BUILD-A-CALCULUS

Experimental dietary research on *in vitro* dental calculus

Universiteit Leiden

Presentation info

- Blue links are to slides with supporting information
  - *To return to the slide you came from, click on the plant symbol in the top right corner*

- Green links are external (websites and articles)
- To return to a previous section (or skip forward), use the links at the bottom of the page.

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  - *This will return you to the beginning of each section*
- Hover over images to obtain more information (and citation)
- For those who are visually impared, alt text has been provided for the pictures
  - *please contact the first author (b.p.bartholdy@arch.leidenuniv.nl) for any other issues viewing the presentation*

Contents


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What?

- This project involves developing a protocol for growing *in vitro* dental calculus
- The model dental calculus system will allow controlled experimentation
- Testing fundamental aspects of research involving dental calculus and diet

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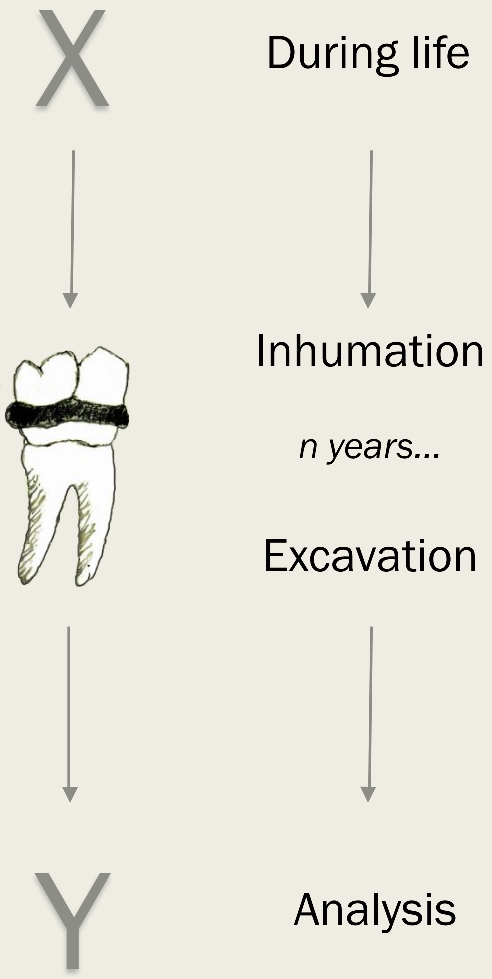
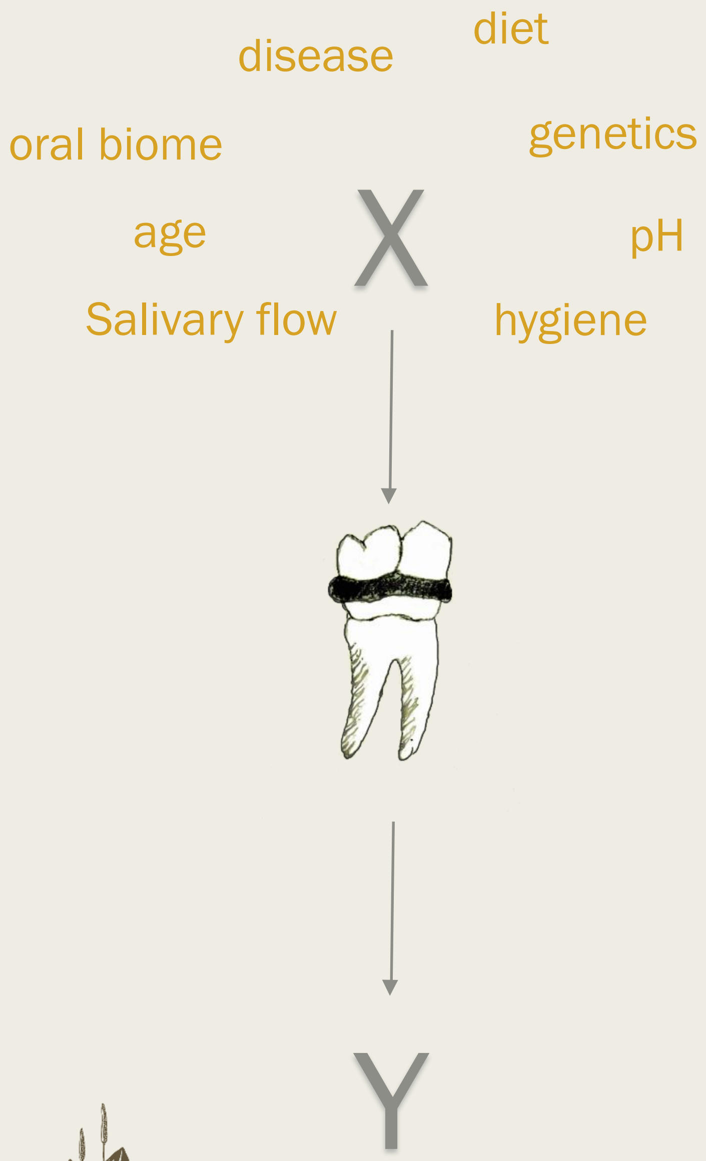
Why?



- Lots of exciting research currently being conducted on calculus
- Certain aspects of calculus knowledge is still limited
  - *Processes of incorporation (Radini et al. 2016)*
  - *Methodological biases*
  - *What we know: dietary reconstructions require caution*
- What is the relationship between X and Y?
  - *In vivo formation to analysis*
- Previous studies associating dietary intake and recovered info
  - *Humans and non-human primates*
  - *Studies reported a high level of stochasticity in dietary information recovered at an individual level*

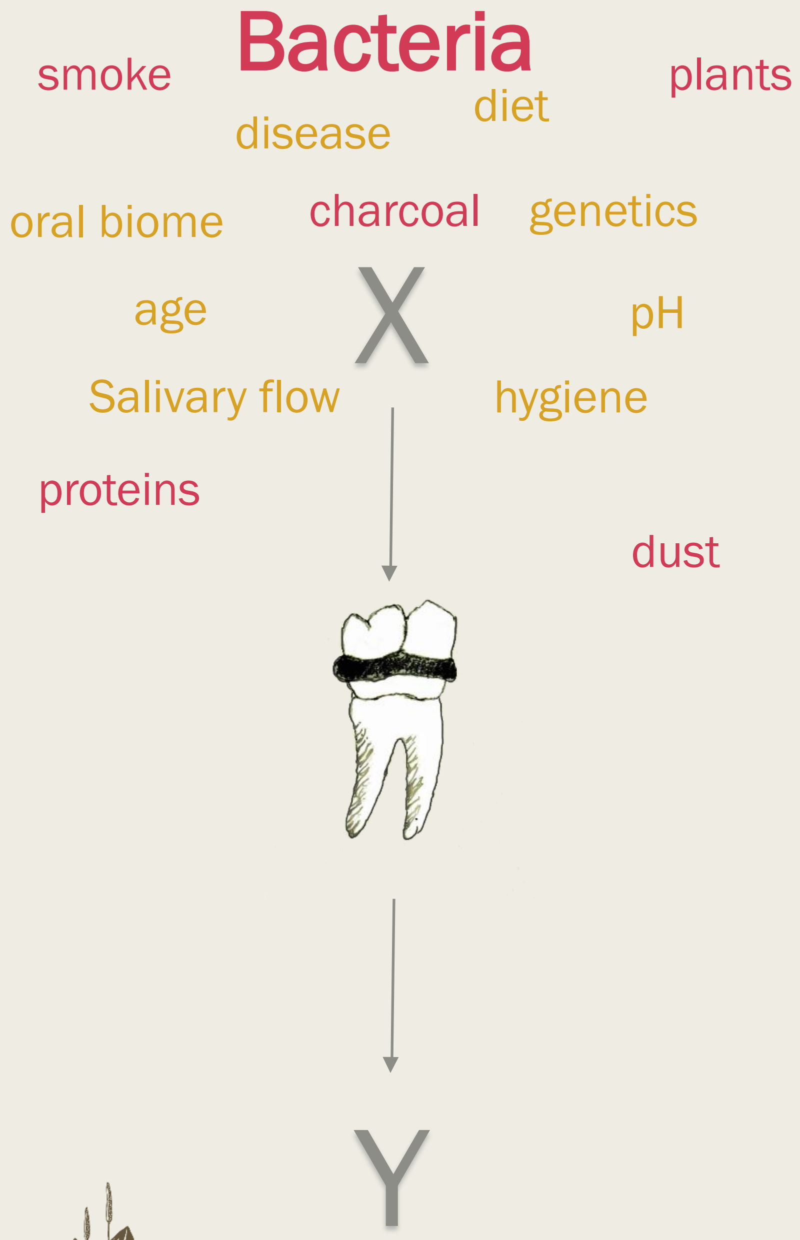
Calculus growth factors



- Multifactorial aetiology
  - *Age, ethnic background, disease (medication), genetic predisposition.*
- Oral conditions
  - *Poor dental hygiene, pH, salivary flow*
  - *Microbiota (Streptococcus spp.)*
- Dietary factors promoting growth
  - *Starch (amylopectin)*
  - *Fat (unsaturated)*
  - *Protein?*

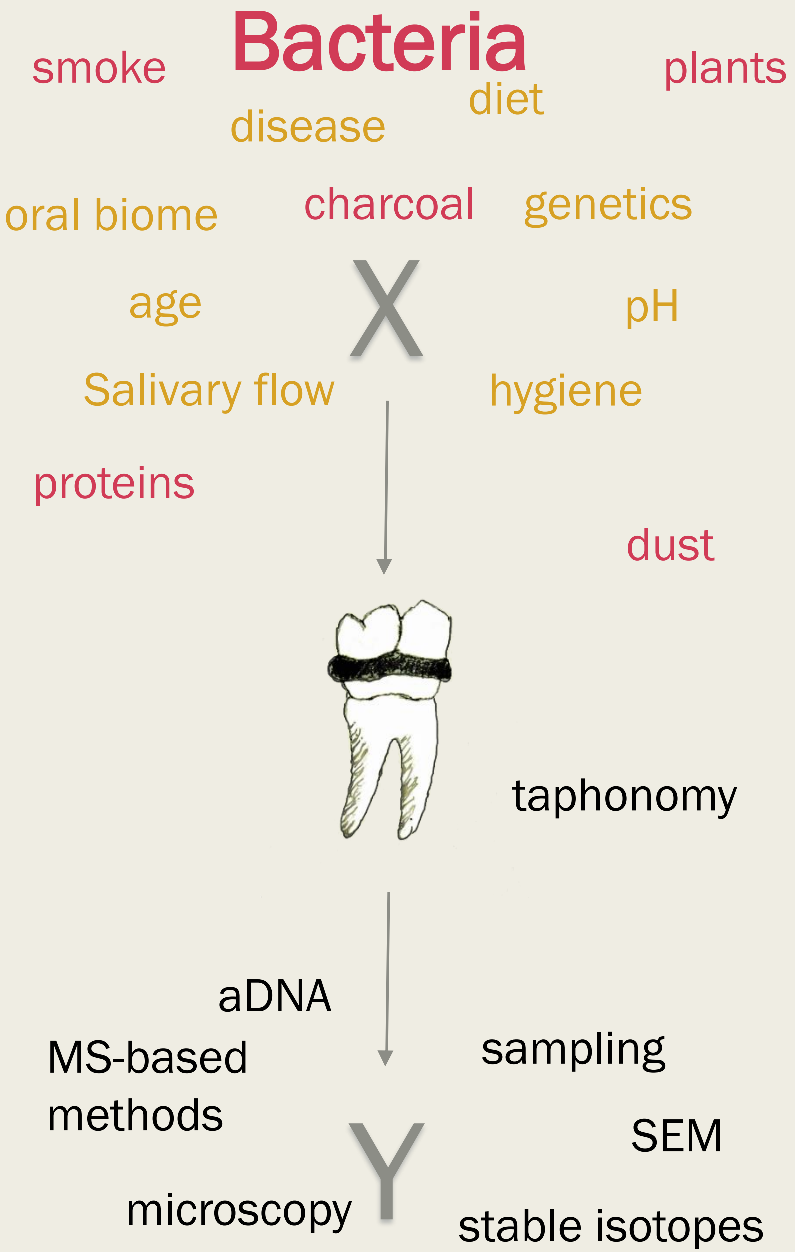
Microremains

- Dietary markers trapped in matrix
  - *Proteins*
  - *Plant micro-remains*
- Non-dietary markers
  - *Plant micro-remains*
  - *Dust*
  - *Smoke*
  - *Charcoal*
- Bacteria
  - *Endogenous*
    - Commensal
    - Pathogens
  - *Exogenous*



Analysis

- Calculus preservation
  - *Taphonomy*
    - Calculus and dietary markers
  - *Sampling methods*
- Methods for analysis
  - *Ancient DNA (aDNA)*
  - *Protein analysis*
  - *Scanning Electron Microscopy (SEM)*
  - *Optical microscopy*
  - *Chromatography*
  - *Stable isotopes*
  - *etc…*



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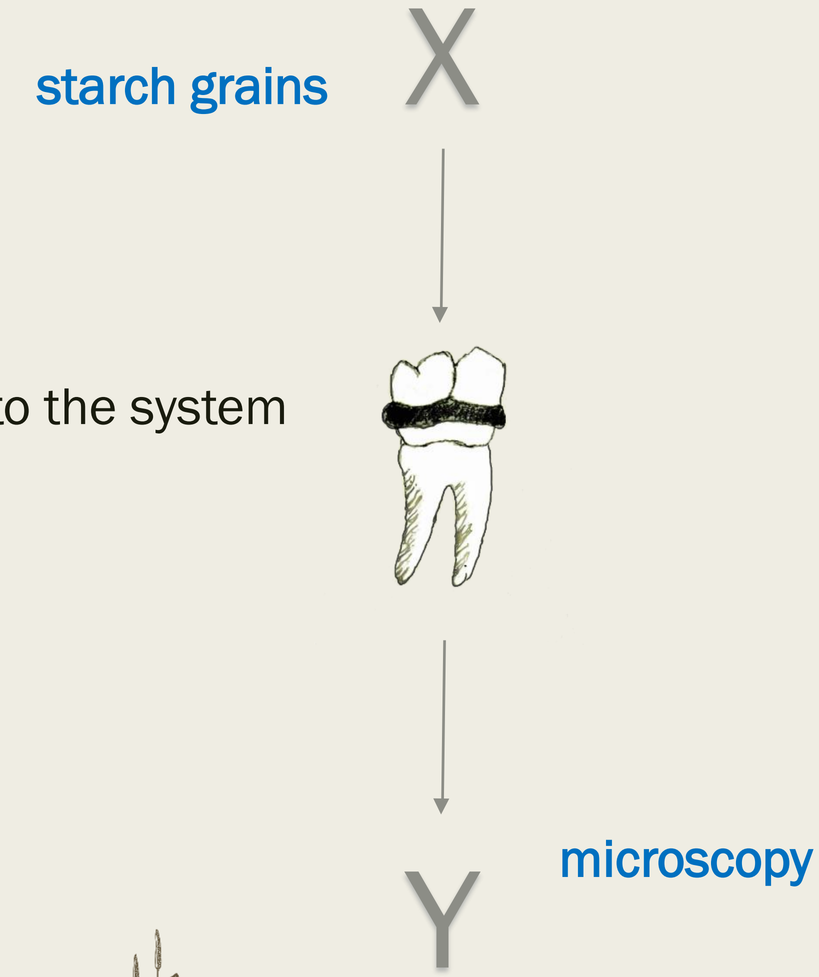
What (again)?

- This project involves developing a protocol for growing *in vitro* dental calculus
- The model dental calculus system will allow controlled experimentation
- Testing fundamental aspects of research involving dental calculus and diet

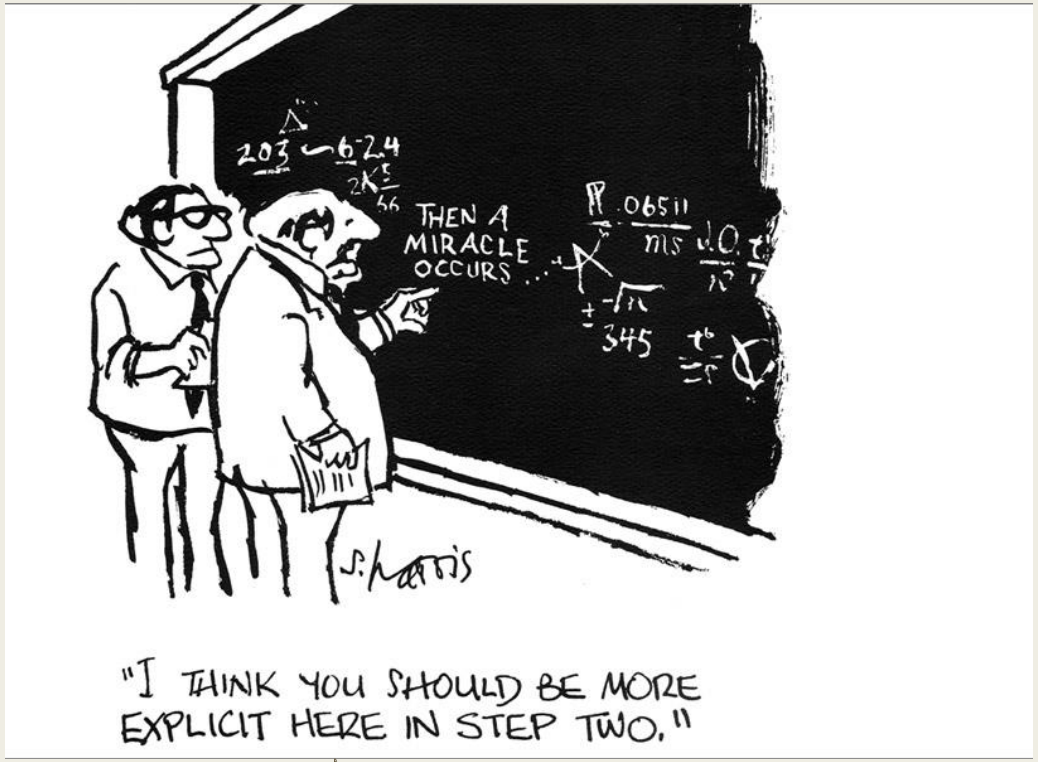
How?

- Addition of known quantities/ratios of dietary markers to the system
  - *Potato and wheat starches*
  - *Sampling and microscopy*



How?

- *In vitro* calculus growth
  - *Multiwell biofilm model*
  - *24-well plate with lid (high throughput)*
  - *Plastic substratum*
- Inoculated with donated saliva
  - *Days 0,3,5*
- Artificial saliva as growth medium
- Aerobic Incubation at 36 C
- Daily ‘feedings’
  - *Sucrose: Promote bacterial growth*
  - *Dietary markers of interest*
  - *‘Encouraged’ mineralisation (from day 15)*
    - Calcium phosphate monoflurophosphate urea (CPMU)
- Duration: 25 days



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How?

Multiwell plate

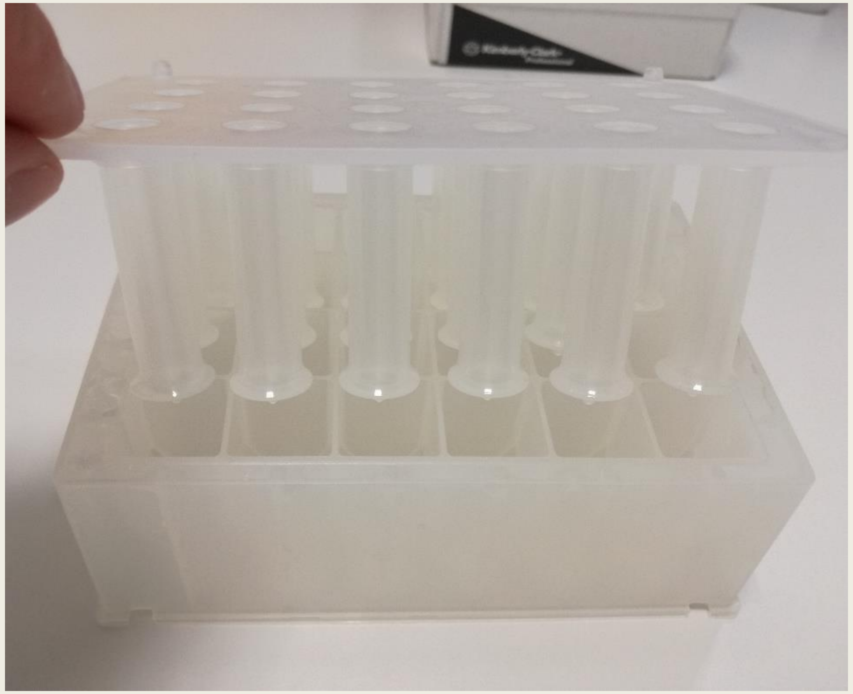
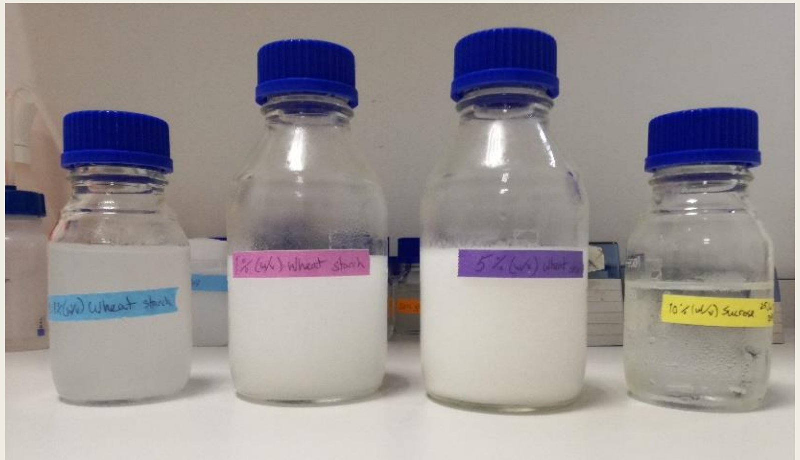


Plate setup

Wheat	Wheat	Wheat	Wheat	Wheat	Wheat
Potato	Potato	Potato	Potato	Potato	Potato
Mix	Mix	Mix	Mix	Mix	Mix
Control	Control	Control	Control	Control	Control

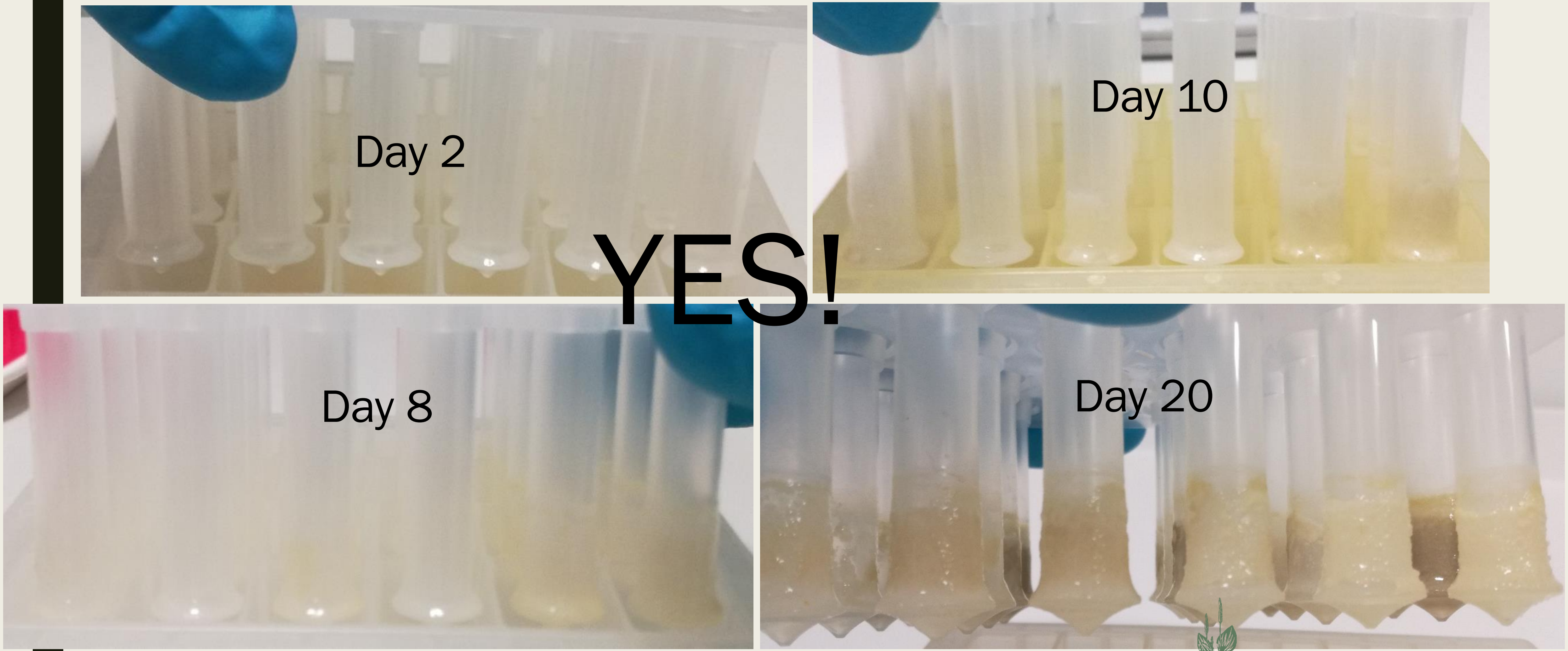


Aqueous starch solutions

Initial questions

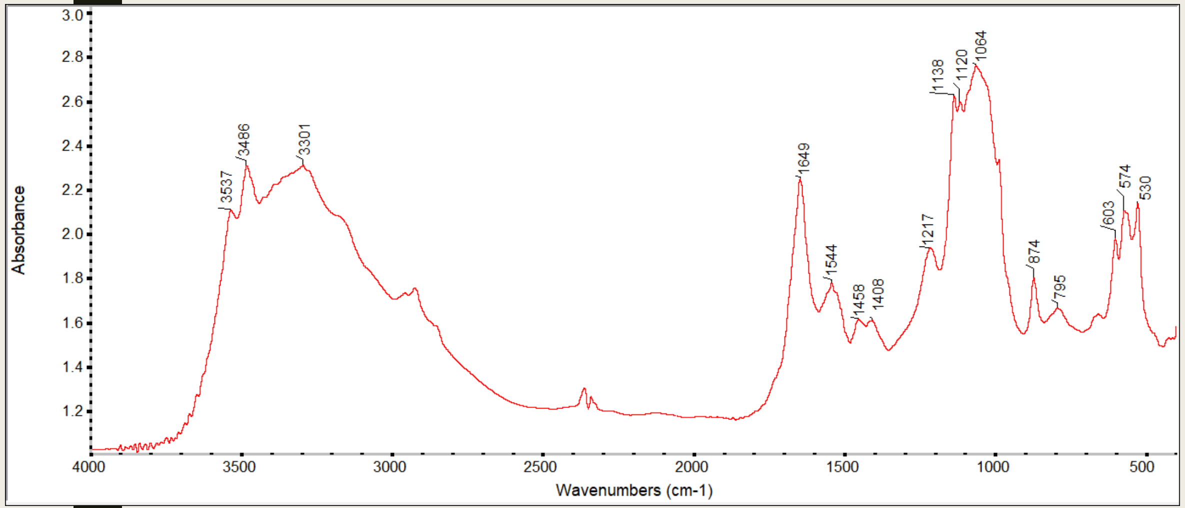
- Will the protocol work?
- Is it calculus?
- Does it incorporate starches?

Does it work?

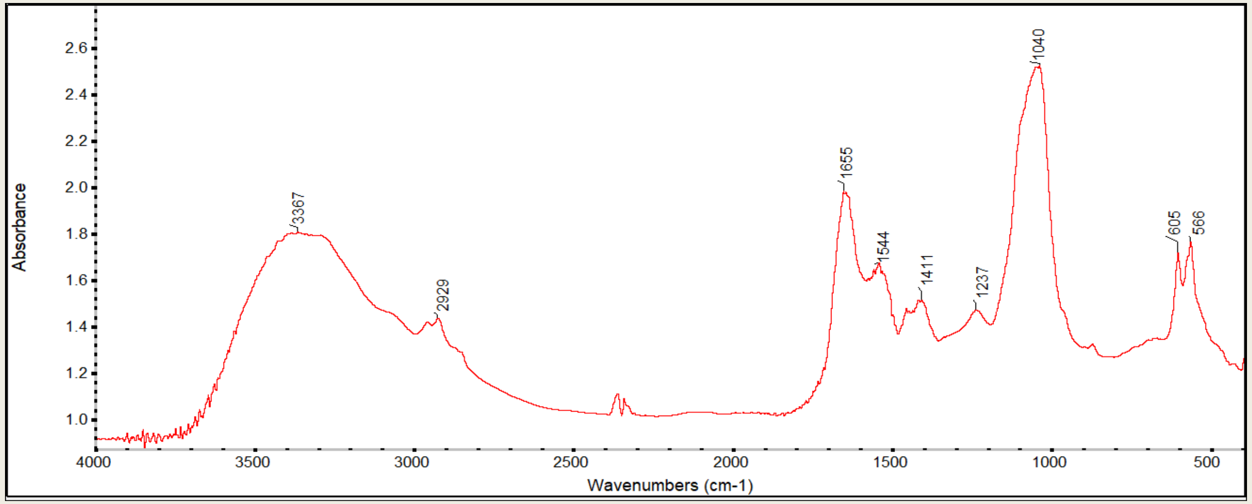


Is it calculus? (FTIR)

Modern reference calculus from donor

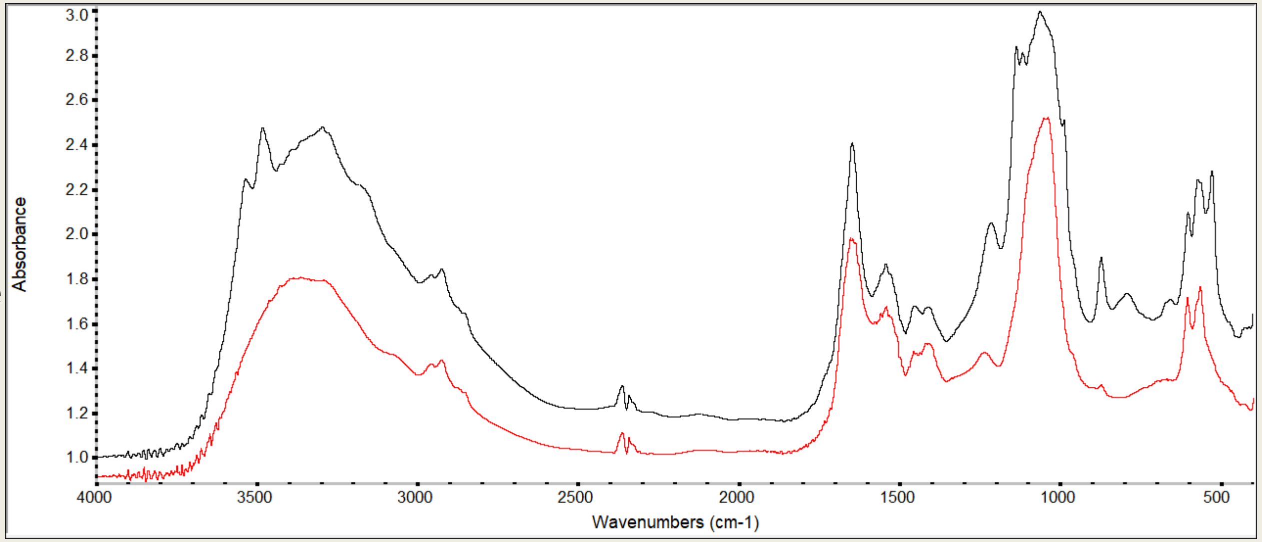


Model calculus



Modern reference calculus

Model calculus

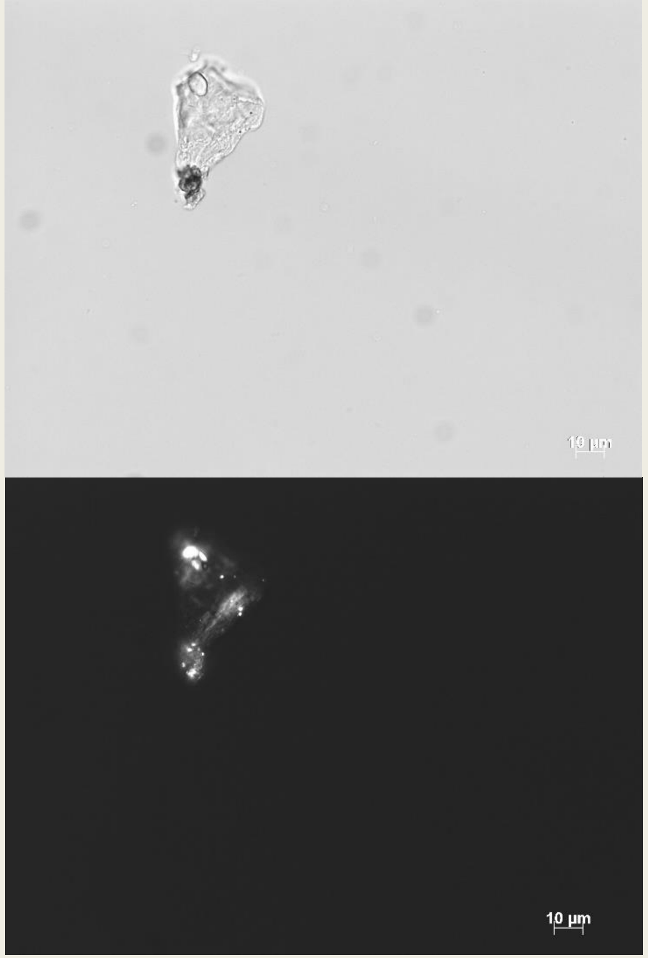


YES!

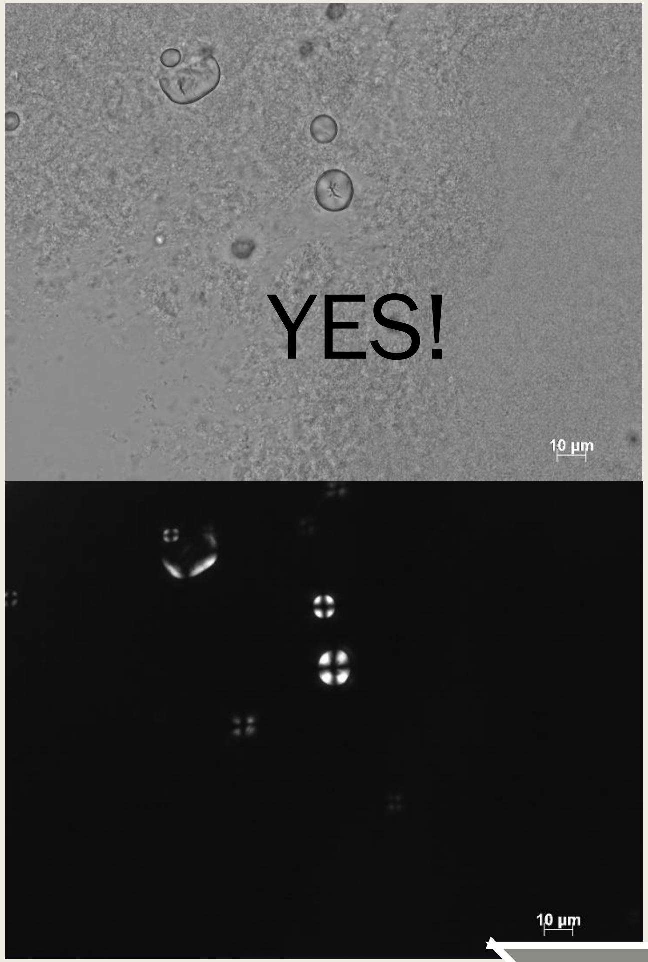
Well, calculus-like…

Does it incorporate starch grains?

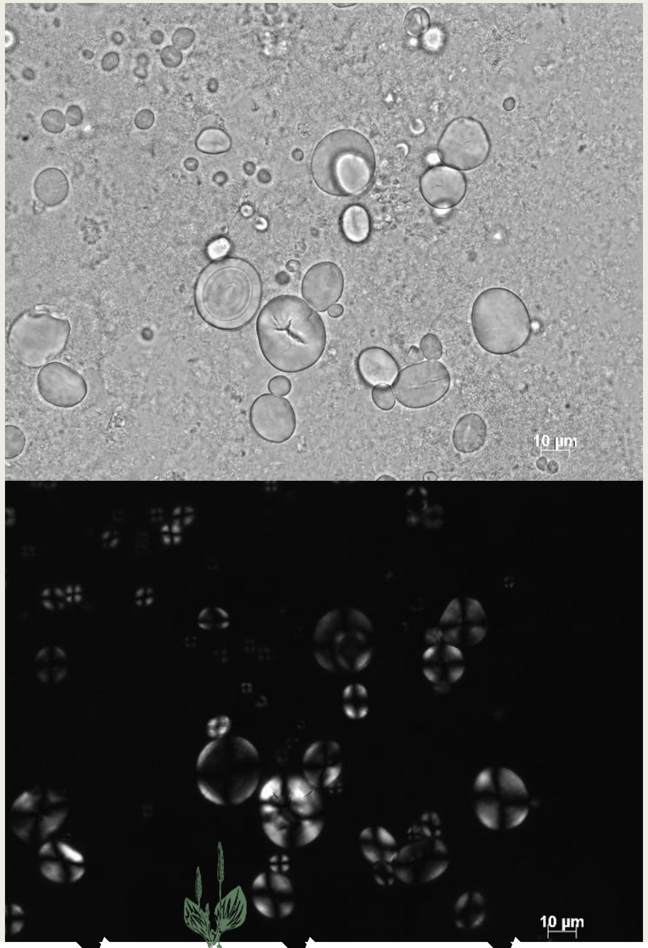
0.1% (w/v) wheat solution



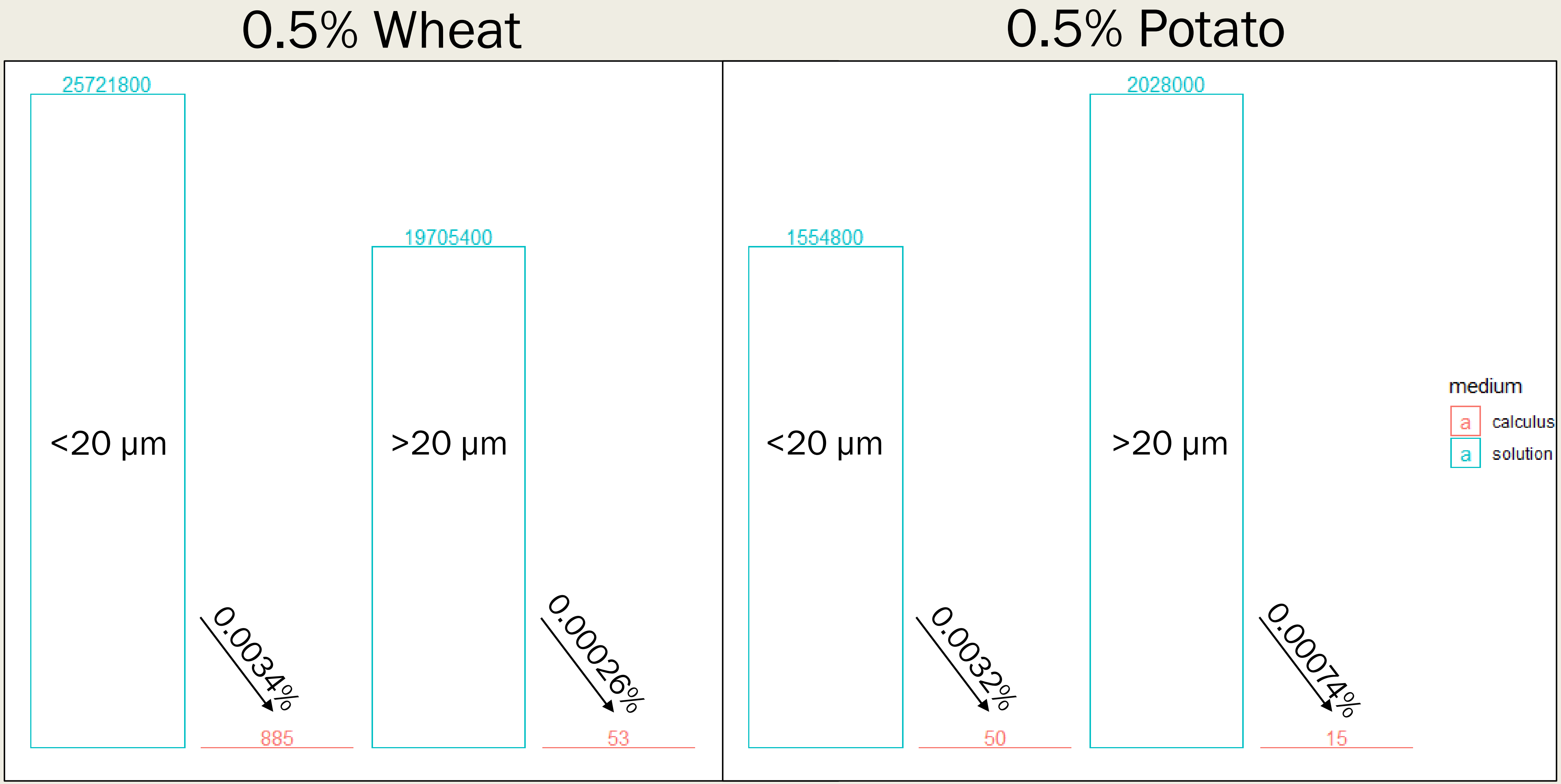
1.0% (w/v) wheat solution



5.0% (w/v) wheat solution

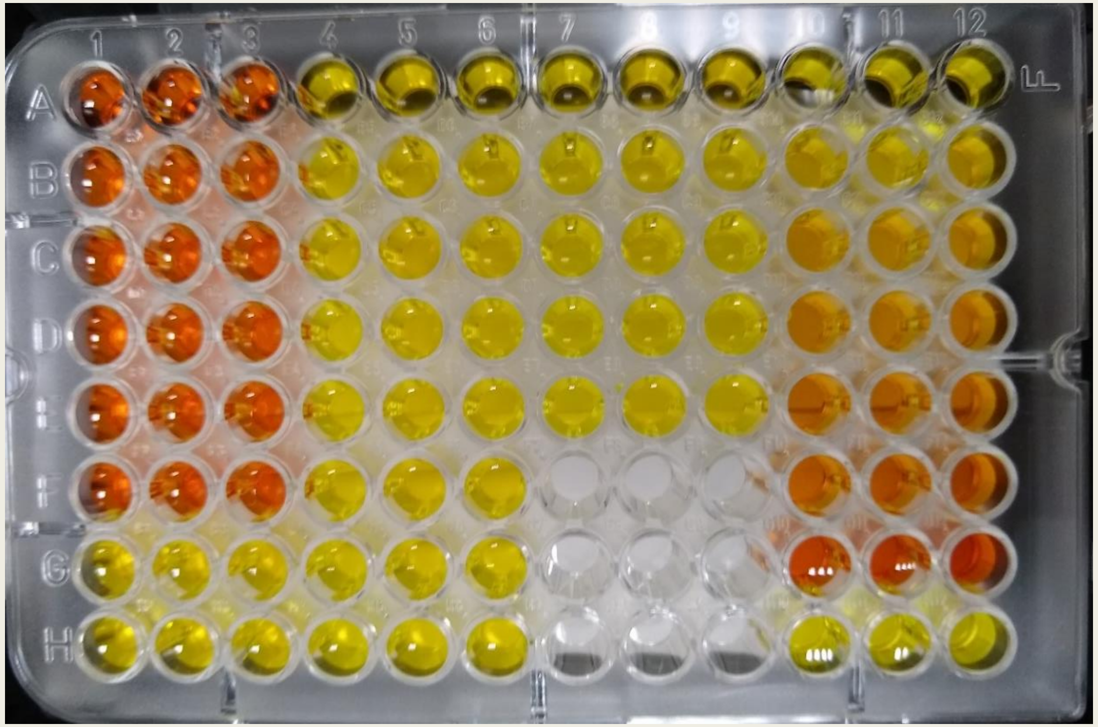


...ish


0.5% Wheat
0.5% Potato
25721800
19705400
1554800
2028000
<20 µm
>20 µm
<20 µm
>20 µm
0.0034%
0.00026%
0.0032%
0.00074%
885
53
50
15
medium
a calculus
a solution

Amylase activity

- No amylase activity detected
  - *Amylase assay conducted on days 5, 6, 7, and 8*
- This means that the starch count will NOT be influenced by hydrolysis from a-amylase
- If the research aim is to explore the effect of amylase, it can be added to the protocol



What does this mean?

- Model calculus system
  - *In vitro growth of calculus (-like substance) allows for controlled experimentation*
  - *Starches were successfully incorporated into the calculus matrix*
  - *The quantity of starch is important (especially for investigator’s sanity)*
    - 1.0% solution was too much to count
    - 0.1% solution was too little for reliable results
    - The current protocol uses a 0.25% solution

What does this mean (cont.)

- Implications for dietary research
  - *Despite the high count of starches in the solutions, very few were incorporated in the calculus*
    - This is consistent with starch studies on archaeological calculus
      - *Preservation may be the least of our worries…*
    - Intake of (non-)dietary markers requires repeated exposure in high quantities
    - Measuring the level of stochasticity will require repeated experiments
    - More insight on the mechanism of starch incorporation is needed to explain the low counts
  - *Does size matter?*
    - Large starch grains (>20 um) underrepresented by a factor of 10

Limitations

- Bacterial make-up still to be determined (in progress)
  - *While the model calculus mimics human calculus in mineral composition, the biofilm microbiota still need to be compared to human oral microbiota*
  - *The lack of amylase may mean a decreased level of α-amylase-binding streptococci (ABS) species present*
    - Or that the bound amylase does not retain sufficient activity
- High variability between samples from the same multiwell plate
  - *Sample (deposit recovered from each peg) weights can range from 3–12 mg*
    - Further protocol optimization needed

Potential for future research

- Optimization of extraction protocols
  - *e.g. HCl vs. EDTA (Tromp et al. 2017)*
- Methods testing
  - *Strengths*
  - *Biases/weaknesses*
  - *Combining protocols*
- Incorporation of dietary starches
  - *Do starch grains get trapped in the matrix or do they adhere to the calculus (via bacteria)?*
  - *Does starch representation differ between processed and native starch grains?*
- How does diet influence calculus growth?
  - *Do certain dietary components inhibit or promote calculus growth?*
- How does enzyme activity affect the recovery of other dietary markers?

Thanks for ~~listening~~ viewing!

- Acknowledgements
  - *HARVEST project (Dr. Amanda Henry)*
    www.harvestproject.eu
  - *Dr. Shira Gur-Arieh*
  - *Dr. Stephanie Schnorr*
  - *Dr. Irina Velsko*
  - *Suzan de Groot*
  - *Rijksdienst voor het Cultureel Erfgoed*
- Questions, comments, etc. are most welcome!
- Contact:
  b.p.bartholdy@arch.leidenuniv.nl

HARVEST

erc European Research Council

References

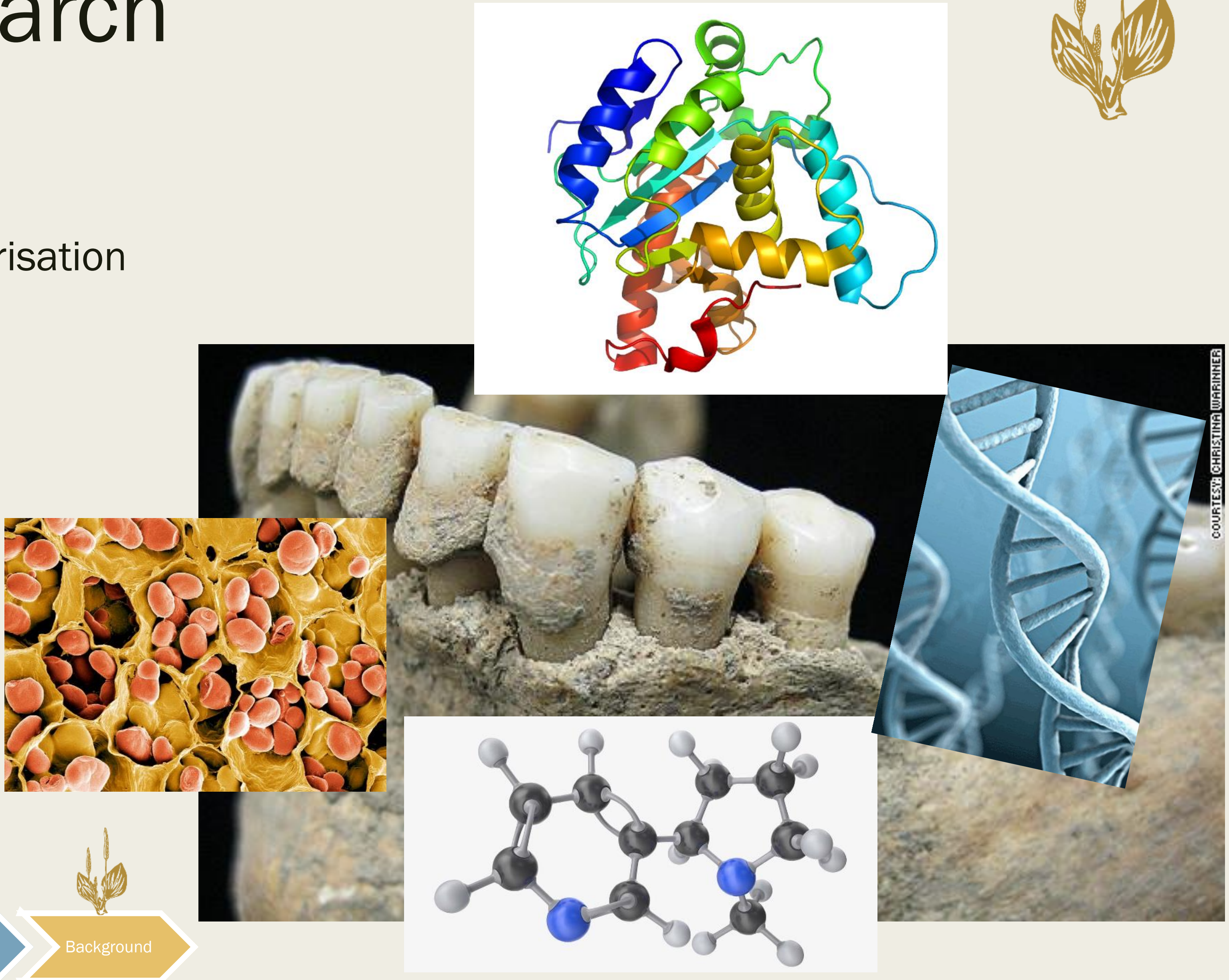
- Dawes et al. 2015
- Hidaka et al. 2008
- Lieverse 1999
- Jin and Yip 2002
- Nikitkova et al. 2013
- Warinner et al. 2014

Saliva

- Whole saliva
  - *A mixture of mucous and serous secretions from the salivary glands*
  - *ca. 99% water*
  - *mucin, proteins, enzymes, minerals, electrolytes*
- Important for the normal functioning of the oral cavity
  - *Lubrication*
  - *Taste*
  - *Buffer (maintains pH)*
  - *Cleansing (removes food particles)*
  - *Initial digestion (enzyme activity)*
  - *Antimicrobial action*
  - *Protection against demineralisation*
- Salivary flow is important for the precipitation of minerals
  - *High salivary flow rates increase the mineral interaction between the saliva and biofilm*

Calculus research

- Oral microbiome characterisation
  - [Mann et al. 2018]
- Dietary reconstruction
  - [Hendy et al. 2018]
- Medicinal use?
  - [Buckley et al. 2014]
- Nicotine use
  - [Eerkens et al. 2018]

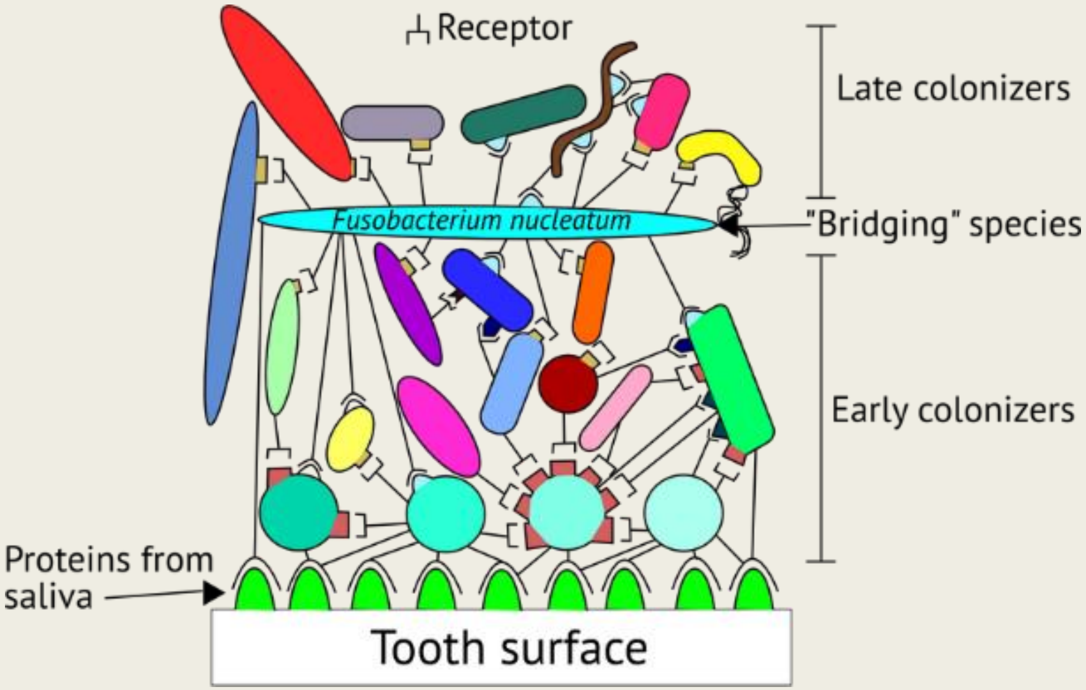


Aetiology

- Differences within and between populations
  - *Microbiome differences*
  - *Access to professional dental care*
  - *Oral hygiene practices*
- Age
  - *With increasing age, increased susceptibility to both caries and calculus*
- Medication
  - *Some medication promotes the formation of calculus*

Oral biofilm formation

- The pellicle is initially formed by salivary proteins
  - *Allowing subsequent bacterial adhesion*
- Lack of oral hygiene will allow bacterial accumulation
  - *Leading to larger plaque deposits*
- Bacterial influence
  - *Especially Streptococcus and Actinomyces spp. are major contributors*
- Both localised and overall pH
  - *Acidic conditions will lead to demineralisation and caries*
  - *Alkaline conditions will lead to mineralisation and calculus formation*
- Mineral deposition
  - *Salivary minerals (Mg, Na, K, **Ca**, Cl, HCO_3, **PO_4**)*
  - *Bacterial mineralisation*
- Attach. Mineralise. Repeat.
  - *calculus forms in layers with multiple mineralisation events*

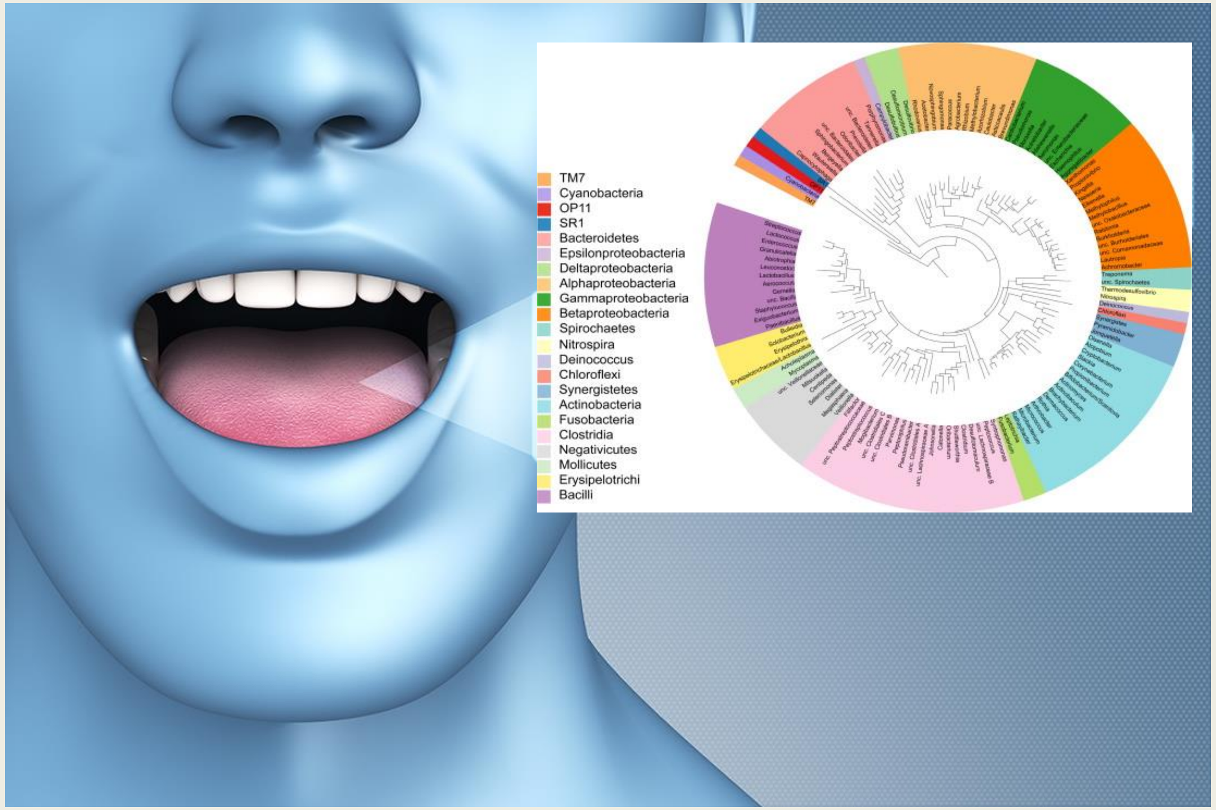


Dietary influence

- Factors promoting calculus growth
  - *Starch*
    - Specifically starches with high amylopectin content
    - Starches high in amylose content more likely to promote caries
  - *Fat*
    - Specifically unsaturated fats
  - *Protein(?)*
    - Increases concentration of urea, promoting a more alkaline environment, which in turn is conducive to calculus growth,
    - but it also suppresses crystal growth
  - *Para-masticatory chewing*
    - Increases salivary flow, which in turn increases the precipitation of minerals,
    - But may also dislodge already developed plaque/calculus

Oral microbiome

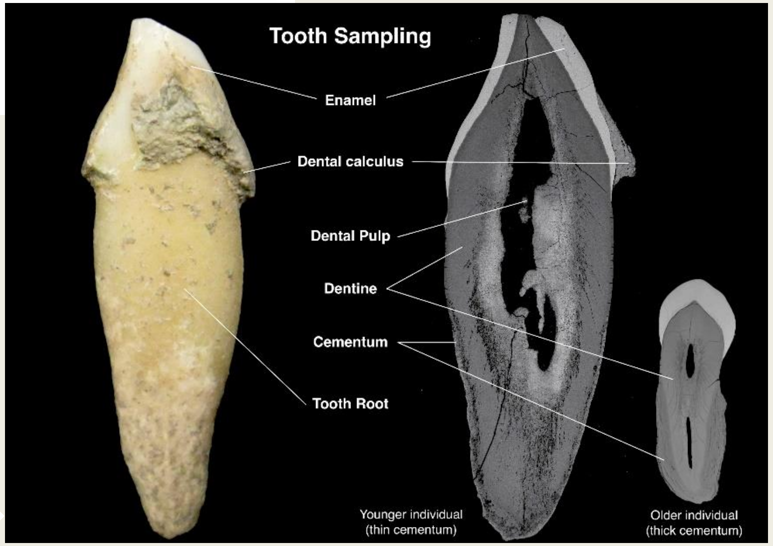
- Microbiome = collection of microorganisms in a certain environment/niche
- Oral bacteria
  - *> 600 species (many unclassified)*
  - *Mixed anaerobic and aerobic*
  - *Mixed temperature tolerance*
  - *Mixed pathogenicity*
  - *Can cause both dental and systemic disease*

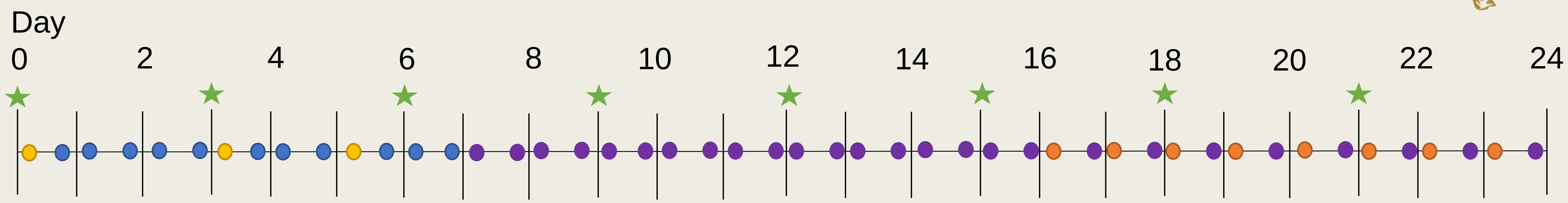


Calculus composition

- Calculus composition
  - *Collagen (organic)*
  - *Octacalcium phosphate, brushite (early stage mineralisation)*
  - *Hydroxyapatite, whitlockite (late stage mineralisation)*
- Supragingival calculus
  - *Mostly harmless*
  - *Forms above the gingival margin (hence the clever name)*
  - *Mineral source: Saliva*
  - *ca. 37% mineral content*
- Subgingival calculus
  - *Associated with periodontitis*
  - *Forms below the gingival margin*
  - *Mineral source: Gingival crevice fluid*
  - *ca. 58% mineral content*

Credit: Jess Beck





- Inoculate with whole saliva
- Sucrose
- Sucrose + starch
- CPMU
- ★ Full media replacement

α–amylase

- Saliva contains the enzyme α-amylase, which is involved in the initial digestion of starches
- α-amylase breaks starches down to smaller sugars (hydrolysis).
- Certain oral bacteria (Streptococci) can bind α-amylase in order to obtain nutrients