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Review

The efficacy of probiotics as pharmacological treatment of cutaneous wounds: Meta-analysis of animal studies

Christos G. Tsiouris[a], Martha Kelesi[a], Georgios Vasilopoulos[a], Ioannis Kalemikerakis[a], Effie G. Papageorgiou[b,*]

[a] Department of Nursing, Faculty of Health and Caring Professions, Technological Educational Institution of Athens, Greece
[b] Department of Medical Laboratories, Faculty of Health and Caring Professions, Technological Educational Institution of Athens, Greece

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ABSTRACT

The aim of the current meta-analysis of animal studies was to evaluate the efficacy of probiotics as pharmacological treatment of cutaneous wounds. A systematic electronic literature search was conducted and in total six animal studies which undertake twelve experiments met our inclusion criteria. We used the percentage (%) of wound area at the end of the first week after initial wounding to evaluate the efficacy of the probiotic treatment. The heterogeneity was estimated as statistically significant ($p < 0.0001$) and therefore the meta-analysis was performed with the random-effect model. Based on the estimated Hedges' g (Hedges, 1982), the administration of probiotics was associated with acceleration of the wound contraction ($g = -2.55$; 95%CI = $-3.59, -1.50$; $p < 0.0001$). The meta-regression analysis showed that the moderator sterile kefir extract has the greater effect on the overall estimated efficacy of probiotic treatment ($g = -5.6983$; $p = 0.0442$) with bacteria probiotic therapies (70% kefir gel, *L. brevis*, *L. fermentum*, *L. plantarum*, *L. reuteri*) following ($g = -2.3814$; $p = 0.0003$). For bacteria dose moderator, the results showed that increase in bacterial dose corresponds to increase of the estimated overall effect size ($g = -10.2056$; $p = 0.0053$). The linear regression test of funnel plot asymmetry showed absence of publication bias. In conclusion, the results indicate that probiotics administration is an effective pharmacological treatment of cutaneous wounds. However, due to the heterogeneity among studies, further research is required.

1. Introduction

The majority of epithelial linings of our body, such as the skin and mucosa, are colonized by a great number of microorganisms that constitute the so-called normal microflora. These microorganisms outnumber 10 times the human body cells. Normal microflora is constituted mainly by commensal bacteria. These bacteria cooperatively interact with their host and they are crucial for its health (Tlaskalová-Hogenová et al., 2011; Patel and DuPont, 2015; Cogen et al., 2008).

Wound healing is a natural biological process that can be affected by many moderating factors. Some of them can lead to improper or impaired wound healing and others can improve wound healing and resolve impaired wounds (Guo and DiPietro, 2010). One of the major factors affecting the healing process is the interaction of the wound with the microbial microflora (Bowler et al., 2001).

Microbial colonization occurs in all wounds, chronic or acute. Understanding the correlation between different microbial communities and wound healing capability is an intense area of research (Scales and Huffnagle, 2013). Recent studies, suggest that changes in local cutaneous microflora, as well as alterations in the gastrointestinal tract microflora, can affect positively or negatively the healing process through various ways, especially through the production of antimicrobial molecules and regulation of immune and inflammatory response (Peral et al., 2009; Rahimzadeh et al., 2014; Poutahidis et al., 2013).

Probiotics are live bacteria or yeasts which exert health-promoting effects to the host (Schrezenmeir and de Vrese, 2001). Preclinical and clinical studies emphasize their efficacy in preventing various infectious, immune-mediated and inflammatory diseases (Wong et al., 2013). Probiotics have the ability to balance the gut microflora and improve the gastrointestinal barrier. In addition, they contribute to the reduction of low-density lipoprotein (LDL) levels and total cholesterol levels, and also suppress inflammation and modulate local and systemic immune functions (Wong et al., 2013; Hakansson and Molin, 2011; Wolvers et al., 2010; Jones et al., 2012a).

According to the evidence so far, probiotics can be useful in the prevention and treatment of difficult healing wounds by regulating the

* Corresponding author at: Technological Educational Institution of Athens, Department of Medical Laboratories, Ag Spyridonos, Egaleo, Athens 12243, Greece.
E-mail address: efipapag@teiath.gr (E.G. Papageorgiou).

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interactions between the host and microbes (Wong et al., 2013). More specifically, studies in laboratory animals showed that certain probiotic bacteria can positively affect the wound healing process by topical administration (e.g. *L. brevis*, *L. plantarum* and L. *fermentum*) or *per os* (*L. reuteri*) (Scales and Huffnagle, 2013; Peral et al., 2009; Poutahidis et al., 2013; Zahedi et al., 2011a; Jones et al., 2012b).

Topical application of specific probiotic species leads to strengthening of immune system response, reduction of inflammation and acceleration of wound healing process (Rahimzadeh et al., 2014; Zahedi et al., 2011a; Nasrabadi and Ebrahimi, 2011; Atalan et al., 2003). More specifically, probiotics bacteria produce exopolysaccharides that have immunostimulatory activity and are able to activate macrophages and lymphocytes (Zahedi et al., 2011a; Foligné et al., 2010; Nayak et al., 2010). The lactic acid bacteria that are used as probiotics, as *L. plantarum,* produce*,* apart from exopolysaccharides, also lactic acid, as the major metabolic end-product of carbohydrate fermentation. Lactic acid has antibacterial properties and inhibits the proliferation of pathogenic microorganisms and therefore lactic acid bacteria or probiotic mixtures in which they found, like Kefir, have been tested for their wound healing properties (Nasrabadi and Ebrahimi, 2011a; Nasrabadi and Ebrahimi, 2011b; Sonomoto, and Yokota, A. 2011; Atalan et al., 2003; Rahimzadeh et al., 2015; Huseini et al., 2012; Farnworth, 2006; Farnworth, 1999). Furthermore, a wound healing, nitric oxide gas (gNO)-producing, probiotic patch using lactic acid bacteria in an adhesive gas permeable membrane has been tested for treating ischemic and infected full-thickness dermal wounds in rabbit models and showed increased wound closure (Jones et al., 2012b).

Dietary intake of lactic acid bacteria has been shown to down-regulate host inflammatory responses, confer more rapid progression of inflammatory events during wound healing and compresses the classical wound repair cascade. In addition, ingestion of lactic acid bacteria leads to rapid collagen deposition, which is very important for proper wound repair (Poutahidis et al., 2013).

Impaired wound healing, as in the case of chronic, ischemic or infected wounds, is a major challenge for both health professionals and patients. The use of common antimicrobial agents is becoming constantly more and more ineffective in the treatment of common pathogens infections, as also it contributes to the emergence, dissemination, and evolution of antibiotic resistance (Gorwitz, 2008; Anstead et al., 2007; Nordmann et al., 2007; Linares, 2001; Davies and Davies, 2010). Therefore alternative pharmacological therapies which do not rely on the use of common antimicrobial agents are becoming more and more needed in the wound management (Jones et al., 2012b). Based on the above-mentioned therapeutic effects of probiotics in wound healing process, by either topical application or *per os* administration, their potential use in the treatment of wounds and ulcers should be taken into account.

Here, we report on a meta-analysis of data from controlled *in vivo* studies testing the efficacy of probiotics as a pharmacological treatment of cutaneous wounds in animal models. We further assessed whether probiotic species, gas NO, route of administration, wound infection, ischemia, treatment day, the frequency of administration, initial wound area and microbial dose per wound, affect the efficacy of probiotic therapy. Also, we examine the heterogeneity of published studies that were included in this meta-analysis and assess the presence of publication bias.

2. Methods

2.1. Search strategy

Using prespecified inclusion and exclusion criteria (see below), we identified all publications reporting experiments in laboratory animals that compare the use of probiotics with a control in cutaneous wounds, by searching (from inception to July 2016) two electronic databases (MEDLINE and EMBASE), with search results limited to those indexed as describing animal experiments.

The structured search strategy used the following format of search terms: (probiotic OR commensal microbiota OR microbiome OR symbiotic OR microbial symbionts OR *lactobacillus* OR *Bifidobacterium* OR *lactobacilli* OR *Saccharomyces* OR Bacteriotherapy OR kefir OR kefir products) AND (wound healing OR wound OR cutaneous wound OR wounds OR burn). No language restriction was imposed. In addition, the reference lists of identified studies were manually checked to identify other potentially eligible trials. This process was performed iteratively until no additional articles could be identified.

2.2. Inclusion and exclusion criteria

We included experiments where functional outcome in a group of animals exposed to cutaneous wound and treated topically or *per os* with probiotics was compared with functional outcome in a control group of animals. We excluded individual comparisons that did not report (or where we could not calculate) the number of animals, the mean outcome, or its standard deviation in each group. Also, we excluded duplicate studies and experiments that have repeated data or did not report outcomes associated with the wound surface and the wound contraction.

2.3. Data extraction and outcome measures

Two authors independently extracted the following data from each experiment: first author, year of publication, animal characteristics, number of animals, probiotic group, route of administration, number of wounds in both treated and control groups, coexisting factors such as infection and ischemia that possibly affect the wound healing process, mean outcome, standard deviation in each group, frequency of treatment administration (No. of Adm./treatment days), initial wound area (day 0, WA_0), wound area on the seventh day after induction of wounds (WA_7), microbial dose per wound and the depth of wounds. If the WA_7 was not given by the study, we extracted the wound area on the sixth day after wounding (WA_6) and examined the treatment day as a moderator variable. We convert wound area measures (WA_7 or WA_6) to a percentage (%) of wound area (WA_7% or WA_6%) considering the initial wound area (day 0, WA_0) as 100%.

It is important to note that we extracted wound area at the end of the first week (7th or 6th day) after initial wounding, because at this time the maximum response of cell proliferation and matrix deposition is occurs, while the inflammation phase is nearing its end (Enoch and Price, 2004). Therefore, based on the importance of cellular events of wound healing that are occur within this one week period (Enoch and Price, 2004; Yussof et al., 2012), we used the percentage (%) of wound area at the end of this first week to evaluate the efficacy of probiotics as pharmacological treatment of cutaneous wounds.

Where a publication reported more than one experiment, or where an experiment reported more than one individual comparison, we considered these separately and extracted data for each, correcting the weighting of these studies in the meta-analysis to reflect the number of experimental groups served by each control group.

2.4. Quality assessment

Study quality of individual studies was assessed according to published criteria (Horn et al., 2001; Antonic et al., 2013; Macleod et al., 2004).

These criteria were:

(i) publication in a peer-reviewed journal
(ii) statements describing control of temperature
(iii) randomization to treatment group
(iv) allocation concealment
(v) blinded assessment of outcome

(vi) sample size calculation
(vii) compliance with animal welfare regulations
(viii) whether the authors declared any potential conflict of interest

Each study was given a quality score out of a possible total of 8 points, and the group median was calculated.

2.5. Statistical analysis

Statistical analysis was conducted using the R programming language (R v3.2.5) and the methodology based on Chen and Peace (2013). For each individual comparison, we calculated effect size (ES). Because the outcome is continuous (WA%), the predictor is dichotomous (treatment with probiotics *versus* control) and means and standard deviations are available, we computed standardized mean differences (SMDs) with 95% confidence intervals (CIs). SMDs and their variances computed for each study to assess the variability of ESs across all included studies and to derive an overall summary effect. To estimate the standardized mean difference we measured g, known as Hedges' g (Hedges, 1982). Especially we computed unbiased estimator g^* defined as:

$$g^* = \left(1 - \frac{3}{4 \bullet df - 1}\right) \bullet \frac{\overline{X_1} - \overline{X_2}}{sd_{pooled}} \quad (1)$$

where df is the degree of freedom (group size minus one), $\overline{X_1}$ and $\overline{X_2}$ are the sample mean scores on the outcome variable (WA%) at post-treatment in the two groups (treated and control respectively) and sd_{pooled} is the pooled standard deviation of both group means, computed as:

$$sd_{pooled} = \sqrt{\frac{(n_1 - 1) \bullet S_1^2 + (n_2 - 1) \bullet S_2^2}{n_1 + n_2 - 2}} \quad (2)$$

where n_1 and n_2 are the sample sizes in each group and S_1 and S_2 are the standard deviations in each group.

Also, we calculated the variance of the g^* as:

$$V_{g^*} = \left(1 - \frac{3}{4 \bullet df - 1}\right)^2 \bullet \left(\frac{2}{\tilde{n}} + \frac{\left(\frac{\overline{X_1} - \overline{X_2}}{sd_{pooled}}\right)^2}{2 \bullet (n_1 + n_2)}\right) \quad (3)$$

where $\tilde{n}$ is the harmonic mean. We used the inverse of the variance to calculate study weights, where larger studies are more precise estimates of the "true" population ES and are weighted heavier in the summary analyses. A Random-Effects model was used in the case of statistically significant heterogeneity and a Fixed-Effect model in the case of statistical significant homogeneity (Borenstein et al., 2009). Where multiple experiments in the same study differed only about the animal sex, we calculated the combined effect size and the pooled standard deviation.

Heterogeneity across studies was tested by using the Q-statistic, τ^2 and I^2-statistic. Studies with an I^2 statistic of 25%–50% are considered to have low heterogeneity, those with an I^2 statistic of 50%–75% have moderate heterogeneity, and those with an I^2 statistic of > 75% have a high degree of heterogeneity (Higgins et al., 2003). An I^2 value > 50% indicates significant heterogeneity (Armitage et al., 2008).

Because animals' characteristics, study design, and other confounding factors were not consistent between studies, we further conducted sensitivity analyses to explore possible explanations for heterogeneity and to examine the influence of various exclusion criterions on the overall pooled estimate. In this meta-analysis, we identified nine moderator variables and we examined the effects of each moderator variable with the mixed-effects model.

The presence of publication bias was assessed by using the Egger's test (Egger et al., 1997). The test statistic was based on a weighted linear regression of the treatment effect on its standard error. A p value < 0.05 was judged as statistically significant, except where otherwise specified.

3. Results and discussion

3.1. Study identification and selection

An initial database search using the prespecified search strategy identified a total of three hundred forty-eight animal studies. Forty-two of them met our prespecified inclusion criteria. Twelve of them were excluded because of duplicate studies and therefore thirty potentially relevant articles were screened. From them seventeen were excluded based on the titles and abstracts and also two full-text articles (Valdez et al., 2005; Halper et al., 2003) were excluded because they did not provide the wound area. A total of eleven full-text articles were included in qualitative synthesis and were reviewed for more detailed evaluation. Five of them were excluded because of duplicate data (Nasrabadi et al., 2011; Nasrabadi and Ebrahimi, 2011; Zahedi et al., 2011b; Huseini et al., 2012; Rahimzadeh et al., 2015). Finally, six studies were included in quantitative synthesis in the present meta-analysis (Rodrigues et al., 2005; Zahedi et al., 2011a; Jones et al., 2012b; Poutahidis et al., 2013; Rahimzadeh et al., 2014; Partlow et al., 2016). The flowchart of study selection is shown in Fig. 1.

3.2. Characteristics of the studies

The main characteristics of the six animal studies included in the meta-analysis and the outcome data of each included trial are summarized in Table 1. In total, bacteria probiotics, yeast probiotics and sterile probiotic extracts were tested. Of the six studies included in the meta-analysis, four used topical applications of probiotic microorganisms (70% kefir gel, *L. brevis*, *L. plantarum*, *L. fermentum*, *S. boulardii*), one used topical applications of sterile kefir extracts and one used oral administration of probiotics (*L. reuteri*). The sterile kefir extract contained the filtered (through a 0.22-micronmillipore filter) supernatants of kefir culture fermentation (48 h and 96 h fermentation) and named as kefir 48 h and kefir 96 h. The antimicrobial and wound healing activity of kefir extracts were positively associated with lactic and acetic acids that bacteria produced (Rahimzadeh et al., 2014). The sterile kefir extracts although they did not contain microorganisms, they contained all the active substances that kefir microorganisms produced and were extracted from the kefir cultures. Therefore, the wound healing properties of kefir extracts are based on the wound healing properties of kefir. For these reasons, although extracts sterility, we consider kefir extract wound therapy as probiotic treatment.

Only one study (Partlow et al., 2016) used pig models to test the wound healing properties of *S. boulardii*, in contrast with the other five studies that used rodent and rabbit models. It is important to mention that pig model is a confounding variable for the estimated overall efficacy of probiotic treatment, because in pig models re-epithelialization dominates over contraction and wound healing is less rapidly than in rodent and rabbit models (Ansell et al., 2012).

The quality assessment of each included study is reported in Table 2. These studies were published between 2005 and 2016. As stated in the Table 2, no study described allocation concealment, blinded assessment of outcome or disclosed a potential conflict of interest. All studies were published in peer-reviewed journals and described a sample size calculation. Except one (Jones et al., 2012b), all studies reported compliance with animal welfare regulations. Statements describing control of temperature were described in three studies (Rodrigues et al., 2005; Zahedi et al., 2011a; Rahimzadeh et al., 2014). Only one study described randomization to treatment group (Partlow et al., 2016). The median quality score was 4 (range 2 to 4).

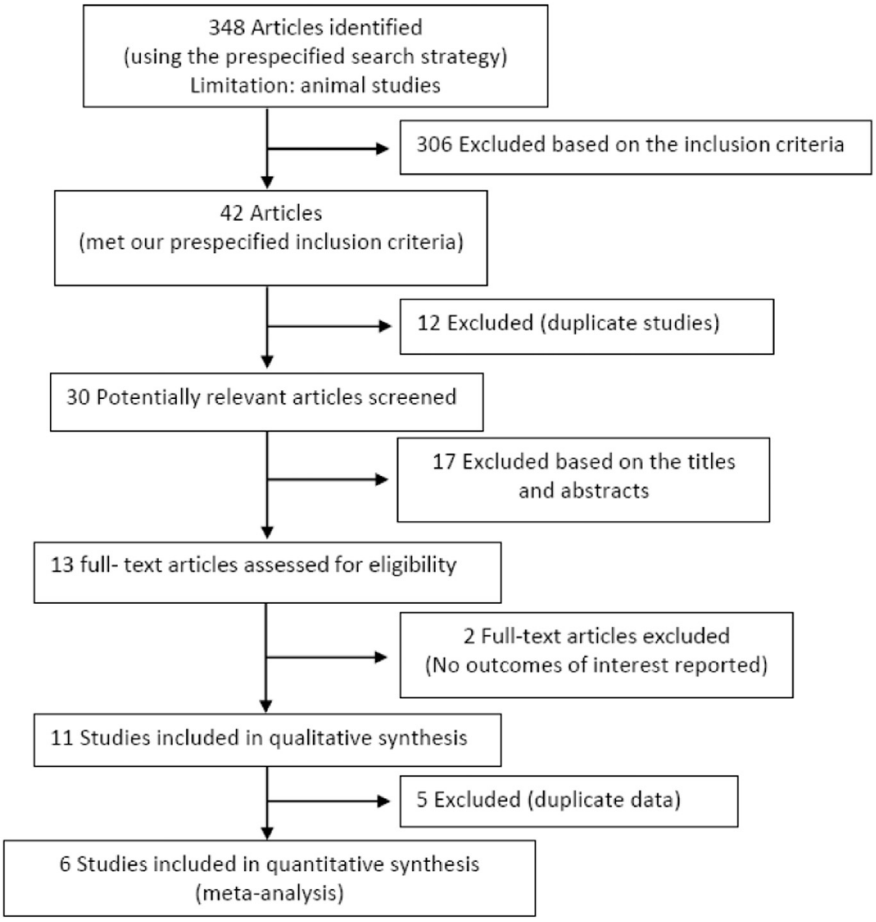


Fig. 1. Flowchart of studies included in meta-analysis.

3.3. Outcomes of the statistical analysis

The test of heterogeneity was statistically significant (p-value < 0.0001), and the quantity of heterogeneity estimated as high degree ($\tau^2 = 2.3138$, $I^2 = 77.6\%$), thus the Random-Effects model was used. The results show that the overall effect of probiotics is $g = -2.55$ (95% CI = -3.59, -1.50) with p-value < 0.0001, indicating that the average wound area% (a week after wounding) for the treated group is on average 2.55 standard deviations lower than that of the control group. Therefore, based on the k = 12 experiments from the 6 studies we included in the meta-analysis, we conclude that the administration of probiotics leads to reduction of wound area and acceleration of wound healing process. The null hypothesis (H_0: Effect of probiotics is no different from that of control) can be clearly rejected (p-value < 0.0001). A graphical overview of the results so far can be obtained by creating a forest plot (Lewis and Clarke, 2001). Fig. 2 shows the forest plot which evaluates the effect of probiotics on the %wound area extent at the end of the first week after wounding.

Subsequently, we performed sensitivity analysis to explore potential source of heterogeneity. Exclusion of 1 study (Partlow et al., 2016) that tested yeast probiotic (*S. boulardii*), yielded moderate increase in heterogeneity ($I^2 = 79.7\%$, $\tau^2 = 3.065$, $p < 0.0001$) and also in the absolute value of g ($g = -3$; 95% CI = -4.31, -1.70). We report these results in Fig. 3.

Specifically, the estimated amount of heterogeneity (among-study variance, τ^2) increased by (3.065–2.314)/ 2.314 = 0.325 = 32.5% and the absolute value of mean g increased by (3–2.55)/ 2.55 = 0.176 = 17.6%. The estimated heterogeneity is in both cases (of inclusion and exclusion) statistically significant (p-value < 0.0001). However, in the case of exclusion of this study (Partlow et al., 2016) the overall effect of probiotic therapy in wound healing decreased by 17.6%, indicating that yeast probiotic *S. boulardii*, is less effective than the others probiotic therapies that we tested in this meta–analysis.

Exclusion of 1 study (Rahimzadeh et al., 2014) that tested sterile kefir extracts instead of kefir live organisms, yielded decrease in heterogeneity ($I^2 = 69.4\%$, $\tau^2 = 1.377$, p-value = 0.0006) and also in the absolute value of g ($g = -2$; 95% CI = -2.9269, -1.0650). We report these results in Fig. 3.

The estimated amount of heterogeneity decreased by (2.314–1.377)/ 2.314 = 0.405 = 40.5% and the absolute value of mean g decreased by (2.55–2) / 2.55 = 0.216 = 21.6%. These results are suggesting that 40.5% of the total amount of heterogeneity can be accounted for by excluding study six from the model. It is important that the exclusion of this study (Rahimzadeh et al., 2014) in meta-analysis decrease the overall effect of probiotics in wound healing by 21.6% indicating that sterile kefir extracts are more effective as probiotic therapy of wounds than the others probiotic therapies that we tested in this meta- analysis.

Exclusion of 1 study with low quality score (Jones et al., 2012b), yielded increase in the absolute value of g ($g = -3.31$; 95% CI = -4.62, -2.01) but heterogeneity remained at the same levels as before ($I^2 = 77.1\%$, $\tau^2 = 2.326$, p-value < 0.0001). The absolute value of mean g increased by (3.31–2.55) / 2.55 = 0.298 = 29.8%. We report these results in Fig. 3.

Exclusion of both 2 studies (Partlow et al., 2016 and Rahimzadeh et al., 2014) that do not evaluate bacteria probiotic treatments but sterile yeast and kefir extracts, yielded moderate decrease in heterogeneity ($I^2 = 73.4\%$, $\tau^2 = 1.975$, p-value = 0.0004) and also in the absolute value of g ($g = -2.34$; 95% CI = -3.56, -1.13). We report

Table 1
Main characteristics and the outcome data of animal studies included in the meta-analysis.

First author, (Year)	Animal characteristics	Treatment tested		Adm. route	No. of wounds (Probiotics/control)	Coexisting factor in wound healing		Treatment day	$\frac{WA\%_{Treated}(\pm SD)}{WA\%_{Control}(\pm SD)}$	Frequency of adm. (No. of Adm. /treatment days)	Initial wound area (WA_0)	Microbial dose /wound	Full-thickness wound
		Probiotic	+ gNO			*S. aureus (infection)*	Ischemia						
Rodrigues et al. (2005)	Male Wistar rats weighting 150–200 g	70% kefir gel.	−	Topical	10 (5/5)	+	−	7th	16.34 (± 5,74) / 113.74 (± 28)	6/7 = 0.86	28.27 mm^2	Not given	+
Zahedi et al. (2011a)	Male Wistar rats weighting 250–280 g	Exp. 1: *L. brevis*	−	Topical	10 (5/5)	−	−	7th	25 (± 6,71) / 57 (± 4,47)	6/7 = 0.86	225 mm^2	$5.5 * 10^{10}$ bacteria	+
		Exp. 2: *L. plantarum*	−	Topical	10 (5/5)	−	−	7th	20 (± 11,2) / 57 (± 4,47)	6/7 = 0.86	225 $m\text{-}m^2$		
Jones et al. (2012b)	Male New Zealand white rabbits 1.5–2.5 kg	Exp. 1: *L. fermentum*	+	Topical	8 (4/4)	−	−	7th	58.67 (± 31.33) / 66.67 (± 12)	6/7 = 0.86	30 mm^2	$2.5 * 10^{10}$ bacteria	+
		Exp. 2: *L. fermentum*	+	Topical	8 (4/4)	+	−	7th	50 (± 16.18) / 75 (± 18.13)	6/7 = 0.86	30 mm^2		
		Exp. 3: *L. fermentum*	+	Topical	8 (4/4)	−	+	7th	25 (± 16.67) / 90 (± 9.33)	6/7 = 0.86	30 mm^2		
		Exp. 4: *L. fermentum*	+	Topical	8 (4/4)	+	+	7th	88.33 (± 20) / 91.67 (± 8.33)	6/7 = 0.86	30 mm^2		
Poutahidis et al. (2013)	C57BL/6 wt mice	*L. reuteri*	−	*Per os*	48 (24/24)	−	−	6th	5.76 (± 1,28) / 25.88 (± 10,39)	6/6 = 1	3.14 mm^2	$3.5 * 10^5$ bacteria	+
Rahimzadeh et al. (2014)	Male Wistar rats, aged 6 months old weighting 200 (± 10) g	Sterile kefir 48 h extract	−	Topical	16 (8/8)	−	−	7th	81.8 (± 4.6) / 96.5 (± 3.5)	6/7 = 0.86	300 mm^2	No	+
		Sterile kefir 96 h extract	−	Topical	16 (8/8)	−	−	7th	54,6% (± 2,8) / 96.5 (± 3.5)	6/7 = 0.86	300 mm^2	No	
Partlow et al. (2016)	Six pigs (three intact females, three castrated males) aged 9 weeks and of mean weight 19.6 kg (± 1.67 kg)	*S. boulardii*	−	Topical	12 (6/6)	−	−	6th	107,14 (± 3,57) / 112,67 (± 7,3)	1/6 = 0.16	1500 mm^2	$5 * 10^9$ yeasts	+
		S. boulardii	−	Topical	12 (6/6)	−	−	6th	103,47 (± 3,2) / 112,67 (± 7,3)	2/6 = 0.33	1500 $m\text{-}m^2$		

Exp., experiment; Adm., administration.

Table 2
Quality assessment of each study.

Authors	Year	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	Score
Rodrigues et al.	2005	+	+	Nk	−	−	+	+	−	4
Zahedi et al.	2011	+	+	Nk	−	−	+	+	−	4
Jones et al.	2012	+	−	−	−	−	+	Nk	−	2
Poutahidis et al.	2013	+	−	−	−	−	+	+	−	3
Rahimzadeh et al.	2014	+	+	Nk	−	−	+	+	−	4
Partlow et al.	2016	+	−	+	−	−	+	+	−	4

Studies fulfilling the criteria of: (1) publication in a peer-reviewed journal, (2) statements describing control of temperature, (3) randomization to treatment group, (4) allocation concealment, (5) blinded assessment of outcome, (6) sample size calculation, (7) compliance with animal welfare regulations, and (8) whether the authors declared any potential conflict of interest.
Ref indicates references; Nk, not known.

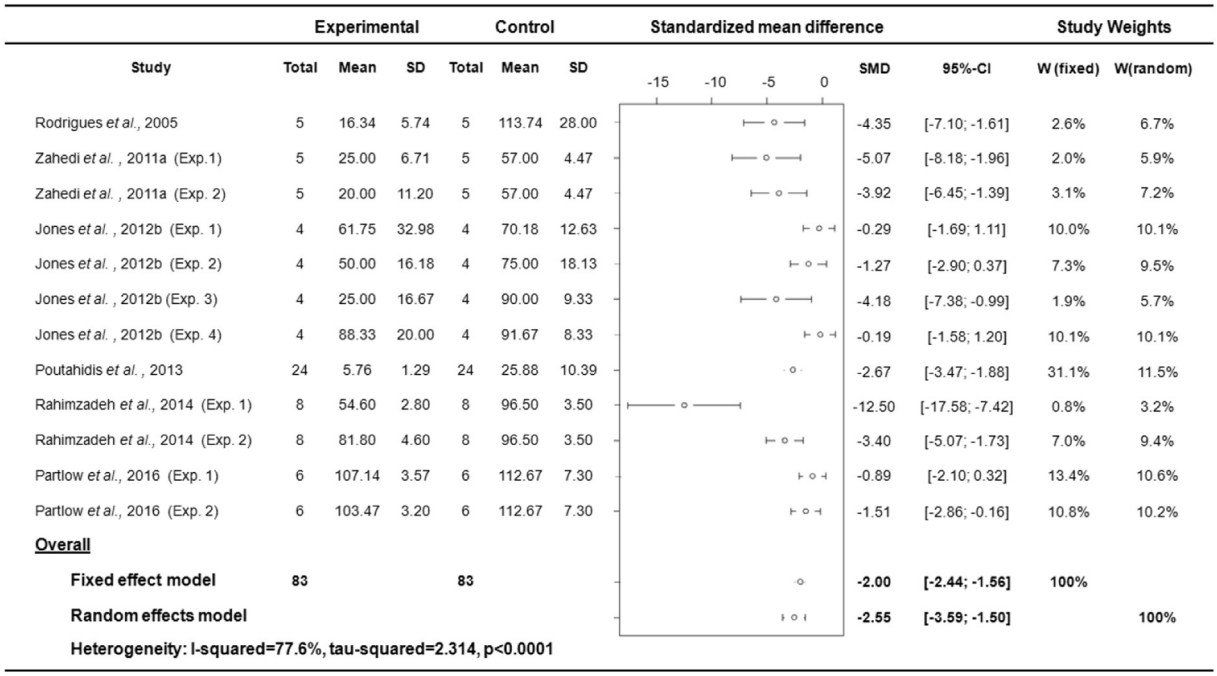
these results in Fig. 3. The estimated amount of among-study variance decreased by (2.314–1.975)/ 2.314 = 0.172 = 17.2% and the absolute value of mean g decreased by (2.55–2.34) / 2.55 = 0.082 = 8.2%. This overall decrease of 8.2% in estimating g can be interpreted as the aggregative result of the increase of g, when we exclude only study six (Partlow et al., 2016), and the decrease of g when we exclude only study five (Rahimzadeh et al., 2014).

Exclusion of 1 study (Poutahidis et al., 2013) that tested oral route administration of probiotics treatment and not topical application as the others five studies, yielded I^2 = 77.8%, τ^2 = 2.996, *p*-value < 0.0001 and absolute value of mean g = 2.63 (− 3.85, − 1.40). We report these results in Fig. 3. The results until now from the sensitivity analyses are described in Table 3.

From the results reported in Table 3, we conclude that the study of Rahimzadeh (Rahimzadeh et al., 2014) that tests sterile kefir extracts, has a significant effect on the estimated summary effect size of probiotic therapy, as with exclusion of this study the estimated g decreased by 21.6%. In addition, from the same table, we can notice that the study Partlow (Partlow et al., 2016) has also a significant effect on the estimated summary effect size but in contrast with Rahimzadeh (Rahimzadeh et al., 2014), exclusion of this study increased the overall estimated g by 17.6%. In both cases of exclusion, heterogeneity changed to a great extent but still remained statistically significant based on the Q test *p*-values. These results indicate that sterile kefir extracts are more effective as pharmacological treatment of wounds than the yeast (*S. boulardii*) probiotic treatment. However, because of the significant heterogeneity existence, we then tested the effectiveness of all different probiotic treatments that are tested in the six studies we included in the meta-analysis, by conducting heterogeneity analysis using mixed- effects models. We also used mixed- effects models to evaluate and others possible moderators of heterogeneity.

At least part of the heterogeneity may be due to influence of moderators. The effectiveness of the probiotics treatment may depend on the probiotic group, gNO, administration route, *S. aureus* (infection), ischemia, treatment day, frequency of administration, Initial Wound Area (WA_0) and microbial dose /wound. We examined these hypotheses by fitting mixed- effects model including these moderators. We analyzed the effects of each moderator variable in a multiple moderator model.

First we examined probiotic group as moderator using a mixed-effects model. Seven probiotic therapies were tested in this meta-analysis: 70% kefir gel, kefir extract, *L. brevis*, *L. fermentum*, *L. plantarum*, *L. reuteri* and *S. boulardii*. We examined as moderators all these seven probiotic treatments. Only 70% kefir gel appears to have a significant influence on the overall effectiveness (*p*-value = 0.0262). Especially, wounds treated with 70% kefir gel have on average 4.3528 standard deviations lower wound area%, compared to the control (g = − 4.3528), by the end of the first week after wounding. The others probiotic therapies don't have statistical significant influence on the overall effectiveness based on the *p*-values. However, if they were significant, kefir extract and *L. brevis* would influenced the overall effectiveness > 70% kefir gel based on the estimated g, with kefir extract appeared to be the most effective (g = − 5.5138). The test for residual heterogeneity is statistically significant (QE = 17.4006, *p*-value = 0.0038), possibly indicating that other moderators are influencing the treatment effectiveness. Although the test for residual heterogeneity is still statistically significant from this meta-regression, the estimated between-study variance dropped to τ^2 = 1.8724 (SE = 1.8159) from the 2.314 which indicates that (2.314–1.8724) = 0.19 = 19% of the total amount of heterogeneity is accounted for by probiotic moderator.



Exp., Experiment; SD, Standard Deviation; SMD, Standardized Mean Difference

Fig. 2. Forest plot evaluating the effect of probiotics on the %wound area extent at the end of the first week after wounding.

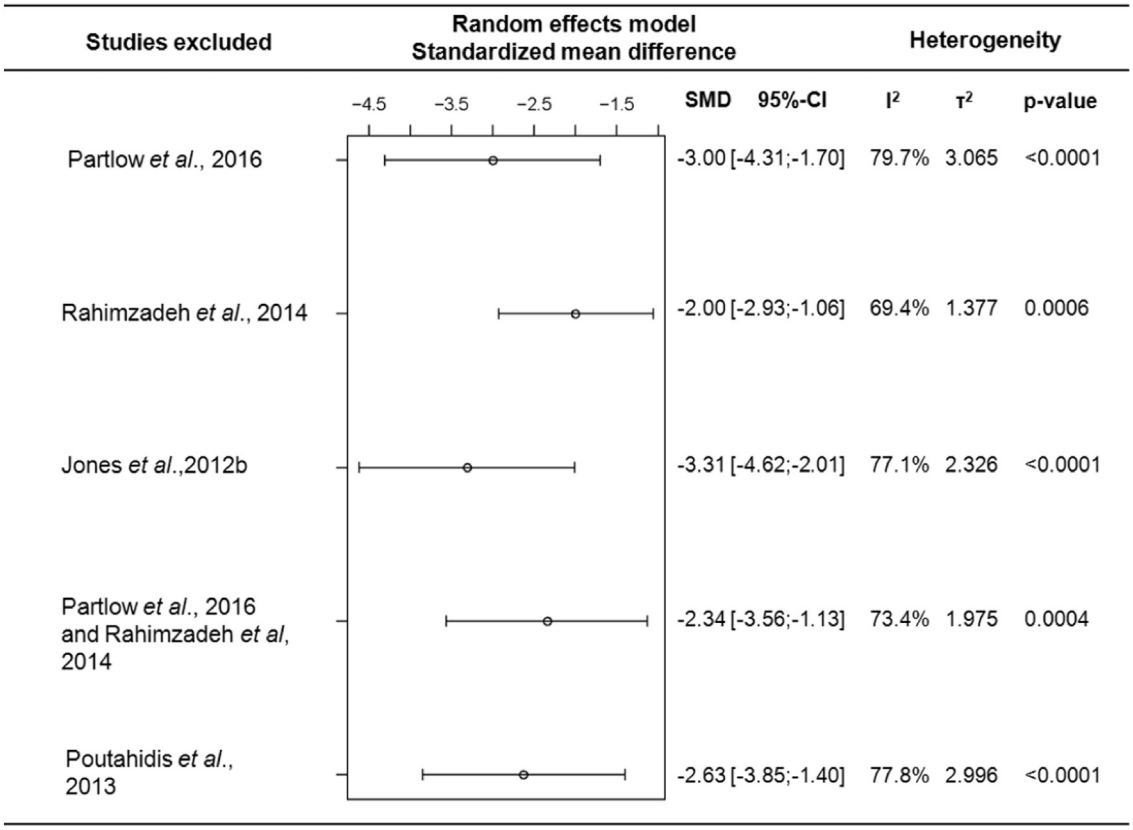


Fig. 3. Sensitivity analysis results.

For further examination of probiotic therapy as moderator, we categorized the seven probiotic treatments into three subgroups: bacteria, extracts and yeasts. Then we examined as moderators these three subgroups of pharmacological therapies. Moderator bacteria appears to have the most statistically significant influence on the overall effectiveness of the treatment ($g = -2.3814$, p-value = 0.0003) with moderator extract following ($g = -5.6983$, p-value = 0.0442). For moderator yeast, the estimated results were not statistically significant ($g = -1.1925$, p-value = 0.3768). Based on the previous results, wounds treated with bacteria have on average 2.3814 standard deviations lower wound area%, compared to the control by the end of first week after wounding. For those treated with probiotic extracts, the wound area was on average 5.6983 standard deviations lower wound area%, compared to the control. Based on estimated SMDs, extracts are more effective treatment compared to bacteria. More specifically probiotic extract treatment was by 5.6983/2.3814 = 2.39 times more effective than probiotic bacteria. Again, the test for residual heterogeneity is statistically significant (QE = 37.8251, p-value < 0.0001), indicating that other moderators model are influencing the overall estimated effectiveness.

Table 3
Sensitivity analyses results.

Studies excluded	Exclusion etiology	τ^2	|Mean g|
Partlow et al. (2016)	Yeast probiotic	+ 32.5%	+ 17.6%
Rahimzadeh et al. (2014)	Sterile kefir extracts (instead of kefir live organisms)	− 40.5%	− 21.6%
Jones et al. (2012b)	Low quality score	+ 0.5%	+ 29.8%
Partlow et al. (2016) and Rahimzadeh et al. (2014)	Not live bacteria probiotics treatments	− 17.2%	− 8.2%
Poutahidis et al. (2013)	Oral route administration (instead of topical application)	+ 29.4%	+ 3.1%

Subsequently, we examined gNO as moderator using a mixed-effects model. Based on the omnibus test (QM = 4.1880, df = 1, p-value = 0.0407) moderator gNO has a statistically significant influence on the effectiveness of the probiotic treatment. The wound area of the wounds treated with probiotics plus gNO was decreased by 1.116 standards deviations compared to the control ($g = -1.116$, p-value = 0.0407) whereas the wound area of the wounds treated with probiotics without presence of gNO was decreased by 3.2302 standards deviations compared to the control ($g = -3.2302$, p-value < 0.0001). The results suggest that the absence of gNO leads to faster wound healing process than in the case of gNO presence. This conflicts with the expected result that NO gas (gNO) is beneficial in promoting wound healing and preparing the wound bed for treatment and recovery (Stenzler and Miller, 2006). This result may be due to the influence of coexisting infection and ischemia in some of the wounds in which gNO was used. So, to test this hypothesis we used mixed effects model to test together gNO, infection, and ischemia as moderators.

Based on the omnibus test (QM = 3.8313, df = 3, p-value = 0.2803) the correlation between gNO, infection, ischemia and the overall effectiveness of treatment cannot be interpreted (p-value > 0.05). So, the correlation of effect size with gNO it can't be inferred with certainty. However, on absence of these three moderators (gNO, infection and ischemia) wounds improved by 3.3663 standards deviations compared to the control ($g = -3.3663$ p-value < 0.0001). The test for residual heterogeneity is significant (QE = 36.2952, df = 8, p-value < 0.0001), possibly indicating that other moderators not considered in the model are influencing the treatment effectiveness.

We then, examined infection, ischemia, administration route, treatment day, administration frequency, Initial Wound Area (WA_0) and bacteria dose separately as moderators using seven mixed- effects models, one for each moderator.

Based on the results, the non-infected wounds treated with probiotics improved by 2.8918 standard deviations compared to control

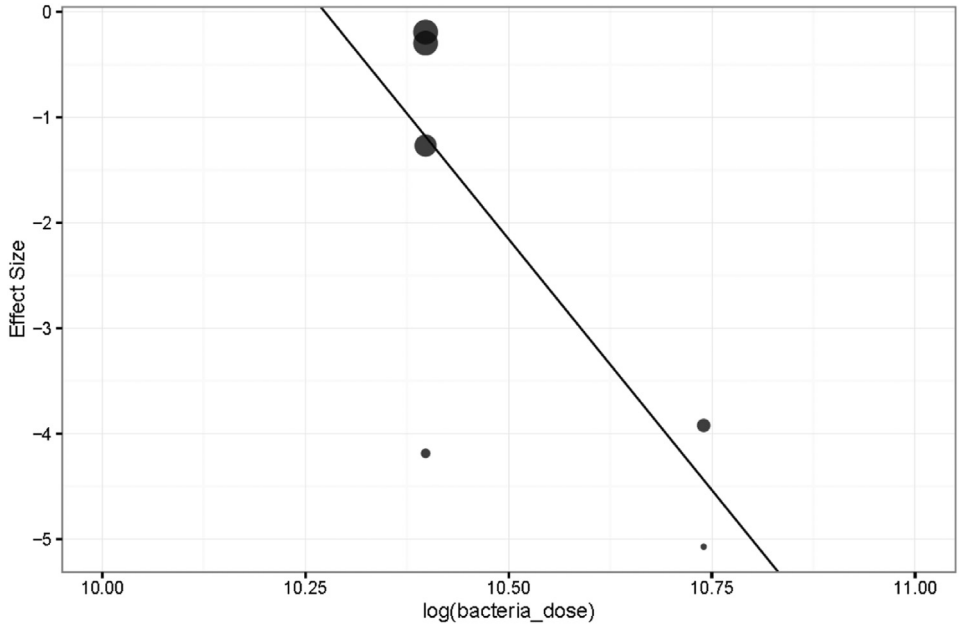


Fig. 4. Scatterplot of the overall effect size as a function of bacteria dose.

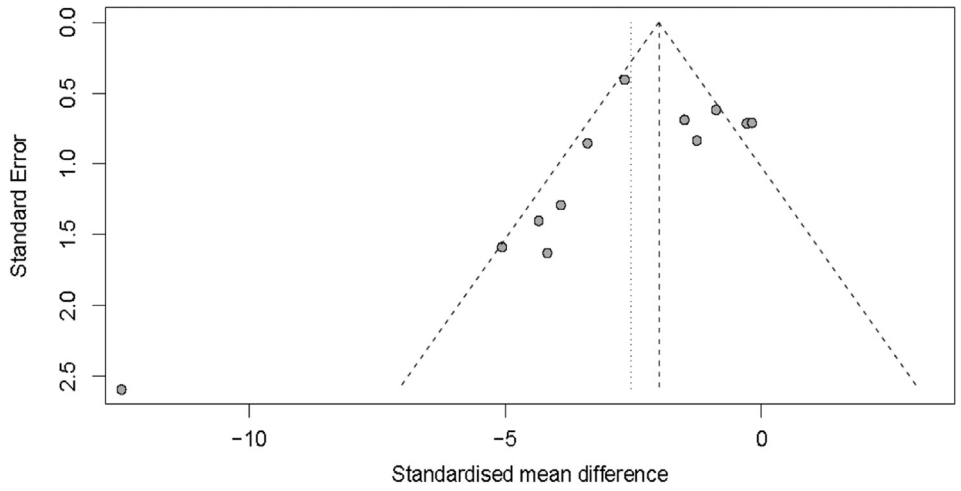


Fig. 5. Funnel plot.

($g = -2.8918$, p-value < 0.0001). The result for infected wounds is not statistically significant ($g = -1.6442$, p-value $= 0.3131$). For ischemia as moderator, the results indicate that the non-ischemic wounds treated with probiotics improved by 2.7361 standard deviations compared to control ($g = -2.7361$, p-value < 0.0001). The results for ischemic wounds were not statistically significant ($g = -1.6514$, p-value $= 0.4660$). For administration route, treatment day, administration frequency and Initial Wound Area (WA_0) the estimated results of SMDs are not statistically significant and thus the coefficient between these moderators and probiotic effectiveness cannot be interpreted. The test for residual heterogeneity in all these mixed effects models is significant (p-value < 0.0001) possibly indicating that other moderators except these six are influencing the treatment effectiveness.

To examine the bacteria dose as moderator, we excluded the studies that did not test live bacteria probiotics. Based on the omnibus test (QM = 7.7819, df = 1, p-value $= 0.0053$) moderator bacteria dose has a statistically significant influence on the effectiveness of the probiotic treatment. For the bacterial dose moderator we used the log(bacteria/wound). More specifically, the mixed effects model estimated that a one unit increase in bacterial dose corresponds to a change of -10.2056 units in term of the average SMD ($g = -10.2056$, p-value $= 0.0053$). Fig. 4 shows the Scatterplot of the overall effect size as a function of bacteria dose. Hedge's g effect size is on the y-axis and decimal logarithm of bacteria dose per wound is on the x-axis. Each point represents a study and the size of the point represents the study weight.

3.4. Publication bias

Publication bias refers to the possibility that experiments showing a statistically significant effect are more likely to be published than experiments with null results which could bias the summary effect. We used the funnel plot to examine for publication bias. The funnel plot of the present meta-analysis is shown in the Fig. 5. Hedge's g effect size (standardized mean difference) is on the x-axis (horizontal axis) and the measure of experiment precision (standard error of ES) is on the y-axis (vertical axis). The outer dashed lines denote a triangular region in which 95% of the studies are expected to lie if selection bias and/or heterogeneity across studies are largely absent.

The vertical dashed line on the right is the estimated summary effect-size based on the funnel plot symmetry. From this figure, we noted that Rahimzadeh Experiment No. 1 (Rahimzadeh_Exp.1) is not following the funnel plot symmetry and has the smallest standardized mean difference of −12.5 on the left. If we take into account the Rahimzadeh_Exp.1 the estimated summary effect-size is represented by the vertical dashed line on the left. Except of Rahimzadeh_Exp.1, the remaining are quite symmetric.

Based on the Linear regression test of funnel plot asymmetry, asymmetry in the funnel plot is not statistically significant (p-value = 0.084 > 0.05) indicating symmetry of the funnel plot and absence of publication bias.

4. Conclusions

To the best of our knowledge, this is the first meta-analysis evaluating the efficacy of probiotics as pharmacological treatment of cutaneous wounds in animal models. In addition, this study highlights the necessity of the probiotic treatment inclusion in wound management.

In our analysis, the focus of attention was at the end of the first week after initial wounding because of the important cellular events of wound healing that are occur within this one week period. The results show that the administration of probiotics is an effective pharmacological treatment of cutaneous wounds, as probiotic therapy accelerates wound healing process. Further analysis showed that sterile kefir extracts have the greater effectiveness as pharmacological treatment of cutaneous wounds compared to bacteria (70% kefir gel, *L. brevis*, *L. fermentum*, *L. plantarum, L. reuteri*) and yeast (*S. boulardii*) treatments. More specifically, bacteria probiotic therapies found to follow in the effectiveness the sterile kefir extracts. Although extracts sterility, we consider the kefir extract wound therapy as probiotic treatment, because kefir extracts contained all the active substances that kefir microorganisms produced and were extracted from the kefir cultures. The results for the yeast probiotic treatment are not statistically significant. However, the estimated effect sizes combined with the sensitivity analysis indicate that yeast (*S. boulardii*) probiotic is less effective treatment than the others.

Wound healing can be affected by many moderator factors and so does the estimated efficacy of probiotic treatments. Therefore, apart from the probiotic group moderator, that we just mentioned, we also tested the effect of other eight moderator variables (gas NO, administration route, infection, ischemia, treatment day, frequency of administration, initial wound area and bacteria dose per wound) on the overall estimated effect size. Based on the results, it can be concluded that bacteria dose per wound has the most statistical significant influence in the overall estimated effect size. Specifically, increase in bacterial dose corresponds to increase of the estimated overall effect size. This correlation can be interpreted by the fact that the more bacteria we administrate, the more active substances for wound healing, such as exopolysaccharides and lactic acid, they produce. However, the main limitation is that we cannot correlate the concentration of active substances that microorganisms produce with the estimated effectiveness of probiotic therapy, based on the outcome data of the animal studies included in the Meta-Analysis. Therefore, further research is required to determine exactly how probiotic treatment affects the wound healing process.

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