DETECTION OF LACTOBACILLUS REUTERI IN SALIVA USING FTA™ ELUTE CARDS AND POLYMERASE CHAIN REACTION-TECHNIQUE

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ABSTRACT

Background: Probiotic bacteria have been used for centuries to obtain a better health and the majority of publications have focused on the gut health. More recent studies do also investigate if the bacteria could have any effect in the oral microflora. Since probiotic products are becoming more common on the market, it is interesting to investigate if these probiotic bacteria exist in the oral cavity and if they exert any therapeutic effect on oral diseases. The aim of this study was to determine an uncomplicated method capable of measuring one bacterium associated with caries prophylactic properties.

Methods: Saliva from three test subjects were collected before and after chewing on a tablet containing Lactobacillus reuteri. DNA from each saliva sample was extracted using FTA™ elute cards and amplified with a PCR. Saliva samples were cultured on Rogosa agar for comparison. Saliva after chewing was diluted for determination of detection level using Rogosa agar counts as standard. Amplified samples were analysed from stained electrophoresis gels.

Results: The PCR method could detect Lactobacillus reuteri in saliva if the content was 250 CFU/mL or higher. An increase in CFU/mL in saliva after chewing can be observed. Saliva could before chewing show in two out of three test subjects no amplifiable DNA whilst after chewing all did.

Conclusions: A method considered uncomplicated that is capable of detecting Lactobacillus reuteri in saliva has been developed. Detection level was 250 CFU/mL.
1. INTRODUCTION

According to the ecological plaque hypothesis, dental caries is a bacterial induced disease resulting from a shift in the oral microflora depending on the dynamic balance between attack and defence factors of the oral cavity. No single bacterial species has been identified to be the unique cause, but *mutans streptococci* (MS) is often found in the biofilm over the caries lesion although not obliged. It suggests that bacteria associated with caries can be found in the dental plaque without disease developing, the bacterial counts could be too low. A change in the local environmental conditions such as repeated sugar intakes together with frequent low pH can cause the oral microflora to shift. Bacteria, such as MS with acidogenic and aciduric properties are favoured and allowed to grow in higher numbers. Under these circumstances MS can often be found associated with caries lesions and therefore are able to disturb the balance between tooth de- and remineralisation, with a net mineral loss. As a consequence of a net mineral loss, a caries lesion develops (Marsh, 2006; Fejerskov & Kidd 2008). The ecological plaque hypothesis also acknowledges the hosts defence mechanisms such as saliva flow as an important role of developing disease or not. Restoring the balance in the oral cavity for treating the disease is significant, both by enhancing the hosts defend mechanisms and change the oral microflora (Marsh & Martin, 2009a).

Today, caries treatment focuses on lowering the frequency of sugar intake and adding fluoride to the saliva (Marsh & Martin, 2009a; Mejáre et al., 2007). Such treatments are not always successful in curing the patient and new approaches for treatment options are developing. One optional caries treatment that is not yet fully investigated, to lower the counts of MS, is through adding probiotic bacteria in the oral cavity and thus changing the oral microflora (Marsh & Martin, 2009a; Mejáre et al., 2007; Meurman, 2005; Meurman & Stamatova, 2007; Takahashi & Nyvad, 2011). A normal oral cavity holds around 500-700 different bacterial species, but not all of them have yet been successfully documented. Of them have about 280 specific species been successfully documented (named and grown in laboratory) (Dewhirst et al., 2010). Approximately 1% of these bacteria are of the Lactobacillus species (Meurman & Stamatova, 2007). Some Lactobacilli and Bifidobacterium species are considered probiotic due to their beneficial effect to the host and are also commonly used as probiotics (Meurman & Stamatova, 2007). These bacteria are sometimes used as dietary supplement in different products available on the market in Sweden. Often in fermented-milk products, as cheese, sour-milk and yoghurt, but also in tablets, drops, chewing gum and fruit juices which are consumed daily by a part of the Swedish inhabitants. *Lactobacillus reuteri* (*L. reuteri*) is considered a probiotic bacterium which could be found in infant formula, gruel and tablets (Livsmedelsverket, 2011; Mejáre et al., 2007; Meurman & Stamatova, 2007; Shornikova et al., 1997). Since probiotics often are administered orally through fermented-milk products that contain calcium and phosphate ions can themselves offer caries prophylaxis through increased remineralisation (Ohlund et al., 2007).

Probiotics signifies “for life” and the World Health Organization has defined probiotics as “bacteria associated with beneficial effects for humans and animals” (World Health Organization: WHO, 2001). Probiotics have been used for centuries to obtain better health. The modern use was introduced by Elie Metchnikoff in the early 1900s. He discovered that lactic bacteria in food reduce harmful microbes in the gut. Lactic bacteria with more beneficial effect on the individual will colonize the gut and as a result of eating this food, life can be extended (Meurman, 2005).
Probiotics have gained much attention for their health-promoting effects and a number of articles have been published. Up until January 2014, more than 11 900 publications about probiotics have been published on PubMed on this theme (search term: probiotic). Among them 380 articles have been published about probiotics and oral health (search term: probiotic oral health) and 118 about probiotics and caries (search term: probiotic caries).

Early probiotic publications are mostly associated with gut health and like the oral cavity the intestine contains a number of different bacteria with different properties. Some of them are beneficial for the health by improving the host’s immune-defense and acting as a barrier or competitor towards intruding harmful bacteria. Whilst some of the bacteria in the gut release toxic substances and thus be considered harmful as for example certain Clostridium bacteria. If this complex balance in the intestine is disturbed, the harmful and toxin-releasing bacteria can cause disease. By adding probiotic bacteria, the disease-causing bacteria can decrease in counts and be replaced. *Lactobacillus rhamnosus GG* (LGG) is one example of a bacterium with demonstrated health promoting effects on the host, by adhering to the intestinal mucosa and releasing antimicrobial substances which can reduce Clostridium counts and stabilize the normal microflora (Gorbach, 2000; Salminen et al., 1998).

The majority of publications have focused on probiotics effect in the intestine, but as the oral cavity is the first part of the gastrointestinal tract they share several resembling properties and it is possible that some probiotics may have similar effects in the oral cavity (Meurman, 2005). For probiotics to have any effect against oral pathogens there are a few desirable properties they need to hold. The bacterium need to possess ability to adhere to the biofilm to compete with the growth space with the pathogens. It is desirable that they can secrete antimicrobial toxins to make the environment non-viable for pathogenic bacteria. The ability to co-aggregate and disturb attachments to the oral biofilm is also desired properties (Gorbach, 2000; Meurman, 2005). LGG appears to hold these properties, but it is not fully investigated if this bacterium has any effect on the oral microflora.

*L. reuteri* has been studied and appears to have probiotic properties which could have an effect on the oral microflora and immune-defence. *L. reuteri* is commonly found in healthy intestine and was primarily isolated from human breast-milk (Shornikova et al., 1997). *L. reuteri* produces a broad-spectrum antimicrobial, reuterin, which is effective over a broad pH range (Nikawa et al., 2004). *L. reuteri* have the possibility to co-aggregate and compete for space and nutrients with MS and can therefore inhibit growth of MS. Lowering MS counts could be thought to offer caries prophylaxis as MS is regularly associated with caries lesions (Haukioja et al., 2006, Meurman & Stamatova 2007, Twetman et al., 2009). There are contradictory studies that demonstrate no decrease in MS count after intake of *L. reuteri* (Keller et al., 2012). Recent published study from Stensson et al., 2013 presents that children who had received *L. reuteri* showed a decrease in caries prevalence (Stensson et al., 2013). It appears that probiotic Lactobacilli do not colonize the host permanently but have to be administered regularly (Keller et al., 2011; Meurman & Stamatova 2007, Ravn et al., 2012).

To investigate if Lactobacilli colonize the oral cavity after administration, a method for detection is needed. There are different methods to detect and specify bacteria in saliva. Method chosen for this study was conventional Polymerase Chain Reaction (PCR). Samples containing DNA was extracted with FTA™ (Flinders Technology Associates, Whatman™) elute cards. The FTA™ elute card is a membrane used for long-term storage and processing of nucleic acids (DNA). The chemical formula that is impregnated on the FTA™ elute card lyset cells, such as bacterial cell membranes and denatures proteins. Remaining DNA are protected from microbial and fungal attacks, making the PCR process more reliable. Using FTA™ elute
cards can consequently lead to fewer sources of error according to manufacturer’s manual, (Whatman, Part of GE Healthcare, 2012). With PCR it is possible to detect bacteria in a small amount. The method is widely used to detect specific DNA sequences by replicating selected DNA sequence. Two specific primers are constructed to fit a unique gene-sequence that only exists in chosen bacterium. The first primer is a start sequence and the second is a stop sequence which decides the size of the selected DNA sequence. After extracting DNA from saliva, primer and other functional components are mixed in a test tube and placed in the PCR-amplifier. The PCR-amplifier performs a replication of the selected specific DNA sequence throughout a series of heat and cool-down cycles. To analyse the results, all samples are applied to an electrophoresis-gel in which electricity will cause the DNA-molecules to move towards the bottom of the gel. Large sequences, with high molecular weight will move slower and appears in the top of the gel and the smaller near the bottom. Each DNA segment has a known molecular weight and when analysing the result, a positive result will appear as a band at the specific known part on the gel. The unit for molecular weight in this context is referred as base pair (bp). If the primer does not fit with any DNA sequence in sample, no amplification will occur and when analysing the results, no band will appear on the gel. The primers can also bind to another DNA sequence which can give a larger or smaller sequence than the desired. This can result in that bands can appear at a higher or lower part of the gel, therefore it is necessary to know how many bp the tested sequence have (Bio-Rad Laboratories, 2014). To be able to detect presence of bacteria, a sufficient amount of DNA is required. One method to get a higher concentration of DNA is to centrifugate the whole amount of saliva to a pellet. The total amount of DNA will be concentrated in the pellet and the sample should contain a higher concentration of the total DNA compared to if only a small amount of whole saliva was to be used instead. Considering this, it could hypothetical be easier to detect L. reuteri in the pellet.

PCR have been widely used for detection of specific bacteria but not often from whole saliva. Several previous studies (Dal Bello & Hertel, 2006; Román Vestman et al., 2013; Román-Méndez et al., 2009) frequently grow bacteria from saliva on agar plates before amplification in PCR. Only one study (Iniesta et al., 2012) to our knowledge has used PCR to detect L. reuteri in saliva without culture step. In this study, FTA™ elute cards were used for DNA extraction from saliva before amplifying in PCR, which to our knowledge never have been published earlier. It is interesting due to time and cost effectiveness to investigate if L. reuteri can be detected directly from saliva. When testing a method, it is necessary to investigate if the method is able to produce repeatable results. It is also desirable to investigate the lowest concentration of the specific bacteria that will produce positive measurable results.

Conventional PCR (qualitative PCR), which is used in the present study, does not quantitate DNA, but only detects presence or not. Investigation of the lowest concentration of bacteria that the sample can hold and still obtain positive results is referred as detection level. To confirm a detection level in this study, same saliva samples were cultured on lactobacilli specific Rogosa agar that allows counts of CFU/mL (colony-forming-units per mL).

Evaluating if probiotic bacteria could be a future treatment method against caries and other diseases is an interesting topic. Since probiotic products are becoming more common on the market, it is interesting to investigate if these probiotic bacteria exist in the oral cavity and if they have any therapeutic effect. The aim of this study was to determine an uncomplicated method capable of measuring one bacterium associated with caries prophylactic properties, L. reuteri.
1.2 Aims
1. The aim of the study was to determine a method capable of measuring *L. reuteri* in saliva with repeatable results. The method should be uncomplicated to perform.

2. To determine a detection level of *L. reuteri* in saliva with a conventional PCR-technique.

1.3 Hypothesis
1. It is possible with conventional PCR-technique to discover the presence of *L. reuteri* in saliva.

2. It will be easier to detect *L. reuteri* in pellet samples compare to whole saliva.

2. MATERIALS AND METHODS

Saliva was collected from three test subjects before and after chewing a tablet (BioGaia ProTectis, Medhouse, Sweden) containing a minimum of $10^8$ freeze dried cells of *L. reuteri* DSM 17938. Saliva was analysed for growth of *L. reuteri* on Rogosa agar. Samples were prepared for amplifiable DNA in; whole saliva, saliva pellet and colonies from Rogosa agar. The tablet used is considered a dietary supplement, (Dolecka et al., 2011). All samples were fresh, not dried or frozen when tested.

2.1 Saliva sample collection
Stimulated saliva was collected from three voluntary test subjects. A one-gram piece of unflavored paraffin wax (Orion diagnostic, Sollentuna, Sweden) was given to the test subjects for chewing under five minutes to obtain stimulated saliva (before chewing). Saliva was collected directly into a sterile tube.

After the first collection the test subjects did another donation (after chewing) where they started with chewing on a tablet containing *L. reuteri*. The tablet was chewed until it was dissolved. Saliva was swallowed before starting with chewing on the piece of paraffin. The same saliva collection procedure was implemented as above. All saliva samples were processed directly.

2.2 Culture growth and analysis
Saliva samples (100 µL) before chewing were spread on a sterile Rogosa agar plate (ROGOSA agar, Merck, Darmstadt, Germany) by using glass beads (5 mm). Saliva samples (100 µL) taken after chewing were serially diluted with sterile water in following order; undiluted saliva, saliva diluted 1/10, 1/100, 1/1000 and 1/10 000. All plates were incubated at 37°C for 72 h under anaerobic conditions (5% CO$_2$ and 95% N$_2$).

Determinations of CFU/mL in saliva were made by counting CFU on Rogosa agar and multiplying with dilution factor.

2.3 Preparation of samples for PCR including DNA extraction
DNA from whole saliva, saliva pellet, colonies from Rogosa agar and chewing tablet were extracted.
2.3.1 Preparation of saliva on FTA™ elute cards
Saliva samples, both before and after chewing were vigorously mixed (vortexed) using vortex-mixer (Sarstedt mono mixer, Germany). 20 µL of each sample was pipetted on to FTA™ elute cards allowed to dry overnight at room temperature.

Discs of 5 mm in diameter were punched out from each FTA™ elute card using a sterile hand-held hole-puncher and transferred into individual tubes of 1.5 mL. The tip of the puncher was cleaned between each punching with 70% ethanol to prevent cross contamination. The punched discs were processed according to manufacturers as described below in 2.3.5.

2.3.2 Preparation of saliva pellet on FTA™ elute cards
Saliva samples, both before and after chewing were centrifuged for 5 min at 5200 x g to make a pellet. Supernatant was removed using a pipette and 100 µL of sterile water was added. The tubes were vortexed and 20 µL of each sample was pipetted on to FTA™ elute cards allowed to dry overnight at room temperature. The pellet samples went through the same preparation procedure as described above in 2.3.1.

2.3.3 Preparation of samples from Rogosa agar plates
Several CFU was taken from each Rogosa agar with a sterile toothpick and placed in a sterile tube containing 100 µL of sterile water. The tubes were vortexed and 20 µL of each sample was pipetted on to FTA™ elute cards allowed to dry overnight at room temperature. Discs were prepared as described in 2.3.1.

2.3.4 Preparation of control samples from chewing-tablet
Control samples were made by mixing one finely ground tablet (same as used for chewing) containing L. reuteri in a sterile tube containing 100 µL of sterile water. The tubes were vortexed and 20 µL of each sample was pipetted on to a FTA™ elute card and allowed to dry overnight at room temperature. Discs were prepared as described in 2.3.1.

2.3.5 DNA extraction from FTA™ elute cards
The discs were washed in a sterile tube by adding 500 µL of sterile water, vortexed 3x 5 s, transferred into clean tubes containing 40 µL of sterile water and placed on a heat-block at 98°C for 20 min. The tubes were vortexed briefly 60 times for one second and the punched discs were removed from the tubes. The extracted DNA was stored at 4°C until PCR-amplification.

2.4 PCR-amplification
The primers (Table 1) used in this study were selected for amplifying species-specific L. reuteri (Dommels et al., 2009) and purchased from Life Technologies Invotrogen, Sweden.

The PCR-amplifications were implemented by mixing following components in a sterile tube before placing them in the PCR-amplifier (2720 Thermal cycler, Applied Biosystems Foster City, CA 94404 USA):
1. 1 PureTaq Ready-To-Go PCR-bead (GE Healthcare, Buckinghamshire, UK).
2. 2 µL primer-solution (L-reu1+L-reu4) in a concentration of 0.5 µM of each primer.
3. 8 µL of extracted DNA sample (see heading 2.3).
4. 15µL of sterile water was added to adjust the reaction mix to a final volume of 25 µL.

Amplifications were performed using the following cycle profile: The samples were heated initially to 95°C for 10 min, followed by 30 cycles of denaturation at 95°C for 15 s, annealing 60°C for 1 min and extension 72°C for 30 s, and an ending step with 72°C for 7 min. Final
hold-temperature 4°C. Amplified samples were stored at 4°C until placement on electrophoresis-gels.

A number of different cycle profiles was run to determine the final cycle profile to achieve best yield of amplification. Factors changed in test cycles were temperature and time.

**Table 1.** Primers used for amplification in the PCR.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequences (5´-3´)</th>
<th>Molecular weight (base pair)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-reu1</td>
<td>5´-CAG ACA ATC TTT GAT TGT TTA G-3´</td>
<td>303 bp</td>
<td>(Dommels et al., 2009)</td>
</tr>
<tr>
<td>L-reu4</td>
<td>5´-GCT TGT TGG TTT GGG CTC TTC-3´</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.5 Detection of specific DNA sequence by gel-electrophoresis

After washing with sterile water, polyacrylamide-gels (BIO-RAD, Sweden) 10% (electrophoresis-gels) were mounted in the electrophoresis chamber and the lanes were filled up to half with Tris Borate EDTA solution containing: tris base, boric acid ochethylenediaminetetraacetate (TBE-gel buffer).

8µL of PCR-amplified sample was mixed with 2 µL tracing-dye-solution (BIO-RAD, Sweden) in a tube, vortexed and centrifuged just a second at 5200 x g to get the sample solution to the bottom of the tube. 4 µL AmpliSize® molecular ruler (BIO-RAD, Sweden) was mixed with 1 µL tracing-dye-solution and added to the first lane.

2 µL of the amplified sample and tracing-dye-solution-mix were pipetted into the lanes on the electrophoresis-gel. The lanes were completely filled up with TBE-gel buffer solution and the electrophoresis-lid was put on. Electrophoresis was run for 75 min at 150 Volt.

2.5.1 Developing of the Electrophoresis-gels

The gels were removed from the electrophoresis-chamber, separated from the plastic-cover then placed into separate glass-containers and were silver stained.

25 mL Fixing solution (benzene sulfonic acid 3 %) + 100 mL 24% ethanol for 30 min.

25 mL Staining solution (silver nitrate 1 %, benzene sulfonic acid 0.35 %) + 100 mL sterile water for 30 min.

100 mL sterile water 1 min for washing.

After washing stage following components were added for 6 min; 25 mL sodium carbonate 12.5% + 125 µl sodium thiosulfate 2% +125 µl formaldehyde 37% +100 mL sterile water. Containers were under this stage light protected by covering with aluminum-foil.

For stopping and preserving, 25 mL Stopping solution (acetic acid 5 %, sodium acetate 25 %, glycerol 50 %) + 100 mL sterile water were added for 30 min.

Between each step the containers were emptied.

After the staining procedure gels were scanned into a computer using a conventional computer-scanner and viewed as jpeg-files on a computer screen.
3. RESULTS

3.1 Rogosa agar
Growth of Lactobacilli on Lactobacilli-specific Rogosa agar from saliva samples, both before and after chewing of a probiotic tablet. Lactobacilli counts increased by a factor of $10^2$-$10^4$ after chewing, (Diagram 1).

![Diagram 1. CFU on Rogosa agar (total Lactobacilli count).](image)

3.2 PCR
Electrophoresis-gels are presented with number of base pair (bp) to the left (vertically), which indicates the molecular weight. On top (horizontally) shows lane-number and test samples.

A total of nine different electrophoresis-gels were made, there were a total of 58 samples tested for *L. reuteri*.

3.2.1 Electrophoresis-gels
Positive saliva samples containing *L. reuteri* demonstrated a band at 303 bp on the electrophoresis-gels. The control-sample in lane 2 was prepared from a tablet containing *L. reuteri*. All gels have this control-sample in lane 2. The first lane contains AmpliSize®-solution and shows a base pair-ladder.
In fig 1, all three test subjects undiluted (1/1) and diluted to 1/10 show strong positive but also additional bands. When diluted to 1/100 all three test subjects show positive bands with no additional bands. When diluted to 1/1000 bands are difficult to read but are considered positive in all three test subjects.

**Fig. 1** Demonstrates amplified saliva samples after chewing (lane 3-14) in different dilutions.
The use of pellet samples demonstrated uneven dilution-series (fig 2). Samples did not show similar strength of band colour in the same dilution between test subjects as they did in fig 1. Undiluted saliva in test subject C was presented with weaker colour intensity than diluted sample to 1/10 in the same test subject. Samples diluted to 1/100 were difficult to read if positive or not in sample B and C. Samples diluted to 1/1000 in test subject B and C showed negative bands. Test subject A diluted to 1/1000 showed positive band.

Fig. 2 Demonstrates amplified pellet samples after chewing (lane 3-14) in different dilutions.
In fig 3, saliva samples (before and after chewing) grown on Rogosa agar, where compared to saliva samples after chewing. Saliva samples before chewing cultured on Rogosa agar (lane 3-5), demonstrated that test subject A and B were negative whilst test subject C showed positive band (lane 4). In lane 9-11, saliva after chewing showed positive bands in all three test subjects. This indicates that *L. reuteri* grew on the Rogosa agar plates.

**Fig. 3** Lane 3-8 demonstrates saliva before and after chewing prepared from Rogosa agar. Lane 9-11 demonstrates saliva after chewing.
In fig 4, where pellet and saliva samples were compared, subject C (lane 5) demonstrated a positive band from saliva, but not from pellet (lane 9).

**Fig. 4** Lane 5 and 7 show saliva sample before chewing. Lane 9 and 11 show pellet sample before chewing.
3.3 Reproducibility of results
Positive control samples (L. reuteri chewing tablet) as well as whole saliva after chewing were tested 34 times, and showed a consistent positive result.

Whole saliva before chewing was tested a total of 6 times, test subject A and B were consistent negative whilst test subject C was consistent positive.

Pellet before chewing were tested a total of 3 times, all three test subjects showed negative results consistently.

Pellet after chewing were tested a total of 14 times, 12 tests showed positive results from all three test subjects. Test subject B and C showed negative results 2 times when diluted 1/1000.

All samples were tested under the same conditions when amplified in PCR. Not all of the counted test results were presented in this study as electrophoresis-gels.

3.4 Determination of detection level
With a concentration over 250 CFU/mL of L. reuteri (Rogosa agar growth), positive results were possible to read. The detection level was therefore 250 CFU/mL for this method.

The optimal detection level or optimal concentration of bacteria, with minimal noise and a clear positive band on the electrophoresis-gel have been determined to 2 500 CFU/mL and higher.

3.4.1 Calculations
Lowest counts of Lactobacilli cultured on Rogosa agar (test subject B) contained 250 000 CFU/mL after chewing. After a dilution of saliva 1000 times (250 CFU/mL) it was still possible to see a positive band using PCR. Test subject B contained 50 CFU/mL before chewing and after chewing 250 000 CFU/mL which results in that almost every CFU was L. reuteri.

4. DISCUSSION

With the PCR method developed in this study it was possible to detect presence of L. reuteri in saliva. Saliva samples with a higher count of L. reuteri than 250 CFU /mL showed positive results. The method was considered uncomplicated to perform. The method demonstrated positive results from all three test subjects after chewing and was considered repeatable in this setting with only three test subjects. In this study, FTA™ elute cards were used for DNA extraction from saliva before amplification, which to our knowledge not have been used earlier for detection of L. reuteri in saliva.

Several studies that applied PCR-technique to detect bacteria in saliva, commonly culture the saliva on agar plates before analyse (Dal Bello & Hertel, 2006; Romani Vestman et al., 2013; Román-Méndez et al., 2009). Only one study, to our knowledge, has used PCR to detect L. reuteri in saliva without culture step (Iniesta et al., 2012). These results are in agreement with the present study that it was possible to detect L. reuteri directly from whole saliva. With FTA™ elute cards used in the present study, DNA extraction was easy and culturing step was
unnecessary. According to previous studies, it was confirmed that *L. reuteri* grew on Rogosa agar and were possible to detect with PCR (Romani Vestman et al., 2013).

It should be possible with the determined method to analyse a high number of saliva samples in a time effective way, compared with other methods mentioned since culturing step is excluded (Marsh & Martin, 2009b).

A surprising finding was that one of the three test subjects showed positive results before chewing, indicating that *L. reuteri* could be a natural resident in the test subjects oral microflora. Iniesta et al. (2012) did also discover that some of the test subjects were colonized with *L. reuteri* before adding the bacteria, but there are contradictory studies that indicates that *L. reuteri* could only be detected after adding *L. reuteri* (Caglar et al., 2009). Considering this it would be desirable to investigate the presence of *L. reuteri* in a normal population.

According to Jacobsen et al. (1999) it could be difficult to differentiate between strains of *L. reuteri* and other Lactobacilli species through PCR (ITS-PCR) (Jacobsen et al., 1999). Iniesta et al. (2012) states that this could be due to that their primer might not have been specific enough to avoid crossed amplification with other Lactobacilli species. Their primers were strain-specific for two certain strains of *L. reuteri* (DSM 17938 and ATCC-PTA-5289). Primers used in the present study were species-specific for *L. reuteri*, which also could be of significance to a source of error regarding crossed amplification. To confirm that no crossed amplification occurred in the present study, same saliva samples (before and after chewing) that were grown on Rogosa agar were amplified in PCR. The results demonstrated that two out of three test subjects were negative before chewing when amplifying CFU from Rogosa agar, whilst after chewing a tablet containing *L. reuteri* all samples were positive. This indicates that *L. reuteri* grew on the Rogosa agar and that the primer did bind to *L. reuteri* DSM 17938. Further research is needed to confirm that the present method only detects *L. reuteri* and do not does cross amplification with other Lactobacilli species.

Bacterial colonies grown on Rogosa agar were counted, first from saliva samples before chewing and saliva after chewing. Diagram 1 demonstrates that test subject B had 50 CFU/mL before chewing and 250 000 CFU/mL after chewing, which concludes that about 249 950 CFU/mL were *L. reuteri*. Dilution series were made to identify at what dilution the band on the electrophoresis-gels became unreadable. The detection level was determined from the test subject with the lowest counts of CFU/mL before chewing, which also showed the weakest band colour intensity.

Diagram 1 and fig 1 demonstrates that the difference between the counts of naturally Lactobacilli in saliva before chewing does not affect the results of saliva after chewing on electrophoresis-gels. The bands intensity were though relevant to the amount of *L. reuteri* in the sample after chewing since test subject B had the lowest count of CFU. It should be noticed that the intensity of the band does not state the concentration of *L. reuteri* in the sample.

Hypothesis two was falsified. Samples from saliva pellet proved to be more complicated for detection of *L. reuteri*. The detection level was also higher when using pellet (fig 2, 4). According to Polgarova et al. (2010), when saliva is centrifuged down to a pellet, a matrix is formed that appears difficult for the PCR or the FTA\textsuperscript{TM} elute card to replicate and dissolve. A high concentration of nucleases and PCR-inhibitors could be the probable cause. This will result in a smaller amount of amplifiable DNA that can be extracted from the FTA\textsuperscript{TM} elute cards and bind to the primer, or that the PCR-inhibitors will disturb the PCR-process (Polgarova et al., 2010). This indicates more reproducible results when using whole saliva samples instead of pellet.
Conventional PCR-technique is a qualitative method which detects presence or not for a specific DNA sequence. The method does not state the quantity (CFU/mL) of bacteria in the sample. By culturing all diluted saliva samples and comparing with the same samples from the electrophoresis-gels, detection level proved to be only 250 CFU/mL or higher. This means that if the saliva sample contains a higher concentration of *L. reuteri* than 250 CFU/mL, a band can be identified at 303 bp on the electrophoresis-gels. This demonstrates that the developed method can detect *L. reuteri* in small counts and thus is considered a sensitive method for detection of *L. reuteri* in saliva.

To obtain more reliable results and confirm the methods reproducibility, a larger test group would be desirable. It is not fully investigated if *L. reuteri* have any therapeutic effect on the oral microflora. Is it an advantage to be colonized, also temporarily, with *L. reuteri*? If it is, how many CFU/mL are necessary for obtaining a beneficial effect on the oral microflora? Can a high count of *L. reuteri* give individuals less counts of MS and with that less caries lesions?

The method developed in this study could be used for investigating the presence of *L. reuteri*, or other Lactobacilli species in a normal population. Since the use of probiotics are increasing and more products are available on the market, it could be of interest to investigate if these bacteria are becoming a part of the normal oral microflora. Does probiotic bacteria promote beneficial health effects to the population?

4.1 CONCLUSIONS

A method has been developed for measuring *L. reuteri* in saliva. This method could detect presence of *L. reuteri* in saliva if the concentration of *L. reuteri* is 250 CFU/mL or higher. The method is considered uncomplicated. PCR analysis of saliva centrifuged to a pellet was inconsistent in detection of *L. reuteri* in saliva.
5. REFERENCES


