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RESEARCH ARTICLE

Supplementation with *Lactobacillus rhamnosus* GG  
normalizes skin expression of genes implicated in insulin  
signalling and improves adult acne

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Running title: *Lactobacillus rhamnosus* GG in adult acne.

## Abstract

Systemic supplementation with probiotics is increasingly being explored as a potential treatment strategy for skin disorders. Because both the gut-skin axis and dysregulation of insulin signalling have been implicated in the pathogenesis of adult acne, we designed the current study to evaluate the effect of supplementation with the probiotic strain *Lactobacillus rhamnosus GG* (LGG) on skin expression of genes involved in insulin signalling and acne improvement in adult subjects. A pilot, randomized, double-blinded, placebo-controlled study was conducted with 20 adult subjects (14 females and 6 males; mean age:  $33.7 \pm 3.3$  years) with acne. Over a 12-week period, the probiotic group ( $n = 10$ ) consumed a liquid supplement containing LGG at a dose of  $3 \times 10^9$  CFU/day (75 mg/day), whereas the placebo group ( $n = 10$ ) received a liquid lacking probiotics. Paired skin biopsies – one obtained before treatment initiation and one obtained at the end of the 12-week treatment period – were analyzed for insulin-like growth factor 1 (*IGF1*) and forkhead box protein O1 (*FOXO1*) gene expression. The clinical criterion for efficacy was the investigator's global improvement rating on a five-point scale. Compared with baseline, the probiotic group showed a 32% ( $P < 0.001$ ) reduction as well as a 65% increase ( $P < 0.001$ ) in *IGF1* and *FOXO1* gene expression in the skin, respectively. No such differences were observed in the placebo group. Patients in the probiotic group had an adjusted odds ratio of 28.4 (95% confidence interval = 2.2–411.1,  $P < 0.05$ ) to be rated by physicians as improved/markedly improved (*versus* worsened or unchanged) compared with the placebo group. We conclude that supplementation with the probiotic strain LGG normalizes skin expression of genes involved in insulin signalling and improves the appearance of adult acne.

**Keywords:** acne; probiotic; *Lactobacillus rhamnosus GG*; supplementation; insulin signalling; skin; gene expression

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4 **1. Introduction**  
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6 Acne is a common chronic inflammatory disorder characterized by a number of different skin  
7 lesions, including comedones as well as painful and inflamed pustules and nodules (Tan and  
8 Bhate, 2015; Das and Reynolds, 2014). The pathogenesis of inflammatory acne is complex  
9 and involves increased sebum production and hyperplasia of the sebaceous glands under  
10 androgenic influence, ductal obstruction from increased keratinocyte desquamation and  
11 proliferation, *Propionibacterium acne* colonization, and inflammatory cell infiltration (Suh  
12 and Kwon, 2015; Tilles, 2014).  
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15 Accumulating evidence suggests that alterations in insulin signalling may play a significant  
16 role in the pathogenesis of adult acne (Cordain *et al.*, 2002; Nagpal *et al.*, 2016; Balta *et al.*,  
17 2015; Kumari and Thappa, 2013; Del Prete *et al.*, 2012). Insulin and insulin-like growth  
18 factor-1 (IGF1) have been shown to exert acne-promoting actions (Melnik and Schmitz,  
19 2009) and raised serum IGF1 levels have been associated with increased risk of post-  
20 adolescent acne in women (Aizawa and Niimura, 1995). In turn, congenital deficiency of  
21 insulin signalling has been linked to lower prevalence rates of acne (Melnik *et al.*, 2011).  
22 IGF1 potently stimulates both sebaceous lipogenesis and androgen receptor signalling by  
23 promoting nuclear extrusion of the androgen receptor through the forkhead box (FOX)  
24 transcription factor O1 (FOXO1) (Agamia *et al.*, 2016; Mirdamadi *et al.*, 2015). A relative  
25 deficiency of the nuclear transcription factor FOXO1 is increasingly being recognized as a  
26 key player in the pathogenesis of acne (Melnik and Schmitz 2103; Melnik, 2010).  
27 Accordingly, downregulation of nuclear FOXO1 expression promotes lipogenesis,  
28 upregulation of inflammatory cytokines, and increased keratinocyte proliferation (Melnik and  
29 Schmitz 2103; Melnik, 2010). Conversely, retinoids commonly used to treat acne have been  
30 shown to restore physiological FOXO1 expression (Melnik and Schmitz 2013; Melnik, 2011).  
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Probiotics are live microorganisms that have recently emerged as natural therapeutics with the potential to improve the insulin signalling pathway (Hulston *et al.*, 2015; Mazloom *et al.*, 2013). For example, *Lactobacillus rhamnosus* GG (LGG) – one of the most extensively studied probiotic strain – has been shown to enhanced insulin sensitivity as well as reduce lipogenesis in experimental animals (Park *et al.*, 2015; Kim *et al.*, 2015). Notably, interest in the use of systemic probiotics in patients with acne is gaining momentum (Kumar *et al.*, 2014; Bowe *et al.*, 2014; Bowe, 2013; Bowe and Logan, 2011). Probiotics may act as modulators of the gut-skin axis by regulating intestinal permeability, which in turn is involved in the fine tuning of systemic inflammation, insulin signalling, and tissue lipid content (Bowe *et al.*, 2014; Bowe and Logan, 2011). Probiotics have been previously studied as an adjunct for patients with acne treated with systemic antibiotics (Jung *et al.*, 2013). However, it remains unknown whether systemic probiotics may exert positive effects on acne when administered as monotherapy.

Because both the gut-skin axis (Bowe *et al.*, 2014; Bowe and Logan, 2011) and dysregulation of insulin signalling (Nagpal *et al.*, 2016; Balta *et al.*, 2015; Kumari and Thappa, 2013; Del Prete *et al.*, 2012) have been implicated in the pathogenesis of adult acne, we designed the current pilot study to evaluate the effect of supplementation with the probiotic strain LGG on skin expression of genes involved in insulin signalling (*IGF1* and *FOXO1*). Our hypothesis was that normalization of insulin signalling in the skin elicited by the probiotic could lead to improvements in the clinical appearance of acne in adult subjects.

## 2. Materials and methods

### *Study subjects*

The study sample consisted of 20 Caucasian adult subjects (14 females and 6 males; mean age:  $33.7 \pm 3.3$  years) with active inflammatory acne on their back. Participants were enrolled

in private practices and dermatology clinics. Patients were excluded if they met one or more of the following criteria: 1) diabetes mellitus, 2) severe physical illnesses, including endocrine diseases, 3) use of oral contraceptives in the last 6 months, 4) pregnancy or breastfeeding, 5) use of steroids, 6) use of probiotics in the last 6 months, and 6) use of oral antibiotics, isotretinoin, benzoyl peroxide, and oral retinoids in the last 6 months. The study protocol was approved by the local ethics committee and complied with the tenets of the Declaration of Helsinki. Written informed consent was obtained from all participants.

*Study design*

This pilot study was designed as a 12-week, double-blinded, placebo-controlled, randomized study. All participants were asked to withdraw any topical product for two weeks before the beginning of the study. In addition, they were not allowed to use any topical or systemic products for acne throughout the entire study period. After the baseline visit, eligible subjects were randomly assigned in a 1:1 fashion to one of the following treatment arm: the probiotic group (n = 10) and the placebo group (n = 10). Randomization was performed using a computer-generated random number table. Patients in the probiotic group consumed for 12 weeks a liquid supplement containing LGG (Sacco s.r.l., Cadorago, Italy) at a dose of  $3 \times 10^9$  CFU/day (75 mg/day). Patients in the placebo group consumed for 12 weeks an identical liquid that did not contain probiotics. The study materials were supplied by Biodue S.p.A. (Tavarnelle Val di Pesa, Italy).

*Study endpoints*

The study had three endpoints, as follows: 1) change in skin expression of *IGF1* and *FOXO1* genes in acne areas from baseline to the end of the study; 2) change in the investigator's global improvement rating (Konishi *et al.*, 2007) on a five-point scale (-1, worsened; 0,

unchanged; 1, improved; 2, markedly improved; and 3, resolved) from baseline to the end of the study; and 3) tolerance (assessed by asking patients about any signs or symptoms of systemic or local adverse reactions).

### *Gene expression data*

Paired 4-mm skin punch biopsies in acne areas in the back – one obtained before treatment initiation and one obtained at the end of the 12-week treatment period – were collected for ribonucleic acid (RNA) extraction and gene expression analyses. RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

Agarose gel electrophoresis was used to check the integrity of RNA, whereas spectrophotometry was utilized to measure RNA quantity. Isolated RNA (1 µg) underwent reverse transcription with the iScript cDNA Synthesis Kit (BioRad, Hercules, CA, USA) and the resulting cDNA was stored at –20°C. All quantitative real-time polymerase chain reactions (qRT-PCR) were performed on a BioRad iQ5 Cyclor (BioRad). The reaction solution (25 µL) consisted of cDNA mixture (40 ng), forward and reverse primers (final concentration 400 nM each, and the iQ SYBR Green Supermix (BioRad). The sequences of the primers were as follows: *IGF1* forward, 5'-CTTCAGTTCGTGTGTGGAGACAG-3'; *IGF1* reverse, 5'-CGCCCTCCGACTGCTG-3'; *FOXO1* forward, 5'-GCCATGTAAGTCCCATCAGGA-3'; *FOXO1* reverse, 5'-ATCGGAACAAGAACGTGGAATC-3'. The amplification conditions were as follows: preheating at 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec, 60°C for 15 sec, and 72°C for 30 sec. The Bio-Rad iQ5 Optical System Software Version 2.0 was used to analyze fluorescence data. The expression of the genes of interest was normalized to the expression of the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a housekeeping gene. The forward and reverse primer sequences used for *GAPDH* were 5'-

GAAGGTGAAGGTCGGAGTC-3' and 5'-GAAGATGGTGATGGGATTTC-3', respectively. Expression levels of mRNA were quantified according to the following formula:  $2^{-\Delta CT}$ , where  $\Delta CT$  (sample) was defined as  $CT$  (gene of interest) –  $CT$  (*GAPDH*).

*Statistical analysis*

Results are given as means and standard deviations or counts and percentages. Independent Student's *t*-tests (continuous variables) and  $\chi^2$  tests (categorical variables) were used to compare parameters between the two study groups. Pre- and post-treatment gene expression data were analyzed using paired Student's *t*-tests after adjustment for age and sex. Logistic regression analysis was used to calculate the crude as well as the age- and sex- adjusted odds ratios (ORs) with their 95% confidence intervals (CIs) for being rated by the physicians as improved/markedly improved (*versus* worsened or unchanged) on the investigator's global improvement rating. All analyses were conducted with the SPSS version 17.0 statistical software (SPSS Inc., Chicago, IL, USA). Two-tailed *P* values <0.05 were considered statistically significant.

**3. Results**

*Changes in skin expression of IGF1 and FOXO1*

The probiotic and control groups did not differ significantly in terms of age and sex (data not shown). In addition, baseline expression levels of *IGF1* and *FOXO1* in the skin of acne areas were similar in the two groups (Table 1). After 12 weeks, the probiotic group showed a 32% ( $P<0.001$ ) reduction as well as a 65% increase ( $P<0.001$ ) in *IGF1* and *FOXO1* gene expression in the skin, respectively. However, no significant changes were observed in the placebo group (Table 1).



### *Changes in the investigator's global improvement rating*

The changes in the investigator's global improvement rating are summarized in Table 2.

Significant differences in the ratings were evident in the two study arms ( $\chi^2 = 10.02$ ; d.f. = 4,  $P < 0.05$ ). Patients in the probiotic group were more likely to be rated by the physicians as improved/markedly improved (*versus* worsened or unchanged) compared with the placebo group (crude OR = 36.0; 95% CI = 2.7–476.3,  $P < 0.05$ ). After adjustment for age and sex, similar results were observed (adjusted OR = 28.4; 95% CI = 2.2–411.1,  $P < 0.05$ ).

### *Tolerance*

The treatment was well-tolerated in all participants. No patient in either group discontinued treatment due to adverse local or systemic effects.

## **4. Discussion**

Because gut microbiota may affect skin physiology (Kumar *et al.*, 2014), oral delivery of probiotics is emerging as a promising approach to address common dermatological conditions (Bowe *et al.*, 2014; Bowe, 2013; Bowe and Logan, 2011). This exploratory clinical study examined the effects of supplementation with LGG – one of the most extensively studied probiotic bacteria – in adult patients with acne. Our results demonstrate that 12 weeks of LGG administration normalized skin expression of insulin signalling genes in acne areas, a molecular change that was accompanied by a significant improvement in acne appearance. In addition, the treatment appeared to be safe and well-tolerated.

Growing evidence indicates that *IGF1* (Melnik and Schmitz, 2009) and *FOXO1* dysregulation (Agamia *et al.*, 2016; Mirdamadi *et al.*, 2015) may be involved in the pathogenesis of acne.

The normalization of their skin expression levels following 12 weeks of LGG supplementation confirms that probiotics may act as clinically useful modulators of the gut-

skin axis. Although the exact mechanisms by which skin *IGF1* and *FOXO1* expression is normalized by LGG remain to be determined, it is possible that this probiotic strain may improve insulin resistance through direct metabolic effects and/or by correcting a state of intestinal dysbiosis (Park *et al.*, 2015; Kim *et al.*, 2013). Alterations in the gut microbiome and gut permeability can trigger innate immunity, with increased levels of circulating endotoxin that can in turn activate Toll-like receptors 2 and 4 (TLR-2 and TLR-4). The activation of TLR-2 and TLR-4 can induce the release of cytokines and the expression of metalloproteinases that ultimately aggravate acne (Dreno *et al.*, 2015; Selway *et al.*, 2013). Additionally, it has been shown that TLR-4 activation can inhibit expression of *FOXO1* in macrophages through Akt signalling, and activate inflammatory pathways via NF- $\kappa$ B, promoting insulin resistance in different tissues (Fan *et al.*, 2010). Notably, LGG has been shown to improve gut permeability (Wang *et al.*, 2012; Sindhu *et al.*, 2014). In this study, modulation of gut microbiota and permeability by LGG (with an associated reduction in endotoxemia and TLR-4 activation) may have lowered *FOXO1* skin expression.

The positive effects of LGG observed in the current study are of particular interest because we included only patients with adult acne (in whom alimentary factors are deemed to play a role) (Agamia *et al.*, 2016; Melnik *et al.*, 2011). In designing the current study, we hypothesized that dysregulation of insulin signalling in the skin could not only play a major role in the pathogenesis of this condition but could also serve as a therapeutic target. To explore this possibility, we focused on the modulation of *IGF1* and *FOXO1* skin expression elicited by LGG, which was selected as a prototypical probiotic modulator of the gut-skin axis.

Interestingly, *IGF1* and *FOXO1* have been suggested to play a crucial role in the pharmacological action of oral isotretinoin (Melnik, 2011), one the most effective drug in the treatment of acne. Although LGG was used in monotherapy in the current study, our results may pave the way for the future investigation of this probiotic in combination with

isotretinoin. Specifically, further research is needed to investigate whether the addition of LGG may further provide an additional decline in the number of inflammatory and total acne lesions as compared with isotretinoin alone.

Several limitations of our study need to be acknowledged. First, this study was designed as an exploratory pilot project and the sample size was small. Although the improvement in acne appearance elicited by LGG supplementation was promising, our data need to be confirmed in clinical trials with larger sample sizes and longer treatment duration. We believe that our current findings provide a strong rationale for such a study. Second, the effect of LGG as an adjunct to acne medical therapy (e.g., antibiotics, retinoids, and isotretinoin) warrants further scrutiny. Third, it is important to note that all of our patients were adult. The question as to whether LGG supplementation may be helpful for adolescent acne requires further scrutiny. Fourth, gene expression studies were performed in acne areas of the back. Owing to this methodological approach, caution should be exercised when extrapolating our findings to other skin areas. Finally, all of the participants were Caucasian, and our findings might not be directly extrapolated to persons with different ethnic backgrounds.

In conclusion, our results demonstrated a normalization of cutaneous insulin signalling and an improved appearance of acne in adult patients who received LGG supplementation for 12 weeks. Further studies are necessary to investigate the exact mechanisms by which gut colonization of supplemented LGG could affect skin homeostasis through the modulation of the gut-skin axis and/or the restoration of an intestinal healthy microbiota.

### **Conflict of interest**

This study was performed based on a collaboration between Enzo Emanuele and Biodue S.p.A. with partial sponsorship and supply of test materials. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Table 1. Changes from baseline to 12 weeks in relative gene expression values of *IGF1* and *FOXO1* in skin biopsies obtained from the acne areas on the back in the two study groups

	Probiotic group (n = 10)		Placebo group (n = 10)	
	Baseline	12 weeks	Baseline	12 weeks
<i>IGF1</i>	2.31 ± 0.91	1.56 ± 0.77*	2.24 ± 0.82	2.11 ± 0.89
<i>FOXO1</i>	0.81 ± 0.15	1.34 ± 0.26*	0.86 ± 0.13	0.90 ± 0.15

Levels of mRNA were determined after adjustment for age and sex with the following formula:  $2^{-\Delta CT}$ , where  $\Delta CT$  (sample) was defined as  $CT$  (gene of interest) –  $CT$  (*GAPDH*).

\* $P < 0.001$  versus baseline.



Table 2. Change from baseline to 12 weeks in the investigator's global improvement rating of adult acne in the two study groups

Rating	Probiotic group (n = 10)	Placebo group (n = 10)
Worsened	0 (0%)	0 (0%)
Unchanged	2 (20%)	9 (90%)
Improved	6 (60%)	1 (10%)
Markedly improved	2 (20%)	0 (0%)
Resolved	0 (0%)	0 (0%)

Data are given as counts and percentages.