estimated on the basis of data obtained in this and previous studies. Decolonisation efficacies were estimated on the basis of data obtained in this study for probiotic-based decolonisation and assuming 100% decolonisation for antibiotic-based or antiseptic-based topical or antibiotic-based comprehensive or systemic (oral application combined with nasal and skin decolonisation) methods at the targeted sites. The effect of probiotic treatment on *S aureus* in the groin, perineum, and axillae was estimated to occur at the same rate as that measured for the nares. Yellow shows *S aureus*. Circle areas represent abundance. For extra-intestinal sites, note comparatively low *S aureus* colonisation as expressed by small yellow circles. Green shows intact microbiome. CFU=colony-forming units.

the participants in the trial are shown in table 1. All participants were assessed for the primary outcome.

Oral administration of probiotic *B subtilis* resulted in significant reduction of *S aureus* in the stool (96·8%; p<0·0001) and the nose (65·4%; p=0·0002), whereas there were no significant differences in the placebo groups (table 2). Direct comparison of decolonisation efficacies in the treatment versus placebo groups by analysing reduction of colonisation yielded significant differences (stool, p<0·0001; nose, p=0·0002; table 2, figure 3). We also detected significant reduction of nasal and stool CFUs when separately analysing individuals who only had colonies in the nose (p=0·0035) or intestines (p=0·0007; table 2). In the analysis of individuals with both nasal and intestinal colonisation, differences in the nose were significant (p=0·021), whereas in stool, values did not reach significance (p=0·083; table 2). Of note, these separately analysed groups with only nasal, only intestinal, or colonisation at both sites were small, because they were not subjected to a previous power analysis, as was done for the primary outcome.

At the end of the intervention period, individuals in the treatment group all had *Bacillus* spp in their faeces at a concentration of about 10^3 CFU/g to 10^5 CFU/g with a geometric mean of about 10^4 CFU/g (9541 CFU and a geometric SD factor of 4·033; appendix p 3). Note that no trial participants had pretrial colonisation with *Bacillus* spp. No *Bacillus* spp was found in the placebo group and representative *Bacillus* spp colonies obtained from the treatment group were confirmed by MALDI-TOF mass spectrometry to be *B subtilis*, substantiating that they originated from the ingested probiotic. *Bacillus* spp were not found in the noses of any participants.

To confirm the absence of deleterious effects on the intestinal microbiome and the specificity of the *S aureus* exclusion mechanism (as opposed to a general effect on the intestinal microbiome), we determined the composition of the intestinal microbiome in all participants who received *B subtilis* probiotic before and after treatment. Common analyses for α-diversity and β-diversity showed absence of substantial microbiome alterations. Furthermore, relative abundances of the major phylae inhabiting the gut, which often show shifts under different diets or drug treatments, were not significantly changed. Moreover, we detected no changes in the most abundant OTUs on the genus level (appendix p 4) and only few changes in any of the detected OTUs (appendix p 5). These results showing absence of significant overall changes in the intestinal microbiome caused by treatment with *B subtilis* probiotic are in accordance with and as expected by the specificity of the quorum-quenching effect of *Bacillus* spp fengycins on *S aureus* as a comparatively negligible component of the intestinal microbiome regarding absolute quantity.

B subtilis, including strain MB40, is being used as a probiotic with demonstrated benefits for gastrointestinal health and has been shown in human studies to be safe.[20,22] Accordingly, in our study no severe adverse effects (severe watery diarrhoea, severe vomiting, dermatitis, or eye irritation) were reported. Moderate adverse effects were rarely reported and were not

significantly more frequent than in the placebo group (table 3).

Discussion

In this study, we did a randomised trial to analyse the value of a *B subtilis* probiotic for *S aureus* decolonisation. Our decolonisation strategy differed from previous approaches in two categorical features. First, we used a probiotic, which is generally considered safe and, in contrast to previous strategies with antibiotics and antiseptics, does not harm the existing microbiota. Furthermore, the specific decolonisation agent that we used was selected on the basis of our previous mechanistic results to virtually only interfere with staphylococcal colonisation,[19] further minimising effects on other members of the microbiota. Second, our strategy was to target intestinal *S aureus* colonisation to eradicate a maximal number of the total colonising *S aureus* population in humans and to base our analysis of efficacy on quantitative rather than qualitative data. This strategy contrasts with previous decolonisation strategies, which generally used topical antibiotic treatment of the nares and occasionally the skin and measured efficacy by analysing how many participants showed *S aureus* eradication over a detection threshold at those sites, notably often neglecting analysis of the faeces.[7]

Our study met the primary outcome of reducing *S aureus* colonisation in the intestine and the nares. Colonisation densities in the intestine were reduced by probiotic treatment by an average factor of about 31. As expected, reduction of colonisation in the nares, as sites distal to the targeted intervention site, was much lower (factor of about 3). This was of minor relevance for our goal to reduce overall *S aureus* colonisation of the human body, but of major importance to our understanding of *S aureus* colonisation dynamics as discussed hereafter.

There were no severe adverse effects, and no other adverse effects were reported at rates significantly higher than in the placebo group. Furthermore, there were no significant effects on the overall composition of the intestinal microbiome. These results show safety and efficacy of the *B subtilis* probiotic in reducing *S aureus* human colonisation, offering a previously unavailable method to eradicate the main, intestinal reservoir of *S aureus* without the considerable dangers of pathogen overgrowth that are associated with systemic oral antibiotic treatment. Based on our data and those from previous studies on *S aureus* CFU densities,[23–27] we estimate that the decolonisation strategy we propose leads to at least about 95% decolonisation, which contrasts with previous strategies aimed at the nose and the skin which, even with 100% eradication at those sites, can only affect a small portion of the total *S aureus* in the human body. Furthermore, we here confirmed our previous findings[19] showing complete association of *Bacillus* colonisation with absence of *S aureus* colonisation in a human population as determined by analysis of faecal CFU. This result suggests that prolonged intake of *B subtilis* could have an even more pronounced effect than that observed in our trial, which was limited by the time of intervention and the dosing requirement of once per day. *Bacillus* spp is a transient coloniser, and it is thus not expected that the effect on *S aureus* colonisation persists long after cessation of oral administration. However, this probiotic strategy allows for long-term application because of the absence of harmful side-effects, which contrasts with antibiotic decolonisation procedures that are similarly short-term in effect, but not amenable to extended use for the aforementioned reasons. Finally, it is important to stress that on the basis of the underlying mechanism that we established in mice,[19] similar efficacy can only be expected from *B subtilis* strains that produce fengycins. According to our in-vitro results, this feature is absent from several frequently used commercially available *B subtilis* probiotic formulae, which is in contrast with the more widespread production of fengycins we previously detected in human isolates of that species.[19]

Our study also has important implications for our understanding of the relative importance of *S aureus* colonisation sites and the dynamics of *S aureus* colonisation. The average number of CFU we detected in only 1 g of faeces from individuals who had *S aureus* colonies was about 1 log higher than that in a total nasal swab (appendix p 2), indicating that total *S aureus* numbers in the gut greatly exceed those in the nose (by about three orders of magnitude given the average weight of human faeces of about 100 g). Although we are not aware of a previous study that measured *S aureus* CFUs in the nares and faeces in the same cohort of individuals, our numbers are in general in accordance with previously obtained data on *S aureus* CFU density in the nares and feces,[23–27] and emphasise the overwhelming importance of the intestinal colonisation site for overall *S aureus* colonisation of the human body in quantitative terms. Furthermore, as

	Probiotic (n=55)	Placebo (n=60)	p value
Fever	0	0	1·00
Infection	0	0	1·00
Nausea and vomiting	4 (7%)	4 (7%)	1·00
Constipation	3 (5%)	2 (3%)	0·67
Headache	0	0	1·00
Muscle pain, cramp, or spasm	0	0	1·00
Upset stomach or heartburn	3 (5%)	2 (3%)	0·67
Gas or bloating	0	2 (3%)	0·50
Unusual stool (loose, discoloured, or more frequent)	3 (5%)	2 (3%)	0·67
Bad taste	4 (7%)	1 (2%)	0·19

p value was established using two-tailed Fisher's exact test.

Table 3: **Adverse events**

mentioned previously, we did not expect a pronounced effect of the gut-targeted decolonisation on nasal colonisation, but the significant reduction of *S aureus* nasal CFU that we observed suggests a dominating role of the intestinal site for *S aureus* colonisation. By contrast, we are not aware of any study that reported reduction of intestinal CFU upon exclusively nose or skin-targeted decolonisation, a scenario that also appears unlikely given the much greater abundance of *S aureus* in the gut. These findings are of particular value, because studies analysing the dynamic interdependence of different sites of *S aureus* colonisation are hardly possible in animals because of the limited extent and duration of experimental *S aureus* nasal colonisation,[28,29] and because the animals eat their faeces. The results indicate that intestinal *S aureus* forms a reservoir for nasal *S aureus* that could originate from repeated anal-to-nasal reintroduction. The higher over-time consistency we observed for intestinal versus nasal colonisation (appendix p 2) is in further agreement with this idea. In that context, it is noteworthy that we also detected a significant reduction of nasal colonisation in individuals in which we detected no previous *S aureus* intestinal colonisation as assessed by faecal CFU counting. However, intestinal colonisation may be undercounted and sometimes remain undetected, because it can only be assessed indirectly by measuring CFU in the faeces, whereas nasal CFU can be directly assessed by swabbing. Although faecal analysis is believed to give an overall adequate assessment of individual or differences in intestinal colonisation after intervention, underlying intestinal *S aureus* might in some cases remain undetected.

Our study has limitations. First, among the non-intestinal *S aureus* colonisation sites we only analysed the nose. We did so because of the traditional focus of *S aureus* colonisation studies on the nose and the comparatively lower colonisation of other non-intestinal body sites.[24,27] Given that the *S aureus* strain composition of those sites is similar,[30] indicating dynamic interdependence, it is likely that the relationship between intestinal colonisation and that of those sites follows dynamics similar to those we have demonstrated for the nose. Second, we did our trial in a rural Thai population, because we wanted to confirm our previous more limited study on *Bacillus* spp and *S aureus* exclusion in the faeces. We believe it is fair to assume a similar trial outcome in *S aureus* carriers from a different geographical area, because the quorum-quenching effect of *Bacillus* on *S aureus* is not strain specific, and we previously established considerable heterogeneity of the *S aureus* strains colonising Thai rural populations.[19] Finally, the intervention groups had somewhat higher average baseline faecal and nasal CFUs than the placebo groups. However, the differences were not significant and unlikely to have had more than a minor effect on the outcome.

In conclusion, our findings suggest that *B subtilis* probiotic could be used to reduce *S aureus* and MRSA colonisation prevalence and thus might have clinical potential to lower infection rates, for example in individuals with a history of recurring *S aureus* infections or in long-term care facilities, such as nursing homes, with notoriously increased *S aureus* colonisation and infection risks. Although no known *S aureus* decolonisation procedure can achieve long-term protection from recolonisation, the probiotic strategy, in contrast to any antibiotic-based strategy, offers the possibility for daily and long-term application, because it does not harm the microbiota nor trigger the development of antibiotic resistance. Furthermore, our data provide support for the notion of a dominating role of the intestinal site for *S aureus* colonisation, suggesting that *S aureus* decolonisation efforts should generally focus on intestinal rather than, or at least in addition to, nasal colonisation.

Contributors

PP and MO conceptualised and designed the study and analysed the data. SK collected human samples and did all microbiological experiments. NR did all procedures at the research clinic, including interviews, intervention, and follow-up with participants. YT prepared the intervention and did the quality control. PP did the microbiome study. SS and DF supervised the procedures at the research clinic. SK and PP accessed and verified all the data reported in this study. All authors had full access to all the data reported in this study and had final responsibility for the decision to submit for publication.

Declaration of interests

We declare no competing interests.

Data sharing

The study protocol and de-identified participant data (questionnaires and summaries) are available from MO (motto@niaid.nih.gov) or SK (sunisa.p@RMUTSV.ac.th) upon request within 1 year of publication. Microbiome sequencing data were deposited at https://www.ncbi.nlm.nih.gov/bioproject/ under BioProject number PRJNA886398.

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