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Experimental Research Article

In vivo study of antioxidant and anti-inflammatory activity of metabiotic extracted from *Lactobacillus acidophilus BF-P-064* in Ethanol-induced peptic ulcer

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ABSTRACT

Introduction: Metabiotics are promising candidates for peptic ulcer (PU) healing by regulating the expression of pro-inflammatory genes and antioxidant capacity.

Methods: We prepared a metabiotic by culturing *Lactobacillus acidophilus* BF-P-064 and evaluated its cytotoxicity and antioxidant properties by MTT and DPPH (α-diphenyl-β-picrylhydrazyl) methods. To investigate the potential of PU healing, we divided 45 rats into 3 groups and induced PU by administering ethanol. The negative-control group was not treated, while the positive-control and metabiotic groups received treatment with a drug and a metabiotic. On different days, the wound area was determined and the histopathological characteristics were evaluated by H&E staining. The expression of pro-inflammatory cytokines (*IL-6, PDGF, VEGF, EGF, TGF-β,* and *TNF-α*) and antioxidant (*SOD* and *GPx*) genes was assayed by qRT-PCR method.

Results: Our results showed that the metabiotic, at a concentration of 60 mg/mL, had no cytotoxic effects on the CaCo2 cell line, and its antioxidant capacity was 72.49 u/mL. The metabiotic caused faster wound contraction by accelerating the infiltration of phagocytic cells, which intensified the inflammatory phase. By increasing the expression of pro-inflammatory cytokines, as well as enhancing antioxidant properties and the expression of SOD and GPx, the metabiotic prevented the induction of oxidative stress in the tissue. So, the proliferation phase started faster, and the healing process improved.

Conclusion: The metabiotic extracted from *L. acidophilus* BF-P-064 can improve the healing process of PU by increasing the antioxidant capacity, improving the pattern of cell infiltration, and the expression of pro-inflammatory cytokines.

Keywords:
Metabiotic
Lactobacillus acidophilus
Peptic ulcer
Antioxidant
Pro-inflammatory cytokines

Introduction

Peptic ulcers (PU), characterized by open sores or lesions that develop in the lining of the stomach, duodenum, or esophagus, remain a significant clinical

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challenge worldwide. Despite advancements in medical treatments, the management of PU and the promotion of effective healing processes continue to be areas of intense research interest (Bereda 2022).

The process of PU healing involves a complex interplay of cellular and molecular factors, among which interleukins (ILs) and reactive oxygen species (ROS) play pivotal roles. Normal cellular metabolism generates ROS, including molecules like superoxide anion (O2-), hydrogen peroxide (H2O2), and hydroxyl radical (OH•), as byproducts (Bousounis, Bergo,Trompouki 2021).

The production of these compounds can increase significantly during times of stress or injury. ROS act as signaling molecules that regulate the healing process. High levels of ROS can impair cellular functions necessary for proper wound healing, and result in prolonged inflammation and the formation of non-healing chronic wounds (Cano Sanchez et al., 2018). The balance between ROS production and antioxidant defenses is critical in determining the outcome of ulcer healing. Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) act as scavengers of ROS, protecting cells from oxidative damage and facilitating the resolution of ulcers (Kamran 2020).

ILs are also key mediators of mucosal inflammation and ulcer repair. They modulate ROS production through various mechanisms. For example, IL-6 and IL-1β can stimulate the expression of NADPH oxidase, a major enzymatic source of ROS in immune cells. Studies have shown that IL-6 stimulates epithelial cell proliferation and migration, which facilitates mucosal restitution (Bryan et al., 2012).

Among the other ILs, Platelet-Derived Growth Factor (PDGF), Epidermal Growth Factor (EGF), Transforming Growth Factor-beta (TGF-β), and Vascular Endothelial Growth Factor (VEGF) play crucial roles in orchestrating different phases of wound repair. Central to the mechanisms underlying their actions is their interaction with ROS, which serves as a key mediator in the wound-healing cascade (Chegini, Rossi, Masterson 1992).

PDGF is a potent mitogen and chemoattractant for various cell types and stimulates cell proliferation, migration, and ECM (Extra cellular matrix) deposition. PDGF also enhances the production of ROS, primarily through the activation of NADPH oxidase in neutrophils and macrophages. EGF is a critical regulator of epithelial cell proliferation and migration, essential processes in re-epithelialization during wound healing. EGF stimulates the expression of antioxidant enzymes such as SOD and CAT, protecting cells from oxidative damage. TGF-β is a multifunctional cytokine involved in various aspects of wound healing, including inflammation, ECM deposition, and tissue remodeling. TGF-β stimulates the production of ROS by activating NADPH oxidase. ROS modulates TGF-β signaling and this reciprocal interaction between TGF-β and ROS influences fibroblast activation, collagen synthesis, and myofibroblast differentiation. VEGF plays a central role in angiogenesis, the formation of new blood vessels essential for providing oxygen and nutrients to the wound site. ROS, which acts as signaling molecules in endothelial cell proliferation and migration, regulates tightly VEGF-induced angiogenesis (Barrientos et al., 2008; Grazul-Bilska et al., 2003; Vaidyanathan 2021).

There is a growing body of knowledge surrounding the healing of PU to pave the way for the development of more efficacious and targeted treatment modalities by administration of natural products such as probiotics and metabiotics. Studies have shown that probiotics, including strains of Lactobacillus and Bifidobacterium, regulate the production of ILs and exert immunomodulatory effects (Tabatabaee Bafroee and Khalili Hadad 2021; Tarapatzi et al., 2022). Metabiotics are non-viable microbial products or metabolic byproducts derived from probiotic microorganisms. Through fermentation or metabolic activity, probiotic bacteria generate these bioactive compounds (Shenderov et al., 2020).

Metabiotics exert various biological activities and health benefits, including immunomodulation, anti-inflammatory effects, antioxidant activity, and promotion of tissue repair. Overall, metabiotics represent a promising therapeutic approach for the management of PU, by harnessing the beneficial effects of probiotic bacteria without the concerns associated with live microorganisms. However, further research is needed to elucidate their mechanisms of action and validate their efficacy in clinical settings (Biswas and Mohapatra 2023; Sadeghi et al., 2023).

In this paper, we aim to explore the intricate interplay between metabiotic derived from *Lactobacillus acidophilus* BF-P-064, interleukins, and PU healing, elucidating the mechanisms by which metabiotic contribute

to the resolution of ulcers.

Materials and Methods

Bacterial culture and metabiotic preparation

Lactobacillus acidophilus BF-P-064 was kindly provided by Bioluence Company and serially diluted in phosphate-buffered saline (PBS) (*Sigma-Aldrich* Co). Diluted samples were cultured in de Man Rogosa Sharpe (MRS) agar (Merck) supplemented with 0.1 mg/L clindamycin (*Sigma*, UK) and 10 mg/L ciprofloxacin (*Sigma*-Aldrich, UK) (Süle et al., 2014). The plates were incubated at 37 °C for 48-72 h under an atmosphere with high levels of CO_2 (air supplied with 5% CO_2). After confirming the purity, a colony of bacteria was transferred to the MRS broth medium and incubated under the same conditions for 24 h. The cells were separated by centrifugation at 6000 rpm for 20 min (*Hettich* Roto, Silenta) and after suspension in the medium containing 13% skimmed milk and 25% glycerol, they were stored in a freezer at -80°C (Cody et al., 2008).

To prepare the metabiotic (cell-free supernatant), the bacterial stock was inoculated in the MRS broth medium, and after 48 h incubation, the metabiotic was separated by centrifugation. To ensure the absence of bacterial cells, the separated metabiotic was cultured on the plate after passing through a 0.2 µm filter (Biswas, Soren,Mohapatra 2024). Finally, the metabiotic powder was prepared by freeze-drying.

Invitro toxicity assay of the metabiotic

Cytotoxicity of the metabiotic was assayed using MTT (3-(4,5-dimethylthiazol-2,5-diphenyl-2 tetrazolium bromide) dye reduction assays. Caco-2 cell line was purchased from the Iranian Biological Resource Center (IBRC C10094). Caco-2 cells were seeded with 1×10^4 cells/well) into 96 well tissue plates and incubated (*Thermo Scientific)* at 37 °C in a humidified CO2 (95% air and 5% CO2) during the overnight. The cells were treated with different metabiotic concentrations (1500, 300, 60, 12, 2.4, 0.48, 0.096, 0.0192, and 0.00384 mg/mL) for 24 h. Then, the MTT was added into the wells and the reaction mixtures were incubated for 4 h. After removing the MTT dye, the formazan crystals were dissolved in dimethyl sulfoxide (*DMSO*, *Merck*, Germany). Eventually, the absorbance was measured at 570 nm using an ELISA Reader (Organon Teknika, Netherlands). The cell viability was calculated using the following equation (Arian et al., 2019; Chiu et al., 2013):

In vitro assay of antioxidant capacity

The antioxidant activity of metabiotic was measured by the 2, 2-Diphenyl-1-picrylhydrazyl (DPPH, *Merck*, Germany) method based on the Xing et al. protocol. Briefly, 1 ml of 0.2 mM solution of DPPH was mixed with an equal volume of metabiotic. The resulting mixture was incubated in the dark at room temperature for 30 min. Then the optical absorbance was determined at 517 nm. It should be noted that distilled water was used as a control sample. The scavenged DPPH was quantified using the following formula (Xing et al., 2015):

$$\text{DPPH radical scavenging activity } (\frac{\text{U}}{\text{mL}}) = \frac{\text{OD}_\text{c} - \text{OD}_\text{s}}{\text{S}} \times 100$$

ODc= Absorbance of the control
ODs= Absorbance of the sample
S= Volume (mL) of the sample

Animal storage and treatment

To investigate the effect of metabiotics on the healing of gastric ulcers, 45 male Wistar rats (4 weeks old, weighing 250–300 g) were purchased from Pasteur Institute (Tehran, Iran). The rats were housed individually in standard cages in a standard normal photoperiod (12 hours light and 12 hours dark cycle) (Dehkohneh, Jafari, Fahimi 2019). All animals had ad-libitum access to water and standard food. After 10 days of adaptation, PU was induced in rats using ethanol (98%) administration. For this purpose, the rats were starved for 24 hours while they had free access to water. Then, the rats were gavaged with the 1 ml ethanol. To ensure wound formation, this process was repeated for 2 consecutive days (Sistani Karampour et al., 2019). Finally, the rats were randomly divided into three groups as follows (n=15):

-The negative control group (Ctrl-): The rats with PU gavaged with 1 mL phosphate buffer solution (pH 7.2)

-The positive control group (Ctrl+): The rats with PU treated with 1 mL of drug constitute of sucralfate (2 mg), omeprazole (1 mg), and magnesium-aluminum (1 mg) (Hunt 1991; Konturek et al., 1989; Schepp and Classen 1989).

-The metabiotic group: The rats with PU which were treated with 1 mL of metabiotic (60 mg/mL)

The present study was carried out by the National Institutes of Health's Guide for the Care and Use of

Laboratory Animals. The protocol was authorized by Lorestan University of Medical Sciences' Committee on the Ethics of Animal Experiments (Permit Number: IR.LUMS.REC.1398.230).

Percentage of wound healing

The day of wound formation was considered as day zero, and after that 3 rats from each group were killed on days 1, 3, 7, 14, and 21 post wounding (PW). The stomachs of the rats were surgically opened along the wider curvature and washed with PBS. The wound area was measured using image analyzing software (Image J, NIH, USA), and the percentage of wound healing was calculated by the following formula (Jamaran et al., 2021):

$$\%\ \text{Healing} = \frac{A_1 - A_n}{A_1} \times 100$$

Where A_1 and A_n are the wound areas on days 1 and n, respectively.

Histopathological study

After determining the area, the wound was divided into two halves for histological and gene expression analysis. For histological analysis, the tissue samples were fixed in paraformaldehyde (4% in PBS, 0.01 M, pH 7.4). Paraffin blocks were prepared based on the Canene-Adams protocol, and the slides with 5 mm thick incisions were prepared and stained with hematoxylin and eosin (H&E) (Slaoui, Bauchet,Fiette 2017). Then, the number of macrophages, neutrophils, fibroblasts, and angiogenesis was evaluated by microscopic examination (*Nikon*, Tokyo, Japan).

Quantitative Real-time PCR

To investigate gene expression, 3 stomach samples from each group were frozen in liquid nitrogen on days 1, 3, 7, 14, and 21. Total RNA was extracted from the sample using a commercial RNA extraction kit (Cinnaclon RNX-Plus Solution) according to the manufacturer's instructions, and its quality and quantity were assessed by spectrophotometry. Reverse transcription was carried out to synthesize complementary DNA (cDNA) from the RNA template, using a WizScriptTM cDNA Synthesis kit according to the manufacturer's protocol. For each qRT-PCR reaction, a master mix was prepared containing 10-100 ng of cDNA, 300 nM of each primer, PCR buffer (1X concentration), dNTPs (200 µM each), Taq DNA polymerase, and $MgCl_2$ (2 mM), made up to a total volume of 20 µl with nuclease-free water. The qRT-PCR thermal cycling program included an initial denaturation step at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 15 seconds. The expression levels of the investigated genes under investigation were adjusted versus GAPDH, and the fold change was calculated using the $2^{-\Delta\Delta Ct}$ formula rather than the reference gene (Wong and Medrano 2005). The sequence of primers and size of products are depicted in Table 1.

Statistical analysis

The quantitative variables were analyzed using GraphPad Prism 8 software with one-way ANOVA and t-test at a significance level of P-value < 0.05.

Results

Metabiotic and cytotoxicity potential

Table 2 indicates the viability of the CaCo2 cell line after 24 h exposure with different concentrations of metabiotic. Our results showed that increasing the metabiotic concentrations up to 60 mg/mL does not cause a significant decrease in CaCo2 cell viability. At higher metabiotic concentrations, cell viability decreases significantly, which indicates metabiotic cytotoxicity at higher concentrations (Fig 1).

Antioxidant capacity of Metabiotic

The DPPH method is commonly used for evaluating antioxidant activity based on radical scavenging. It is a fast and simple way to determine antioxidant activity but this method does not consider bioavailability and membrane permeability in complex cell environments. The metabiotic of *L. acidophilus* BF-P-064 exhibited almost strong DPPH radical scavenging activities (72.49 ± 2.475 U/mL).

Assessment of ulcer size and percentage healing

Microscopic examination of wound healing showed that the use of drugs and metabiotics significantly improves the wound healing process. Interestingly, the percentage of wound closure in the group treated with metabiotic was better than the group receiving the drug until the third day (p value= 0.002). On the seventh day, the peptic ulcer by oral ethanol gavage was completely

TABLE 1: Primer sequences and product sizes for qPCR-amplified genes

Gene name	Primer (5'to 3')	Product size (bp)	References
pdgf-F	AGCCAAGACACCTCAAACTC	225	(Ritz et al., 2010)
pdgf-R	TAAATAACCCTGCCCACACTC		
egf-F	AGGCTGCACCCACGACAGAA	93	(Assar et al., 2021)
egf-R	CTTTGGTCTGCATTCACATC		
gpx-F	CAGTTCGGACATCAGGAGAAT	290	(Kapoor and Kakkar 2012)
gpx-R	AGAGCGGGTGAGCCTTCT		
sod-F	ACTGGTGGTCCATGAAAAAGC	387	(Zhang et al., 2015)
sod-R	AACGACTTCCAGCGTTTCCT		
tgf-β-F	CCTGGATACCAACTATTGCTTCAG	82	(Kianmehr, Rezaei,Boskabady 2016)
tgf-β-R	CAGACAGAAGTTGGCATGGTAG		
tnf-α-F	CCAGGAGAAAGTCAGCCTCCT	214	(An et al., 2020)
tnf-α-R	TCATACCAGGGCTTGAGCTCA		
il-6-F	CGAAAGTCAACTCCATCTGCC	150	(Wang et al., 2022)
il-6-R	GGCAACTGGCTGGAAGTCTCT		
β -actin-F	AGAGCTATGAGCTGCCTGACG	80	(Lu et al., 2016)
β -actin-R	AGAGCTATGAGCTGCCTGACG		

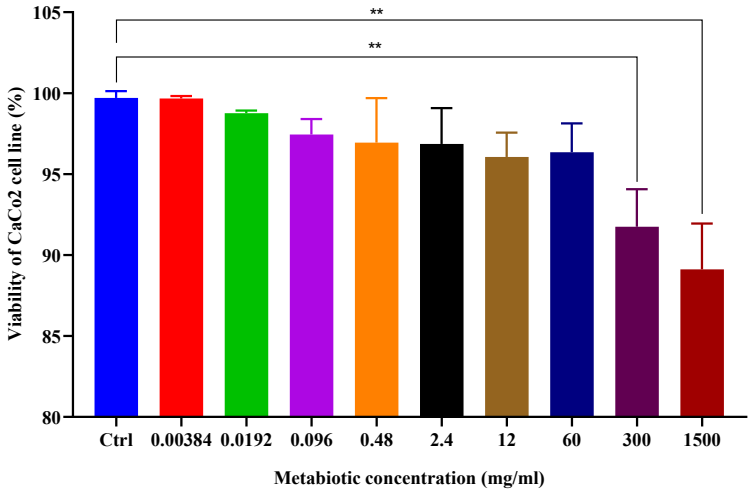

FIGURE 1. Viability of CaCo2 cells after 24 hours exposures to different concentration of metabiotic

closed in two treated groups, but in the Ctrl- group, the healing process continued until the 21st day (Table 2).

Histological analysis

To investigate the wound healing process, after sampling and preparation of tissue sections, the slides were stained with H&E stain and evaluated with a light microscope for the presence of polymorphonuclear cells (PMNs), mononuclear cells, and angiogenesis (Table 2 and Fig 2). The recruited neutrophils and macrophages initiate the inflammation phase in the healing process. Our results showed that in the first 3 days post-wounding (PW); The number of neutrophils and macrophages in the Ctrl+ and metabiotic group was significantly higher than in the Ctrl- group. In the following days, the number of these cells showed a significant decrease, which indicated the end of the inflammation phase and the beginning of the proliferation phase. In the negative

TABLE 2: Histological features of wound healing in rat with ethanol induced peptic ulcer. Mean ± SEM of wound healing percentage, neutrophils, macrophages, and fibroblasts

Day	1	3	7	14	21
% Wound Healing (Mean ±SEM)					
Ctrl-	0.120 ± 0.006^{c}	32.330 ± 1.453^{c}	57.330 ± 4.333^{b}	89.330 ± 2.333^{b}	99.670 ±0.333^{a}
Ctrl+	0.350 ± 0.058^{b}	60.140 ± 2.385^{b}	97.030 ± 0.353^{a}	99.870 ± 0.030^{a}	99.875 ± 0.133^{a}
Metabiotic	0.623 ± 0.015^{a}	93.830 ± 3.346^{a}	99.740 ± 0.257^{a}	99.980 ± 0.023^{a}	99.930 ± 0.073^{a}
Neutrophile number (Mean ±SEM)					
Ctrl-	36.800±0.800^{b}	11.000±0.316^{c}	27.000±1.870^{a}	21.000±0.894^{a}	13.200±1.157^{a}
Ctrl+	50.400±1.860^{a}	37.200±1.240^{a}	16.800±1.624^{b}	8.600±0.927^{b}	2.800±0.969^{b}
Metabiotic	45.200±0.489^{a}	29.800±1.356^{b}	13.600±2.227^{b}	8.800±0.583^{b}	3.600±0.927^{b}
Macrophages number (Mean ±SEM)					
Ctrl-	3.200±0.374^{b}	12.000±0.836^{c}	25.200±1.562^{a}	14.800±1.157^{a}	11.000±0.316^{a}
Ctrl+	10.800±0.374^{a}	29.200±1.019^{a}	13.000±1.140^{c}	10.600±0.509^{a}	2.800±0.583^{ab}
Metabiotic	9.400±0.244^{a}	24.000±1.673^{b}	20.000±1.788^{b}	11.200±0.860^{a}	7.000±0.447^{a}
Fibroblast number (Mean ±SEM)					
Ctrl-	0.130±0.007^{a}	3.200±0.583^{b}	11.000±0.836^{b}	23.200±1.280^{a}	23.800±1.462^{a}
Ctrl+	0.238±0.007^{a}	26.800±1.496^{a}	32.800±1.319^{a}	20.800±1.854^{a}	12.800±1.428^{b}
Metabiotic	0.254±0.005^{a}	26.000±1.483^{a}	32.800±1.113^{a}	24.200±2.653^{a}	12.000±1.378^{b}
Angiogenesis score (Mean ±SEM)					
Ctrl-	0.020±0.019^{a}	5.800±0.489^{a}	22.600±1.720^{b}	22.000±0.948^{b}	38.400±2.619^{a}
Ctrl+	0.020±0.020^{a}	9.600±0.748^{a}	50.000±3.065^{a}	58.200±3.215^{a}	12.200±1.655^{c}
Metabiotic	0.200±0.007^{a}	9.000±0.632^{a}	46.000±2.588^{a}	49.200±2.835^{a}	23.000±1.760^{b}

Different letters in each column indicate a significant difference between the groups.

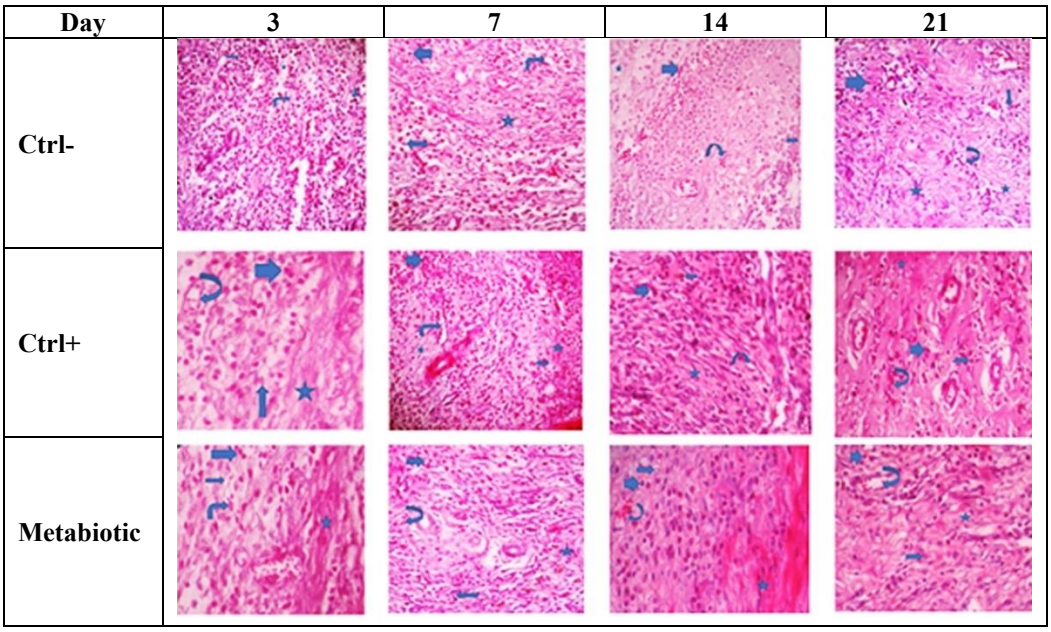

FIGURE 2. The microscopic view of a gastric ulcer healing site in Ctrl-, Ctrl+ and Metabiotic groups on day 3, 7, 14, and 21 post wounding (H&E staining, × 250). Thick arrow demonstrates the infiltration of polymorphonuclear neutrophils; Narrow arrow demonstrates the infiltration of mononuclear inflammatory cells; Star demonstrates the presence of fibroblasts; and curved arrow demonstrates the rate of angiogenesis.

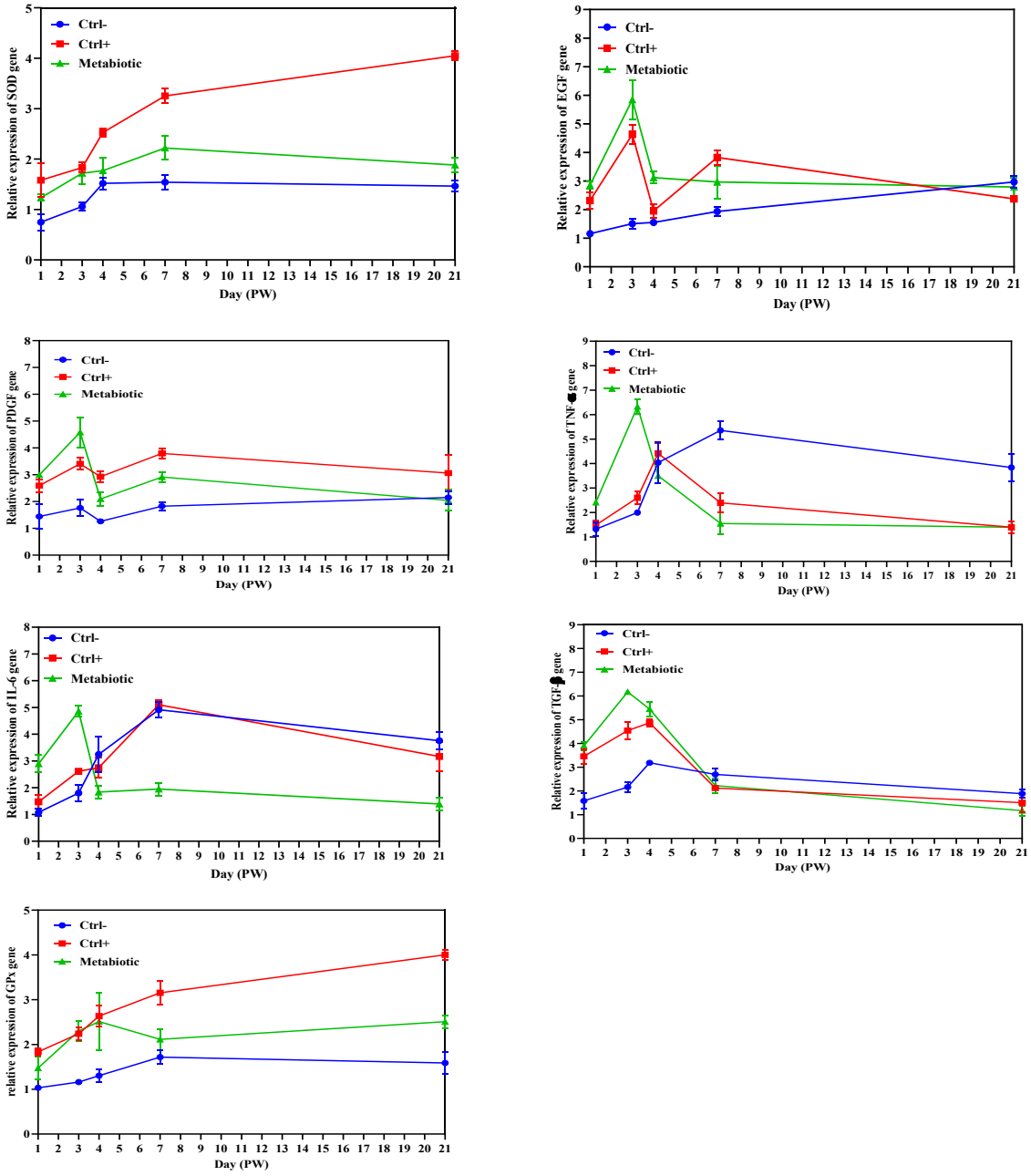

FIGURE 3. Relative mRNA expression levels of PDGF, EGF, GPx, SOD, TGF, TNF-α, and IL-6 on days 1, 3, 7 ,14, and 21 post wounding (PW). Quantitative RT-PCR was performed on total RNA gastric wound sample of rats in Ctrl-, Ctrl+, And Metabiotic groups. All dCt values were corrected for the efficiency of the respective primer sets in relation to the β-actin housekeeping gene.

control group, the number of these cells reached a maximum on day 7 (PW) and decreased after that. These results show that the inflammation process in the Ctrl+ and metabiotic groups started faster and with more intensity and also resolved faster.

Fibroblasts are the prevailing cell population in the proliferative phase. The proliferation and migration of these cells are the necessary and rate-limiting steps in the repair process. In the Ctrl+ and metabiotic groups, the number of these cells gradually increased and reached a maximum on the 7th (PW) was significantly more than the Ctrl- group. In the following days, the number of fibroblasts decreased in these two treated groups. This pattern was also observed in the process of angiogenesis so that during the first 14 days (PW), the maximum angiogenic process was observed in the two treated groups.

Gene expression assay by qRT-PCR

Inflammatory responses are produced by pro-inflammatory cytokines such as IL-6. These cytokines are produced by fibroblasts and macrophages residing in the wound site following the formation of a fibrin clot. Our results showed that the use of metabiotics increased the expression of IL-6 compared to other groups. Interestingly, in the following days, a significant decrease in the expression of this cytokine was observed compared to the mentioned groups. It should be noted that there was no significant change in the expression of this gene in the two negative and positive control groups. Expression of IL-6 causes inflammatory cells such as PMN and monocytes to be recruited and release other pro-inflammatory cytokines such as TNF and TGF-β. As we expected, the expression of these two cytokines was significantly higher in the metabiotic group in the first 3 days. The expression of them decreased significantly in the following days, which indicated a faster clearing of inflammation. In the two control groups, the peak of expression of these cytokines was seen on the seventh day after wound formation (Fig 3).

PDGF is another pro-inflammatory cytokine released by macrophages and endothelial cells, which causes chemotactic and activation of PMNs, macrophages, and fibroblasts. Administration of drugs and metabiotics caused the expression of this cytokine to increase significantly compared to the Ctrl- group. There was no significant difference in the expression level of PDGF in Ctrl+ and metabiotic and it reached its peak of production on the 3rd PW. EGF is another pro-inflammatory cytokine whose expression in groups showed a pattern similar to that of PDGF (Fig 3).

SOD and GPx encode two antioxidant enzymes that can prevent the damaging effects of ROS. Our results showed that the use of drugs and metabiotic increased the expression of these two genes in the entire wound healing period compared to the control group. Surprisingly, there was no difference in the expression of SOD in the drug and drug groups, but the expression of GPx by metabiotic was significantly more than in the ctrl+ group (Fig 3).

Discussion

Recent literature has increasingly emphasized the role of probiotics and their derivatives in wound healing, shedding light on their multifaceted mechanisms of action. Some studies have demonstrated the antioxidant properties of probiotic cell-free supernatants, underscoring their potential to mitigate oxidative stress during wound repair.

Halper showed that the supernatant obtained from *Lactobacillus* sp. can intensify the inflammatory phase and increase the expression of TNF and angiogenesis (Halper et al., 2003). Bharti showed that *L. plantarum* and *L. acidophilus* cell-free supernatant have strong ROS scavenging activities (Bharti et al., 2017). *Chen* evaluated the antioxidant properties of cell-free extracts of 10 different *Lactobacillus* spp. The highest reported antioxidant property was related to *L. rhamnosus* GG with 77% u/ml. Bharti showed that *B. amyloliquefaciens* can have antioxidant activity equivalent to 67% at a concentration of 10^8 CFUs/mL (Bharti et al., 2017). *Yang* reported that probiotics can help heal gastrointestinal ulcers (Yang et al., 2021). The experiments of Virchenko proved that lactic acid bacteria (LAB) can treat gastrointestinal ulcers through the balance between oxidant and pro-oxidant (Virchenko et al., 2015). *Nagaoka* reported that polysaccharides produced by *B. breve* and *B. bifidum* can heal gastrointestinal ulcers caused by acetic acid and ethanol. He mentioned that these polysaccharides can improve the expression of growth factors such as VEGF and EGF (NAGAOKA et al., 1994). *Wang* reported that treatment with *B. longum and L. plantarum* can increase catalase activity in the A7R5 cell line and improve intracellular SOD activity (Wang et al., 2021). *Noureen* investigation has demonstrated that the rats treated with a supernatant of *L. brevis* exhibited an enhancement in SOD, CAT, and GST activity in all tissues, as well as GSH in the liver and serum (Noureen et al., 2019). While these studies have provided valuable insights into the antioxidant capacity of probiotics, they often lack comprehensive investigations into the underlying signaling pathways and gene expression profiles associated with wound healing.

Our study elucidates the intricate interplay between metabiotic derived from *L. acidophilus* BF-P-064 and the healing process of PU. By employing a multifaceted approach encompassing cytotoxicity assays, antioxidant capacity assessments, animal experiments, histopathological analyses, and gene expression assays, we uncovered significant mechanistic insights.

The interaction between various cellular and molecular components involved in wound healing is a complex

and coordinated process. Macrophages and neutrophils are key players in the inflammatory phase of wound healing. Upon tissue injury, neutrophils are recruited to the wound site, where they phagocytose pathogens and cellular debris, and release pro-inflammatory cytokines such as TNF-α and IL-6. Macrophages, which are recruited subsequently, further amplify the inflammatory response by releasing cytokines and chemokines. This initial inflammatory phase is crucial for clearing pathogens and initiating tissue repair processes. PDGF is another potent mitogen, primarily released by macrophages. PDGF enhances the production of ROS, which serve as signaling molecules in various cellular processes involved in wound repair. TNF, released by neutrophils and macrophages, enhances the expression of adhesion molecules on endothelial cells, facilitating leukocyte recruitment to the site of injury. Additionally, TNF regulates the production of other cytokines and growth factors involved in wound repair(Efron and Moldawer 2004; Werner and Grose 2003).

Our results showed that the metabiotic of *L. acidophilus* BF-P-064 stimulates the recruitment of neutrophils and monocytes/macrophages to the wound site. The metabiotic up-regulates the pro-inflammatory cytokines gene expression such as PDGF, TNF-α, and IL-6. The increase in the expression of these pro-inflammatory cytokines is associated with an increase in the concentration of ROS, which has a dual function. The excess ROS level leads to oxidative stress and has destructive effects on the wound-healing process. Elevated and sustained levels of ROS are seen in chronic and non-healing wounds. It causes the continuous transcription of pro-inflammatory cytokines, induction of matrix metalloproteases, and breaks down the extracellular matrix (ECM) proteins. So, the production of ROS and the activity of antioxidant enzymes like GPx and SOD must tightly regulate, ensuring proper redox balance and minimizing oxidative damage (Wattamwar and Dziubla 2012).

Today, various strategies are used to prevent the destructive effects of oxidative stress in wound treatment, which mainly include the use of enzymatic and non-enzymatic antioxidant compounds (Saadatzadeh et al., 2013). In this experiment, we found that the administration of metabiotic increased the expression of SOD and GPx genes.

The antioxidant activity of metabiotics is attributed to compounds such as glutathione, butyrate, and folate (Dizman et al., 2021; Wang et al., 2012) .Our results showed the metabiotic of *L. acidophilus* BF-P-064 has an antioxidant activity equal to 72 u/ml. Besides, it can increase the expression of *GPx* and *SOD* during the proliferation phase. So, it causes a faster PU healing which was evident in the microscopic and macroscopic observations.

Conclusion

In conclusion, our study represents a significant advancement in our understanding of the molecular mechanisms underlying metabiotic-mediated wound healing. By addressing the gaps and shortcomings in previous literature, we provided insights into the benefit of metabiotics for PU healing. It can be concluded that the metabiotic of *L. acidophilus* BF-P-064 can accelerate PU healing by recruitment of phagocytic cells to the wound site and ameliorating oxidative stress.

Conflict Of Interest

The authors have no conflicts of interest to declare that are relevant to the content of this article.

Ethics Approval

The protocol was authorized by Lorestan University of Medical Sciences' Committee on the Ethics of Animal Experiments (Permit Number: IR.LUMS.REC.1398.230).

References

An J-H, Li Q, Bhang D-H, Song W-J, Youn H-Y. TNF-α and INF-γ primed canine stem cell-derived extracellular vesicles alleviate experimental murine colitis. Scientific reports 2020; 10: 2115. https://doi.org/10.1038/s41598-020-58909-4

Arian S, Kaboosi H, Heshmatipour Z, Koohpar Z K, Pyravii-Ghadikolaii F. Anti-proliferative effects of two new Lactobacillus strains of human origin on Caco-2 cell line. Iranian Red Crescent Medical Journal 2019; 21. https://doi.org/10.5812/ircmj.84683

Assar D H, Elhabashi N, Mokhbatly A-A A, Ragab A E, Elbialy Z I, Rizk S A, et al. Wound healing potential of licorice extract in rat model: Antioxidants, histopathological, immunohistochemical and gene expression evidences. Biomedicine & Pharmacotherapy 2021; 143: 112151. https://doi.org/10.1016/j.biopha.2021.112151

Barrientos S, Stojadinovic O, Golinko M S, Brem H, Tom-

ic-Canic M. Growth factors and cytokines in wound healing. Wound repair and regeneration 2008; 16: 585-601. https://doi.org/10.1111/j.1524-475X.2008.00410.x

Bereda G. Peptic Ulcer disease: definition, pathophysiology, and treatment. Journal of Biomedical and Biological Sciences 2022; 1: 1-10.

Bharti V, Mehta A, Mourya G K, Ahirwal L. In vitro antioxidant potential of cell free supernatant of probiotic bacteria. FPI Journal 2017; 1: 187-193. https://doi.org/10.5530/fpi.1.4.4

Biswas I, Mohapatra P K D. Recent advancement in metabiotics: A consortium with bioactive molecules after fermentation by probiotic bacteria with multidisciplinary application potential and future solution in health sector. Bioresource Technology Reports 2023: 101583. https://doi.org/10.1016/j.biteb.2023.101583

Biswas I, Soren J P, Mohapatra P K D. Preparation of metabiotic curd and optimization of tannase and gallic acid production by probiotic Lactiplantibacillus plantarum PKI15. Biocatalysis and Agricultural Biotechnology 2024: 103041. https://doi.org/10.1016/j.bcab.2024.103041

Bousounis P, Bergo V, Trompouki E. Inflammation, aging and hematopoiesis: A complex relationship. Cells 2021; 10: 1386. https://doi.org/10.3390/cells10061386

Bryan N, Ahswin H, Smart N, Bayon Y, Wohlert S, Hunt J A. Reactive oxygen species (ROS)-a family of fate deciding molecules pivotal in constructive inflammation and wound healing. Eur Cell Mater 2012; 24: e65. https://doi.org/10.22203/eCM.v024a18

Cano Sanchez M, Lancel S, Boulanger E, Neviere R. Targeting oxidative stress and mitochondrial dysfunction in the treatment of impaired wound healing: a systematic review. Antioxidants 2018; 7: 98. https://doi.org/10.3390/antiox7080098

Chegini N, Rossi M J, Masterson B J. Platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and EGF and PDGF beta-receptors in human endometrial tissue: localization and in vitro action. Endocrinology 1992; 130: 2373-2385. https://doi.org/10.1210/endo.130.4.1312455

Chiu Y-H, Lu Y-C, Ou C-C, Lin S-L, Tsai C-C, Huang C-T, Lin M-Y. Lactobacillus plantarum MYL26 induces endotoxin tolerance phenotype in Caco-2 cells. BMC microbiology 2013; 13: 1-9. https://doi.org/10.1186/1471-2180-13-190

Cody W L, Wilson J W, Hendrixson D R, McIver K S, Hagman K E, Ott C, et al. Skim milk enhances the preservation of thawed− 80 C bacterial stocks. Journal of microbiological methods 2008; 75: 135-138. https://doi.org/10.1016/j.mimet.2008.05.006

Dehkohneh A, Jafari P, Fahimi H. Effects of probiotic Lactobacillus paracasei TD3 on moderation of cholesterol biosynthesis pathway in rats. Iranian Journal of Basic Medical Sciences 2019; 22: 1004.

Dizman N, Hsu J, Bergerot P G, Gillece J D, Folkerts M, Reining L, et al. Randomized trial assessing impact of probiotic supplementation on gut microbiome and clinical outcome from targeted therapy in metastatic renal cell carcinoma. Cancer medicine 2021; 10: 79-86. https://doi.org/10.1002/cam4.3569

Efron P A, Moldawer L L. Cytokines and wound healing: the role of cytokine and anticytokine therapy in the repair response. Journal of Burn Care & Rehabilitation 2004; 25: 149-160. https://doi.org/10.1097/01.BCR.0000111766.97335.34

Grazul-Bilska A T, Johnson M L, Bilski J J, Redmer D A, Reynolds L P, Abdullah A, Abdullah K M. Wound healing: the role of growth factors. Drugs Today (Barc) 2003; 39: 787-800. https://doi.org/10.1358/dot.2003.39.10.799472

Halper J, Leshin L, Lewis S, Li W. Wound healing and angiogenic properties of supernatants from Lactobacillus cultures. Experimental Biology and Medicine 2003; 228: 1329-1337. https://doi.org/10.1177/153537020322801111

Hunt R H. Treatment of peptic ulcer disease with sucralfate: A review. The American journal of medicine 1991; 91: S102-S106. https://doi.org/10.1016/0002-9343(91)90459-B

Jamaran S, Jafari P, Marjani A, Akbari N, Feizabad M M. Novel wound dressing based on postbiotic/chitosan film accelerates cutaneous wound healing. Jundishapur Journal of Microbiology 2021; 14. https://doi.org/10.5812/jjm.120806

Kamran S. Evaluation of wound healing potential, antioxidant activity, acute toxicity and gastroprotective effect of 2-pentadecanone in ethanol induced gastric mucosal ulceration in rats. Journal 2020. https://doi.org/10.3923/ijp.2019.944.952

Kapoor R, Kakkar P. Protective role of morin, a flavonoid, against high glucose induced oxidative stress mediated apoptosis in primary rat hepatocytes. 2012. https://doi.org/10.1371/journal.pone.0041663

Kianmehr M, Rezaei A, Boskabady M H. Effect of carvacrol on various cytokines genes expression in splenocytes of asthmatic mice. Iranian journal of basic medical sciences 2016; 19: 402.

Konturek S, Brzozowski T, Drozdowicz D, Nauert C. Gastroprotection by an aluminium-and magnesium hy-

droxide-containing antacid in rats. Scandinavian journal of gastroenterology 1989; 24: 1113-1120. https://doi.org/10.3109/00365528909089264

Lu W, Li B, Chen J, Su Y, Dong X, Su X, Gao L. Expression of calcitonin gene-related peptide, adenosine A2a receptor and adenosine A1 receptor in experiment rat migraine models. Biomedical Reports 2016; 4: 379-383. https://doi.org/10.3892/br.2016.591

Nagaoka M, Hashimoto S, Watanabe T, Yokokura T, Mori Y. Anti-ulcer effects of lactic acid bacteria and their cell wall polysaccharides. Biological and Pharmaceutical Bulletin 1994; 17: 1012-1017. https://doi.org/10.1248/bpb.17.1012

Noureen S, Riaz A, Arshad M, Arshad N. In vitro selection and in vivo confirmation of the antioxidant ability of Lactobacillus brevis MG 000874. Journal of applied microbiology 2019; 126: 1221-1232. https://doi.org/10.1111/jam.14189

Ritz M-F, Graumann U, Gutierrez B, Hausmann O. Traumatic spinal cord injury alters angiogenic factors and TGF-beta1 that may affect vascular recovery. Current neurovascular research 2010; 7: 301-310. https://doi.org/10.2174/156720210793180756

Saadatzadeh A, Fazeli M R, Jamalifar H, Dinarvand R. Probiotic properties of lyophilized cell free extract of Lactobacillus casei. Jundishapur journal of natural pharmaceutical products 2013; 8: 131. https://doi.org/10.17795/jjnpp-8564

Sadeghi A, Ebrahimi M, Kharazmi M S, Jafari S M. Effects of microbial-derived biotics (meta/pharma/post-biotics) on the modulation of gut microbiome and metabolome; general aspects and emerging trends. Food Chemistry 2023; 411: 135478. https://doi.org/10.1016/j.foodchem.2023.135478

Schepp W, Classen M. Omeprazole in the acute treatment of gastric ulcer. Scandinavian Journal of Gastroenterology 1989; 24: 58-62. https://doi.org/10.3109/00365528909091246

Shenderov B A, Sinitsa A V, Zakharchenko M, Lang C. Metabiotics: Springer, 2020. https://doi.org/10.1007/978-3-030-34167-1

Sistani Karampour N, Arzi A, Rezaie A, Pashmforoosh M, Kordi F. Gastroprotective effect of zingerone on ethanol-induced gastric ulcers in rats. Medicina 2019; 55: 64. https://doi.org/10.3390/medicina55030064

Slaoui M, Bauchet A-L, Fiette L. Tissue sampling and processing for histopathology evaluation. Drug Safety Evaluation: Methods and Protocols 2017: 101-114. https://doi.org/10.1007/978-1-4939-7172-5_4

Süle J, Kõrösi T, Hucker A, Varga L. Evaluation of culture media for selective enumeration of bifidobacteria and lactic acid bacteria. Brazilian journal of Microbiology 2014; 45: 1023-1030. https://doi.org/10.1590/S1517-83822014000300035

Tabatabaee Bafroee A S, Khalili Hadad B. Evaluating the effect of supernatant collected from the culture of bifidobacterium bifidum on the increase of angiogenesis in acute wound healing. Iranian Journal of Medical Microbiology 2021; 15: 552-568. https://doi.org/10.30699/ijmm.15.5.552

Tarapatzi G, Filidou E, Kandilogiannakis L, Spathakis M, Gaitanidou M, Arvanitidis K, et al. The probiotic strains bifid obacterium lactis, lactobacillus acidophilus, lactiplantibacillus plantarum and saccharomyces boulardii regulate wound healing and chemokine responses in human intestinal subepithelial myofibroblasts. Pharmaceuticals 2022; 15: 1293. https://doi.org/10.3390/ph15101293

Vaidyanathan L. Growth factors in wound healing-a review. Biomed. Pharmacol. J 2021; 14: 1469-1481. https://doi.org/10.13005/bpj/2249

Virchenko O, Falalyeyeva T, Beregova T, Spivak M, Lazarenko L, Demchenko O. Effects of mono-, poly-and composite probiotics on the ulceration caused by restraint stress. Fiziolohichnyi Zhurnal (Kiev, Ukraine: 1994) 2015; 61: 35-41. https://doi.org/10.15407/fz61.01.035

Wang C-Y, Yang C-C, Hsiao L-D, Yang C-M. Involvement of FoxO1, Sp1, and Nrf2 in upregulation of negative regulator of ROS by 15d-PGJ 2 attenuates H 2 O 2-induced IL-6 expression in rat brain astrocytes. Neurotoxicity Research 2022: 1-19. https://doi.org/10.1007/s12640-020-00318-6

Wang Y, Fang Z, Zhai Q, Cui S, Zhao J, Zhang H, et al. Supernatants of Bifidobacterium longum and Lactobacillus plantarum strains exhibited antioxidative effects on A7R5 cells. Microorganisms 2021; 9: 452. https://doi.org/10.3390/microorganisms9020452

Wang Y, Liu Y, Sidhu A, Ma Z, McClain C, Feng W. Lactobacillus rhamnosus GG culture supernatant ameliorates acute alcohol-induced intestinal permeability and liver injury. American Journal of Physiology-Gastrointestinal and Liver Physiology 2012; 303: G32-G41. https://doi.org/10.1152/ajpgi.00024.2012

Wattamwar P P, Dziubla T D. Modulation of the wound healing response through oxidation active materials. Engineering Biomaterials for Regenerative Medicine: Novel Technologies for Clinical Applications 2012: 161-192. https://doi.org/10.1007/978-1-4614-1080-5_7

Werner S, Grose R. Regulation of wound healing by growth factors and cytokines. Physiological reviews 2003; 83: 835-870. https://doi.org/10.1152/physrev.2003.83.3.835

Wong M L, Medrano J F. Real-time PCR for mRNA quan-

titation. Biotechniques 2005; 39: 75-85. https://doi.org/10.2144/05391RV01

Xing J, Wang G, Zhang Q, Liu X, Gu Z, Zhang H, et al. Determining antioxidant activities of lactobacilli cell-free supernatants by cellular antioxidant assay: a comparison with traditional methods. PloS one 2015; 10: e0119058. https://doi.org/10.1371/journal.pone.0119058

Yang R, Zhao X, Wu W, Shi J. Potential of probiotics for use as functional foods in patients with non-infectious gastric ulcer. Trends in Food Science & Technology 2021. https://doi.org/10.1016/j.tifs.2021.02.070

Zhang S, Xue J, Zheng J, Wang S, Zhou J, Jiao Y, et al. The superoxide dismutase 1 3′ UTR maintains high expression of the SOD1 gene in cancer cells: The involvement of the RNA-binding protein AUF-1. Free Radical Biology and Medicine 2015; 85: 33-44. https://doi.org/10.1016/j.freeradbiomed.2015.04.012