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1 **Topical cream with live lactobacilli modulates the skin microbiome and reduce acne symptoms**

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17

18 Summary

19 The skin is home to an important part of our commensal microbiota, despite it being a cool, acidic
20 and desiccated environment. Tailored microbiome modulation approaches with, for example
21 probiotics, are highly challenging for this body site. Here we show by next-generating sequencing
22 that *Lactobacillus* taxa -especially those known to be dominant in the human vagina- are
23 underestimated members of the skin microbiota. Specific *Lactobacillus* strains were selected in the

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24 lab and formulated in a viable form in an oil in water-based topical cream. Facial application by
25 patients with mild-to-moderate acne symptoms was able to reduce inflammatory lesions and
26 comedone formation. This was associated with a temporary modulation of the skin microbiome,
27 including a reduction in relative abundance of staphylococci and an increase in lactobacilli. Skin
28 microbiome modulation by addition of carefully formulated lactobacilli seems to be new therapeutic
29 option to reduce antibiotic use for common acne symptoms.

30

31 **Introduction**

32 Being the most extensive interface of the human body with the environment, the skin acts as a home
33 to an important part of our commensal microbiota. Similar to the gut, the skin microbiota have
34 essential roles in the education of our immune system and the protection against invading pathogens
35 and other foreign substances. With recent advances in DNA sequencing approaches, our knowledge
36 has been improved on the biogeography of the skin microbiota at different body sites[1]. We are now
37 transitioning from these descriptive, observational studies towards a better understanding of the
38 functional roles of the commensal microbiota, allowing the design of tailored modulation
39 approaches. However, compared with the richer environment of our intestines, the skin lacks many
40 nutrients beyond basic proteins and lipids, with sweat, sebum and the stratum corneum being main
41 resources[2]. In addition, the skin is a cool, acidic and desiccated environment and skin cells are
42 frequently renewed and shed, so that strategies targeting the skin microbiome are highly
43 challenging. For example, probiotics, i.e. live micro-organisms that, when applied in adequate
44 amounts, promote a health effect on the host[3], have not yet been widely considered for direct
45 application on the skin.

46 One of the most common skin diseases is acne vulgaris, a chronic inflammatory skin condition of the
47 sebaceous follicles and glands. The pathogenesis of acne vulgaris is multifactorial, with increased
48 sebum production, alteration in the quality of sebum lipids, dysregulation of the hormone

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49 environment and follicular hyperkeratinization as contributing factors. In addition, specific strains of
50 the facultative anaerobe *Cutibacterium acnes* (formerly known as *Propionibacterium acnes*[4]) are
51 involved in the inflammation of the skin, especially by secreting lipase enzymes that are able to
52 metabolize sebum into free fatty acids which may lead to skin irritation[5]. Yet, the observation that
53 almost all adults are colonized with *C. acnes* but only a minority have acne, highlights that other
54 bacteria such as *Staphylococcus* species can be linked to acne pathogenesis as pathobionts or disease
55 modulators[6]. Therefore, both oral and topical antibiotics such as doxycycline, minocycline and
56 clindamycin are frequently used by acne patients[7], but because of rising problems of antibiotic
57 resistance, various alternative therapies need to be developed[8].

58 Here we explored the potential of topically applied, live probiotic lactobacilli to beneficially modulate
59 cutaneous microbial interactions and host inflammatory responses in subjects with mild-to-moderate
60 acne symptoms. Lactobacilli were selected based on their long history of safe use in fermented
61 foods[9], the gastro-intestinal[10], urogenital tract[11] and nasal cavity[12], but it was unsure whether
62 lactobacilli could also thrive and have health-promoting activities on the skin.

63 **Results and discussion**

64 **Prevalence of *Lactobacillus* on the skin.** Because lactobacilli are not considered to be commensals of
65 the skin, we first monitored the prevalence of lactobacilli on the skin of healthy volunteers. Their
66 relative abundance was explored through 16S amplicon sequencing via Illumina MiSeq (separate runs
67 for V1V2 and V4 variable regions) of facial skin samples (cheek) of 30 volunteers (15 male and 15
68 female), who did not display acne-related symptoms. In the samples of all female volunteers and 12
69 male volunteers *Lactobacillus* sequences were found (**Figure 1a)**. *Lactobacillus* species generally did
70 not occur in the top five of most abundant taxa present on the skin. However, some volunteers
71 showed a relative high abundance of *Lactobacillus* taxa (amplicon sequence variants or ASVs), up to
72 6.4% (based on V1V2 16S sequencing) or 14.3% (by V4 16S sequencing) (**Figure 1b, Extended data**
73 **Figure 1a-b**). The relative abundance of *Lactobacillus taxa* based on both runs (V1V2 and V4) was

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74 also 10-fold higher in women compared to men, with an average relative abundance of 0.8% (1.4% in
75 women and 0.2% in men, Kruskal-Wallis p= 0.0005). **(Extended Data Figure 1b)**. *Lactobacillus* taxa
76 can thus be considered as endogenous members of the skin microbiota, although their relative
77 abundance is lower than *Staphylococcus*, *Corynebacterium, Cutibacterium* (often still classified as
78 *Propionibacterium*), and *Streptococcus*, which were the most the dominant taxa in our dataset for
79 both variable regions sequenced **(Extended Data figure 1 a)**.

80 To confirm our in-house generated data and investigate whether our results are facial site-specific,
81 the presence of lactobacilli was also substantiated in publicly available skin metagenome shotgun
82 datasets by using the curatedMetagenomicData R-package recently described by Pasolli *et al.* [13]. In
83 total, 466 samples from three different studies[14, 15 and 16] were analyzed. Of these samples, 38%
84 (177/466) showed the presence of at least one *Lactobacillus* species **(Figure 1a)**, but only 29 samples
85 showed a relative abundance higher than 1%. Yet, high relative abundances up to 52% on the skin
86 were also observed (average relative abundance based on curated metagenomics was 3.79%) (**Figure**
87 **1b**). We also included 16S amplicon data from the Human Microbiome Project (V3V5)[17] where the
88 relative abundance was 24.9% on average due to some outliers having up to 90% relative abundance
89 (**Figure 1b**). The relative abundance of *Lactobacillus* sequences in the skin samples was also
90 compared to the publicly available data of other human body sites (both 16S and curated
91 metagenome) (**Figure 1b**). As expected, the vagina showed the highest relative abundance of
92 *Lactobacillus* taxa, but the skin turned out to be the second most important niche for these taxa.
93 Moreover, to have a better idea of the phylogenetic diversity of all *Lactobacillus* taxa present, we
94 also plotted all data on a phylogenetic tree of the *Lactobacillus* genus complex[18] (**Figure 1c)**. These
95 data indicate that taxa typically associated with the human vagina, *Lactobacillus crispatus, L. iners, L.*
96 *gasseri* and *L. jensenii* were also found as the most prevalent lactobacilli on the skin (**Figure 1d and**
97 **1e**). Also members of the more niche-flexible *Lactobacillus* taxa[19], *i.e.* from the *L. plantarum/L.*
98 *pentosus* group and *L. casei/paracasei/rhamnosus* group, were frequently detected **(Figure 1d and**
99 **1e)**. The occurrence of *Lactobacillus* taxa on the skin is in agreement with the fact that after normal

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100 delivery through the birth canal, these bacteria originating from the mother are among the first to
101 colonize neonate skin[20]. The data presented here **(Figure 1 a-e)** indicate that these *Lactobacillus* taxa
102 are still present in adults, but do not stay dominant in the different human body skin sites studied.
103 Yet, despite their low relative abundance, they could still play a role as keystone microbes, recently
104 redefined as taxa exerting a considerable influence on microbiome structure and functioning
105 irrespective of their abundance across space and time[21]. Therefore, we subsequently aimed to
106 manipulate biotic interactions of lactobacilli on the skin.
107
108 **Rationale *in vitro* strain selection.** *Lactobacillus* strains were selected from our in-house available
109 laboratory collection (**Extended Data Table 1)** for tailored application in patients with mild-to
110 moderate acne symptoms. A thorough screening approach was applied based on the rationalization
111 that the strains had to be safe, applicable (being robust and showing niche-flexibility as described for
112 lactobacilli by Duar *et al.*[19]) and have the capacity to exert the desired beneficial functions on the
113 human skin including microbiome modulation, immune modulation and epithelial barrier
114 enhancement (**Figure 2a)**. Key properties were substantiated with laboratory tests, genome
115 screening and information available in the literature. Three *Lactobacillus* strains were selected i.e.
116 *Lactobacillus rhamnosus* GG, *Lactobacillus plantarum* WCFS1 and *L. pentosus* KCA1. The rationale for
117 these strains was based on their genome availability[22–24], knowledge of their host interaction capacity
118 [25,26], their robustness and growth capacity[22,24,26] (**Extended Data Figure 2a**), in addition to information
119 on their safety in humans after oral[27,28,29], nasal[30] and vaginal[31] high-dose application. *L. rhamnosus*
120 GG was also selected because of previous reports on its capacity to inhibit the toxic effects of *S.*
121 *aureus* on epidermal keratinocytes[32], its strain-dependent capacity to promote re-epithelialization[33]
122 and to augment tight-junction barrier function in human primary epidermal keratinocytes[34], and our
123 previous experience with this probiotic strain[25]. For microbiome modulation, *C. acnes* was targeted
124 as model pathobiont associated with the inflammatory character of acne vulgaris. *S. aureus* was also
125 targeted as an important pathogen causing skin inflammation. When the activity of spent culture

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126 supernatant of our collection of *Lactobacillus* strains was screened for antimicrobial effects on the
127 growth of *C. acnes* in suspension, all *Lactobacillus* strains tested inhibited the growth of *C. acnes*
128 ATCC6919 and *S. aureus* ATCC29213, but *L. pentosus* KCA1 (vaginal origin) and *L. plantarum* WCFS1
129 (saliva origin) were among the bacteria tested able to exert the highest inhibition **(Figure 2b and**
130 **Extended Data Figure 2b)**. Other related strains tested, including *Staphylococcus epidermidis* 12228,
131 did not inhibit *C. acnes* growth **(Extended data Figure 2)**. In addition, these strains were able to
132 significantly reduce the lipase activity of *C. acnes* (Figure 2c). These lipase enzymes are involved in
133 inflammation of the skin induced by *C. acnes*, because they metabolize sebum into free fatty acids
134 which may lead to skin irritation[5]. Furthermore, because lactic acid has a strong antimicrobial
135 activity[35], as well as a documented dose-dependent capacity to ameliorate the appearance of
136 keratoses and acne in dermatology[36], we also substantiated lactic acid production by the selected
137 lactobacilli (**Figure 2d).** Furthermore, we validated that the three selected lactobacilli did not exhibit
138 toxic or overt inflammatory responses on primary skin cells **(Figure 2e)**, in agreement with genome
139 predictions[22–24] and laboratory validation of antibiotic resistance profiles according to the guidelines
140 of the European Food Safety Authority (EFSA)[37].

141

142 **Viable *Lactobacillus* formulation in O/W cream.** We then aimed to design a topical formulation
143 suitable for the application of live bacteria in a sufficient dose on the skin. The selected bacteria were
144 freeze-dried for stability reasons[38] and embedded in the core of 2-compartment microcapsules
145 **(Figure 3a)**. Various processing conditions were optimized as described in the Methods section and
146 schematized in **Figure 3a**, resulting in capsules of 1500 - 2000 µm diameter with a core of suspended
147 freeze-dried bacteria that can be released upon applying mechanical pressure, such as rubbing on
148 the skin (**Figure 3b)**. Ingredients were selected so that they did not significantly impact on the growth
149 capacity of the skin commensals and pathobionts (tested for *S. epidermis, S. aureus,* and *L. crispatus*)
150 **(Extended Data Figure 2 c-d).** This formulation and encapsulation approach significantly improved

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151 the viability for storage at 4°C and even at 25°C, compared to non-encapsulated freeze-dried bacteria
152 when suspended in a carrier oil-in-water (O/W) cream **(Figure 3c)** and this for up to 6 months (**Figure**
153 **3d**).

154 Subsequently, the skin irritation potential was checked for 20 volunteers with skin patch tests
155 according to Basketter *et al.*[39]. No erythema, dryness or edema was observed in any of the
156 volunteers studied (skin irritation index: 0.00) (**Extended Data Table 2).** For comparison, adapalene
157 products, which are naphthoic acid derivatives with retinoid activity and documented efficacy in the
158 treatment of mild-to-moderate acne vulgaris, have a mean cumulative irritation index between 0.25-
159 1[40]. Also the widely used combined clindamycin–benzoylperoxide treatment for moderate acne has
160 been reported to frequently induce dry skin, flaky/peeling skin, irritated skin, itchy skin and redness
161 in acne patients[41].

162

163 ***Lactobacillus* skin microbiome modulation.** Subsequently, we applied the topical cream twice daily
164 in an open-label 'proof-of-concept' trial to ten volunteers for eight weeks twice daily at 10^8 CFU per
165 application (± 1 gram/application) **(Figure 4a)**. Patients with mild-to-moderate acne symptoms that
166 were not using antibiotics or another acne treatment were included by the responsible dermatologist
167 **(Extended Data Table 3)**. The impact of the *Lactobacillus* cream on their facial skin microbiome was
168 monitored by 16S amplicon sequencing at four different time points, over a period of 10 weeks
169 **(Figure 4a)**. In this way, the skin baseline microbiome before, during and after the treatment was
170 compared. The skin acne microbiome of these patients at the time of inclusion was especially
171 characterized by an increased relative abundance of *Staphylococcus* taxa (p= 0.0058, Wilcoxon rank
172 sum test) when compared to the healthy controls **(Figure 4b) (Extended Data Figure 4** for 3 specific
173 *Staphylococcus* ASVs**)**. No significant difference in relative abundance of *Lactobacillus* taxa was
174 observed between our patients and the reference samples at time of inclusion **(Extended Data**
175 **Figure 3a-b)**. However, we did observe a significantly reduced relative abundance of *Streptococcus*

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176 *salivarius,* a taxon also belonging to the lactic acid bacteria with lactic acid production as core
177 function (**Extended Data Figure 4**). After application of the cream with the lactic-acid producing
178 lactobacilli, the facial skin samples of our acne patients at visit 2 and visit 3 clearly clustered
179 separately on a PCoA plot (**Figure 4c)**. Interestingly, in 7 of 10 patients at visit 2 (4 weeks) and 8/10
180 patients at visit 3 (8 weeks), *Lactobacillus* ASVs were found in relative high abundances (between
181 20.9% and 92.8%), while in three patients at visit 2 and two patients at visit 3, their relative
182 abundance was below 5% (between 0.015 and 1.1 %) (**Figure 4d and Extended Data Figure 3**)**.** ASV
183 analysis via EZ taxon[42] and comparison with the whole genome sequences[22–24] for rRNA copy variants
184 confirmed that the detected ASVs matched the applied lactobacilli. Interestingly, the three probiotic
185 strains appeared to persist on the skin in similar numbers (**Figure 4d**). To substantiate that the
186 lactobacilli detected on the skin were still viable, samples were also plated on *Lactobacillus*-selective
187 MRS agar. Most samples at visit 2 (7/9) and visit 3 (6/7) were culture-positive, indicating that – at
188 least some of- the lactobacilli applied were metabolically active on the skin (**Figure 4d**). At visit 4 (two
189 weeks after the stop of the treatment), most *Lactobacillus* ASVs had disappeared and also growth in
190 MRS medium was markedly reduced, further substantiating that the lactobacilli detected originated
191 from the applied topical cream. We then explored whether the presence of lactobacilli during
192 treatment had impacted on the pathobionts of acne (*C. acnes* and *Staphylococcus* taxa). The relative
193 abundance of both pathobiont taxa dropped indeed at visit 2 and 3 and increased again at visit 4 ($p < 0.05$ for visit 3 versus visit 1 – Wilcoxon test for *Staphylococcus*) **(Figure 4b**).
194

195 ***Lactobacillus* improvement of acne symptoms.** Subsequently, the acne symptoms were clinically
196 scored as the presence of inflammatory lesions and comedones. This analysis showed an overall
197 improvement of the acne symptoms in all patients treated with the *Lactobacillus* cream, as reflected
198 by a significant reduction in inflammatory lesions at visit 2 and 3 compared to visit 1, and a significant
199 reduction in comedone counts at visit 2 **(Figure 5a)**. A significant association between comedonal
200 counts and both *Staphylococcus* and *Propionibacterium/Cutibacterium* was also found **(Figure 5b),**
201 but not for the inflammatory lesions **(Extended Data Figure 5).** Of note, when the treatment

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202 stopped, the acne scores increased again, indicating that the applied lactobacilli and associated
203 microbiome - staphylococcal modulation – did not persist, in agreement with the fact that the
204 exogenously applied lactobacilli could not permanently colonize (**Figure 4 d**). On the other hand, this
205 increase in acne scores when the *Lactobacillus* application stopped, further suggests a possible causal
206 association between the applied lactobacilli and the acne symptom reduction.

207

208 **Conclusion**

209 Acne vulgaris is a common reason for long-term antibiotic use, with dermatologists prescribing
210 antibiotics more commonly than any other physician group[7]. Here, we applied a multiphasic and
211 multidisciplinary approach to substantiate that *Lactobacillus* strains have potential as skin probiotics
212 to target acne. First, we provided detailed information that lactobacilli (and other lactic acid
213 producing taxa) are unneglectable endogenous members of the human skin microbiota, with relative
214 abundances in between those of human vaginal[43] and stool[10] samples. Of interest, phylogenetic
215 placement of the *Lactobacillus* sequences detected in our data (amplicon sequence variants) and the
216 curatedMetagenomicData recently described by Pasolli et al.[13] showed that the dominant
217 *Lactobacillus* taxa (*L. crispatus, L. iners, L. gasseri, L. jensenii)* of the vaginal community are also
218 among the most prevalent *Lactobacillus* taxa for the skin. Previous studies have briefly acknowledged
219 the presence of lactobacilli in the skin microbiota [44,45], however such detailed analysis of specific
220 *Lactobacillus* taxa in the skin niche had not yet been performed. Yet, we also showed that to apply
221 selected lactic acid bacteria on the skin, other properties such as robustness to (processing) stress
222 conditions and growth capacity are required, in addition to safety and lack of (transferable) antibiotic
223 resistance properties (as rationalized in **Figure 2a)**. Following this rationalized scheme, we did
224 manage to translate our results directly from *in vitro* lab tests with skin cells and pathogens to human
225 volunteers, without the need for animal testing. Spent-culture supernatant of the selected *L.*
226 *rhamnosus* GG, *L. plantarum* WCFS1 and *L. pentosus* KCA1 could inhibit the growth of *C. acnes* and *S.*

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227 *aureus in vitro,* could survive the formulation in capsules in an O/W cream and were found in similar
228 amounts after 1/1/1 application on the facial skin of patients with mild-to-moderate acne symptoms.
229 Twice daily topical application of this cream with the live lactobacilli was able to reduce inflammatory
230 acne lesions and comedone formation in the ten patients included in the open-label pilot study, and
231 was associated with a reduction in *Staphylococcus* relative abundance (as summarized in **Figure 6)**.
232 Our 16S rRNA ASV-based comparison of the acne facial microbiome of 30 healthy volunteers and 27
233 patients with acne symptoms suggests indeed that *Staphylococcus* taxa are increased in acne
234 patients and that *Staphylococcus* could thus form an interesting acne target to further investigate.
235 ASV level analysis of the sequenced V4 region of the 16S rRNA gene did not allow identification of the
236 *Staphylococcus* taxa up to species/strain level, so that no distinction between *S. epidermidis* and *S.*
237 *aureus* and specific more virulent strains could yet be made. On the other hand, microbiome
238 comparison of the skin of subjects with a healthy skin and patients with mild-to-moderate acne
239 vulgaris also pointed to other lactic acid producing bacteria such as *Streptococcus salivarius* as being
240 potentially beneficial against acne.

241 Our findings are consistent with the growing body of evidence that lactic acid bacteria such as
242 lactobacilli can be applied at multiple human body sites to target the microbiome, epithelial barrier
243 function and immune system in various conditions[35]. In this study, we now add support for the skin
244 as topical therapeutic area. Evidently, compliance of this probiotic therapy by the patients will be a
245 key aspect to monitor – and possibly improve- in the future. The promising results from our proof-of-
246 concept study with live lactic acid-producing microbes should now also be confirmed in larger-scale
247 and longitudinal studies, in addition to more molecular studies towards underlying antimicrobial and
248 anti-inflammatory mechanisms and probiotic effector molecules (**Figure 6)**. Together, these studies
249 will contribute to a new era of skin therapeutics based on microbiome modulation, as well as more
250 fundamental and mechanistic insights on the keystone core functions of lactic acid bacteria for skin
251 health.

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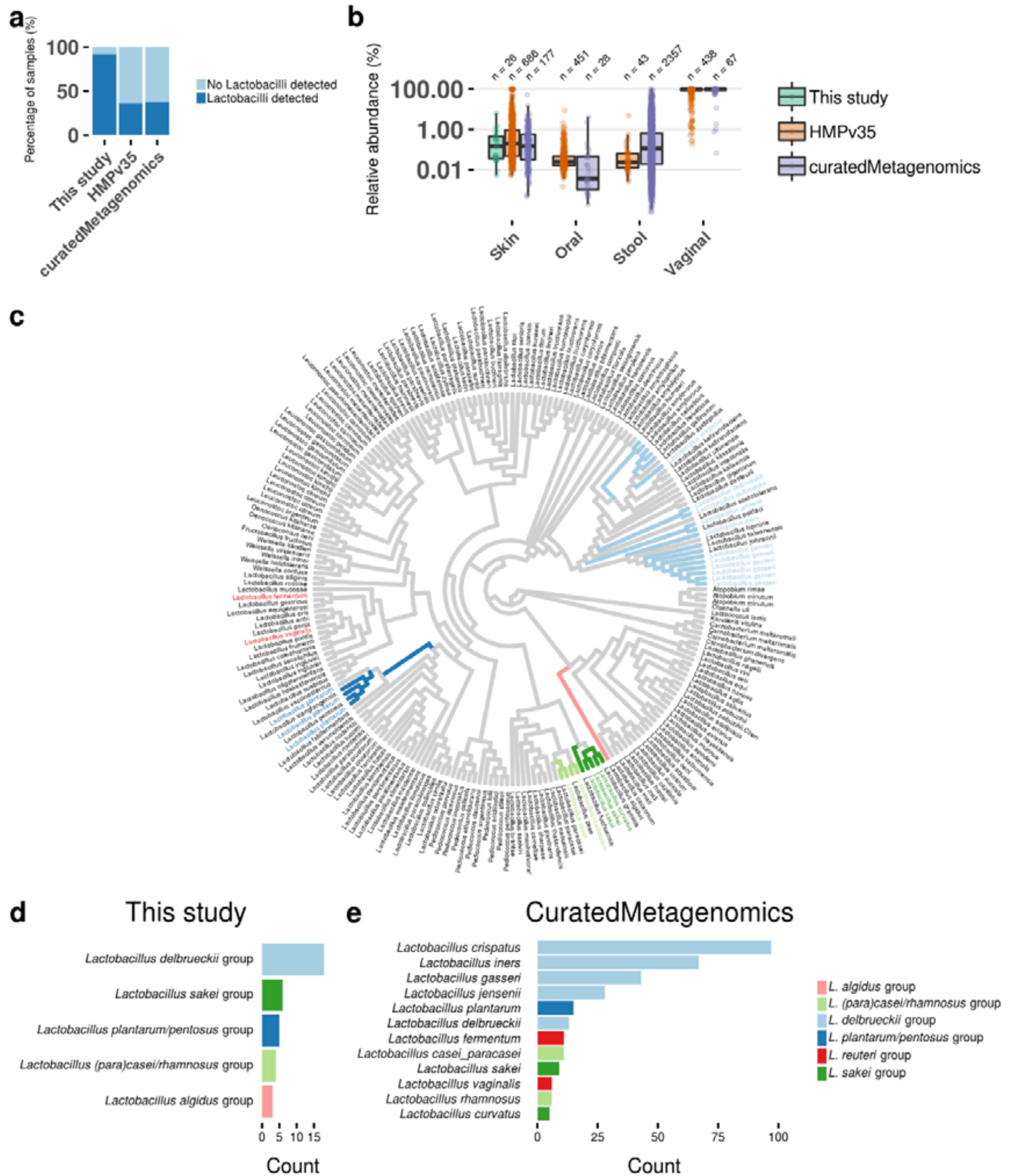
252 **Figures and figure legends**



253
254 **Figure 1 – *Lactobacillus* taxa in skin samples of 16S rRNA amplicon and shotgun metagenomic data**

255 (a) Presence and absence of *Lactobacillus* in skin samples of this study, the Human Microbiome
256 Project (HMPv35)[46] and shotgun metagenomic datasets from three studies[14, 15 and 16,] accessed
257 through the curatedMetaganomics R Package. (b) Comparison of relative abundance of *Lactobacillus*
258 in different niches of the three used datasets. The y-axis is represented in log scale. (c) 16S rRNA
259 cladogram of the *Lactobacillus* Genus Complex. Branches are colored based on phylogenetic
260 placement of Lactobacillus ASVs from this study and the phylogenetic group (as described by Duar *et*
261 *al.*[19]) they belong to. Tip labels are colored based on the 12 most abundant *Lactobacillus* species
262 found in the skin shotgun metagenomic datasets. (d and e) The most abundant *Lactobacillus*
263 members in this study (d) and the skin shotgun metagenomic datasets (e) colored according to the
264 phylogenetic group of the *Lactobacillus* Genus Complex they belong to.

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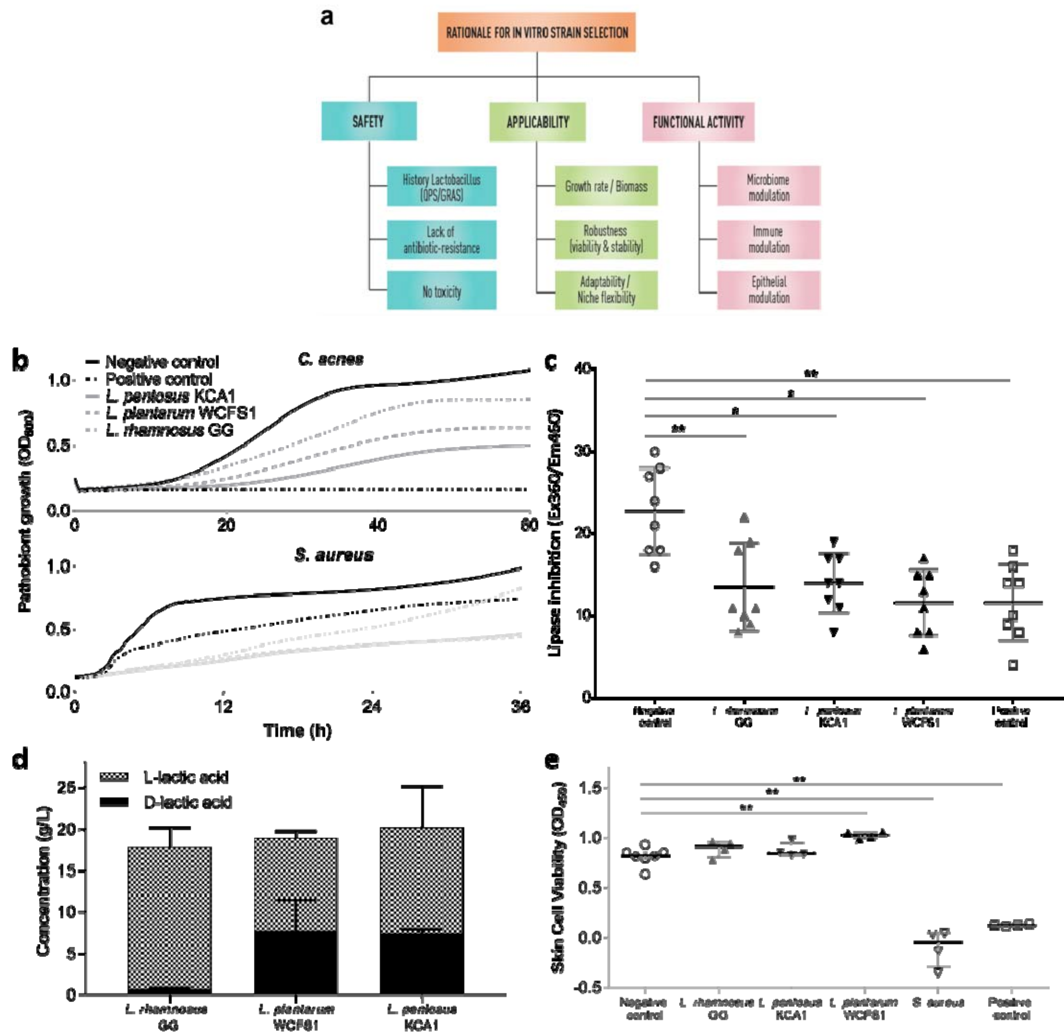

265

266 **Figure 2 - *In vitro* selection of *Lactobacillus* strains for targeted application against acne vulgaris.**

267 (a) Schematic overview of the rationale for the selection. Each criterion needs to be taken into
268 account upon selection. Laboratory or genomic prediction tests exist for each criterion. More
269 information can be found in the main text. (b) Antimicrobial activity of the spent-culture supernatant
270 of the selected *Lactobacillus* strains against the two pathobionts tested, *C. acnes* and *S. aureus,* and
271 compared to the positive control (10 mg/mL Clindamycin, a common antibiotic used in acne). MRS at
272 pH4, which is comparable to the pH of the spent supernatant of lactobacilli, was used as a negative
273 control. (c) Inhibition of lipase activity of *C. acnes* by the spent-culture supernatant of the selected
274 *Lactobacillus* strains*,* and compared to the positive control (10 mg/mL Clindamycin) and the negative
275 control (MRS). (d) Concentration of L-lactic acid and D-lactic acid as key antimicrobial and skin-
276 modulating molecules produced by the selected lactobacilli after overnight incubation in MRS broth.
277 (e) Skin cell viability results of NHEK cells after addition of the selected lactobacilli compared to the
278 negative control, keratinocyte growth medium 2, and positive controls, *S. aureus* and Triton-X,
279 measured at 450 nm using an XTT assay. Statistical analysis were performed using a Mann-Whitney
280 test where * = $p<0.05$ and ** = $p<0.01$.

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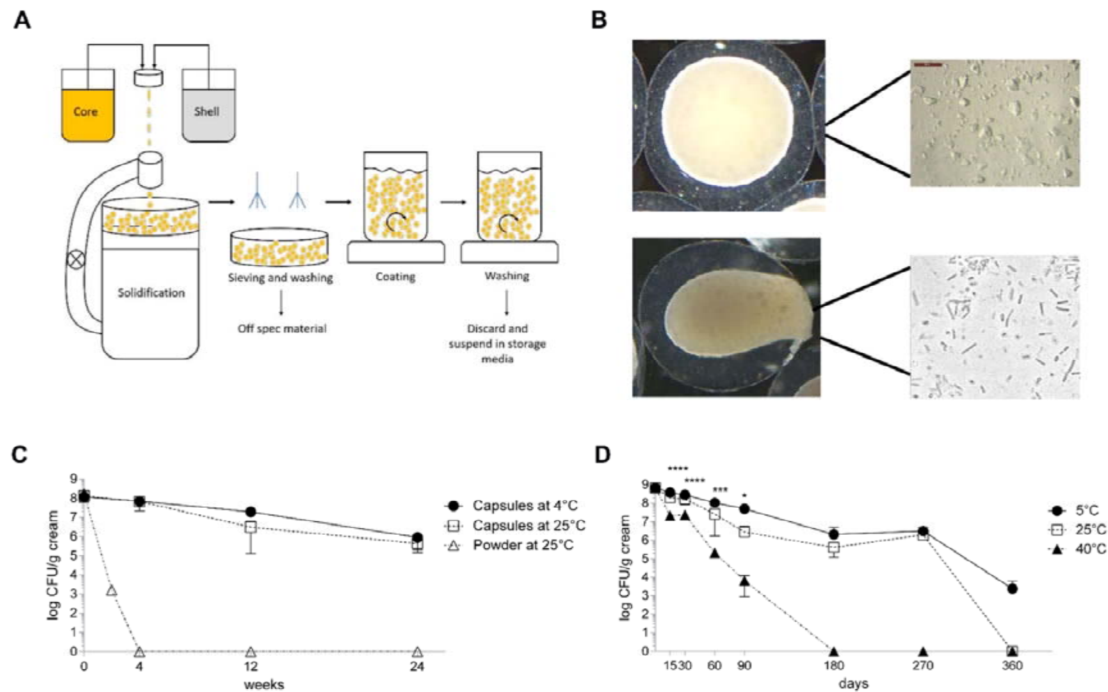

281
282 **Figure 3 - Formulating live lactobacilli in a topical cream**

283 (a) Schematic representation of the micro-encapsulating process with the bacteria in the core
284 suspension and an outer shell made by the shell solution. (b) Resulting micro-capsules with a core of
285 freeze-dried bacteria suspended in oil compared to microcapsules in which a force is applied just
286 before application on the skin, hereby releasing the bacteria and activating them through water-
287 uptake. (c) Survival of the bacteria in the microcapsules after different days compared to non-
288 encapsulated freeze dried bacterial powder in an O/W cream. (d) Survival of the encapsulated
289 bacteria in o/w cream tested according to the International Council for Harmonisation of Technical
290 Requirements for Pharmaceuticals for Human Use Q1A(R2). Statistical analysis was performed using
291 a Two-way ANOVA where * = $p<0.05$, *** = $p<0.001$ and **** = $p<0.0001$.

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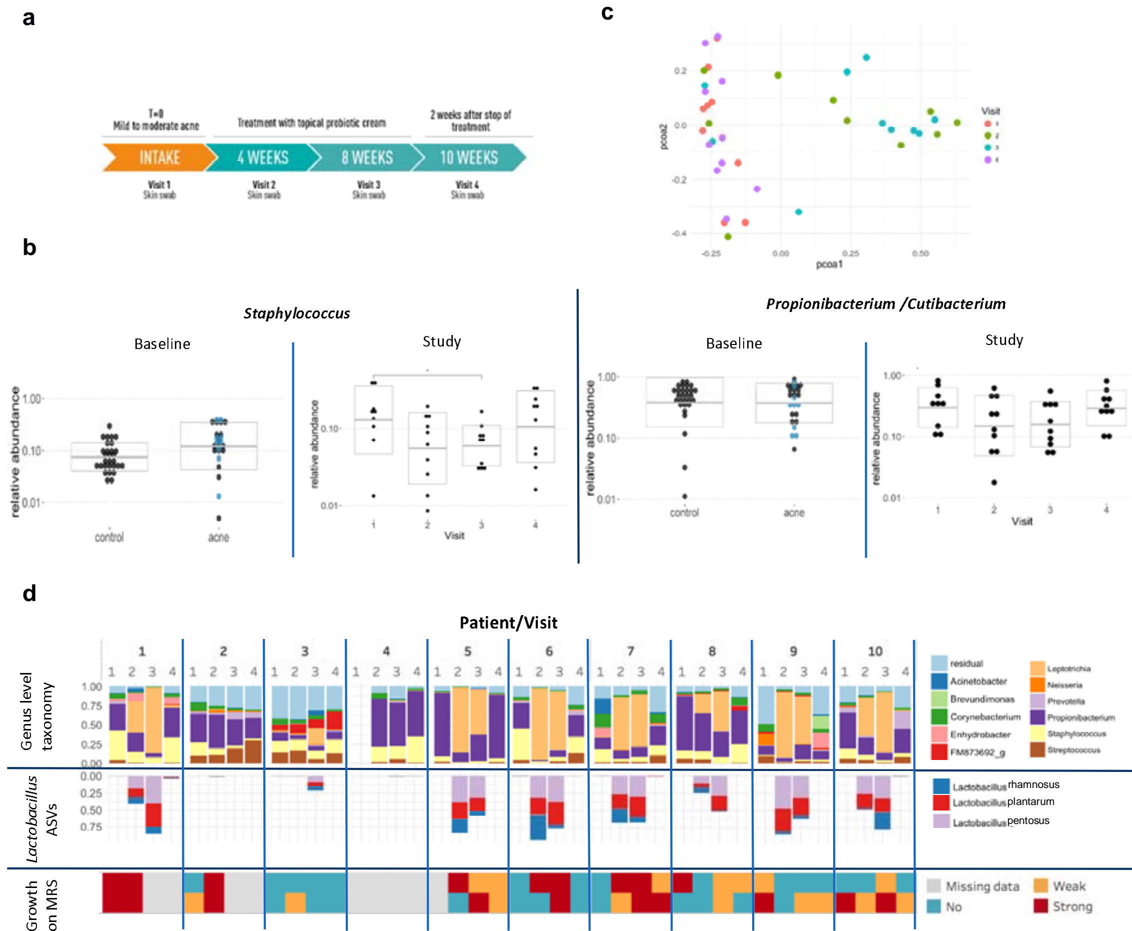

292

293 **Figure 4 – Impact of the *Lactobacillus* cream on the skin microbiome**

294 (a) Schematic overview of the POC intervention study with the O/W cream containing the selected
295 and formulated lactobacilli, the visits at which a skin swab was taken and dermatological symptom
296 analysis was performed by the dermatologist. The cream was applied twice daily for 8 weeks with a
297 minimal dose of 10^6 CFU/application. (b) Relative abundance of *Staphylococcus* and
298 *Propionibacterium/ Cutibacterium* respectively at baseline and over the four visits of the study
299 (right). For the baseline, skin samples of the 30 healthy volunteers without acne symptoms (cfr.
300 Figure 1) and 27 patients with mild-to-moderate acne symptoms were compared. Of these 27 acne
301 patients, 10 patients (indicated with blue dots) were included in the *Lactobacillus* intervention Study
302 (Study) shown at the right side of each panel. For the study visits, $p< 0.05$ for visit 3 versus visit 1
303 based on Wilcoxon ranks test is indicated with a star. (c) PCOA plot distributing samples according to
304 beta-diversity (Bray-Curtis distance). Similar samples are located closely to each other, and colored
305 by visit. (d) Microbial communities during the study period with the genus-level taxonomy indicated
306 (top), relative abundance of the three *Lactobacillus* ASVs resulting from the cream (middle) and
307 observed growth on MRS medium (top row on agar, bottom row growth in MRS broth) after addition
308 of the skin samples (bottom). Other *Lactobacillus* ASVs were not observed at a higher relative
309 abundance than 1%. Samples were ordered by participant and by visit.

310

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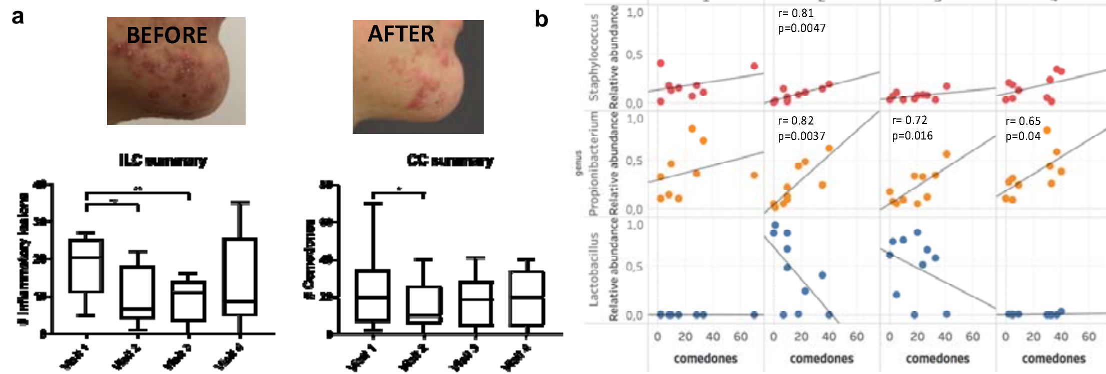

Figure 5- Effect of the *Lactobacillus* cream on acne symptoms and correlation with microbiome data

(a) Evolution of counts of inflammatory lesions (left) and comedones (right) over the course of the study, grouped by visit. All 10 patients included in the pilot study showed a clinical improvement after the application of the cream as exemplified with a picture of the acne spot area of one patients at visit 1 versus visit 2. Statistical analysis were performed using a Wilcoxon matched-pairs signed rank test where * = $p<0.05$ and ** = $p<0.01$ (b) Correlation of relative abundances of *Staphylococcus* (top), *Propionibacterium/Cutibacterium* (middle) and *Lactobacillus* (bottom) to comedonal counts, per visit. Pearson correlation coefficient and p-values are indicated where $p<0.05$.

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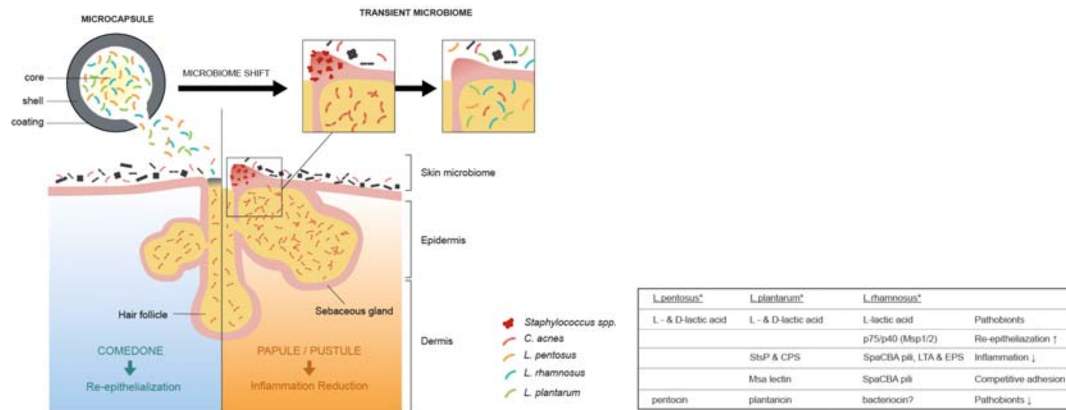

L.pentosus*	L.plantarum*	L.rhamnosus*	
L - & D-lactic acid	L - & D-lactic acid	L-lactic acid	Pathobionts
		p75/p40 (Msp1/2)	Re-epithelazation ↑
	StsP & CPS	SpaCBA pili, LTA & EPS	Inflammation ↓
	Msa lectin	SpaCBA pili	Competitive adhesion ↑
pentocin	plantaricin	bacteriocin?	Pathobionts ↓

Figure 6 - Schematic overview of the main findings of this study on how live lactobacilli formulated in a topical cream modulate skin microbiome and improve acne symptoms.

Specific *Lactobacillus* strains were selected and formulated in capsules in an oil (O) in water (W) cream that release the probiotics upon rubbing on the skin. Microbiome analysis (16S amplicon sequencing), as well as counting of comedone and inflammatory lesions substantiated that these lactobacilli could reduce inflammation and comedone formation, as well have transient impact on the skin microbiome, especially by decreasing the relative abundance of staphylococci and *C. acnes* as acne pathobionts. The postulated mode of action (also indicated with * in table) includes their antimicrobial activity against pathobionts by lactic acid (this study), competitive exclusion[47],[32] and possibly bacteriocins[48,24], their capacity to reduce inflammation, e.g. by the serine-threonine rich protein StsP of *L. plantarum* WCFS1[49] or SpaCBA pili of *L. rhamnosus* GG[50,51], and their capacity to promote re-epithelialization[33,34], by e.g. the secreted proteins Msp1 (p75)/Msp2 (p40) for *L. rhamnosus* GG[29,52]. Yet, the involvement of these probiotic effector molecules remains to be further substantiated in follow-up work.

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336 **Methods**

337 **Bacterial growth.** *Lactobacillus* strains were grown at 37°C in de Man, Rogosa and Sharpe (MRS)
338 medium (BD Difco, Erembodegem, Belgium). *Propionibacterium acnes* ATCC6919 was inoculated in
339 reinforced clostridial broth (LabM Limited, Heywood, UK), supplemented with 0.2% Tween20 and
340 cultured microaerobically (5% CO_2) at 37°C. *Staphylococcus aureus* ATCC29213 was grown in Mueller-
341 Hinton broth at 37°C. Solid media contained 1.5% (w/v) agar. Time-course experiments were also
342 performed analysing the antimicrobial activity of spent culture supernatant (SCS) of the selected
343 *Lactobacillus* strains against C. *acnes* and *S. aureus* ATCC29213 (cfr. [53]). Additionally, the impact of
344 this SCS (10%) on the lipase activity of *C. acnes* was determined as previously described [5].
345 **Human skin cell culture.** Normal human epidermal keratinocytes (NHEK) cells from juvenile foreskin
346 from pooled donors were purchased from Promocell (Heidelberg, Germany) and cultured according
347 to manufacturer's recommendations in Keratinocyt Growth medium 2 (Promocell, Heidelberg,
348 Germany). Cytotoxicity of probiotic strains was assessed using the 2,3-Bis(2-methoxy-4-nitro-5-
349 sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT, Sigma-Aldrich) cell viability assay. NHEK cells were
350 seeded at a density of 5000 cells/well in 96-well plates and cultured until confluent. Overnight
351 cultures of probiotic strains or *S. aureus* were added to the wells with or without NHEK cells at 10^6
352 CFU/well and incubated for 2 h at 5% CO2 and 37°C. Triton X-100 (0.5%) was used as a positive
353 control.

354 **Collection of skin samples and total microbial DNA extractions.** Skin samples were collected by
355 brushing the cheek (control group) or the affected area on the face (patients) with a FloqSwab
356 (Copan) over an area of ± 10 cm^2 or around the lesions. Swabs were then transferred to a falcon
357 containing 800µl Bead solution of QIAamp PowerFecal DNA kit (Qiagen). Samples were stored at 4°C
358 until further processing (maximally 14 days). Before DNA extraction, samples were vortexed for 1
359 minute, after which the Bead solution was transferred to the bead tube. Subsequent steps of the
360 DNA extraction were executed according to manufacturer's instructions.

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361 **Illumina MiSeq *16S rDNA* gene amplicon sequencing.** The primers used for Illumina MiSeq
362 sequencing were based on the previously described 27F-338R or 515F-806R primers[54] and altered for
363 dual-index paired-end sequencing, as described earlier[55] (**Extended Data Table 4**). Separate runs
364 were carried out for V1V2 and V4 *rRNA* gene variable regions. Quality control and processing of reads
365 was performed using the R package DADA2, version 1.6.0 [56]. Denoised reads (amplicon sequence
366 variants or ASVs) were merged and read pairs with one or more conflicting bases between the
367 forward and reverse read were removed. Chimeric sequences were removed using the function
368 "removeBimeraDenovo". Finally, ASVs were classified from the kingdom to the genus level using the
369 EzBioCloud 16S database[42]. A species annotation was added to each ASV by listing the species of all
370 16S sequences in the database that showed an exact match to the ASV sequence. Contaminants
371 were identified using the approach of Jervis-Bardy *et al* [57]. ASVs with a strong negative correlation
372 between relative abundances and total sample read counts were considered contamination. For each
373 ASV, this correlation was calculated and tested for significance. ASVs with a p-value less than 0.0001
374 were removed. Samples were filtered by removing those with less than 1000 reads left after all read
375 and ASV filtering steps.

376 **Biostatistical and bioinformatics analysis.** Processing of the ASV table, ASV annotations (e.g.
377 classification) and sample annotations (metadata) were performed using the in-house R package
378 "tidyamplicons", publicly available at github.com/SWittouck/tidyamplicons. For the analyses at the
379 genus level, ASV read counts were aggregated at the genus level or, if unavailable, at the most
380 specific level at which taxonomic annotation was available.

381 **Analysis of public datasets.** Processed OTU-table and sample metadata from the Human Microbiome
382 Project (HMPv35)[46] and the shotgun metagenomic datasets were retrieved using the MicrobeDS R
383 package and curatedMetagenomics R package[16] respectively. All data was loaded, processed and
384 visualized in the R-environment using Phyloseq. All scripts are available at
385 https://github.com/LebeerLab/skin_acne_study.

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386 **qPCR for estimation of absolute bacterial concentrations.** qPCR was performed in duplicate on a 20-
387 fold dilution (to avoid interference of PCR inhibitors) of total DNA isolated from the samples, using
388 the StepOnePlus real time qPCR system (Applied Biosystems®, Foster City, California, USA), SYBR®
389 Green chemistry (PowerUp™ SYBR® Green Master Mix, Applied Biosystems®, Foster City, California,
390 USA) and primers as indicated in **Extended Data Table 4**. Standard curves were used to estimate
391 bacterial concentrations in the samples and derived from serially diluted DNA from an overnight
392 culture of *Lactobacillus crispatus* LMG12005 isolated similarly as the samples. Bacterial concentration
393 was determined by plating.

394 **Formulation of lactobacilli in microcapsules and O/W cream.** A single colony of the three selected
395 probiotic strains was grown until stationary phase and lyophilized. The lyophilized bacterial powder
396 was grinded and milled (Frewitt, Switzerland) to obtain a fine powder (± 10^{11} CFU/gram) and
397 subsequently encapsulated via a core-shell encapsulation approach. Briefly, the strains were mixed in
398 equal amounts and homogeneously suspended to obtain a stable oil-based feed core suspension.
399 The shell feed solution contained a hydrocolloid alginate polymer as gelling agent. Both liquid feeds
400 were pumped to a concentric nozzle, to obtain a concentric fluid flow. The laminar liquid flow was
401 broken up by a vibrational unit to obtain spherical -droplets that were solidified upon falling in a
402 calcium-based solidification solution, forming the capsules. The collected capsules (10^9 - 10^{10}
403 CFU/gram) were washed and suspended in an oil-in-water cream containing the following
404 ingredients: Aqua, Glycerin, Polyglyceryl-3 Rice Branate, Propylheptyl Caprylate, Cetyl Alcohol, Nylon
405 6/12, Caprylic/Capric Triglyceride, Squalane, PEG-400, Cyclopentasiloxane, Cetearyl Alcohol, Prunus
406 Amygdalus Dulcis Oil, Allantoin, Limnanthes Alba Seed Oil, Tocopheryl Acetate, Xanthan Gum,
407 Cyclohexasiloxane, Helianthus Annuus Seed Oil, Algin, p-Anisic Acid, Silica Dimethyl Silylate, Disodium
408 EDTA, Sodium Hydroxide, Hydrochloric Acid. The ingredients of this cream, mainly the emulsifiers
409 and preservatives, were selected to be compatible with the micro-capsules and bacteria, both during
410 storage and upon release of the probiotics. Hereto, the impact of the topical cream without the
411 capsules on the growth of four skin reference bacteria (*S. aureus, S. epidermidis, L. crispatus* and *C.*

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412 *acnes*) was evaluated at a concentration of 1, 10 and 100 mg/ml, by a time-course analysis of OD600
413 measurements as described above. Mechanical force (rubbing on the skin) was confirmed to break
414 the capsules, releasing the inner core material containing the suspended probiotics. Skin irritation
415 tests with the O/W cream containing the freeze –dried lactobacilli were performed as described
416 previously[39].

417 **Proof-of-concept human in patients with acne vulgaris.** A proof-of-concept clinical trial was
418 performed on patients with mild-to-moderate acne vulgaris included after careful assessment of the
419 responsible dermatologist by counting of comedones and inflammatory lesions **(Extended Data**
420 **Table 3)**. Patients were men between 12-25 years. Exclusion criteria were use of oral antibiotics
421 within 4 weeks prior to start of the study and use of systemic retinoids within 6 months prior to start
422 of study. Subjects provided written informed consent before the study began. Patients were asked to
423 apply the topical probiotic cream (containing 10^8 CFU of each *Lactobacillus* strain per application of 1
424 g of the topical cream). Patients were asked to apply the cream twice daily for 56 days (8 weeks). The
425 patients were seen by a dermatologist at start (before the therapy) (visit 1), week 4 (visit 2), week 8
426 (visit 3) and week 10 (visit 4). A skin swab was taken at each visit, total DNA was extracted and
427 amplified for 16S amplicon sequencing as described above. Moreover, a clinical scoring was
428 performed and a photograph taken at each visit.

429

430 **Clinical trial registration.** The protocol of this study was in accordance with the Declaration of
431 Helsinki, and was approved by the ethics committee of the University Hospital of Antwerp (Belgium)
432 before initiation of the study. The study was given the approval number B300201628507 (Belgian
433 registration) and registered online at clinicaltrials.gov with unique identifier NCT03469076.

434

435 **Data availability.** Sequencing data are available at the European Nucleotide Archive with the
436 accession number PRJEB27311.

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595

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600 *plantarum* YUN-V2.0 and *L. rhamnosus* YUN-S1.0 in their commercial ACN product. Part of the results
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