A Tale of 3 Mummies:  
A Microbiome Analysis of Life in the Peruvian Andes 1,000 Years Ago  
Raul Cano, Ph.D.  
Professor Emeritus, California Polytechnic State University  
Director, Microbiome Research, ATCC-CTM  
April 14, 2016
About ATCC

- Founded in 1925, ATCC is a non-profit organization with headquarters in Manassas, VA
- World’s premiere biological materials resource and standards development organization
- ATCC collaborates with and supports the scientific community with industry-standard biological products and innovative solutions
- Strong team of 400+ employees; over one-third with advanced degrees
A Tale of 3 Mummies: A Microbiome Analysis of Life in the Peruvian Andes 1,000 years ago
Cuzco, Peru — Geography
Collaborators and Funding

- **Tasha Santiago-Rodriguez:** Sample processing, data analysis, and MOST everything else
- **Franco Rollo:** My friend and gatekeeper
- **Gino Fornaciari:** Mummy Czar
- **Stefania Luciani:** Ancient DNA isolation
- **Isolina Marota:** Continue Franco Rollo’s legacy (and lab)
- **Gary Toranzos:** Public health emphasis
Selected Andean Chronologies
Inca Accomplishments

- Excellent farmers, builders, and managers
- Roads and aqueducts
  - Built > 19,000 miles of roads (over mountains)
  - Built canals and aqueducts to carry water to dry areas
- Advancements in medicine and how to make them – use of medicinal plants
- Arts and science
- Accomplished travelers
Inca Diet

- Crops cultivated across the Inca Empire included quinoa, maize, beans, grains, potatoes, sweet potatoes, peppers, tomatoes, peanuts, cashews, squash, cucumber, cotton, carob, and avocado.

- Livestock was llama and alpaca herds; meat eaten as charqui.

- Drank chicha a lot! (so I’m told)
Questions

- Suitability for analysis
- Microbiome/metagenome structure
  - Microbial diversity
  - Metabolic diversity
  - Correlations?
- Medical and cultural aspects
  - AMR/MDR genotypes
  - Infectious diseases
  - Diet
What Got It All Started!

Iceman colon, circa 1998
Cast of Characters
The Protagonists: Three Natural Mummies

**FI3**
14th Century male
25-30 years old
Good preservation
Leishmaniasis

**FI9**
11th Century female
18-23 years old
Well preserved
Cardiomegaly

**FI12**
14th Century female
20-25 years old
Good preservation
Bronchopneumonia
# Supporting Cast

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Medici Family</th>
<th>Aragonese Family</th>
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<tbody>
<tr>
<td></td>
<td>NASD3</td>
<td>NASD22</td>
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<td></td>
<td>NASD12</td>
<td>NADS27</td>
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<td>NASD29</td>
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<table>
<thead>
<tr>
<th>Personage</th>
<th>Francesco I</th>
<th>Ferdinando I</th>
<th>Gian Gastone</th>
<th>Ferrante I d'Aragona</th>
<th>Luigi Carafa</th>
<th>Unknown</th>
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<tr>
<td>Lifespan</td>
<td>1541-1587</td>
<td>1549-1601</td>
<td>1671-1737</td>
<td>1431-1494</td>
<td>1511-1576</td>
<td>16th Century</td>
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<tr>
<td>Comments</td>
<td>Grand Duke</td>
<td>Grand Duke</td>
<td>Gran Duke</td>
<td>King of Naples</td>
<td>Prince of Stigliano</td>
<td>¿?</td>
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<tr>
<td>COD</td>
<td>Malaria</td>
<td>Tertian fever</td>
<td>Small Pox</td>
<td>Colon carcinoma</td>
<td>Natural causes</td>
<td>Cirrhosis</td>
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</table>
Analysis Workflow

16S μbiome → Sequences (Illumina MiSeq) → Shotgun

FastQC → High Quality Sequences (PHRED ≥ 30)

Taxonomic grouping:
- ClaMS
- QIIME
- CLC

Search databases:
- DiaGrid
- CAZy
- CARD

Metabolic pathways:
- MG-RAST
- PIECRUST
- CLC

Analysis
QC/QA of Sequences

ANCIENT DNA MAY BE DAMAGED – SEQUENCES OF POOR QUALITY
Genetic Information is Lost Over Time

Information Loss as a Function of Coprolite Age

- **Observed Species Count**
- **Shannon Diversity Index ($H'$)**

Age of Coprolite in Years (based on $^{14}$C dating)

- 1620
- 1640
- 1660
- 1680
- 1700
- 1720
- 1740

Genetic information decreases over time, as indicated by the observed species count and Shannon diversity index.
Assurance of good NGS results

Per base sequence quality

Per sequence quality scores

P = 0.00010
Rarefaction Curves
Assurance of sufficient sample size
## Mock Community for Assessing DNA Extraction

<table>
<thead>
<tr>
<th>Taxon</th>
<th>ATCC® No.</th>
<th>Inoculum CFU/mL</th>
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<tbody>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>6501™</td>
<td>1.00E+06</td>
</tr>
<tr>
<td><em>Clostridium butyricum</em></td>
<td>3627™</td>
<td>1.00E+06</td>
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<tr>
<td><em>Escherichia coli</em></td>
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<td>1.00E+06</td>
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<tr>
<td><em>Salmonella enterica</em></td>
<td>8388™</td>
<td>1.00E+06</td>
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<tr>
<td><em>Klebsiella pneumoniae</em></td>
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<tr>
<td><em>Staphylococcus aureus</em></td>
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<td>1.00E+06</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>19433™</td>
<td>1.00E+06</td>
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<tr>
<td><em>Citrobacter freundii</em></td>
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<td>1.00E+06</td>
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<td><em>Streptomyces griseus</em></td>
<td>23345™</td>
<td>1.00E+06</td>
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<tr>
<td><em>Micrococcus luteus</em></td>
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<tr>
<td><em>Lactobacillus gasseri</em></td>
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### OTUS

<table>
<thead>
<tr>
<th>OTUS</th>
<th>Percentage</th>
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<tr>
<td>Lactobacillus gasseri</td>
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<td>Micrococcus luteus</td>
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<td>Bacillus subtilis</td>
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</table>
Source Tracker Results (from QIIME 1.9.0)

Assessing Environmental Contamination

A. FI3

B. FI9

C. FI12

Legend:
- Purple: Gut
- Blue: Oral
- Green: Skin
- Red: Soil
- Gray: Unknown
Mummy FI9
A Peruvian natural mummy of a young woman 20±3 years old from Cuzco (Peru), housed in the Museum of Anthropology and Ethnology of Florence, was autopsied.

The funerary equipment is typical of the Andean highlands, Late Intermediate Period (1000-1476 A.D.)
After preliminary X-rays showing good preservation of the body, the mummy was then autopsied through the posterior thorax to avoid damage.
Alpha Diversity: FI9

OBSERVED SPECIES

<table>
<thead>
<tr>
<th></th>
<th>A_COLON</th>
<th>T_COLON</th>
<th>D_COLON</th>
<th>PALEOFECES</th>
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SHANNON DIVERSITY INDEX

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<td>4.097717179</td>
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</table>
## Taxonomic Diversity

### Circa 1999: TRFL-P + clone and sequence

<table>
<thead>
<tr>
<th>BLAST ID</th>
<th>No. Clones</th>
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<tr>
<td>Clostridium botulinum E</td>
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<td>Clostridium botulinum G</td>
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<tr>
<td>Clostridium perfringens</td>
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<td>Clostridium sp.</td>
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<tr>
<td>Clostridium algidicarnis</td>
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<tr>
<td>Eubacterium pectinii</td>
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</tbody>
</table>

### Circa 2014: NGS + QIIME

#### A. Coprolite
- Clostridium (7.7%)
- Turicibacter (89.2%)

#### B. Descending colon
- Clostridium (81.4%)
- Turicibacter (5.1%)

#### C. Transverse colon
- Turicibacter (2.7%)
- Clostridium (96.2%)

#### D. Ascending colon
- Turicibacter (19.7%)
- Clostridium (68.3%)
Metagenome Binning (ClAMS) – FI9
Paleopathology of Peruvian Mummies
Macroscopically, we (Gino) found a megavisceral syndrome in the form of cardiomegaly, megaesophagus, gastric ectasia, and megacolon, with enormous amounts of feces.
Light microscopy showed massive fat substitution, fibrosis of the myocardium, and coronary thrombosis.

The mummy mega-visceral syndrome strongly suggested a case of Chagas’ disease, chronic phase, caused by the protozoan parasite *Trypanosoma cruzi*.
- Giemsa staining evidenced rare roundish intratissutal nests, about 15-20 µm large, in the myocardium, containing several ovular formations (1-2 µm) with small nuclei.

- The findings correspond morphologically to intratissutal nests of amastigotes of *Trypanosoma cruzi*.

An intramyocardial nidus of amastigotes of *Trypanosoma cruzi* (Giemsa, 1000X)
Phylogenetic Studies – Mixed Infections

A. *Trypanosoma cruzi*

B. *Leishmania donovani*
PCoA and Heatmap: FI9

- Descending colon
- Paleofeces
- Ascending colon
- Transverse colon

Taxa Bar Marks:
- Acidobacteria
- Actinobacteria
- Aplanocytoplasma
- Aquificae
- Armatimonadetes
- Bacillariophyta
- Bacteroidetes
- Basidiozymyces
- Betaproteobacteria
- Chlamydia
- Chlorobi
- Chloroflexi
- Chlorophyta
- Chloroidea
- Chromera
- Chrysoniches
- Chloridiales
- Cyanobacteria
- Deferribacteres
- Deinococcus-Thermus
- Deltaproteobacteria
- Elusimicrobia
- Euryarchaeota
- Firmicutes
- Fusobacteria
- Gemmatimonadetes
- Glomerales
- Kyrinymyctes
- Korarchaeota
- Lentisphaerae
- Microsporidia
- Nanoarchaeota
- Nepocallimastigomycota
- Nitrospirae
- Phaeobacteria
- Planctomycetes
- Platyhelminthes
- Poribacteria
- Proteobacteria
- Spirochaetes
- Streptophyta
- Synergistetes
- Tardigrada
- Thaumarchaeota
- Thematogae
- unclassified (archaeal)
- unclassified (bacteria)
- unclassified (eukaryota)
- unclassified (fungi)
- unclassified (other sequences)
- unclassified (unclassified)
- unclassified (viruses)
- Verrucomicrobia

EDC
Principal Pathogens Detected

- *Trypanosoma cruzi* (Chagas disease)
- *Leishmania donovani* (Leishmaniasis)
- *Clostridium*
  - *botulinum*
  - *difficile*
- Human papillomavirus
  - HPV-21
  - HPV-49
Trypanosoma cruzi

A. *T. cruzi* large subunit

B. Phylogenetic comparisons
HPV – Sequence Homology and Phylogeny

A. Sequence homology

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B. Phylogenetic relationships
Diet and the Microbiome
## Significant “Hits” in Peruvian Mummies WGS

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<th>Crop plant</th>
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<td>Coca</td>
<td>517</td>
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<tr>
<td>Beans</td>
<td>1122</td>
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<tr>
<td>Potatoes</td>
<td>865</td>
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<tr>
<td>Peanuts</td>
<td>912</td>
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<tr>
<td>Pepper</td>
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<td>Tomatoes</td>
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<tr>
<td>Corn</td>
<td>589</td>
</tr>
<tr>
<td>Rice</td>
<td>435</td>
</tr>
<tr>
<td>Avocado</td>
<td>911</td>
</tr>
<tr>
<td>Quinoa</td>
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<td>Sweet potatoes</td>
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<table>
<thead>
<tr>
<th>Animals</th>
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<td>Alpaca</td>
<td>618</td>
</tr>
<tr>
<td>Llama</td>
<td>459</td>
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<tr>
<td>Chimney swift</td>
<td>155</td>
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<tr>
<td>Bony fishes</td>
<td>377</td>
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</table>
Gut Microbiome Comparisons: Two Diets
Principal Dietary Composition – Comparative Study

###Livestock
- Alpaca: 4
- Goat: 17
- Llama: 3
- Pig: 14
- Sheep: 18
- Corn: 20
- Potato: 19
- Quinoa: 42
- Rice: 11
- Sweet Potato: 0
- Tomato: 3
- Wheat: 0

###Vegetables
- Italian
- Peruvian
Dietary Impact on Microbial Prevalence

![Graph showing dietary composition of Italian and Peruvian diets](image)

- Italian Nobility Mummies
- Peruvian Inca Mummies

Bacteroides: High Fiber Diet
Prevotella: High Animal Tissue and Fat

Vereades, et al. 2014, *Frontiers in Microbiology*
Dietary Impact on Microbial Prevalence ... Continued
Alpha Diversity: Italian v. Peruvian Mummies

**SHANNON DIVERSITY INDEX**

- Italian: 4.56
- Peruvian: 1.90

**OBSERVED SPECIES**

- Italian: 1044.33
- Peruvian: 396.67
Carbohydrate Metabolism
Human-Encoded GH: By Anatomical Site of FI9

**FI9 - MICROBIOME CONTRIBUTION OF GHs**

- **ASCENDING**
  - Human-encoded: 41.28%
  - Microbiome-encoded: 58.72%

- **TRANSVERSE**
  - Human-encoded: 7.06%
  - Microbiome-encoded: 92.94%

- **DESCENDING**
  - Human-encoded: 15.73%
  - Microbiome-encoded: 84.27%
Human-Encoded GH: By Anatomical Site

RELATIVE ABUNDANCE OF HUMAN-ENCODED GHs IN FI9

- Colon

GH1
GH10
GH13
GH18
GH31
GH35

Values:
- GH1: 0.0030
- GH10: 0.0017
- GH13: 0.0441
- GH18: 0.0047
- GH31: 0.1624
- GH35: 0.0003

Colon:
- GH1: 0.0001
- GH10: 0.0002
- GH13: 0.0071
- GH18: 0.0018
- GH31: 0.0066
- GH35: 0.0019

Metabolism (BlastX v PIECRUST)

A. Metagenome
- Xenobiotics degradation and metabolism
- Transport and catabolism
- Nucleotide metabolism
- Metabolism of terpenoids and polyketides
- Metabolism of other amino acids
- Metabolism of cofactors and vitamins
- Lipid metabolism
- Glycan biosynthesis and metabolism
- Folding, sorting and degradation
- Energy metabolism
- Carbohydrate metabolism
- Biosynthesis of other secondary metabolites
- Amino acid metabolism

B. 16S
- Xenobiotics degradation and metabolism
- Transport and catabolism
- Nucleotide metabolism
- Metabolism of terpenoids and polyketides
- Metabolism of other amino acids
- Metabolism of cofactors and vitamins
- Lipid metabolism
- Glycan biosynthesis and metabolism
- Folding, sorting and degradation
- Energy metabolism
- Carbohydrate metabolism
- Biosynthesis of other secondary metabolites
- Amino acid metabolism

Percentage of sequences

- FI3
- FI9
- FI12
- Modern
NASD 22 v. NASD 27
Final Comments (Finally!)

- Ancient DNA must be suitable for analysis
  - Sample collection and processing
  - Taphonomy
  - Damage analysis recommended

- Can help answer questions by providing a time reference
  - Mindful of information loss
  - Ask the correct and appropriate questions
Thank you for your interest

Questions?
Thank you for joining today!

Register for more ATCC “Excellence in Research” webinars, or watch recorded webinars, at www.atcc.org/webinars.

- **April 21, 2016**
  10:00 AM, 3:00 PM EST
  Steven Budd, M.S., M.B.A., *Product Line Business Specialist*, ATCC
  Best Practices in Cryopreservation

- **April 28, 2016**
  10:00 AM, 3:00 PM EST
  Frank Simione, M.S., *Director, Standards, Standards Resource Organization*, ATCC
  The ATCC Story: A Ninety Year Celebration

Please email additional questions to:

tech@atcc.org