Original Article

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In Vitro Study of the Effects of Postbiotic Mouthwash Prototype with Cannabis Extracts on the Reduction of Inflammatory Cytokine: Tumor Necrosis Factor Alpha

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Abstract:

Objective: This study evaluated the effects of postbiotic mouthwash containing cannabis extracts (CBD) on the reduction of tumor necrosis factor alpha (TNF-α) levels *in vitro*.

Material and Methods: Seven mouthwash formulations were prepared using CBD at concentrations ranging from 0.25% to 1.0% v/v. TNF-α reduction was assessed using a lipopolysaccharide (LPS) model to activate the THP-1 human monocytic cell line (ATCC, TIB202). The enzyme-linked immunosorbent assay (ELISA) technique was employed to quantify TNF-α.

Results: All formulations reduced TNF-α levels compared to the control. The most effective formulations (Preparations 1–4) achieved approximately 91% inhibition. Formulations 5–7 showed inhibition levels of 49.3%, 80.9%, and 82.5%, respectively.

Conclusion: The postbiotic mouthwash containing CBD effectively reduced TNF-α levels, indicating its potential for clinical application in inflammation management.

Keyword: cannabis, CBD, proinflammatory cytokine, mouthwash, postbiotic, TNF-alpha

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Introduction

Oral surgery is a specialized branch of dentistry that involves surgical procedures performed on the mouth, teeth, and jaw, including tooth extraction, wisdom teeth removal, dental implants, bone graft surgery, and more. Oral surgery plays a crucial role in the healing process of individuals since the procedure can cause oral wounds and stimulate the body's response to trauma[1]. Swelling is a common side effect after surgery, resulting from tissue injury during the procedure. The peak time for swelling can be up to 48 hours after surgery[2].

Swelling is part of the body's wound healing process, which can be divided into 3 main stages: hemostasis and inflammation, proliferation, and remodeling[3]. The duration of wound healing is influenced by white blood cells and inflammatory mediators. Neutrophils and macrophages play significant roles by releasing mediators that drive the healing process[1] However, the release of excessive inflammatory mediators compromises wound healing. Elevated local and systemic levels of tumor necrosis factor alpha (TNF-α) are associated with impaired healing, as TNF-α can slow wound repair in humans[4].

Beyond traditional anti-inflammatory drugs, beneficial microorganisms have gained attention as alternative treatments for inflammatory conditions such as inflammatory bowel disease and dermatitis. Postbiotics, non-viable microorganisms and their metabolites, promote health and recovery when present in appropriate amounts. However, different species and strains yield distinct outcomes[5,6].

Lactobacilli are gram-positive, rod-shaped bacteria found in various body sites. Known for their acid-resistant properties and lactic acid production, Lactobacillus species exhibit anti-inflammatory effects. Clinical studies have shown that they can reduce TNF-α levels[7]. Comparative research highlights *Lactobacillus paracasei (L. paracasei)* as particularly effective in modulating inflammation and lowering TNF-α levels[8].

There is growing interest in using herbs and natural extracts to minimize drug side effects and reduce treatment costs. Hemp-derived cannabinoids have notable antibacterial properties, which is significant since bacterial infections trigger inflammatory mediators, particularly in post-oral surgery patients with compromised hygiene[1].

Cannabinoids show greater antibacterial activity against gram-positive than gram-negative bacteria[9-11] by interfering with bacterial quorum sensing (AI-2 signal cascade)[12]. Cannabis extracts (CBD) extracts also inhibit gram-negative bacterial membrane formation and enhance antibiotic effects[11]. These attributes position cannabinoids as promising agents for antibacterial and plaque-prevention applications.

Currently, research studies are underway to harness the potential of postbiotics in mitigating inflammation. It was found that the supernatant of *L. paracasei* MSMC39-1, which can be extracted from the feces of healthy newborns, has exhibited promising anti-inflammatory effects. These effects were evidenced by the reduction of TNF-α levels in culture supernatants, as demonstrated in studies conducted at HRH Princess Maha Chakri Sirindhorn Medical Center (MSMC), Srinakharinwirot University[13].

Previous research by our team demonstrated that postbiotic supernatants significantly reduce TNF-α levels. However, combining postbiotics with other beneficial compounds for additional clinical effects—, such as targeting harmful flora or accelerating wound healing—, remains unexplored. This study aimed to evaluate a mouthwash prototype containing *L. paracasei* MSMC39-1 postbiotic supernatant and cannabis extract in reducing TNF-α levels. Ultimately, this formula could lower inflammatory complications and bacterial overgrowth in oral surgery, improving treatment outcomes.

Material and Methods

The experimental method in this research was structured into 5 distinct parts, as follows:

Part 1: Preparation of *Lactobacillus paracasei* MSMC39-1 supernatant[13]

Lactobacillus paracasei MSMC39-1 was cultivated under anaerobic conditions, then diluted in a liquid medium to achieve a cell concentration of 10^9 cells/ml. This culture was then incubated in an oxygen-free environment at 37°C for 48 hours. After incubation, the supernatant was separated by centrifugation at 4000 x g for 10 minutes, filtered using sterile 0.22µm filter paper (Sigma, USA) and stored at -20 ºC. In this study, postbiotics were cultured in MRS broth, and the cell-free culture supernatant was used as postbiotics.

Part 2: Preparation of a mouthwash formula containing postbiotic supernatant and cannabis-derived ingredients

Mouthwash formulas were prepared using *Lactobacillus paracasei* MSMC39-1 supernatant and cannabis-derived ingredients. The optimum concentration of 10% postbiotic was selected based on previous studies[14,15]. Each formulation (10 ml) was designated as Formula 1 through Formula 7, with the compositions detailed in Table 1:

- Formula 1: 0.25% cannabidiol (CBD), 10% postbiotic supernatant, and other mouthwash ingredients.
- Formula 2: 0.5% CBD, 10% postbiotic supernatant, and other mouthwash ingredients.
- Formula 3: 1% CBD, 10% postbiotic supernatant, and other mouthwash ingredients.
- Formula 4: 10% postbiotic supernatant and other mouthwash ingredients.
- Formula 5: 0.25% CBD and 10% postbiotic supernatant.
- Formula 6: 0.5% CBD and 10% postbiotic supernatant.
- Formula 7: 1% CBD and 10% postbiotic supernatant.

The positive control contained 10% postbiotic supernatant alone, and 0.9% normal saline solution (NSS) served as the negative control.

Note: The percentages of active ingredients represent the final concentrations.

Part 3: cultivation of human monocytic cell line type THP-1 (ATCC, TIB202)

The THP-1 human monocytic cell line (ATCC, TIB202) was cultured in RPMI 1640 medium (Gibco-Invitrogen, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco-Invitrogen, USA) in flat-

Table 1 Shows the composition of mouthwash formulations 1-7 containing postbiotic L. *paracasei vitreous*. Strain MSMC39-1 and Cannabidiol, 10 ml

Formula (unit)	Total amount in the formula							Roles
	1	2	3	4	5	6	7	
5% CBD (ml)	0.5	1	2	-	0.5	1	2	Main ingredient
Glycerine (ml)	2.5	2.5	2.5	2.5	-	-	-	Moisturizing agent
Polysorbe 80 (µl)	100	100	100	100	-	-	-	Solvent agent
0.9% Sodium saccharin (µl)	333	333	333	333	-	-	-	Flavoring agent
Postbiotic supernatant (ml) Positive control	1	1	1	1	1	1	1	Main ingredient, Solvent
Normal saline 0.9% (µl) Negative control	5,567	5,067	4,067	6,067	8,500	8,000	7,000	Solvent

CBD=cannabis extracts

bottomed 96-well culture plates (Corning, USA). The cells were incubated at 37 °C in a 5% CO_2 environment.

Part 4: Testing the effects of mouthwashes containing Postbiotics and Cannabis-derived Ingredients and the stimulation of TNF-α secretion

The THP-1 cells were stimulated to secrete TNF-α by adding 10 µl of purified lipopolysaccharide (LPS) from *Escherichia coli* O127:B8 (Sigma, USA) to achieve a final concentration of 100 ng/ml.

Postbiotic-containing mouthwash and hemp-derived ingredients test

Each mouthwash formulation was tested in triplicate wells. Treated cells were incubated at 37 °C with 5% CO_2 for 3.5 hours. Cell viability was assessed using a hemocytometer under an inverted microscope. The supernatant was collected, centrifuged at 3,000 x g for 5 minutes at 4 °C, and stored at –80 °C for analysis of TNF-α inhibition using the ELISA method (R&D Systems, USA).

Part 5: Analysis of TNF-α secretion inhibition and statistical analysis

Calculation of inhibition of TNF-α secretion percentage

The percentage inhibition of TNF-α secretion was computed using the following formula: *Percent inhibition of TNF-α secretion=100 ×(1 – (sample solution÷negative control).*

Where:

**Sample solution: Absorbance at 450 nm from supernatant treated with each mouthwash.

**Negative control: Absorbance at 450 nm from untreated supernatant.

Statistical Analysis

All collected data values were subjected to calculations using statistical software, GraphPad Prism version 9.3.1. The statistical analysis was performed using the Student's t-test, employing a two-tailed distribution with a 95% confidence level.

Results

The impact of the absorbance level of a mouthwash formula containing the postbiotic L. paracasei vitreous MSMC39-1 and cannabis extracts on the levels of the inflammatory mediator TNF-α

The absorbance levels of mouthwash formulations containing postbiotic *L. paracasei* MSMC39-1 and cannabis extracts were evaluated for their effects on TNF-α secretion.

To induce TNF-α secretion, purified lipopolysaccharide (LPS) was introduced to THP-1 human monocytic cells, followed by exposure to different mouthwash formulations. After 3.5 hours of incubation, TNF-α levels were measured using the ELISA method.

All 7 mouthwash formulas reduced TNF-α levels compared to the control (0.9% saline, absorbance 0.94325). Compared to the positive control (10% postbiotic supernatant, absorbance 0.3955), reductions were more pronounced.

Among the 7 formulations, 6 showed significant TNF-α inhibition. Formula 5, containing 0.25% cannabidiol and 10% postbiotic supernatant, was the least effective, with an absorbance value of 0.47825.

Formulas 1–4 exhibited strong TNF-α inhibition:

- Formula 1: 0.25% CBD+10% postbiotic (absorbance 0.0847)
- Formula 2: 0.5% CBD+10% postbiotic (absorbance 0.0848)
- Formula 3: 1% CBD+10% postbiotic (absorbance 0.08415)
- Formula 4: 10% postbiotic (absorbance 0.08175)

Formula 6 (0.5% CBD+10% postbiotic, absorbance 0.1798) and Formula 7 (1% CBD+10% postbiotic, absorbance 0.16495) demonstrated moderate TNF-α

reduction. Notably, Formula 6 showed slightly better performance than Formula 7.

Statistical analysis confirmed that all mouthwash formulations, except Formula 5, significantly reduced TNF-α levels at a 95% confidence level when compared to the positive control. Formula 5's absorbance was significantly higher than the positive control (p-value<0.05). Table 2 and Figure 1 summarize these results.

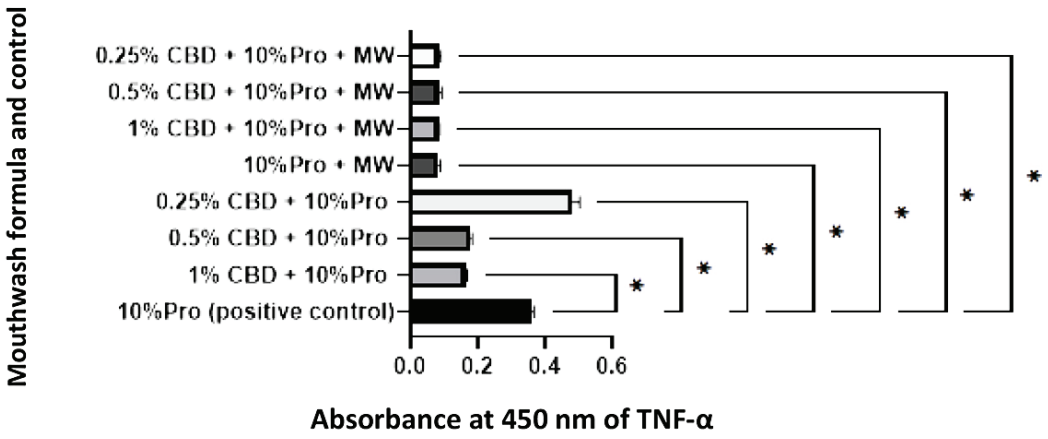
Percentage inhibition of TNF-α secretion

Based on the absorbance data, formulas 1–4 exhibited approximately 91% TNF-α inhibition, comparable to the negative control (0.9% saline). Formula 5 demonstrated the lowest inhibition at 49.3%, significantly below the positive control (61.88%). Formula 7 (82.51%) outperformed Formula 6 (80.94%).

Table 2 presents absorbance readings at 450 nm and TNF-α secretion inhibition percentages for formulas 1–7, positive control, and negative control

Formula	Component	Mean absorbance (OD)	TNF-α reducing percentages (%)
1	0.25% CBD+10% post+MW	0.08470	91.02
2	0.5% CBD+10% post+MW	0.08480	91.01
3	1% CBD+10% post+MW	0.08415	91.08
4	10% post +MW	0.08175	91.33
5	0.25% CBD+10% post	0.47825	49.30
6	0.5% CBD+10% post	0.17980	80.94
7	1% CBD+10% post	0.16495	82.51
8	10 % Pro (positive control)	0.35955	61.88

TNF-α=tumor necrosis factor alpha, CBD=cannabis extracts, OD=optical density



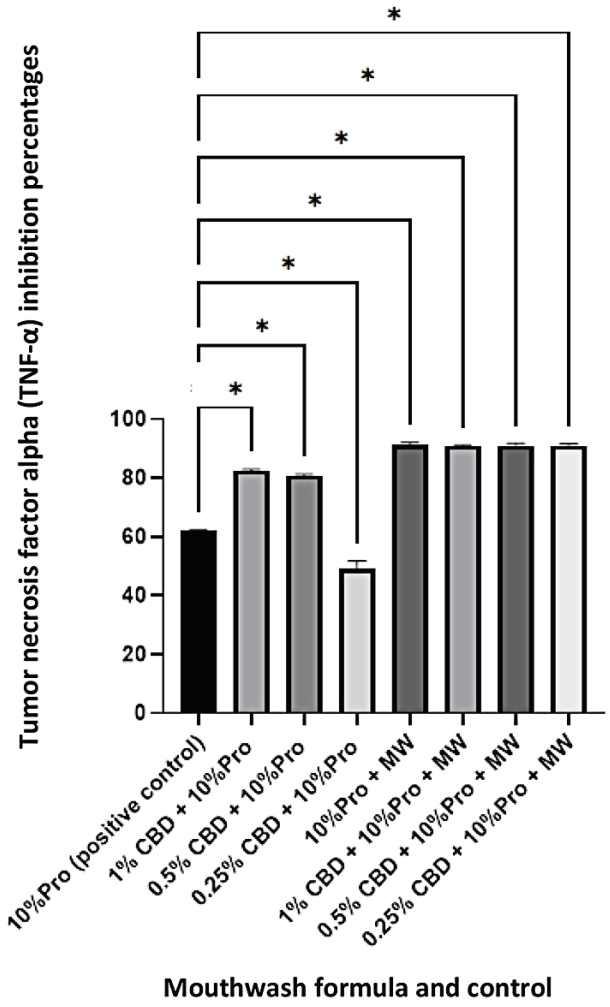
TNF-α=tumor necrosis factor alpha, CBD=cannabidiol, MW=mouthwash

Figure 1 Illustrates absorbance at 450 nm for all formulations and controls, indicating lower TNF-α levels with reduced absorbance. Results show substantial TNF-α inhibition (p-value<0.05) for all but Formula 5

Statistical analysis confirmed consistent patterns across all formulations. All formulas, except Formula 5, showed significant TNF-α inhibition (p-value<0.05) compared to the positive control. Formula 5 showed a significant decrease in inhibition (p-value<0.05). Figure 2.

Discussion

Oral surgery involves surgical procedures on the internal structures of the mouth, including tooth extraction, wisdom tooth removal, dental implant surgery, bone grafting, gum dressing, and implant placement. These procedures



CBD=cannabidiol, MW=mouthwash

Figure 2 displays Tumor necrosis factor alpha (TNF-α) inhibition percentages for formulas 1–7. A higher percentage indicates greater suppression of TNF-α-mediated inflammation. The pattern confirms significant TNF-α inhibition for all formulas, except Formula 5, which exhibited a reduced effect (p-value<0.05)

are closely linked to wound healing, which involves 3 stages: hemostasis and inflammation, proliferation, and remodeling. Each stage is influenced by various white blood cells and inflammatory mediators[16].

Neutrophils and macrophages play pivotal roles in wound healing by releasing inflammatory mediators that stimulate the repair process. However, excessive or imbalanced secretion of these mediators can impede healing. This emphasizes the importance of maintaining a delicate balance for optimal wound healing[17,18].

TNF-α, a proinflammatory cytokine, is crucial in the inflammatory process. It triggers prostaglandin synthesis, leading to pain, swelling, redness, and elevated temperature[19,20]. Elevated TNF-α levels can delay wound healing, highlighting its significant role in inflammation and recovery[21].

Research on postbiotics has shown their potential to reduce inflammation. The *L. paracasei* MSMC39-1 strain has been demonstrated to lower TNF-α levels both *in vitro* and *in vivo*[15,22]. It has also been effective in reducing inflammatory mediators in liver fibrosis[23] and acne[24].

Chlorhexidine-based mouthwashes are commonly prescribed post-surgery for their broad-spectrum antibacterial properties. However, their effectiveness in reducing TNF-α is limited, and prolonged use can cause altered taste perception and tooth staining[25]. This study aimed to develop a mouthwash prototype combining *L. paracasei* MSMC39-1 and CBD, which has antibacterial properties[26] and may reduce bacterial-induced inflammation. This combination is particularly relevant for post-surgical patients with compromised hygiene.

Experimental quantification of TNF-α posed a challenge due to its picogram-scale concentration. Absorbance readings were used to represent TNF-α levels, as higher absorbance indicates elevated inflammatory mediators.

The results showed significant TNF-α inhibition across most formulations, although not all reduced every type of inflammatory mediator. Formula 5, which contained only 0.25% CBD and 10% postbiotic supernatant, exhibited the least inhibition. Conversely, formulations containing glycerin, polysorbate 80, and sodium saccharine alone produced low inflammatory mediator levels, possibly due to cytotoxic effects on THP-1 cells.

Comparisons with conventional mouthwashes highlight safety concerns. Studies have shown that chlorhexidine and essential oil-based mouthwashes reduce fibroblast viability, even at low concentrations[27,28]. This underscores the need for gentler alternatives.

Mouthwash formulas 5 (0.5% CBD+10% post) and 6 (1% CBD+10% post) demonstrated significant TNF-α reductions. Inhibition percentages of 53.84% and 76.10% (relative to saline control) align with previous findings by Ladda et al.[13] where *L. paracasei* MSMC39-1 reduced TNF-α by modulating the NF-κB pathway through interactions with TLR-2 and TLR-4, preventing nuclear translocation of NF-κB subunits.

These results are consistent with the studies by Jantararussamee et al.[23] and Sathikulpakdee et al.[24] that demonstrated the strain's anti-inflammatory effects on liver fibrosis and acne. CBD, derived from cannabis trichomes, has shown antibacterial and TNF-α-inhibiting effects[29]. Silva et al.[30] confirmed CBD's attenuation of NF-κB signaling in macrophages at 15 μM, though higher concentrations caused cytotoxicity.

An ideal mouthwash reduces TNF-α without cytotoxicity. Encouragingly, the 1% CBD+10% postbiotic formula achieved this balance. Future *in vivo* studies and clinical trials are recommended to validate its therapeutic potential.

Conclusion

The combination of the postbiotic strain *L. paracasei* MSMC39-1 and CBD was evaluated for reducing TNF-α levels *in vitro*. Notably, mouthwash Formula 4 (10% postbiotic supernatant and other mouthwash components)

demonstrated the most pronounced reduction in TNF-α levels. Conversely, Formula 5 (0.25% CBD and 10% postbiotic supernatant) exhibited the least reduction.

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Conflict of interest

The authors declare that they have no conflict of interest.

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