

INSTITUTE OF GRADUATE STUDIES, MAHSA UNIVERSITY
Defense of Research Proposal
24 January 2024

Title: Local Delivery of *Lactobacillus Reuteri* Probiotic as an Adjunct to Non-Surgical Periodontal Treatment in Patients with Stage II, III, and IV Periodontitis and Type 2 Diabetes Mellitus: A Prospective Split-Mouth Clinical Trial.

Part A

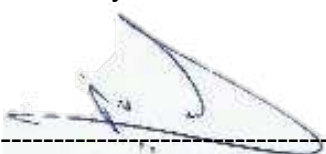
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Date : 24 January 2024

1. Full Tittle of The Research

Local Delivery of *Lactobacillus Reuteri* Probiotic as an Adjunct to Non-Surgical Periodontal Treatment in Patients with Stage II, III, and IV Periodontitis and Type 2 Diabetes Mellitus: A Prospective Split-Mouth Clinical Trial.

2. Study Background

Periodontitis is characterised by loss of periodontal attachments which comprise of periodontal ligaments, cementum, alveolar bone and gingiva due to host mediated inflammations, associated by microbial invasions (Tonetti et al., 2018). World Health Organization (WHO) reported that nearly 10% of global population are affected with severe periodontal disease, which make it the 11th most prevalent condition in the world, according to Global Burden of Disease Study 2016 (Vos et al., 2016). This disease impacts roughly 50% of adults aged over 30 in its milder manifestation and surpasses a prevalence of 60% in individuals aged 65 and older (Eke et al., 2016). Even in Malaysia, the latest report of National Adult Oral Health Survey in 2010 showed that more than 90% of adolescents, aged 15 to 17 years old were affected with periodontal disease (Mohd-Dom et al., 2013), and this is definitely an awaking alarm. Aside from being listed as the most common cause of tooth loss worldwide (Zohrabian & Abrahams, 2015), periodontitis also contributes to the negative impact on the quality of life, especially via alterations of aesthetic and oral functions (Ferreira et al., 2017). Therefore, effective treatment is a must to restore back the quality of life to optimal.

Diabetes mellitus encompasses a clinically and genetically diverse set of metabolic disorders characterised by abnormally elevated levels of glucose in the bloodstream (Mealey & Ocampo, 2007). International Diabetes Federation (IDF) reported that the global prevalence of diabetes has more than tripling the 2000 figure to the current (2021) from an estimated 151 million to 537 million. Conversely, the prevalence of diabetes in Malaysia is on a persistent upward trajectory. According to the 2019 National Health and Morbidity Survey (NHMS), the prevalence stands at 18.3% (3.9 million) for adults over 18 years old, reflecting a 4% rise from 2015, with nearly half (48.6%) of cases remaining undiagnosed. Alarming, around 5.0% of young Malaysians aged 18-29 years are also affected by diabetes, indicating a worrying trend. As per the registry dataset, the National Diabetes Registry (NDR) recorded a total of 1,614,363 registered patients, with 99.3% of them being diagnosed with Type 2 Diabetes Mellitus (T2DM). In Malaysia, a diagnostic threshold for T2DM based on HbA1c is considered met when it equals or exceeds 6.3% (45 mmol/ mol). The diagnostic threshold of $\geq 6.3\%$ (≥ 45 mmol/ mol) is derived from the Clinical Practice Guidelines for the Management of Type 2 Diabetes Mellitus published in 2020 by the Malaysian Endocrine and Metabolic Society (MEMS) in collaboration with the Ministry of Health, Malaysia. The recommended target goal for most adults with T2DM is to maintain an A1c level that is below 7%. The interaction between periodontal disease and diabetes has been extensively documented, supported by compelling evidence. The correlation between periodontal disease and diabetes is often described as a "two-way relationship," where diabetes elevates the risk of periodontal disease, and in turn, periodontal disease negatively influences glycemic control while exacerbating the severity of diabetes-related

complications (Grossi & Genco, 1998). Consequently, promoting periodontal health should be considered an integral aspect of diabetes management.

Scaling and root planing are the most widely used treatments for removing plaque and calculus in periodontitis patients (Rabbani et al., 1981). Currently, Lang et al. (2019) also acknowledged non-surgical periodontal debridement to be a gold standard for treatment of periodontitis prior considering any surgical therapy afterwards. In a systemic review by Van Der Weijden & Timmerman (2002), subgingival debridement found to be effective in reducing probing pocket depth (PPD) besides improving the clinical attachment level (CAL). Additionally, other studies also showed that root surface debridement (RSD) resulted in an improvement in plaque score, bleeding score, pocket depth and clinical attachment level (Garrett et al., 1999; Palmer et al., 1999). However, despite all these improvements, complete removal of subgingival deposit is difficult (Rabbani et al., 1981) especially in deep pockets, causing failure of treatment provided.

Hence, local delivery of antimicrobial therapy was introduced for controlling and treating periodontal disease, especially for deep pockets. It is proven effective according to systematic review by Tan et al. (2020), as an adjunct to chronic periodontal treatment. Currently, few types of synthetic antimicrobial agents were used; tetracycline, minocycline, metronidazole and chlorhexidine (Reddy et al., 2016), (Jain et al., 2012), (Leiknes et al., 2007), (Sakellari et al., 2010).

However, until now, doubts persist regarding the use of *Lactobacillus reuteri* probiotic as an adjunct therapy for deep pocket management and via this study, it's effectiveness as an adjunct to non-surgical periodontal therapy can be evaluated.

3. Problem Statement

Tan et al. (2020) in their systematic review have proven the effectiveness of local antimicrobial therapy as an adjunct to mechanical debridement in improving pocket depth and clinical attachment level. These local antimicrobial therapies work by helping to eradicate the periodontopathic bacteria which are difficult to be eliminated by mechanical debridement alone, especially on initial deep pockets of > 5mm (Gellin et al., 1986; Rabbani et al., 1981).

A systematic review conducted recently demonstrated a positive impact when employing a combined probiotic containing two strains of *Lactobacilli reuteri* systemically alongside scaling and root planing (Martin-Cabezas et al., 2016). Furthermore, it was demonstrated that the utilisation of these probiotic lozenges resulted in a statistically significant and more substantial decrease in *Porphyromonas gingivalis* (Teughels et al., 2013) and in the proportions of obligate anaerobes for a period of up to 180 days (Tekce et al., 2015). However, despite all these, the study of *Lactobacillus reuteri* as an adjunct in treating patients with type 2 diabetes mellitus and periodontal disease with deep pockets is none to be found yet.

4. Research Questions

1. Does the application of *Lactobacillus reuteri* probiotic subgingivally in periodontal pockets after root surface debridement (RSD) results in significant improvement of

bleeding on probing (BoP) among patients with type 2 diabetes mellitus and periodontal disease?

2. Does the application of *Lactobacillus reuteri* probiotic subgingivally in periodontal pockets after root surface debridement (RSD) results in significant improvement of probing pocket depth (PPD) among patients with type 2 diabetes mellitus and periodontal disease?

3. Does the application of *Lactobacillus reuteri* probiotic subgingivally in periodontal pockets after root surface debridement (RSD) results in significant improvement of clinical attachment level (CAL) among patients with type 2 diabetes mellitus and periodontal disease?

4. Does the application of *Lactobacillus reuteri* probiotic subgingivally in periodontal pockets after root surface debridement (RSD) results in significant improvement of plaque index (PI) among patients with type 2 diabetes mellitus and periodontal disease?

5. Does the application of *Lactobacillus reuteri* probiotic subgingivally in periodontal pockets after root surface debridement (RSD) results in significant improvement of gingival index (GI) among patients with type 2 diabetes mellitus and periodontal disease?

5. Hypothesis

We hypothesise that the local delivery of *Lactobacillus reuteri* probiotic on periodontitis patients with type 2 diabetes mellitus, as an adjunct to non-surgical periodontal treatment may have a beneficial effect on the clinical parameters (BoP, PPD, CAL, PI and GI).

6. Objectives

6.1. General Objective

This study is to evaluate the effectiveness of local delivery of *Lactobacillus reuteri* probiotic on periodontitis patients with type 2 diabetes mellitus, as an adjunct to non-surgical periodontal treatment.

6.2. Specific Objectives

To compare:

1. The bleeding on probing (BoP) between the group with *Lactobacillus reuteri* probiotic application after root surface debridement (RSD) with group of mechanical debridement alone at 3 months.
2. The probing pocket depth (PPD) between the group with *Lactobacillus reuteri* probiotic application after root surface debridement (RSD) with control group of mechanical debridement at 3 months.
3. The clinical attachment level (CAL) between the group with *Lactobacillus reuteri* probiotic application after root surface debridement (RSD) with control group of mechanical debridement at 3 months.
4. The plaque index (PI) between the group with *Lactobacillus reuteri* probiotic application after root surface debridement (RSD) and control group with mechanical debridement at 3 months.

5. The gingival index (GI) between the group with *Lactobacillus reuteri* probiotic application after root surface debridement (RSD) and control group with mechanical debridement at 3 months.

7. Scope and Limitations

This study will focus on the applications of *Lactobacillus reuteri* probiotic as local delivery agent in management of periodontitis. This agent will act as an adjunct in non-surgical periodontal therapy and will be applied subgingivally into the pocket after root surface debridement procedure. This new invention using probiotics will give advantages in this field. However, there are certain limitations that might affect the outcome of this study. For example, it is impossible to draw any firm conclusions, given the wide variety of delivery vehicles (liquid drops, lozenges, powder etc.).

8. Rationale of Study

To date, effectiveness of local drug delivery as an adjunct has been proven (Tan et al., 2020), but studies regarding effectiveness of probiotic as an adjunct is still lacking. There has been no study conducted specifically focusing on the effectiveness of *Lactobacillus reuteri* probiotic as an adjunct in subgingival debridement clinically for patients with type 2 diabetes mellitus. In the past decade, there has been a growing interest in utilising probiotics to improve periodontal health. Initially, experimental studies in animal models explored the use of *Lactobacillus spp.* and *Bifidobacterium spp.*, revealing a reduction in periodontitis (Teughels et al., 2007). Subsequent studies investigated the

application of probiotics in individuals with periodontal disease and concluded that the depth of periodontal pockets in humans also decreases following the use of probiotics as part of periodontal treatment (Lee et al., 2015; Iniesta et al., 2012).

There were also reported positive effects of the probiotic at both the microbiological and immunological levels. Vivekananda and colleagues demonstrated a significant reduction in counts of *Aggregatibacter Actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Prevotella intermedia* through the use of *Lactobacilli reuteri* lozenges (Vivekananda et al., 2010). The utilisation of *Lactobacillus reuteri* probiotic has also demonstrated a notable reduction in pro-inflammatory markers, including TNF- α , IL-1 β , IL-8, and MMP-8, as evidenced by studies (Ince et al., 2015; Szkaradkiewicz, Stopa, & Karpinski, 2014; Twetman et al., 2009). Additionally, an increase in anti-inflammatory markers such as TIMP-1 was observed (Ince et al., 2015).

Amid all these, *Lactobacillus reuteri* probiotic application clinically in type 2 diabetes mellitus patients has not yet been studied. Therefore, this study is conducted to assess its effectiveness, clinically.

9. Literature Review

Since decades, scaling and root planing has been introduced and used as the most effective treatment for removing plaque and calculus subgingivally for management of periodontitis (Rabbani et al., 1981). This mechanical action of debridement will help in removing the bacterial loads on the rough surface of the calculus. However, its complete removal is almost impossible especially on a deep pocket and this leads to the

introductions of different types of chemical antimicrobial agents that can be used locally as an adjunct.

Nevertheless, the use of these chemical antimicrobial agent sometimes can present with unpredictable unwanted effect. The shift towards a less pathogenic subgingival microbiota composition, even when complemented with antiseptics or antibiotics, appears to be only temporary (Quirynen et al., 2002). There is also the potential for the emergence of antibiotic-resistant bacterial strains (Kapoor et al., 2012). Due to the side effects associated with chemical antimicrobial agent approaches, efforts have begun to investigate the use of probiotics as a method for modulating the composition of pathogenic biofilms, particularly when combined with scaling and root planing (Teughels et al., 2008).

The World Health Organisation (WHO) defines probiotics as live cultures of microorganisms that, when administered in sufficient doses, confer health benefits on the host (www.who.int/entity/foodsafety/fs_management/en/probiotic_guidelines.pdf). Research has underscored success in various medical domains, particularly in the treatment of diseases associated with the gastrointestinal tract and oropharyngeal infections (Teughels et al., 2008; Devine & Marsh, 2009). Nevertheless, the precise role of probiotics in modulating periodontal diseases is not yet fully comprehended.

Teughels et al. (2007) reported that the application of beneficial bacteria in beagle dogs after scaling and root planing (SRP) led to a delayed and reduced re-colonisation of periopathogens, along with decreased inflammation. The use of probiotic lactic acid bacteria may enhance gingival health by reducing gingival bleeding and lowering the levels of periodontal pathogens associated with diabetes and other diseases (Kang et al., 2020). At the local level, the production of lactic acid, which penetrates the bacterial membrane,

causing a decrease in pH in the bacterial cytoplasm, thereby inhibiting the proliferation of *Porphyromonas gingivalis*, *Streptococcus mutans* (Sookkhee, Chulasiri & Prachyabrued, 2001) and *Prevotella intermedia* (Köll-Klais et al., 2005).

When talking about the application of probiotic bacteria, whether in the form of a rinsing liquid or a solid substance, within the periodontal pocket disrupts the relationship between these probiotics and periodontal pathogens (Pangsomboon et al., 2006). This process results in a reduction of harmful bacteria within periodontal pockets, highlighting the effectiveness of probiotic bacteria. Numerous strains of probiotic bacteria, including *Lactobacillus spp.*, *Bifidobacterium spp.*, and *Saccharomyces spp.* (Elahi et al., 2005; Gröschl et al., 2009), have been identified and are considered the most important for the treatment of periodontitis.

Probiotic organisms are believed to operate through diverse mechanisms, including the exclusion and competition with potential pathogens for nutrients and epithelial cell adhesion, the production of antimicrobial substances targeting periodontopathogens, local and systemic immunomodulation, and the enhancement of mucosal barrier function (Teughels, Loozen & Quirynen, 2011). Among these mechanisms, systemic immunomodulation is a key target of probiotics, regulating the production of both anti-inflammatory and pro-inflammatory cytokines (Williams, 2010).

Lactobacillus reuteri is recognised for its production of an antimicrobial substance called reuterin, which effectively inhibits a broad spectrum of pathogenic bacteria. Additionally, strains of *Lactobacillus reuteri* have exhibited the capability to impede the binding of pathogenic bacteria to host tissues (Stamatova & Meurman, 2009). The potential mechanisms of action exhibited by *Lactobacillus reuteri*, including the production

of antimicrobial substances and the prevention of pathogenic bacterial binding, could form the foundation for its direct or indirect anti-plaque properties (Krasse et al., 2006; Stamatova & Meurman, 2009). The substantial enhancements observed in probing pocket depth and attachment levels could potentially be attributed to the resolution of inflammation through the anti-inflammatory effects exerted by probiotics (Krasse et al., 2006; Twetman et al., 2009). Since periodontitis itself is a chronic inflammatory disease, the anti-inflammatory effects of *Lactobacillus reuteri* probiotic definitely make a contribution.

Current in the field, the study by Teughels et al. (2013) indicated that, the adjunctive use of *Lactobacillus reuteri* lozenges throughout 12 weeks led to significant additional clinical improvements, particularly in initially moderate to deep pockets, compared to scaling and root planing (SRP) alone. In 2020, Laleman et al. showed an added favourable clinical impact, with the application of *Lactobacillus reuteri* probiotics for 12 weeks, specifically in the form of lozenges, resulted in statistically significantly lower probing pocket depth (PPD) compared to the control lozenges after 24 weeks. This difference was also particularly pronounced in moderate and deep pockets. Nevertheless, no statistically significant differences were observed for any of the examined clinical parameters in the probiotic lozenge group between the quadrants where the control drops were applied and the quadrants where the probiotic drops were locally applied. Despite this, a pilot study by Minić, Pejčić & Bradić-Vasić (2022) asserted that incorporating the local adjunct application of probiotics for a period of 5 days, alongside scaling and root planing (SRP) during periodontal treatment have demonstrated a remarkable clinical effect in reducing the depth of periodontal pockets after 30 days.

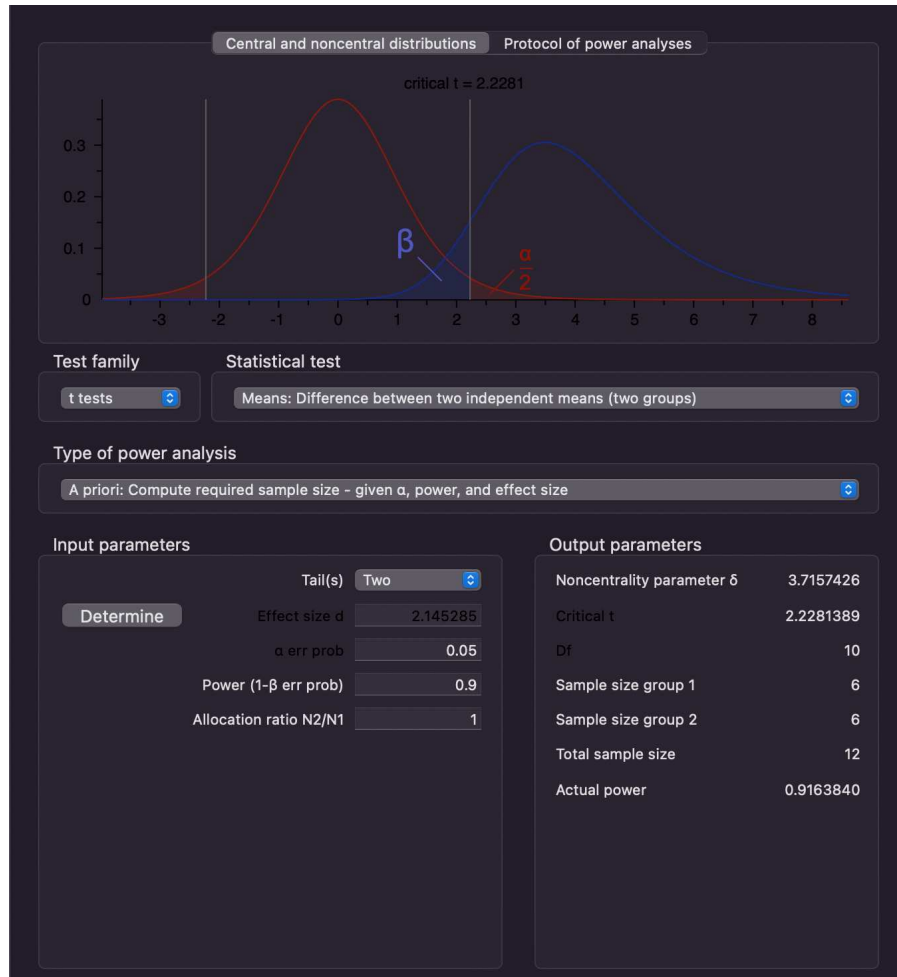
Despite all the benefits listed, the effectiveness of *Lactobacillus reuteri* probiotic apply locally in helping treating periodontitis with deep pocket is still unclear. With the proven benefits, this study will eventually help open a pathway for the usage of probiotics as an adjunct for management of periodontitis in future.

10. Methodology

10.1. Preparation of *Lactobacillus Reuteri* Probiotic

For this study, we will be using the BioGaia probiotic drops containing a minimum of 100 million live patented lactic acid bacterium *Limosilactobacillus reuteri* (*L. reuteri* DSM 17938) per dose which is 'HALAL' certified and is fit for consumption by human and also recommended for oral intake.

10.2. Sample Size Calculations



Sample size calculation done using G-power sample size calculator version 3.1.9.6 (Kang, 2021). The sample size calculation is based on the previous study by Minić and colleagues on the effect of the local probiotics in the therapy of periodontitis: a randomised prospective study (Minić, Pejčić, & Bradić-Vasić, 2022). The statistical test was set as “means: difference between two independent means (two groups)”, with an alpha error of 5%, and 90% statistical power. The minimum total sample size required is 12. Considering the probability of drop out rate 10%, 14 patients will be included.

$$\underline{12 + (12 \times 0.10) = 14}$$

10.3. Subjects

All periodontitis patients who attended MAHSA Dental Clinic are eligible for this study. Screening will be done by main examiner and 14 subjects who fulfil the inclusion and exclusion criteria will be recruited with consent. Dental Panoramic Tomogram (DPT) will be then taken to confirm the presence of bone loss with expert periodontist and alginate impression will also be taken to fabricate acrylic stents with reference marks for both arches on each patient for pocket localisation to determine exact measurement sites in every visits.

Inclusion Criteria
<ul style="list-style-type: none">• Periodontitis patient aged between 18 and 65 years.• Periodontitis patient with controlled type 2 diabetes mellitus ($HbA1c < 7\%$).• Periodontitis patient present with Stage II, Stage III and Stage IV periodontitis, based on New 2017 Classification of Periodontal and Peri-Implant Disease and Conditions (Papapanou et al., 2018) with at least 20 teeth present.• No history of antibiotics or probiotics for any purpose and periodontal therapy within the past 6 months.

Exclusion Criteria

- Involvement in another clinical trial.
- Pregnancy or lactation.
- Smoker/ alcoholic.
- Completely edentulous individuals.
- Individuals with a history of systemic diseases, such as acquired immune deficiency syndrome, cardiovascular disorders, hepatic diseases (including hepatitis b and c) and renal diseases.
- Individuals with overlapping teeth.
- Individuals that reported to have undergone periodontal therapy within the past 6 months.
- Individuals that reported to have used antibiotics, probiotics, non-steroidal anti-inflammatory drugs and/ or steroid use within the past 6 months.
- Individuals with physical limitation that might hinder proper oral hygiene procedures.

10.4. Study Design

This is a single blinded, prospective, randomised split-mouth clinical trial, where quadrants will be divided randomly into control and test groups.

Baseline records of periodontal clinical parameters will be taken for all subjects which include; bleeding on probing index (BoP), probing pocket depth (PPD), clinical

attachment level (CAL), plaque index (PI) and gingival index (GI). All the clinical records will be kept in locked case and delivered after the end of the clinical trial. University of North Carolina (UNC-15) probe will be used to measure the periodontal pocket depth and CAL.

All patients, after the baseline parameters recorded, will then receive motivation and oral hygiene instructions on standard effective oral hygiene practice which include tooth brushing, flossing, interdental brush usage and single tufted brush, followed by full mouth root surface debridement (RSD) under local anaesthesia on all teeth with pocket of ≥ 5 mm (Heitz-Mayfield, 2005; Matesanz-Pérez et al., 2013) using ultrasonic scaler and Gracey's curette, until the root surface feels smooth and clean to an explorer tip of UNC-15 probe (Kaner et al., 2007). For each patient, complete full mouth professional mechanical plaque removal (PMPR) and RSD will be done within 24 hours. All these will be done by main examiner, YKZ.

Both right (quadrant 1 and 4) and left (quadrant 2 and 3) halves will be assigned randomly to one of the two treatment modalities. A simple randomisation method will be employed to assign 14 participants into two groups (Group 1 and Group 2 corresponding to right halves: test and left halves: control or right halves: control and left halves: test) alternatively. A series of fourteen unique random numbers will be generated using the Numbers RAND() formula, one for each. Patients will then be alternately assigned to Group 1 and Group 2, starting with Group 1, done by supervisor, FHA. The treatment allocation cards will be sealed in envelopes until the clinical procedure.

The subgingival applications of either control (sterilised distilled water) or test (*Lactobacillus reuteri* probiotic) drops will be done based on the randomisation sequences done by FHA before. The allocated group for every subject will only be told to YKZ on the

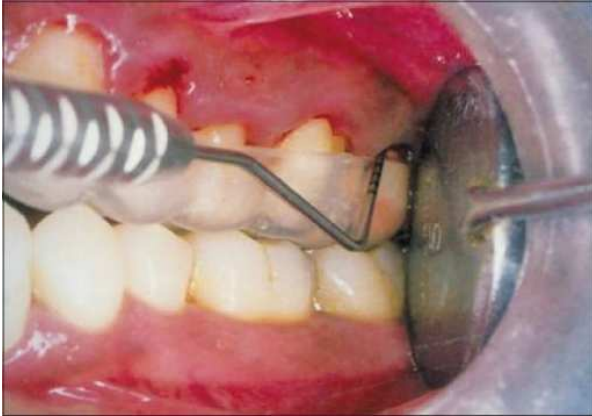
day of applications. Post-operative instructions will be given after the applications to all subjects. Each subject will also be given the same oral hygiene kit containing soft bristle toothbrush, single tufted brush, interdental brush, fluoridated toothpaste and dental floss. The applications will be repeated again in both groups after initial applications on every 7, 14, 21 and 28 days (Sufaru et al., 2022), all by student YKZ.

Follow up visit to evaluate all clinical parameters will be done on every subject, at three months (Segelnick & Weinberg, 2006) after treatment (RSD) by main examiner, YKZ.

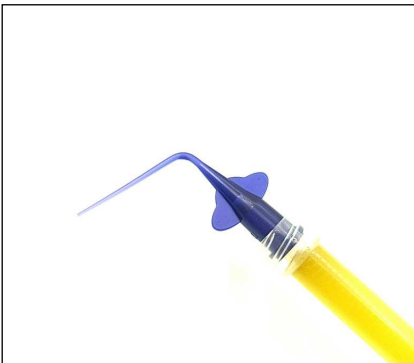
10.5. *Lactobacillus Reuteri* Probiotic Applications

The subgingival applications of control (sterilised distilled water) and test (*Lactobacillus reuteri* probiotic) drops will be done after RSD on all pockets of $\geq 5\text{mm}$ (Matesanz-Pérez et al., 2013; Passariello et al., 2012), on each groups, in the same day after RSD completion. The subgingival applications will be done using 1mL disposable syringe attached with blunt soft Intra Vascular cannula tip (22 Gauge). Isolation will be done using cotton roll and saliva suction will be used to avoid saliva contaminations as possible. The control/ *Lactobacillus reuteri* probiotic drops will be introduced into the pocket, until overfill is observed at the gingival margin (Griffiths et al., 2000; Unsal et al., 1994). Subjects will then be instructed not to eat, drink or rinse for at least 30 minutes. Only tooth brushing will be instructed to subject and to avoid sticky food and chewing hardly on the applied area. The control and *Lactobacillus reuteri* probiotic drops applications will be repeated every 7, 14, 21, and 28 days (Sufaru et al., 2022), following the same duration procedure. Oral hygiene instruction will also be re-emphasised on each

visit. Follow up visit for assessment of clinical parameters (BoP, PPD, CAL, PI and GI) will be done at 3 months interval for both groups.



Picture showing example of acrylic stent with marking. *Journal of Indian Society of Periodontology - Vol 13, Issue 2*



Picture showing a 1mL disposable syringe attached with blunt soft flexible tip (0.25mm) which will be used for subgingival applications of control and *Lactobacillus reuteri* probiotic drops.

10.6. Clinical Parameters

Bleeding on Probing (BoP)	(Mühlemann & Son, 1971)
Probing Pocket Depth (PPD)	(Ramfjord, 1967)
Clinical Attachment Level (CAL)	(Ramfjord, 1967)
Plaque Index (PI)	(Löe, 1967)
Gingival Index (GI)	(Löe, 1967)

List of parameters that will be recorded.

1. Bleeding on Probing (BoP); bleeding on probing will be recorded using Sulcus Bleeding Index (SBI) introduced by (Mühlemann & Son, 1971).

Score	Criteria
0	gingiva of normal texture and colour, no bleeding.
1	gingiva apparently normal, bleeding on probing.
2	bleeding on probing, change in colour, no oedema.
3	bleeding on probing, change in colour, slight oedema.
4	either: (a) bleeding on probing, change in colour, obvious oedema; or (b) bleeding on probing, obvious oedema.
5	bleeding on probing and spontaneous bleeding, change in colour, marked oedema.

Graduated University of North Carolina (UNC-15) probe will be used to probe the pocket up until the bottom on four sites of tooth surfaces (the labial, lingual, mesial and distal sites). Then, the bleeding on probing score will be recorded separately on

each four sites according to sulcus bleeding index score as above. Bleeding on probing score of the individual tooth gain by adding all the bleeding score on four sites, divided by four and the bleeding score for an individual gain by adding all the individual tooth score, divided by total numbers of teeth present.

2. Probing Pocket Depth (PPD); the measurement from the gingival margin to the bottom of the pocket will be measured using graduated University of North Carolina (UNC-15) probe to the nearest mm on all six sites of each tooth (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual and disto-lingual) (Ramfjord, 1967).
3. Clinical Attachment Level (CAL); the measurement from the cemento-enamel junction (CEJ) to the base of the pocket will be measured using graduated University of North Carolina (UNC-15) probe to the nearest mm on all six sites of each tooth (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual and disto-lingual). If the gingival margin located above the CEJ level, then CAL will be gain by subtracting the probing pocket depth with the distance of gingival margin to the CEJ. If the gingival margin located below the CEJ, then the CAL measurement gain by adding the probing pocket depth with the gingival recession measured (Ramfjord, 1967).

4. Plaque Index (PI); plaque index will be recorded using Silness and Loe, 1967 index.

Score	Criteria
0	No plaque in the gingival area.
1	A film of plaque adhering to the free gingival margin and adjacent area of the tooth. The plaque may only be recognized by running a probe across the tooth surface.
2	Moderate accumulation of soft deposits within the gingival pocket, on the gingival margin and/ or adjacent tooth surface, which can be seen by the naked eye.
3	Abundance of soft matter within the gingival pocket and/ or on the gingival margin and adjacent tooth surface.

Four surfaces of each tooth will be evaluated (buccal/ labial, mesial, distal and lingual/ palatal) and given the score accordingly. To get the score for each tooth, all score from the four area will be added and divided by four. Finally, to get the individual plaque index, all the tooth score will be added and divided by the number of teeth present (Löe, 1967).

5. Gingival Index (GI); Gingival index will be recorded using Loe and Silness 1967 index (Löe, 1967).

Score	Criteria
0	Normal gingiva.
1	Mild inflammation — slight change in color, slight oedema. No bleeding on probing.

2	Moderate inflammation — redness, oedema and glazing. Bleeding on probing.
3	Severe inflammation — marked redness and oedema. Ulceration. Tendency to spontaneous bleeding.

To assess gingival index score, the qualitative changes of the gingiva soft tissue will be examined by direct vision and gentle probing using UNC-15 probe up until the gingival crevice only. Each tooth will be evaluated at 4 sites; buccal/ labial, mesial, distal and lingual/ palatal, and will be given the score according to the criteria listed. To get the score for individual tooth, all the score at the four sites will be added and divided by four. Meanwhile, for individual score, all gingival index score for each tooth will be added and divided by the total number of teeth present.

10.7. Group Allocations

Both right (quadrant 1 and 4) and left (quadrant 2 and 3) halves of all 14 participants, split mouth design will be assigned randomly to one of two treatment modalities via computer generated random sequence done by supervisor. A simple randomisation method will be employed to assign 14 participants into two groups (Group 1 and Group 2 corresponding to right halves: test and left halves: control or right halves: control and left halves: test) alternatively. A series of random numbers will be generated using the Numbers RAND() formula, done by supervisor, FHA. The RAND() function in Numbers produces uniformly distributed random numbers between 0 and 1. Fourteen unique random numbers will be generated, one for each participant in the study. The assigned random numbers will be sorted in ascending order from smallest to largest. This

step ensured that the patients will be arranged in a random sequence. Patients will then be assigned to two groups (Group 1 and Group 2) alternatively, starting with Group 1. This process will start with Group 1 and continue with Group 2, then back to Group 1, and so forth, until all patients were assigned to a specific group. The treatment allocation cards were sealed in envelopes until the clinical procedure.

The random allocations sequences will be concealed in a sequential sealed opaque envelope by FHA and will only be opened on the day of intervention's applications. Subgingival applications of either control drops and *Lactobacillus reuteri* probiotic drops will be done by YKZ.

Groups	Treatment
Control Halves	<p>On day 0, baseline record will be taken. Then, subjects will receive motivation and oral hygiene instructions on standard effective oral hygiene practice which include tooth brushing, flossing, interdental brush usage and single tufted brush, followed by professional mechanical plaque removal (PMPR) and root surface debridement (RSD). Following this, the control quadrants will be receiving application of the control drops (sterile distilled water) immediately after RSD together with post-operative instructions. Oral hygiene kit will also be given. (YKZ)</p> <p>Reapplications of control drops together with oral hygiene review visit will be done on day 7, 14, 21 and 28 days after the first subgingival control applications. (YKZ)</p> <p>Follow up visit for assessment of BoP, PPD, CAL, PI and GI will be done at 3 months interval from the baseline. (YKZ)</p>

Test Halves	<p>On day 0, baseline records will be taken. Then, subjects will receive motivation and oral hygiene instructions on standard effective oral hygiene practise which include tooth brushing, flossing, interdental brush usage and single tufted brush, followed by professional mechanical plaque removal (PMPR) and root surface debridement (RSD). Following this, the control quadrants will be receiving application of the test drops (<i>Lactobacillus reuteri</i> probiotic) immediately after RSD together with post-operative instructions. Oral hygiene kit will also be given. (YKZ)</p> <p>Reapplications of test drops together with oral hygiene review visit will be done on day 7, 14, 21 and 28 days after the first subgingival control applications. (YKZ)</p> <p>Follow up visit for assessment of BoP, PPD, CAL, PI and GI will be done at 3 months interval from the baseline. (YKZ)</p>
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Treatment for root surface debridement will be done under local anaesthesia on all subjects by single clinician, YKZ for all 14 patients at Day 0, right after baseline parameters recorded and oral hygiene instructions and motivations given. All patients will be recall back after 3 months to assess the clinical parameters (BoP, PPD, CAL, PI and GI) by YKZ.

10.8. Examiner Calibration

To ensure reliability of the parameter's measurements, intra and inter-examiner calibration will be done prior the study.

1. Intra-Examiner Calibration

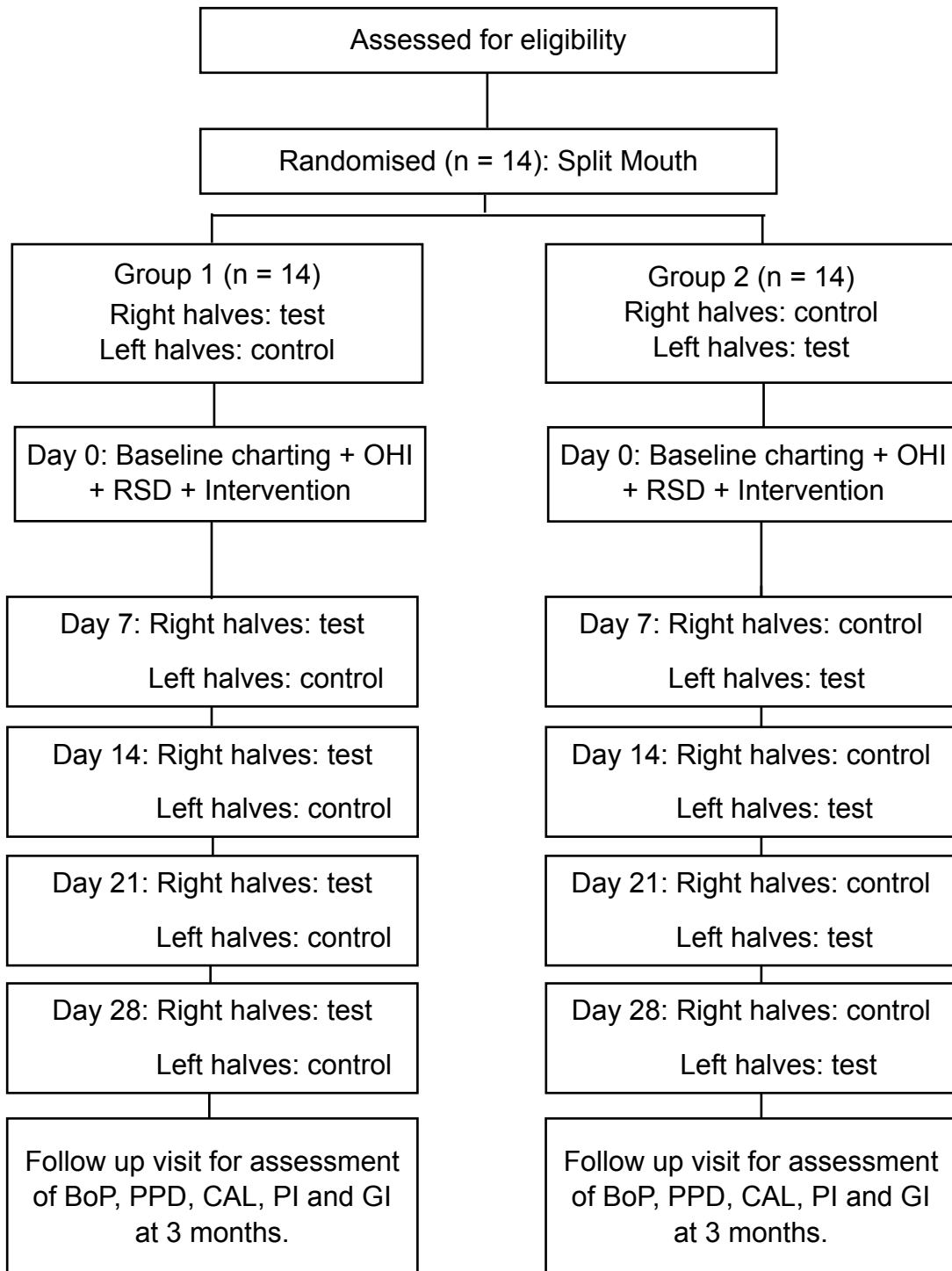
The examiner (YKZ) will assess the clinical parameters of five subjects in two separate sessions within 2 hours interval (Drucker et al., 2012; Polson, 1997). For periodontal pocket depth and clinical attachment level measurements, graduated UNC-15 probe with 1mm interval will be used. Only one quadrant will be assessed on each subject. Examiner will probe all six sites of each tooth in the chosen quadrant. Probing depth is measured in nearest mm between gingival margin up to the base of the pocket, while clinical attachment level is measured between the cemento-enamel junction (CEJ) to the base of the pocket. The accepted measurement difference was within $\pm 1\text{mm}$ (Hill et al., 2006). For bleeding on probing, plaque index, and gingival index; it will be recorded using Sulcus Bleeding Index (SBI) by Mühlemann & Son (1971), plaque index and gingival index by Löe (1967) accordingly, as stated above. Intra-examiner calibration is accepted when the intra-class correlation coefficient (ICC) value is ≥ 0.75 (Koo & Li, 2016) for each parameters.

2. Inter-Examiner Calibration

For this calibration, the supervisor, Professor Dr Fouad Hussain Al-Bayat, a periodontal specialist and an expert examiner will supervise and examine the inter-examiner agreement.

Three subjects (Polson, 1997) will be examined by both examiner (YKZ and FHA) within 2 hours interval (Drucker et al., 2012; Polson, 1997). For periodontal pocket depth and clinical attachment level measurements, graduated UNC-15 probe with 1mm interval will be used. Examiners will probe all six sites of each tooth in the chosen quadrant. Probing depth is measured in nearest mm between gingival margin up to the base of the pocket, while clinical attachment level is measured between the cemento-enamel junction (CEJ) to the base of the pocket. The accepted measurement difference was within $\pm 1\text{mm}$ (Hill et al., 2006). For bleeding on probing, plaque index, and gingival index; it will be recorded using Sulcus Bleeding Index (SBI) by Mühlemann & Son (1971), plaque index and gingival index by Löe (1967) accordingly, as stated above. Inter-examiner calibration is accepted when the ICC value is ≥ 0.75 (Koo & Li, 2016) for each parameters.

10.9. Study Flow Chart



11. Statistical Analysis

All data will be collected in numerical form and will be analysed using the IBM SPSS Statistics Version 26.0 software. Histogram plot of data will be first used to check for normality of data distribution. Then, either Independent t-Test or Mann-Whitney Test will be used to compare data between two groups. The variables will be presented as mean and standard deviation for parametric test or median and interquartile range for non-parametric test. Significant level will be set at p-value $p < 0.05$. Meanwhile data within groups to evaluates statistically significant changes from baseline to follow up visits will be analysed either using Paired t-Test or Wilcoxon Signed Ranked Test.

Parameter	Visit	Control group	Test group	p-Value
BoP	Baseline			
	3 months			
PPD (mm)	Baseline			
	3 months			
CAL (mm)	Baseline			
	3 months			
PI	Baseline			
	3 months			
GI	Baseline			
	3 months			

Clinical Parameters for Groups 1 and 2 (mean \pm SD or median (IQR)) at different time intervals

(*denotes statistically significant difference, $p < 0.05$)

12. Budget

	Quantity	Cost/ Unit	Total Cost
Disposable syringe (1ml) (100's/ box)	1	RM 50.00	RM 50.00
Flexible tip (0.25mm) (100's/ box)	1	RM 200.00	RM 200.00
Gracey curettes	28	RM 209.25	RM 5859.00
Sharpening kit	1	RM 2442.00	RM 2442.00
Small cassette	3	RM 592.50	RM 1777.50
<i>Lactobacillus reuteri</i> probiotic	20	RM 134.50	RM 2690.00
Acrylic stents	20	RM 200.00	RM 4000.00
Oral hygiene kit	20	RM 100.00	RM 2000.00
		T O T A L COST	RM 19,018.50

13. Gantt Chart

	2023	2024				2025				2026			
	NOV- DEC	JAN- MAR	APR- JUN	JUL- SEP	OCT- DEC	JAN- MAR	APR- JUN	JUL- SEP	OCT- DEC	JAN- MAR	APR- JUN	JUL- SEP	OCT- DEC
Drafting proposal													
Defence of research proposal (DRP)													
Ethics application													
Data collection preparation													
Data collection													
Thesis write-up													
Submission research paper													
Thesis submission for viva													
Research sends for publication													

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Appendix 1: Patient Information Sheet

1. Full title of the study

Local Delivery of *Lactobacillus Reuteri* Probiotic as an Adjunct to Non-Surgical Periodontal Treatment in Patients with Stage II, III, and IV Periodontitis and Type 2 Diabetes Mellitus: A Prospective Split-Mouth Clinical Trial.

2. Name of investigator and institution

Name of Investigator : Dr Yeu Kai Zhe (**YKZ**)

Name of supervisor : Professor Dr Fouad Hussain Al-Bayaty

Institution : Faculty of Dentistry, MAHSA University

3. Introduction

You are invited to participate in a research study because you have periodontitis that requires non-surgical periodontal treatment. This document outlines the specifics of the research trial. It is crucial that you understand the purpose of the research and its associated commitments. Take the necessary time to read through and carefully consider this information before deciding on your willingness to participate. If you have any uncertainties or require additional details, feel free to ask. Once you are confident in your understanding and wish to take part, please sign the informed consent form. To facilitate

your participation, you may need to share your health history with your doctor (YKZ), and it is important to provide accurate information to avoid potential harm to yourself.

4. What is the purpose of the study?

The purpose of the study is to evaluate the effectiveness of local delivery of *Lactobacillus reuteri* probiotic on periodontitis patients with type 2 diabetes mellitus, as an adjunct to non-surgical periodontal treatment. To date, the efficacy of local drug delivery as an adjunct has been established, whereas research on the effectiveness of probiotics as an adjunct remains limited. Specifically, there is a lack of studies focusing on the clinical effectiveness of *Lactobacillus reuteri* probiotic as an adjunct in subgingival debridement for patients with type 2 diabetes mellitus.

Over the past decade, there has been a growing interest in using probiotics to enhance periodontal health. Subsequent studies investigated the application of probiotics in individuals with periodontal disease, concluding that the depth of periodontal pockets in humans also decreases with probiotic use as part of periodontal treatment. Positive effects of probiotics have been reported at both the microbiological and immunological levels. *Lactobacillus reuteri* probiotic has also shown a notable decrease in pro-inflammatory markers and an increase in anti-inflammatory markers. Despite these findings, the clinical application of *Lactobacillus reuteri* probiotics in patients with type 2 diabetes mellitus has not yet been explored.

A total of 14 subjects like you will be participating in this study. The whole study will last about 18 months and your participation will be about 3 months duration.

5. What kind of study procedures will I receive?

If you agree to participate in the study, the doctor (YKZ) may need to perform some tests and examinations to determine if you are suitable for the study. If you are deemed suitable, you will be assigned to both of the treatment groups below (split-mouth study design). Both right (quadrant 1 and 4) and left (quadrant 2 and 3) halves will be assigned randomly to one of the two treatment modalities. The study products do not contain porcine, bovine or animal components.

5.1. Test group:

- a. Baseline charting
- b. Oral hygiene instructions (OHI) + Professional mechanical plaque removal
- c. Root surface debridement (RSD)
- d. *L. reuteri* probiotic subgingival applications on all pockets of $\geq 5\text{mm}$

5.2. Control group: (Looks like the study treatment but has no active medication.)

- a. Baseline charting
- b. Oral hygiene instructions (OHI) + Professional mechanical plaque removal
- c. Root surface debridement (RSD)
- d. Sterilised distilled water subgingival applications on all pockets of $\geq 5\text{mm}$

The applications will be repeated again in both groups after initial applications on every 7, 14, 21 and 28 day.

6. What are my responsibilities when taking part in this study?

It is important that you answer all of the questions asked by the study staff honestly and completely. If there are any changes in your condition or circumstances during the study, you must promptly inform the study doctor (YKZ). Certain medications may be restricted during your participation, and the doctor (YKZ) will provide guidance on this. Notify your study doctor (YKZ) immediately if you make any alterations to your existing treatments, even those which you have been taking for a long time.

It is very important that your study doctor (YKZ) be informed very rapidly of any eventual changes to your health during your participation in the study. For your own security, it is important that you follow your study dental surgeon's instructions throughout the entire duration of the study.

7. What kind of treatment will I receive after my participation in the trial?

Whether you complete the study or withdraw early, your doctor (YKZ) will discuss the best options for your future treatment with you.

8. What are the potential risks and side effects of being in this study?

The risks for probiotic adjunctive non-surgical periodontal therapy, while generally considered safe, may pose some potential risk factors. Some potential risk factors may include:

a. Temporary discomfort; Some individuals may experience temporary discomfort or pain following non-surgical periodontal procedures, such as scaling and root surface debridement. This discomfort typically subsides within a few days.

b. Gingival and/ or root sensitivity; Some individuals may experience increased sensitivity in the gums or teeth, particularly to hot or cold temperatures, after non-surgical periodontal therapy.

c. Bleeding gums; Individuals might notice temporary bleeding from the gums, especially during and immediately after the procedure. This is usually minor and resolves with proper post-operative care.

d. Recession of gums; In rare instances, there may be a risk of gingival recession, where the gums pull away from the teeth. This is more likely in individuals with thin or fragile gum tissue.

e. Infection; While rare, there is a potential risk of infection, particularly if proper hygiene practices are not followed after the procedure.

f. Allergic reactions; Some individuals may be allergic to probiotic used during adjunctive non-surgical periodontal therapy, leading to allergic reactions (skin rashes or itchiness).

If you require additional information regarding risks and side effects, please consult your study doctor (YKZ). The trial staff will promptly update you on any new discoveries or modifications to the study product that may impact your health or willingness to continue in this study. If needed, you may be requested to provide your consent to participate again.

9. What are the benefits of being in this study?

Participating in this study, you will receive a complete set of oral hygiene kit. While participating, you may or may not experience direct benefits. Nonetheless, the data and information collected will contribute to advancing the treatment or management of individuals with the same disease or condition.

10. What are my alternatives if I do not participate in this study?

Yes, you have the right to refuse to take part in the study at any time. You do not have to participate in this study to get treatment for your periodontal disease. Your participation in the research is entirely voluntary, and you can choose not to participate without any obligation or negative consequences.

11. Who is funding the research?

This study is supported by postgraduate research grant from the MAHSA University for all study materials, equipments, and procedures.

12. Will my personal information be kept private?

All your information obtained in this study will be kept and handled in a confidential manner, in accordance with applicable laws and/ or regulations. When publishing or presenting the study results, your identity will not be revealed without your expressed consent. Individuals involved in this study, qualified monitors and auditors, and governmental or regulatory authorities may inspect and copy your medical records, where appropriate and necessary.

Data from the study may be archived for the purpose of analysis, but your identity will not be revealed at any time.

13. Who should I call if I have questions?

If you have any questions about the study or you want more information about the treatment, please contact the study doctor:

Name : Dr Yeu Kai Zhe

Call : +60179569319

Email : dperio23116011@mahsastudent.edu.my

Appendix 2: Informed Consent Form

Full title of the study: Local Delivery of *Lactobacillus Reuteri* Probiotic as an Adjunct to Non-Surgical Periodontal Treatment in Patients with Stage II, III, and IV Periodontitis and Type 2 Diabetes Mellitus: A Prospective Split-Mouth Clinical Trial

By signing below I confirm the following:

- a. I have received both oral and written information for the study mentioned above, and I have read and understood the provided information.
- b. I have had sufficient time to consider participating in the study and have been afforded the opportunity to ask questions. All of my inquiries have been answered to my satisfaction.
- c. I acknowledge that my participation in this study is voluntary, and I reserve the right to withdraw at any time without providing a reason. I understand that my decision to withdraw will not impact my future treatment. Currently, I am not involved in any other research study. I understand the associated risks and benefits, and I willingly provide my informed consent to participate under the specified conditions. I am aware of the necessity to follow to the study doctor's (investigator's) instructions throughout my participation in the study.
- d. I understand that study staff, qualified monitors and auditors, and governmental or regulatory authorities, have access to my record in order to make sure that the study is conducted correctly and the data are recorded correctly. All personal details will be treated as STRICTLY CONFIDENTIAL.

e. I will be provided with a signed and dated copy of this subject information/
informed consent form to take home.

Subject:

Signature : I/C number :

Name : Date :

Investigator conducting informed consent:

Signature : I/C number :

Name : Date :

Impartial witness: (Required if subject is illiterate and contents of patient information
sheet is orally communicated to subject)

Signature : I/C number :

Name : Date :