BUILD-A-CALCULUS

Experimental dietary research on *in vitro* dental calculus
Presentation info

- Blue links are to slides with supporting information
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- Green links are external (websites and articles)
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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 impaired, alt text has been provided for the pictures
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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.
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...

Contents

![Diagram showing relationships between Bacteria, saliva, and ancient DNA with related terms like charcoal, genetics, pH, etc.](image-url)
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 monofluorophosphate urea (CPMU)*
- *Duration: 25 days*
**How?**

- Multiwell plate
- Plate setup

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Aqueous starch solutions

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![Image of a Multiwell plate](image1)

![Image of Aqueous starch solutions](image2)
Initial questions

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

Day 2

Day 8

Day 10

Day 20

YES!
Is it **calculus**? (FTIR)

Modern reference calculus from donor

Model calculus

YES!

Well, calculus-like...
Does it incorporate starch grains?

- 0.1% (w/v) wheat solution
- 1.0% (w/v) wheat solution (YES!)
- 5.0% (w/v) wheat solution
...ish

0.5% Wheat

<20 µm

19705400

>20 µm

25721800

0.0034%

885

0.0006%

63

0.5% Potato

<20 µm

1554800

>20 µm

2028000

0.0032%

50

0.00074%

16
Amylase activity

- No [amylase](#) activity detected
  - Amlylase 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
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    www.harvestproject.eu
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- Questions, comments, etc. are most welcome!
- Contact:
  b.p.bartholdy@arch.leidenuniv.nl
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, HCO₃, PO₄) - 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.