Natural anti-acne extract aids healthy skin microbiota

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Abstract

Although a number of facts on the physiopathological mechanisms of acne are now well established, it is still unclear what triggers the inflammatory disease of the sebaceous follicle. Very recently, there has been a paradigm shift in the understanding of the role of *Cutibacterium acnes* (*C. acnes*, formerly *Propionibacterium acnes*) in the pathophysiology of acne. Firstly, the aim of this study was the characterization of the skin microbiota with a focus on *Cutibacterium acnes* (*C. acnes*) phylotypes in subjects with acne. Secondly, microbiota changes after 28 days' treatment of berries Rhodomyrtus tomentosa active ingredient (RT), rich in acylphloroglucinols, polyphenols, and organic acids were analyzed. Clinical evaluations (lesion counts) were performed at baseline (D0) and after 28 days (D28) of twice-daily application of RT active ingredient.

Acne is one of the most common skin diseases worldwide affecting up to 85% of the population.[1] At the pathophysiological level, two factors play a crucial role: the sebaceous gland and Cutibacterium acnes. More precisely, located in the sebaceous gland-rich sites of skin, *C. acnes* is a gram-positive aero-tolerant anaerobic bacterium belonging to the *Actinobacteria* phylum. Several mechanisms have been proposed by which *C. acnes* aggravates acne, including augmentation of lipogenesis, comedone formation, and host inflammation.[2] Recently, different strains of *C. acnes* were identified, showing that its subtypes could also be important in acne development.[3] Despite controversial data in line with the population samplings, anatomic sites and typing methods, most of studies report that strains from the type IA1 preferentially colonize skin with acne while others are not or poorly present in acne lesions (IB, II and III).[4] Dagnelie *et al.*[5] also reported that inflammatory severe acne of both the face and back is associated with diversity loss of *C. acnes* phylotypes, with a high predominance of phylotype IA1, both on the face (72.7%) and the back (95.6%). Thus, it seems that the severity of acne may be more related to the selection of its subtypes than to its proliferation. It is also important to note that other factors such as androgens and hormonal fluctuation or imbalance, poor nutrition, stress, pollution and habits are important in the development and persistence of the disease.[6] Moreover, although *C. acnes* is best-known for its connection with acne, it has been shown that other bacteria might also (indirectly) contribute to the inflammatory process. In fact, culture-based studies have reported that *Cutibacterium granulosum* is highly abundant in the comedones and pustules of acne patients, and displays stronger virulence (i.e., lipase activity) than *C. acnes*.[7]

Topical retinoids, benzoyl peroxide (BPO), which suppresses bacterial

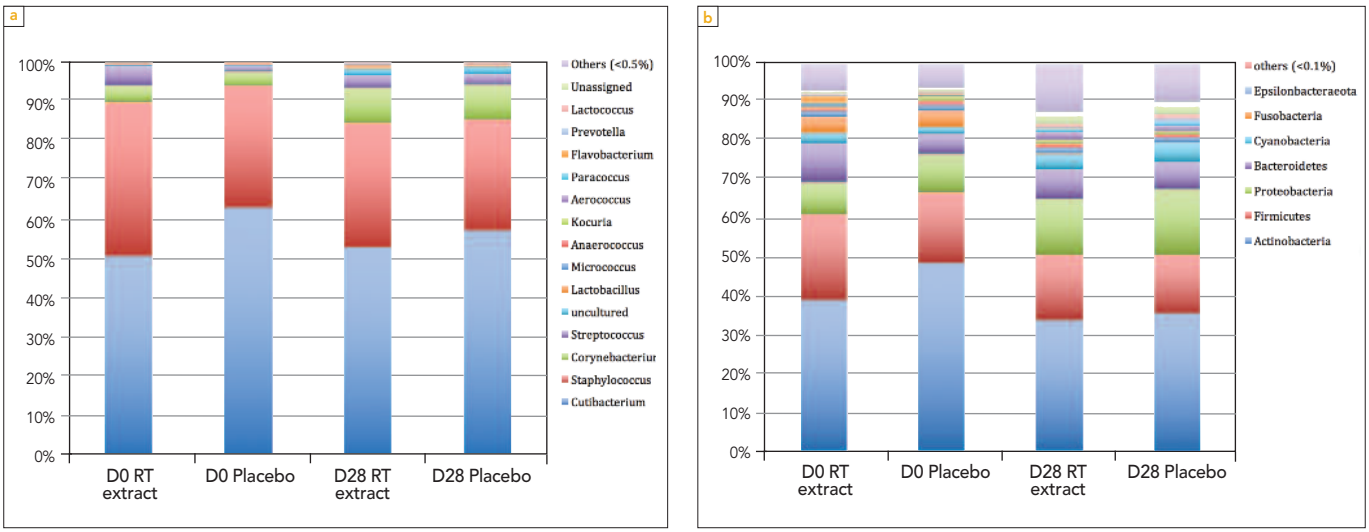

Figure 1: Mean Relative Phylum (A) and Genera (B) abundances for each group of samples. For the sake of clarity, only Phylum with mean relative abundance superior than 0.1% and Genera with mean relative abundance superior than 0.5% are represented.

proliferation, hyperkeratinization and inflammation, and systemic antibiotics are currently the first-line treatments for mild-to-moderate acne. Although highly effective, topical treatments affect skin barrier integrity and are often associated with side effects such as dryness, irritation, itching and redness.[8] In this context, novel therapies are in high demand and an ethnopharmacological approach to discover new plant sources of anti-acne therapeutics could contribute to filling this void in effective therapies. Exploitation of natural resources, especially medicinal plants and their derived products are considered as promising alternative agents for the treatment of diseases.

Among them, *Rhodomyrtus tomentosa* (*RT*) is a flowering plant belonging to the family Myrtaceae, native to southern and southeastern Asia. All parts of this plant (leaves, roots, buds, and fruits) have been used in traditional Vietnamese, Chinese and Malaysian medicine for a long time. In traditional medicine, *RT* fruits have been used to treat diarrhea and dysentery, and to boost the immune system.[9] It contains various phytochemical compounds in many parts of the plant: ellagitannins, stilbenes, anthocyanins, flavonols and phenolic acids being the phenolic compounds identified in the fruit. Recently, the acylphloroglucinol rhodomyrtone from *RT* fruit was evidenced as a potential inhibitor of inflammation. Moreover, rhodomyrtone showed strong antibacterial activity against a wide range of gram-positive pathogenic bacteria, as well as anti-biofilm property against *Staphylococci* causing severe infections. More specifically, rhodomyrtone inhibits *C. acnes* proliferation.[10]

After having characterized the microbiota profile of skin areas with acne lesions, we investigated whether 28 days application of fruit *Rhodomyrtus tomentosa* extract (*RT*) modify this microbiota, with a focus on *C. acnes* phylotypes.

Methodology

Participants

Seventeen volunteers were included by a dermatologist. Written informed consent and photography were obtained from each subject before enrolment.

They were aged 17.3 ± 1.0 years with mild to moderate acne (Global Acne Severity Scale (GEA) II to III), presenting at least five papules and pustules on the face (not located on the nasal pyramid),[26] and at least six closed and open comedones on the face, including at least three on the forehead. Exclusion criteria were presence of any cutaneous lesion affecting the face apart from ongoing acne (i.e. vitiligo, psoriasis and seborrhoeic dermatitis), or any chronic or acute progressive disease, which may interfere with the study. Patients who applied facial topical treatments during the preceding month including anti-acneic agents (retinoids, zinc, benzoyle peroxide), antimicrobial agents (antibiotics, antiseptic), corticosteroids or nonsteroid anti-inflammatory drugs (NSAID) or patients who took systemic anti-acneic treatment during the preceding month with antibiotics (tetracycline, macrolides, macrolide derivatives) or zinc, or patients treated with NSAID, corticosteroid, or antibiotics other than anti-acneic taken during the preceding month were not included. We also excluded patients treated with oral retinoids treatment or any hormonal treatment for contraceptive or anti-acneic purpose initiated or modified during the 12 preceding weeks. During treatment, none of the aforementioned treatments was allowed.

Two visits were performed for subjects: at inclusion (D0), and at the end of the study (D28). At baseline, microbiological sampling were performed on 17 acne patients for microbiota studies. Besides, the dermatologist performed a clinical examination of the face at D0 and D28. Concomitant treatments, adverse events and treatment compliance were recorded.

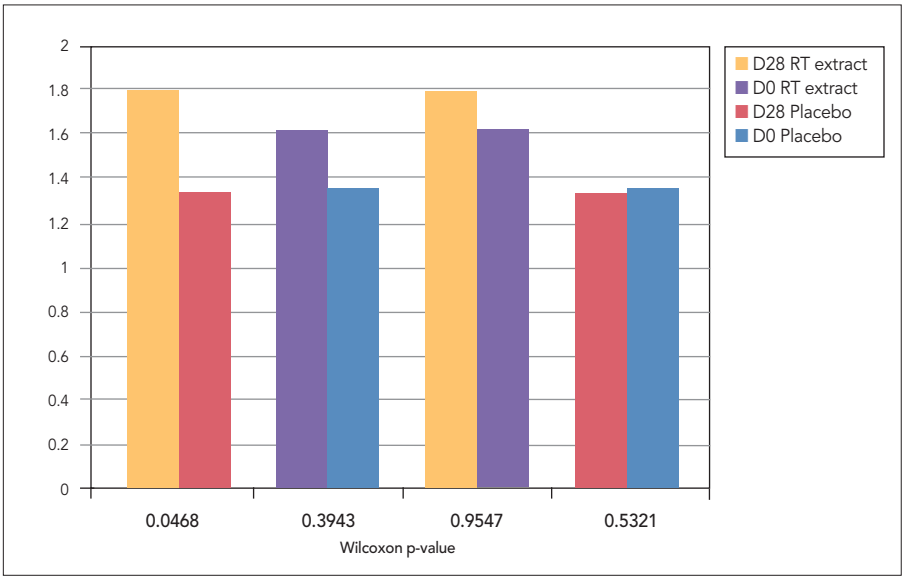

Figure 2: Shannon diversity indice comparaison at the genus level comparison using Wilcoxon test.

Study product

The study products were a gel containing *Rhodomyrtus tomentosa* fruit extract and a placebo based on the same gelling agent acrylates/C10-30 alkyl acrylate crosspolymer and where the dry mater active was replace by demineralized water. Each formulation provided the same penetration capacity thanks to the presence of the surfactant PEG-8 Caprylic/Capric Glycerides (1%). They were applied twice daily, on each half-face. Subjects were instructed not to change their hygiene habits or to apply other skin care products or topical drugs during the study on their face.

Microbiota sampling

On the first day of study (Day 0), skin samplings (swabs) were performed for each volunteer. Sampling were collected by the same investigator on the cheeks, forehead, temple or chin of each patient's face (depending on the location of acne lesions). Then, volunteers were asked to apply active formula on one face side, and placebo formula on the other side, twice a day for 4 weeks. New samplings were performed at the end of treatment on day 28 (Day 28) for each volunteer and face side.

Swab treatment, DNA extraction and purification and 16S rDNA amplification/sequencing

After sampling, swabs were immediately placed in PowerBead tubes containing cell lysing solution and beads, and were stored at -80°C until DNA extraction was performed. DNA extraction and purification were performed using Qiagen DNeasy PowerSoil Kit (Cat # 12888).

16S metagenomics 16SrDNA variables regions V1 to V3 were amplified using primers pair 27F (AGAGTTTGATCCTGGCTCAG) and 534R (ATTACCGCGGCTGCTGG). 16s DNA sequencing was performed on Illumina Miseq using paired-end technology. All sequences processing was performed using QIIME2 suite (https://qiime2.org/) using default settings. After quality filtering, reads pairs were merged by overlapping and clustered and chimeric sequences were removed. OTU (operational taxonomic units) were assigned to sequences against Silva 132 rDNA database.

To evaluate whether the active ingredient affects populations of five bacterial species (*Staphylococcus epidermidis, Staphylococcus aureus, Staphylococcus hominis, Cutibacterium acnes, and Cutibacterium granulosum*, and

one genus (*Corynebacterium*), genus/species quantification using quantitative (real time) PCR (qPCR) were done. All qPCRs were performed with LightCycler 480 II (Roche) thermocycler using LightCycler® Multiplex DNA Master (Roche, ref 07339577001) core qPCR kit and fluorescent TaqMan probe chemistry for species specific qPCR, and LightCycler® 480 SYBR Green I Master (Roche, ref 04707516001) for genus specific qPCR. For each qPCR, standards made of known amount of corresponding genomic DNA extracted from pure strains (105 to 10 genome equivalent) were integrated to qPCR assay.

Cutibacterium acnes.
Variable *C. acnes* region described by Scholtz et al.[11] was amplified and sequenced on Illumina Miseq using paired-end technology using mofidified primers. All sequences processing was performed using QIIME2 suite (https://qiime2.org/) using default settings. After quality filtering, reads pairs were merged by overlapping and clustered and chimeric sequences were removed. SLST (Single Locus sequence Type) were assigned to sequences against SLST reference database (http://medbac.dk/slst/pacnes).

Clinical evaluations
They were conducted by the same investigator on Day 0 and Day 28 and included the scoring of lesions (blackheads, microcysts, papules and pustules) on each hemi-face (except nasal pyramid, the vermillion border, the crease of the chin and the rim of the scalp), and the reporting of local tolerance issues, acne signs and symptoms. The variations (D28-D0) in the number of lesions are calculated for each kind of lesions. Local tolerance issues and self-assessment of the products were also evaluated.

Statistical analysis

Raw data were used to determine taxa abundances and α-diversity indices after removing OTU with less than 2 counts. Filtered and normalized data were used to compare populations between treatments and days (β-diversity) and to compare taxa abundances between same samples' groups. Abundances for five taxonomic levels (Phylum to Genus) were calculated by summing sequences numbers assigned to same taxa for the considered level. Shannon diversity indice was calculated using Past 3.20 software. Indices increase with diversity.

Concerning the genomic quantitative study of major genus/species of skin microbiome, statistics were performed on normalized data. Normalization was done for each sample by dividing observed taxon abundance by the sum of all taxa abundances. Statistical comparison of taxa abundances was performed uniquely for taxa identified in at least half of tested samples. Missing values (i.e. samples where no species or genus was detected) were replaced by a random value sampled around the minimum value +/- 50%. Taxa abundances were compared using either parametric paired T-test or non-parametric Wilcoxon and Sign rank tests. Normality of data distribution was verified using Shapiro-Wilk test.

SLST types and phylotypes abundances and diversities were calculated for each sample and compared to evaluate treatment effect between groups made of volunteers treated with active formula and volunteers treated with placebo formula and treatment effect overtime using non-parametric Wilcoxon Test. For all hypothesis testing, p-value threshold was set at 0.05 to determine whether observed differences were statistically significant.

Table 1: Inflammatory and non-inflammatory lesions variation between D0 and D8.

Parameters	Variation	*p value*
Blackheads	-3.5±1.1	0.007
Papules	-1.6±0.4	0.001
Global non-inflammatory lesions	-4.1±1.4	0.009
Global inflammatory lesions	-2.2±0.6	0.001

Results

The RT active ingredient contains polyphenols (10% per dry matter), organic acids (2% per dry matter), rhomomyrtone (5 ppm) and piceatannol (500 ppm).

As shown in Figure 1, major phyla were *Actinobacteria* (avg. 56%), followed by *Firmicutes* (avg. 32%), *Cutibacterium* being the most abundant genus (avg. 40%), followed by *Staphylococcus* (avg. 18%), *Corynebacterium* (avg. 12%) and *Streptococcus* (avg. 7%) both at D0 and D28, with no significance differences between placebo and RT extract treatment. When diversity index was compared between treatment using non-parametric Wilcoxon test, diversity expressed by Shannon index appeared to be statistically higher (p-<0.05) after 28 days of treatment with RT active ingredient when compared with D0 as shown in Figure 2.

At D0, *Cutibacterium acnes* was the major species in term of mean abundance, followed by *Staphylococcus epidermidis*. *Staphylococcus hominis* was also present with a mean abundance close to that of *S. epidermidis*. At D28, a significant increase in *Corynebacterium genus* (p< 0.008) was noted only for the Placebo treatement. *Cutibacterium granulosum* prevalence was decreased from 53% to 29% after the RT active ingredient treatment.

In most samples and all groups, A1 was the most abundant SLST type, and IA1 the most abundant Phylotype, followed by Phylotypes II, IB, IA2, IC and III. This repartition was the same whatever the treatment except for RT treatment at D28, which for the 2 latter were inversed (Fig 2). Diversity expressed by Simpson index appears to be statistically higher after 28 days of treatment with active cosmetic when compared with D0 (Data not shown).

RT active ingredient induced significant decrease in blackheads, papules, general non-inflammatory lesions, and global inflammatory lesions (Table 1). This was not the case for the Placebo. 83% of subjects noted that RT extract treatment reduced imperfections (36% after the placebo treatment, 77% observed that it eliminated sebum excess (53% for the placebo treatment), and 71% that it tightened the pores (30% for the placebo).

Discussion

The aim of this exploratory study was to determine the microbiota on the surface of skin with acne lesions and to evaluate changes in the microbiota profile and C. acnes phylotype biodiversity after 28 days of twice daily application of RT extract or placebo. This study reveals that prior to the application of RT extract, the skin surface microbiota in acne subjects was dominated by *Actinobacteria* followed by *Firmicutes* and *Proteobacteria*. At genus level, *Cutibacterium* (*Actinobacteria*) was the most abundant genus followed by *Staphylococcus* (*Firmicutes*) and *Corynebacterium* (*Actinobacteria*), with no changes after *RT* active ingredient treatment. This finding is consistent with literature for healthy face skin, comparable repartition being also reported for patients suffering from acne.[12]

At D28, *Corynebacterium* significantly increased after Placebo treatment, this genus being identified as dominant mediators of skin immunity and inflammation, and recognized as pathogens, particularly among immunocompromised hosts.[13] Of the five bacterial species evaluated, *Cutibacterium acnes* was the major species in terms of mean abundance, followed by *Staphylococcus epidermidis*, both these taxa being detected in all tested samples at D0. *Staphylococcus hominis*, a skin commensal bacteria and opportunistic pathogen, was detected in almost all samples (84%), its abundance not being affected by the

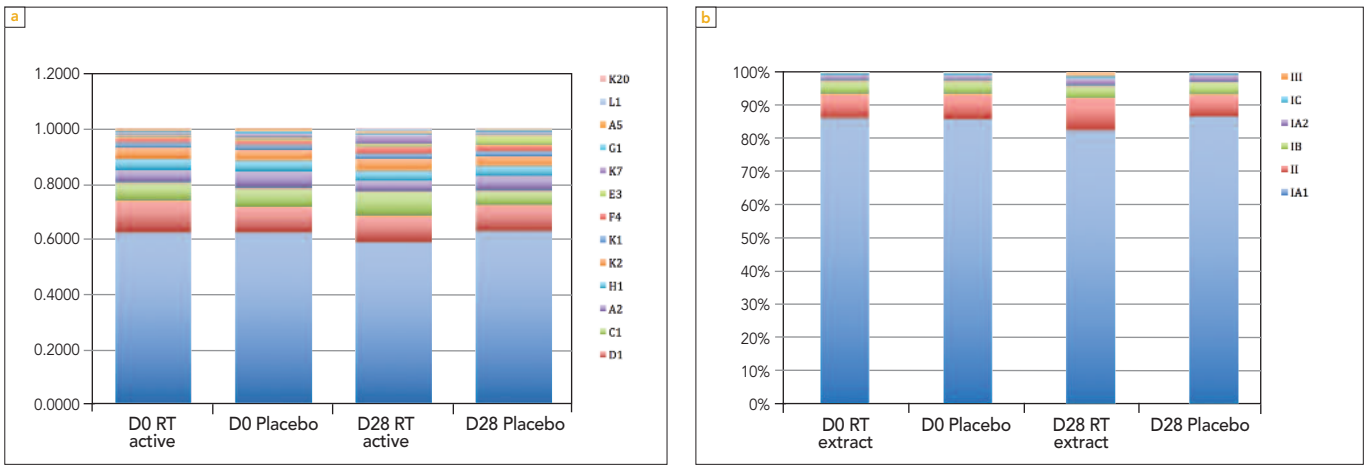

Figure 3: Mean relative SLST type (A) and Phylotype (B) abundance during experimentation.

treatment. At the same time, *Cutibacterium granulosum* prevalence was decreased by 52% after the RT active ingredient treatment. It is worth mentioning that excess *C.acnes* colonization might not be an important factor in acne pathogenesis with some studies reporting little difference in the comparative amount of *C. acnes* in individuals with and without acne. Generally speaking, a consensus has raised in scientific community to consider that *C. acnes* proliferation is not the trigger of acne that is most probably driven by an imbalance in skin microflora and/or a disequilibrium between *C. acnes* phylotypes. *Staphylococcus epidermidis* may also play a role in acne by controlling *C. acnes* proliferation and inhibiting *C. acnes*-induced inflammation.[14] Early culture-based studies reported that *C. granulosum* is more prevalent in comedones and pustules compared to uninvolved follicles of acne patients Moreover, *C. granulosum* was reported to possess greater lipase activity compared to *C. acnes*.[15]

C. acnes strains are classified into six main phylotypes, designated IA1, IA2, IB, IC, II, and III by MLST. A Japanese experimentation, using the SLST method, as in our study, showed that phylotype IA1 was predominant in each acne severity category (with 60%, 57.1% and 63.3% of strains in the severe, moderate and mild acne groups, respectively)16. Phylotype II is described as less abundant on acne skin than healthy skin. At D0, phylotype IA1 was also the most represented one, with a predominance of SLST type A1, followed by Phylotypes II, IB, IA2, IC, and III. After 28 days of RT active ingredient treatment, the 2 latter were inversed. The changes were not observed after Placebo treatment (Fig 3). Higher incidence of phylotype IA1 on acne occurrence or severity may be due to its higher capacity to adhere to skin form biofilms. Another hypothesis is that phylotype IA1 and II strains show different hyaluronate lyase activity, this from phylotype IA1 being less active, partial degradation of hyaluronic acid may produce various oligosaccharides that could be related to the inflammatory process induced by acne. Finally, more than 75% of the subjects noted that RT extract treatment reduced imperfections and eliminate sebum excess, 71% reported pore tightening effects.

Conclusion

This study opens up new areas of research into innovative alternative treatment for mild acne by using botanical therapy. Using 16S rRNA profiling and a single-locus sequence typing (SLST) scheme for *C.acnes* our data confirm the correlation between the presence of some phylotypes and acne. We also showed that the *RT* active ingredient acts as microbiota-regulating agent by selectively reduced virulent phylotypes of *C. acnes* by decreasing Phylotype IA1 along with an increasing of other phylotypes, with at the same time an increasing of *C. acnes* strains diversity. These modifications induced beneficial clinical effects. Thus, *RT* extract can be applied for treatment of skin diseases such as acne. Various and novel treatment focusing on *C. acnes* acne-associated phylotypes are worthy of further investigation for acne management. PC

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