

Formulation Development of a Topical Probiotic Gel for Antidermatophytosis Effect

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Received 2016 January 06; Revised 2016 February 07; Accepted 2016 February 17.

Abstract

Background: Topical drug delivery is a painless route of drug administration. Skin is one of the most easily accessible parts of human body for topical administration and also is a main route of topical drug delivery system. There are different skin diseases and infections caused by fungus. An antifungal compound is a pharmaceutical fungicide used to treat mycoses such as dermatophytes which have the ability to attack keratinized tissues and used keratin cause dermatophytosis, the most common human contagious fungal disease. Because of drug resistance, side effects and cytotoxicity associated with long-term treatment with antifungal drugs, finding new useful drugs to treat fungal infections is necessary.

Objectives: The purpose of the present study was to formulate topical gel containing stabilized extract of *Lactobacillus* as an anti-dermatophytic preparation and evaluate its physicochemical properties.

Materials and Methods: *Lactobacillus casei* was cultured in MRS anaerobically and then its extract was collected by separating the bacterial cells with centrifugation. Firstly, antifungal activity of the extract against four dermatophyte species (*Trichophyton rubrum*, *Trichophyton verocosum*, *Microsporum canis*, and *Microsporum gypseum*) was done using the well-plate method. The macrodilution method was used to determine the minimum inhibitory concentration (MIC) of the probiotic extract in the range of 0.0625 mg/mL to 1 mg/mL. To select the best gel formulation, one general formula was considered and then corrected by changing the polymer ratio. Gel formulations were characterized for monotonousness, probiotic extract content, pH determination, viscosity measurement, grittiness and antifungal activity in comparison to topical cream of clotrimazole 1%.

Results: The results showed the same effect of the probiotic extract against 4 pathogens; however, the greatest average zone of inhibition was measured 34 mm against *T. rubrum*. The MIC test showed that stabilized probiotic extract had more antidermatophyte effect compared to supernatant ($P < 0.01$). Formulation containing 5% probiotic extract (F5) was chosen as the best antidermatophyte topical gel because of its more stability. Also, all formulations were studied for antifungal activity compared to clotrimazole, and F5 was found to be significantly more effective than clotrimazole ($P < 0.01$).

Conclusions: The results of this study show that the beneficial effect of *Lactobacillus casei* is related to its metabolites. A formulated gel shows a significant antidermatophytosis activity compared to clotrimazole.

Keywords: Lactobacillus, Probiotic, Topical Gel, Dermatophytes

1. Background

Microsporum, *Trichophyton* and *Epidermatophyton* are fungi that caused dermatophytosis. Dermatophytes are the pathogenic members of the keratinophilic soil fungi. *Microsporum* and *Trichophyton* are human and animal pathogens, while *Epidermatophyton* is a human pathogen. They have the ability to attack keratinized tissues and used keratin and cause dermatophytosis (1, 2). Resulting diseases of these fungi often called Tinea. According to the World health organization (WHO), dermatophytes affect about 25% population of the world (3, 4). Although many antifungal drugs have been developed in recent years and

have become available for the treatment of dermatophytosis, they are limited in two anti-fungal families: the allyl-amin and the azole. Because of their resistance and side effects in clinical, they can lead to failure in the treatment of mycoses (5, 6). The azole antifungal agents such as itraconazole or ketoconazole can be substrates and inhibitors of the P-glycoprotein, which excretes toxins and drugs into the intestines (7). Polyene antimycotics administration is limited by infusion-related toxicity, an effect postulated to result from proinflammatory cytokine production. The acute toxicity of amphotericin-B deoxycholate includes fever, rigors, nausea, hypertension, vom-

iting and hypoxia, and chronic side effect is nephrotoxicity (8). Synthetic fungicides can cause pollute in environment by their slow biodegradation in the environment. These years have seen a more interest to study about natural products, because they can be more efficacious than other already drugs in use and for diseases control and less harmful in environmental pollution. Probiotics, which are normal resident flora of intestinal tract, can be formulated into different types of products. The term “probiotic” was first proposed as microbial derived factors promoting the growth of other microorganisms in 1965 by Lilly and Stillwell (9). Probiotics have many useful effects such as lactose intolerance, prevention of atopic dermatitis, gastrointestinal disorders and urogenital infection. Probiotics have two mechanisms for their beneficial effects: 1) their live cells are caused direct effects, and 2) their metabolites; biogenics or metabolites are caused indirect effects. Lactic acid bacteria (LAB) are widely used in food and feed fermentation, contributing to the flavor, structure, safety and stability of food or feed products (10). Lactic acid bacteria are commonly used as probiotics (11, 12), which have potential for production of metabolites including enzymes, vitamins, organic acids, bacteriocins, and other metabolites (13). Several of these low molecular metabolites have been isolated and shown to have the ability to blunt or inhibit fungal growth (14, 15). Topical drug delivery is a useful painless route of drug administration, it offers an easy way for patients, and prevents the hepatic first pass metabolism of the drug (16, 17). Within the major groups of a topical drug delivery system, the use of topical gel formulation has expanded in pharmaceutical field because of its favorable properties such as being thixotropic and greaseless, easily spreadable and removed, emollient and water soluble (18). Hydroxy propyl methyl cellulose (HPMC) is recommended in the gel formulation because of its nontoxic nature, ease of compression and good bioadhesiveness (19).

2. Objectives

The purpose of this study was to investigate the effect of probiotic cell free extract on the dermatophytes and formulate a topical gel with effective concentration of probiotic extract as antidermatophyte preparations.

3. Materials and Methods

3.1. Microorganisms

The probiotic strain was *Lactobacillus casei* (PTCC 1608). The freeze dried sealed glass ampoules of microorganisms used in this study were obtained from Persian type culture collection (PTCC). The microorganisms were as follows: *Microsporium canis* PTCC 5069, *Microsporium gypseum*

PTCC 5070, *Tricophyton rubrum* PTCC 5143, and *Tricophyton verocosum* PTCC 5056.

3.2. Microbial Culture and Preparation of Fungal Inoculums

Bacteria were cultured in the MRS broth medium for 24 hours at 37°C and maintained on MRS agar, anaerobic glove box with 95% N₂, 5% H₂, 6% O₂, and 5% CO₂. These fungi were revived on satorad dextrose broth (SDB) and then cultured on satorad dextrose agar (SDA) at 22°C for 21 days at 22°C to obtain adequate growth.

3.3. Growth Rate Determination of Probiotics on Culture Media

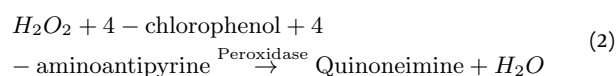
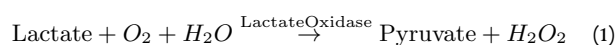
For estimation of the growth rate of *Lactobacillus casei*, 1 mL (equal to 10² cfu/mL) of the fresh cultured *Lactobacillus* was inoculated in 100 mL of the MRS broth. After mixing, flask was incubated at 37°C for 24 hours and viable cells count was performed.

3.4. Preparation of Cell-Free Supernatant

The *Lactobacillus* strain was grown anaerobically in 100 mL of the MRS broth for 24 hours at 37°C. Supernatant was obtained by centrifuging the medium at 3000 rpm for 15 minutes. Centrifuged supernatant was passed through a sterile 0.22 μ-pore-size filter unit (Millex GS Millipore). The filtrate was collected as the mixture of metabolites and then was kept at 4°C (20).

3.5. Lactic Acid Assay

Quantitative detection of lactic acid in acquired supernatant was performed using an enzymatic lactate reagent kit (chem. Enzyme). This assay method was based on two stage oxidative reactions as follows:



The absorbance of quinoneimine as the final colored product was measured at 500 nm wavelength (UV detector, S-3100, Scinco, Korea). The lactic acid level of supernatant was expressed as mg/dL. A standard curve was prepared using lactic acid with 99% of purity as the reference standard in different concentrations. The assay test was repeated in triplicate (20).

3.6. Long Term Stability Study

The acquired supernatant was stored for 9 months at 4°C. The value of pH was determined using a digital PH-meter (SL.901) during storage course and results were recorded.

3.7. Antidermatophytosis Effect of Supernatant

The agar well-plate method was used to determine an antidermatophytosis effect of acquired supernatant and its lyophilized form. This method needed fungal suspension with these characteristics; in λ_{\max} 580 nm with spectrophotometer (UV detector, S-3100, Scinco, Korea) the absorbance must be between 0.15 and 0.17 nm. Two bores were made on the pathogenic seeded medium in each petri-dish, and respectively, poured by 100 μ L of fresh supernatant on 0 day (Sup-0) and the lyophilized probiotic extract (LPE). After keeping at 4°C for diffusion, the plates were incubated for 7 days at 22°C and a zone of inhibition was observed and measured (20).

3.8. Determination of the Minimum Inhibitory Concentration of Both Probiotic Extracts Against Dermatophytes Strains

The conventional macro serial dilution method was used to determine the minimum inhibitory concentration (MIC) of both probiotic extracts, fresh supernatant (Sup-0) and LPE with respect to four fungal strains. A stock solution of Sup-0 was prepared in sterile SDB (100 mg/mL), which was further diluted in SD-b to reach a concentration range of 6.25 mg/mL to 100 mg/mL. The lyophilized probiotic extract was also dissolved in SD-b to reach a concentration range of 0.0625 mg/mL to 1 mg/mL.

Final concentration of fungi in individual tubes was adjusted to about in 5×10^6 CFU/mL control tubes contained culture media with fungal strain (in 5×10^6 CFU/mL) and only culture media without any antifungal agent. Other test tubes included culture media with fungal strain (5×10^6 CFU/mL) and LPE, culture media with fungal strain (5×10^6 CFU/mL) and Sup-0.

After 7 - 10 days incubation at 22°C - 25°C, the test tubes were examined for possible fungal turbidity and MIC of both compounds for each test fungi was determined respectively as lowest concentration that could stop visible bacterial growth for 7 and 10 days (21).

3.9. Preparation of Hydroxy Propyl Methyl Cellulose Topical Gel

First, of total water needed was heated to 60°C, then HPMC powder was slowly added to preheated water with constant stirring. Then remaining water and precalculated amounts of LPE were added. The mixture was stirred until a homogenous appearance was attained. Then, each solution was left over night in the refrigerator until a transparent gel was formed (22).

3.10. Physical Examination

After the gels were set in the container, the homogeneity of each gel was tested by visual inspection. They were also examined for their physical appearance and the presence of any aggregation.

3.11. Determination of pH

Two gram of each gel formulation was dissolved in 10 mL of deionized water and their pH was measured using a digital PH-meter (SL.901). Each test was performed in triplicate and the results were reported as mean \pm SE.

3.12. Viscosity

A Brookfield viscometer (model DV-II, spindle 64, 20 rpm) was utilized to measure the viscosity of gel formulations and evaluate their rheological behavior (23).

3.13. Grittiness

Each formulation was microscopically evaluated if any appreciable particle was seen under a light microscope (24).

3.14. Thermal Cycle Test

The portion were stored at 5°C for 48 hours and then at 25°C for 48 hours. The procedure was repeated 6 times and then their stability and appearance were evaluated (25).

3.15. Thermal Change Test

Three 20 g portion of each formulation were stored at 4 - 6°C, 25°C and 45 - 50°C. After 24 hours, one and three months, their stability and appearance were evaluated (25).

3.16. Freezing and Thawing

Twenty gram portion of each formula were stored periodically at 45°C - 50°C and 4°C for 48 hours each. The procedure was repeated six times and then the samples were checked regarding their appearance and stability (25).

Comparison of Antidermatophytotic Effect of Gel Formulations With Clotrimazole Cream

The agar well plate method as describe previously, was used in this evaluation. Gels and cream must be diluted separately with distilled water (2: 3D.W). Then, equivalent amount of gels and cream (200 μ L) were poured in wells separately. After 7 - 10 days incubation at 22°C, the zone of inhibition for each fungus was analyzed separately.

4. Results

4.1. Growth Kinetic of *Lactobacillus casei*

Figure 1 shows that *Lactobacillus casei* took approximately 8 hours to reach the log phase with generation time (TG) in 1 hour.

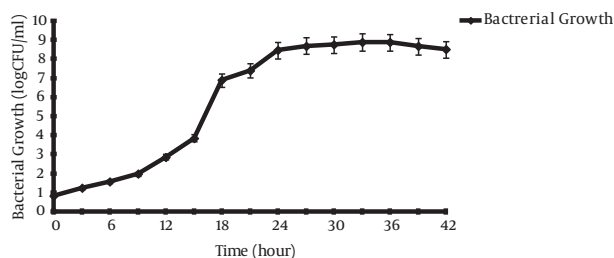


Figure 1. The Kinetics of Growth of *Lactobacillus casei* in MRS Media Incubated at 37°C for 42 Hours Under Defined Microaerophilic Condition

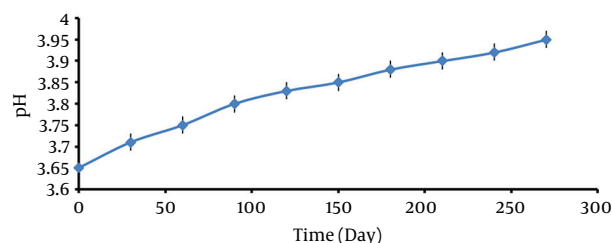


Figure 2. Long Term Stability of Supernatant of *Lactobacillus casei* Stored at 4°C for 9 Months

4.2. Long-Term Stability of Supernatant

Evaluation of pH of the acquired cell free supernatant showed a few variations during 9 months storage in 4°C, but even these low changes could bias the stability of supernatant properties (Figure 2).

4.3. Antidermatophytosis Effect of the Supernatant

Figure 3 shows that, the antifungal activity of LPE was significantly higher than that of other groups, fresh supernatant (Sup-0) with probiotics viable cells and there was a significant reduction in effectiveness of antifungal properties of supernatant by time lapse as it was lowest in Sup-90. In all the samples, the greatest inhibition zone was toward *Trichophyton rubrum*, and the narrowest zone was related to *Microsporum gypseum*.

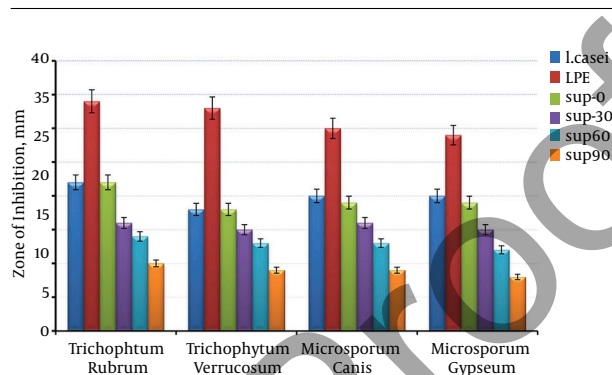
4.4. Determination of Minimum Inhibitory Concentrations

Minimum inhibitory concentrations of Sup-0 and LPE were tabulated in Table 1. It was found that the MIC of LPE for *Trichophyton rubrum*, *Trichophyton verocosum*, *Microsporum canis* and *Microsporum gypseum* was 0.625 mg/mL and the MIC of Sup-0 for these fungi was 6.25 mg/mL.

4.5. Different Gel Formulations

F1, F2 and F3 were chosen as the best formulations. F4, F5 and F6 were eliminated because of their very low viscosity, and F7, F8 and F9 formulations were eliminated because of their high viscosity (Table 2).

Figure 3. Comparison of Antifungal Activity Between Different Samples of Probiotic Extract



LPE, lyophilized probiotic extract; Sup-0, Fresh probiotic extract; Sup-30, probiotic extract 30 days post extraction; Sup-60, probiotic extract 60 days post extraction; Sup-90, probiotic extract 90 days post extraction.

4.6. Physicochemical Evaluation of Gel

The result for pH, viscosity, homogeneity and grittiness were shown in Tables 3 - 5.

4.7. Stability of Gel

The appearance of formulated gel remained clear and no significant change was observed in stability after a 3-month period.

4.8. Antidermatophytosis Effect of the Lyophilized Probiotic Extract Gel

Figure 4 shows antifungal effect of different gel formulations. It was related to the content of LPE as formula contained 5% of LPE had a greatest inhibition zone. Also, there was no significant difference between 5% LPE gel and clotrimazole cream ($P > 0.05$).

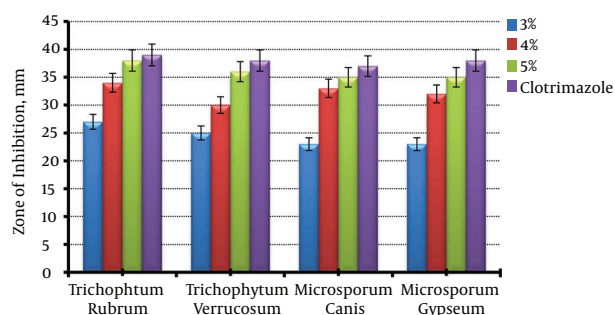


Figure 4. Comparison of Antidermatophytosis Effect of Formulated Gel With Clotrimazole 1%

Table 1. Minimum Inhibitory Concentrations of Fresh Supernatant (Sup-0) and Lyophilized Probiotic Extract

Indicator Pathogens	<i>Trichophytum rubrum</i>	<i>Trichophytum verrucosum</i>	<i>Microsporium canis</i>	<i>Microsporium gypseum</i>
Concentration range of Sup-0, mg/mL				
25	-	-	-	-
12.5	-	-	-	-
6.25	-	-	-	-
3.125	+	+	+	+
1.56	+	+	+	+
Concentration Range of LPE, mg/mL				
2.5	-	-	-	-
1.25	-	-	-	-
0.625	-	-	-	-
0.312	+	+	+	+
0.156	+	+	+	+

Table 2. Different Gel-Based Formulations

Ingredients	Formulation of Gels								
	F1	F2	F3	F4	F5	F6	F7	F8	F9
HPMC	2.5	2.5	2.5	2	2	2	3	3	3
Distilled Water	97	96	95	97	96	95	97	96	95

Abbreviation: HPMC, hydroxy propyl methyl cellulose.

Table 3. Evaluation Data of Viscosity of Lyophilized Probiotic Extract Gel

Formulation Code	Cp, η (cp) $\times 10^6$			Mean \pm SD	Storage Time
	cp ₁	cp ₂	cp ₃		
F1	14.9	15	15.2	15.03 \pm 0.15	After a week
	14.8	14.7	14.5	14.66 \pm 0.15	After a mount
	14.7	14.5	14.4	14.53 \pm 0.15	After 3 mounts
F2	14.5	14.6	14.7	14.6 \pm 0.1	After a week
	14.5	14.3	14.4	14.4 \pm 0.1	After a mount
	14.4	14.1	14.2	14.23 \pm 0.15	After 3 mounts
F3	14	14.1	14.3	14.13 \pm 0.15	After a week
	13.9	14	14.2	14.03 \pm 0.15	After a mount
	13.9	14	14.1	14 \pm 0.1	After 3 mounts

5. Discussion

It has been shown that 20% to 30% of human infections are due to dermatophytes and there are several reports indicating their resistance to common antifungal agents. In

the other hand, the commonly used antifungal agents including "azol" and "alylamine" compounds such as itraconazole and terbinafine are hepatotoxic and they cause many drug interactions (7).

Table 4. Evaluation Data of pH of Lyophilized Probiotic Extract Gel

Formulation Code	pH			Mean \pm SD	Storage Time
	pH ₁	pH ₂	pH ₃		
F1	5.48	5.48	5.48	5.48 \pm 0	After 48 hours
	5.38	5.38	5.39	5.38 \pm 0.005	After a week
	5.39	5.38	5.40	5.39 \pm 0.01	After a month
	5.39	5.38	5.38	5.38 \pm 0.005	After 3 months
F2	5.39	5.40	5.41	5.4 \pm 0.01	After 48 hours
	5.30	5.31	5.30	5.30 \pm 0.005	After a week
	5.30	5.32	5.31	5.31 \pm 0.01	After a month
F3	5.31	5.30	5.31	5.30 \pm 0.005	After 3 months
	5.32	5.33	5.33	5.32 \pm 0.005	After 48 hours
	5.27	5.28	5.29	5.28 \pm 0.01	After a week
	5.22	5.22	5.23	5.22 \pm 0.005	After a month
	5.21	5.21	5.22	5.21 \pm 0.005	After 3 months

Table 5. Evaluation Data of Homogeneity and Grittiness

Formulation	Homogeneity	Grittiness
F1	+++	-
F2	+++	-
F3	+++	-

In recent years, to overcome the difficulties due to dermal infections, many researchers have been focused on discovering new efficient topical agents with natural origin and fewer side effects. In 2010, Karska-Wysocki et al. investigated antimicrobial effect of *Lactobacillus acidophilus* and *Lactobacillus casei* on methicillin resistant *Staphylococcus aureus* (MRSA). They showed that *Lactobacillus acidophilus* produces antimicrobial compounds that inhibit MRSA growth to the extent that after 24 hours incubation of probiotic bacteria and pathogens at 37°C, there was 99% decrease in pathogens population. Also, *Lactobacillus casei* by producing different compounds such as lactic acid has bactericidal effect on MRSA pathogens (25). In 2012, Gou et al. studied the anti-fungal effect of 4 *Lactobacillus* types against *Microsporum canis*, *Microsporum gypseum* and *Epidermophyton floccosum* by the agar diffusion method. According to their findings, *Lactobacillus reuteri* had the maximum antifungal effect in comparison to the other types (26).

Our findings showed that the maximum acid production was 18 to 24 hours after cultivation of the bacteria. Also, after a 9-month period, stability study showed about 0.3% to 0.35% increases in pH, which had a significant impact on antipathogenic efficacy of the extract. Therefore, to enhance the probiotic supernatant stability and also to concentrate and reinforce its efficacy, lyophilization was utilized as a proper method. According to our findings, LPE, in comparison with supernatant was more stable, and also had more pathogenic effect. Well-diffusion study results (Figure 3) demonstrated that aging may decrease the antifungal activity of total extract and due to equal antifungal efficacy of Sup-0 and probiotic, it may be utilized as a proper substitute. There was a significant difference between antidermatophytic efficacy of LPE and the supernatant ($P < 0.05$) and it showed the bigger growth inhibition zone in culture media. Also, as indicated in Table 1, LPE even in lower concentrations, was significantly more potent in growth inhibition than the plain probiotic extract ($P < 0.01$). Macroscopic evaluation of LPE containing gel showed its homogeneity and there were no rigidity and air bubbles (Table 5). Also, there was no considerable pH change during three months of storage (Table 3) and it can be deduced that by increasing the extract content of the gels, the formulation becomes more acidic.

Rheological evaluation showed no significant change in gel viscosities, although it was an inverse relation be-

tween LPE content and viscosity (Table 4). Figure 4 shows an antifungal effect of different gel formulations. It can be seen that all of the formulations inhibit growth of common dermatophytes, although 5% LPE gel was significantly more effective ($P < 0.05$). Also, there was no significant difference between 5% LPE gel and clotrimazole cream ($P > 0.05$). Therefore, due to safety and lack of adverse effects of probiotic containing products, they may be considered as a proper substitute in the treatment of dermatophytosis.

From this study, it can be concluded that the prepared probiotic topical gel, due to its efficacy and safety can be used for the treatment of dermatophytosis. However, it is needed for in vivo application to examine clinically the prepared gel containing LPE in more detail.

Footnote

Funding/Support: This study was supported by a research grant from nanotechnology research center, Jundishapur University of Medical Sciences, Ahvaz, Iran.

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