

Lecture 2: Caries Research Themes and Techniques



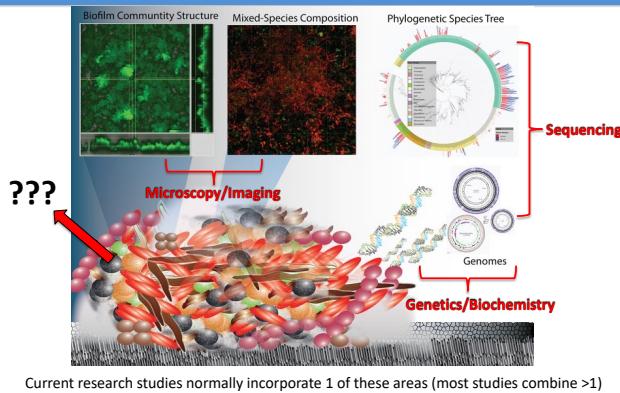
1

Koch's Postulates (1890's)

- Used to determine microbial causality for disease
- Present in disease state, but not in health (i.e. foreign)
- Can isolate species from disease samples and grow in pure culture
- Can introduce the species into a healthy organism and recreate disease
- Can isolate species from the experimental infection
- Paradigm: one pathogen = one disease
- Consider Caries: **Any problems when applying Koch's Postulates?**

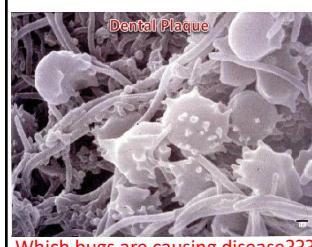
2

Major Caries Research Foci



3

Caries research – the beginning

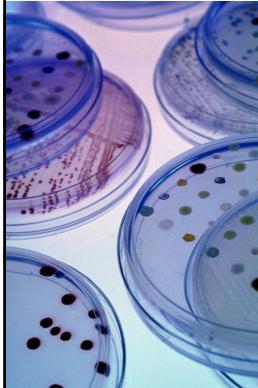


Which bugs are causing disease???

- We know there are many species in dental plaque
- The obvious question from the beginning: which ones cause disease (i.e. pathogenic)?
- What criteria do you use to designate an organism as a pathogen?
- Early caries research: identify the cariogenic species to better understand how the disease develops

4

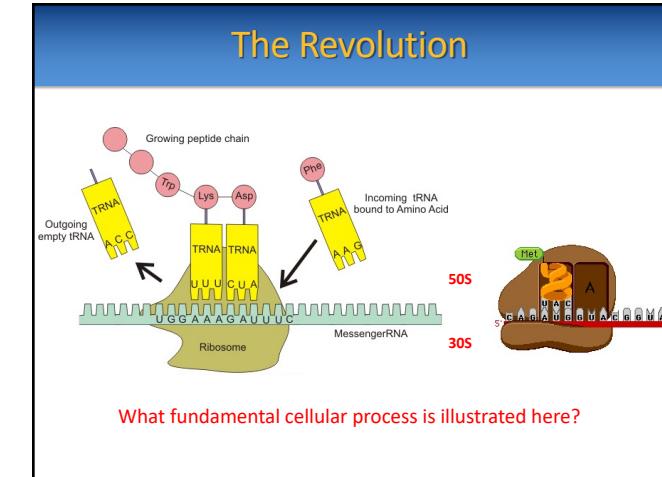
The Bad Old Days....



- In the not too distant past, it was necessary to isolate colonies on agar plates and then determine the species through a series of biochemical characterizations
- Take plaque samples from health vs. disease and look for colonies that are characteristic of each (i.e. morphological examinations, biochemical tests, Bergey's Manual)
- Hugely time consuming
- Not fun!
- Inherent limitation?**

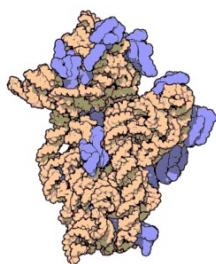
5

The Revolution



6

Sequencing – 16S rRNA



3-D atomic structure of the 30S subunit of the ribosome (16S rRNA in orange + 21 proteins in blue) – ribonucleoprotein complex
Parts of the gene encoding the 16S rRNA are identical in all bacteria

7

Sequencing: 16S rRNA Gene



CONSERVED REGIONS: unspecific applications

VARIABLE REGIONS: group or species-specific applications

Green regions are sequences shared by all or nearly all bacterial species

Grey regions are sequences that have species specific signatures

By knowing grey region sequences, we can identify which species the organism must be

8

16S rRNA Gene Sequencing

Amplify the 16S gene from your bacterial isolate and determine its sequence

CONSERVED REGIONS: unspecific applications
VARIABLE REGIONS: group or species-specific applications

No biochemical tests required!!!
What is the fundamental limitation with this approach?

9

Sequencing: Culture-Independent

Amplify the 16S gene directly from plaque samples and sequence everything

CONSERVED REGIONS: unspecific applications
VARIABLE REGIONS: group or species-specific applications

Product is a mixture of everything that was originally in plaque.
Sensitivity is determined by the amount of sequence information that can be generated (and cost).

10

Sequencing: Next Generation

- 454 Pyrosequencer
- Introduced in 2005
- Latest versions generate 400-700 Megabase
- Typical sequencing methods generate roughly 1 Kilobase
- For a 500 bp section of the 16S gene, this would result in up to 1.4 million individual reads, which are cataloged by the computer to determine the bacterial composition of the sample (including relative abundances for each species)

11

Sequencing: State of the Art

- Illumina: top company in the field
- Best instrument yields 6 Tb (**Terabase**) of DNA sequence per run
- That is 12 billion 500 bp 16S gene copies sequenced
- The human genome contains a total of ~6 Gb
- 1st human genome completed in 2003 cost \$3 billion
- As of 2014, Illumina is selling sequencing platforms for commercial human genome sequencing that delivers 30X coverage for <\$1000/genome (3 million-fold cost reduction in nearly a decade)

12

The personal genome era has arrived...

23 pairs of chromosomes.
One unique you.

- No membership fee
- No monthly subscription
- Includes access to all US and International DNA ancestry connections in 23andMe's database
- Receive 60+ personalized genetic reports

order now \$199

13

Metagenomics

- Sequencing technology is improving to the point that it is now feasible to sequence the entire collection of genes found within dental plaque (metagenome), rather than just the 16S gene
- We can now ask the question of whether certain bacterial *genes* predispose one to disease
- **Is this information as important for understanding caries as knowing species composition? Why or why not?**

Analyze genetic content of the entire population in biofilm specimens

14

Imaging: Fluorescent

- Sequencing technology gives valuable information about species abundance or certain genetic information, but it is not well suited for characterizing spatial phenomena (i.e. species distribution, protein localization, etc.)
- The development of many new types of fluorescent probes has revolutionized how science is performed

15

Imaging: Fluorescent Proteins

- A variety of naturally fluorescent proteins have been adapted for research purposes
- Originally discovered in jellyfish (2008 Nobel Prize)
- Used for genetically engineering fluorescence into other organisms (i.e. requires genetic techniques)
- Localization of cells or even particular proteins can now be visualized within living organisms
- **Is this useful for studying clinical specimens directly?**

16

Imaging: Fluorescent Proteins

Excitation spectrum (left): Shows three peaks labeled EX1, EX2, and EX3. The x-axis is 'Wavelength'.

Emission spectrum (right): Shows three peaks labeled EM1, EM2, and EM3. The x-axis is 'Wavelength'.

Legend: Ex = excitation, Em = emission.

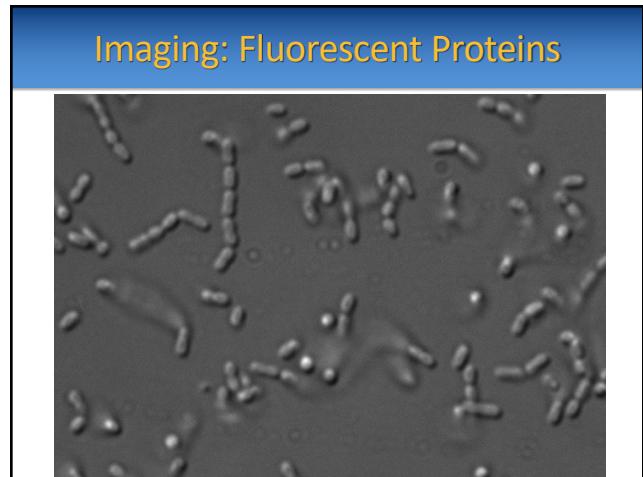
Many fluorophores (like the example above) can be excited by more than 1 wavelength of light. Compare the results using 3 different wavelengths (EX1 – EX3)?

Fluorophores have electrons that can be excited to a higher energy state by absorbing photons from a specific range of wavelengths. A return back to the normal energy state releases photons in a characteristic longer wavelength.

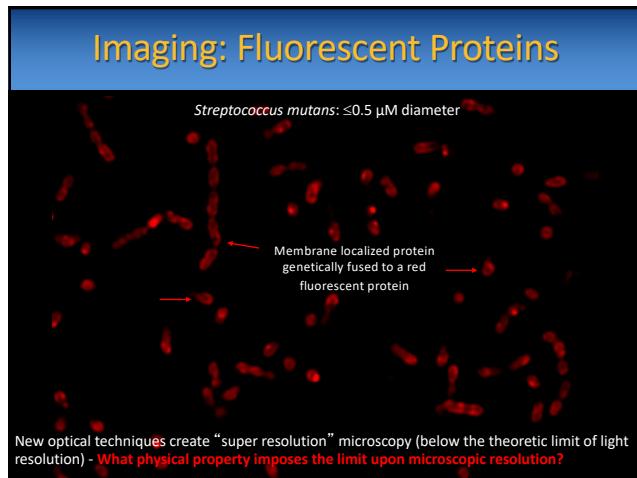
*Fluorescent proteins (and dyes) are activated by light – you must shine a light on them to see light in return. (Think blacklights...)

Protein	Colour Class	Excitation Peak (nm)	Emission Peak (nm)
EBFP2	Blue	383	448
CFP	Cyan	420	455
mCerulean	Cyan	433	475
eGFP	Cyan-green	462	485
mCherry	Red	552	565
YFP	Yellow	518	527
mYellow	Yellow	515	528
mOrange	Orange	519	529
YRFP	Yellow	517	530
mKO	Orange	548	559
TagBFP	Orange	555	564
Strawberry	Red	574	586
Tomato	Red	579	587
mCherry	Red	587	600
Tomato	Red	598	605

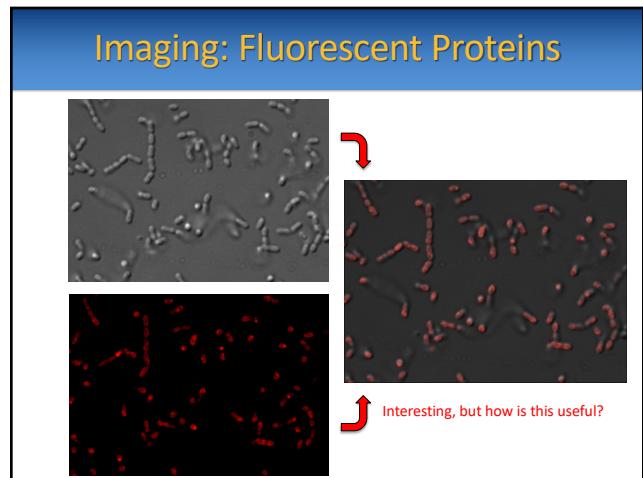
17



18



19



20

Imaging: Fluorescent Proteins

Normal (Wild-type)

Hypothesis: The membrane protein M anchors the cytoplasmic protein R at the membrane via direct protein interactions.

Prediction: What happens to R when we delete M?

The harder question: how do I demonstrate that this happens through a direct interaction between M and R?

21

Imaging: Fluorescence *In situ* Hybridization (FISH)

0	100	200	300	400	500	600	700	800	900	1000	1100	1200	1300	1400	1500	bp
V1	V2	V3	V4	V5	V6	V7	V8	V9								

CONSERVED REGIONS: unspecific applications
VARIABLE REGIONS: group or species-specific applications

A

16S rRNA Structure

Use FISH to image bacteria in their native context (i.e. genetic manipulation is not required).

FISH allows you to label particular species within a mixed population using fluorescently labeled single-stranded DNA probes that are complementary to a sequence in the 16S RNA.

Since RNA is single stranded it can hybridize to itself. Single stranded regions can be targeted by FISH.

How does FISH compare with fluorescent proteins for the analysis of bacteria in clinical specimens?

22

Imaging: FISH

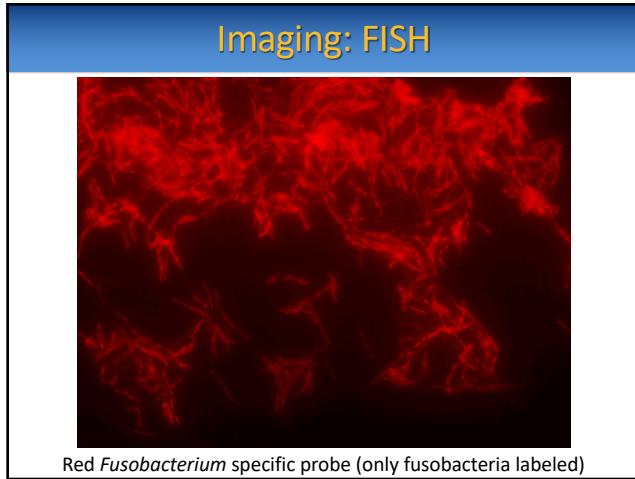
Cultured Dental Plaque (Interactions with *Fusobacteria*)

23

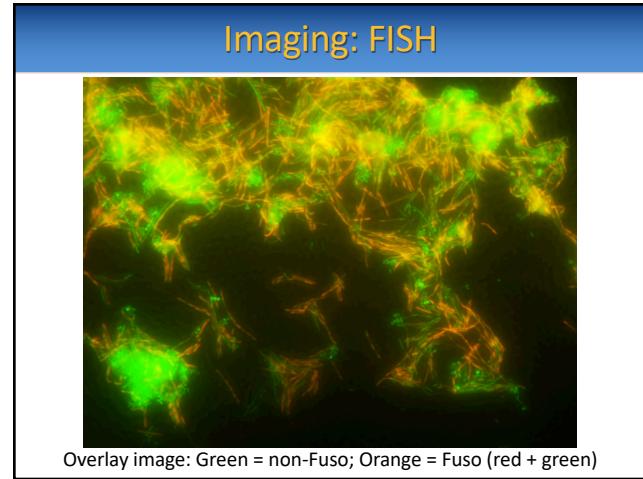
Imaging: FISH

Green 16S RNA Universal Probe (all bacteria labeled)

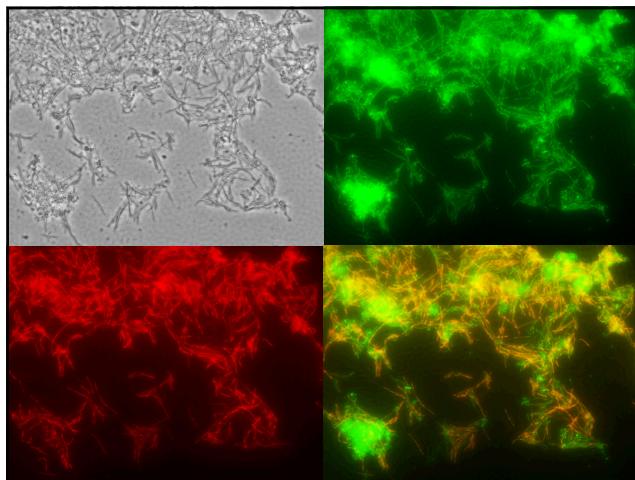
24



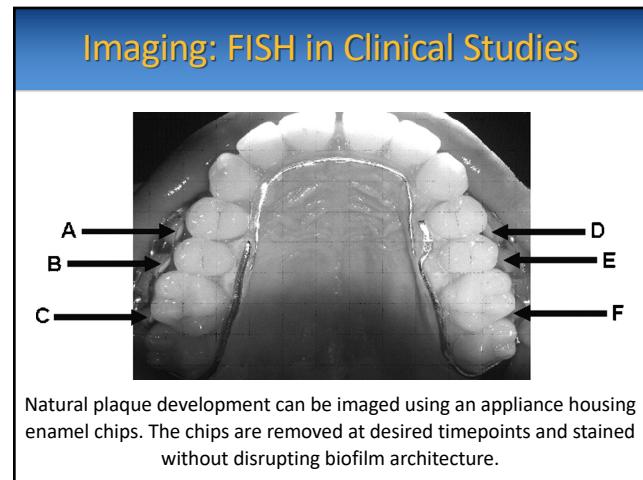
25



26

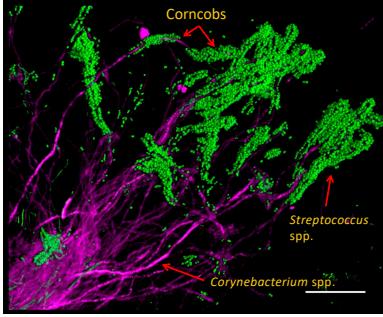


27



28

Imaging: FISH in Clinical Studies



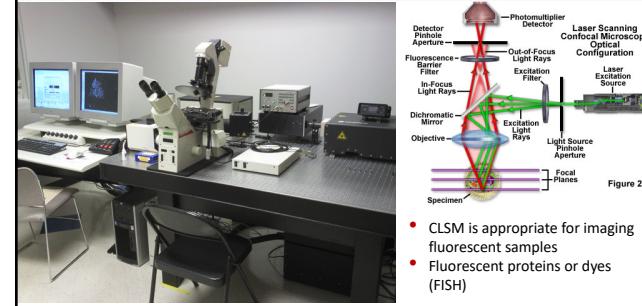
What is the term we use to describe specific adherence between different bacterial species?

Imaging plaque in its native context can reveal clinically relevant associations between different species of the flora.

Cornobs
Streptococcus spp.
Corynebacterium spp.

29

Imaging: Confocal Laser Scanning Microscopy (CLSM)



A combination of different lasers and light filters allows multiple fluorophores of different colors to be imaged in a single sample. The laser scans line by line through the sample for 3-D renderings.

- CLSM is appropriate for imaging fluorescent samples
- Fluorescent proteins or dyes (FISH)

Figure 2

30

Imaging: CLSM Analysis of Biofilm

Top row: wild-type
Middle row: various mutants
Bottom row: complemented mutants
Why are mutations complemented?

Green: normal cells
Red: dead/dying cells
Yellow: mixture

Each square on the grid = 25 μM^2

WT
J1
J2
J1c
J2c
J1J2c

CLSM images allow biofilms to be quantified (thickness, roughness, viability, etc.).

31

Imaging: Luciferase (Bioluminescence)

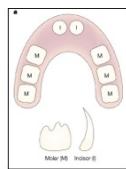


Photinus pyralis ("Firefly")

- Firefly: naturally produces bioluminescence via the luciferase enzyme
- The firefly luciferase was the first to be adapted for research use (many others exist)
- Luciferases can be genetically encoded as research tools (like fluorescent proteins)
- Luciferases produce light via enzyme-substrate reactions (best signal to noise ratio)
- Luciferases are particularly useful for studies requiring quantitative data
- Marine organisms are an abundant source of new luciferases and fluorescent proteins
- In the deep ocean, would you expect organisms to primarily encode luciferases or fluorescent proteins?

32

Imaging: Luciferase

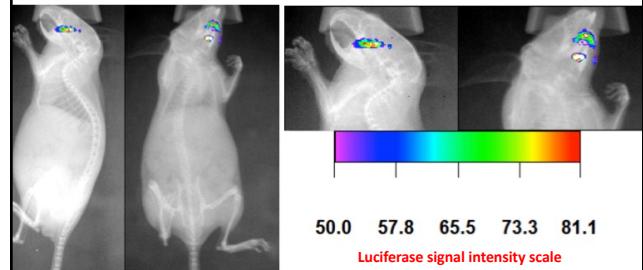


Nature Reviews Genetics. 2004 July; 5: 499–508

- To infer the role certain genes in determining cariogenicity, it is useful to have an animal model
- For the past couple years, our lab has been developing a luciferase-based system
- We use mice because they are cheap to house, widely available, and they have a limited number of teeth

33

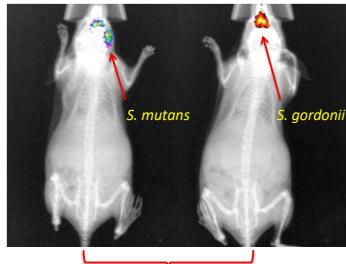
Imaging: Luciferase



- We can quantify the *S. mutans* that have colonized the mice and even determine which specific teeth have been colonized (in this example, maxillary molars).
- We can follow population changes in *S. mutans* over time.
- Why might luciferases perform better than fluorescent proteins for animal imaging?

34

Imaging: Luciferase



Same mouse imaged twice for 2 separate luciferase signals

We can also infect with multiple species expressing different luciferases to study questions related to oral ecology.

35

Imaging: Luciferase

How could we use luciferase to demonstrate that an *S. mutans* gene of interest is being expressed while *S. mutans* is growing in a mouse oral cavity?

36

Imaging: Luciferase

We can also genetically engineer *S. mutans* such that a particular gene of interest is only expressed when we want. Luciferase can confirm that the expected gene expression pattern is occurring.

In the above example, all 6 mice were infected with the same genetically engineered strain of *S. mutans*.

37

The Mutans Group

- *S. mutans* – found in virtually all human adults; cariogenic
- *S. criceti* – hamsters; cariogenic
- *S. ratti* – rats; cariogenic
- *S. devriesei* – horses; cariogenic
- *S. downei* – monkeys; cariogenic
- *S. ursoris* – bears; unknown cariogenicity
- *S. orisuis* – pigs; unknown cariogenicity
- *S. dentirosetti* – fruit bats; unknown cariogenicity
- Mutans group species can be found in the oral cavities of all types of mammalian omnivores or herbivores (bats, pigs, horses, bears, apes, etc.) and these animals can have caries too!
- This is very ancient niche that has persisted throughout evolution

38

The Mutans Group

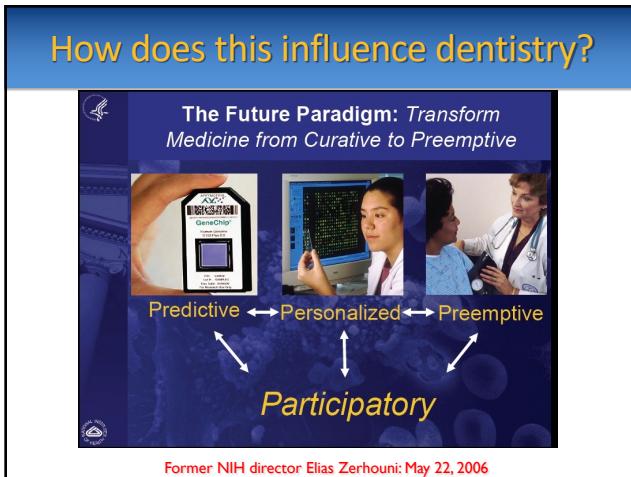
- Not only is *S. mutans* a specialist of humans, this trait extends to the entire group of Mutans streptococci found in all types of distantly related mammals
- Presumably, there are some common traits among these species that have allowed them to coevolve with their mammalian hosts
- Why is *S. mutans* so successful in humans?
- Can we use this knowledge to our advantage therapeutically?

39

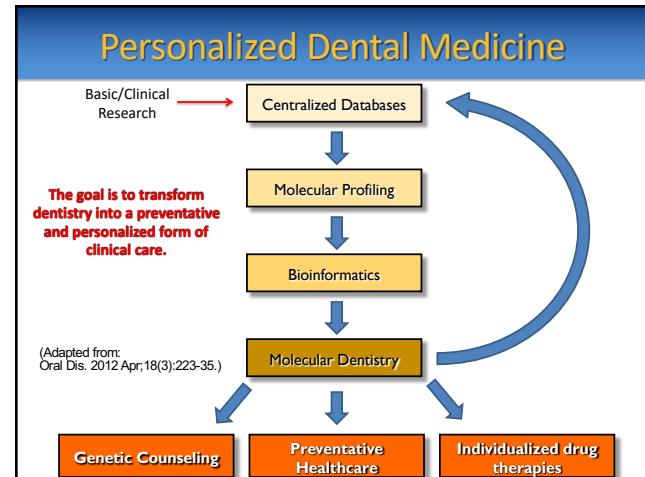
How do we use this info?

- Do you expect typical antibiotic treatments to reverse dysbiosis? Why or why not?
- Possible future therapeutic approaches:
 - Species-specific antibiotics (STAMPs or rationally designed small molecule inhibitors)
 - Nonlethal species-specific inhibitors – Any guesses what genes or processes could be targeted?
 - Agents to boost the competitiveness of the non-cariogenic flora, such as Mitis group streptococci
 - Immunization – caries vaccines have been tried with limited success
 - probiotics – could flora be used as a probiotic?

40



41



42



43



44