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Local domestication of microbes via cassava beer fermentation

Cassava beer, or *chicha*, is typically consumed daily by the indigenous Shuar people of the Ecuadorian Amazon. This traditional beverage made from cassava tuber (*Manihot esculenta*) improves nutritional quality and flavor while extending shelf life in a tropical climate. Bacteria responsible for *chicha* fermentation could be a source of microbes beneficial to human health, but little is known regarding the microbiology of *chicha*. We investigated bacterial community composition of *chicha* batches using Illumina high-throughput sequencing. Fermented *chicha* samples were collected from seven Shuar households in two neighboring villages in the Morona-Santiago region of Ecuador, and the composition of the bacterial communities within each *chicha* sample was determined by sequencing a region of the 16S ribosomal gene. Members of the genus *Lactobacillus* dominated all samples, demonstrating that *chicha* is a source of organisms related to known probiotics. Significantly greater taxonomic similarity was observed between communities in *chicha* samples taken within a village than those from different villages. Community composition varied among *chicha* samples, even those separated by short geographic distances, suggesting that ecological and/or evolutionary processes, including human preference, may be responsible for creating locally adapted and regionally resilient ferments. Our results suggest that traditional fermentation may be a form of domestication that provides endemic beneficial inocula for consumers.

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Local domestication of microbes via cassava beer fermentation

Alese M. Colehour[1,2]
James F. Meadow[2]
Melissa A. Liebert[1]
Tara J. Cepon-Robins[1]
Theresa E. Gildner[1]
Sam S. Urlacher[3]
Brendan J.M. Bohannan[2]
J. Josh Snodgrass[1]
Lawrence S. Sugiyama[1]

[1] Department of Anthropology, University of Oregon, Eugene, OR, USA
[2] Institute of Ecology and Evolution, University of Oregon, Eugene, OR, USA
[3] Department of Human Evolutionary Biology, Harvard University, Cambridge, MA, USA

Corresponding Author: Alese M. Colehour, alese@uoregon.edu, @MicrobeMagick

ABSTRACT

Cassava beer, or *chicha*, is typically consumed daily by the indigenous Shuar people of the Ecuadorian Amazon. This traditional beverage made from cassava tuber (*Manihot esculenta*) improves nutritional quality and flavor while extending shelf life in a tropical climate. Bacteria responsible for *chicha* fermentation could be a source of microbes beneficial to human health, but little is known regarding the microbiology of *chicha*. We investigated bacterial community composition of *chicha* batches using Illumina high-throughput sequencing. Fermented *chicha* samples were collected from seven Shuar households in two neighboring villages in the Morona-Santiago region of Ecuador, and the composition of the bacterial communities within each *chicha* sample was determined by sequencing a region of the 16S ribosomal gene. Members of the genus *Lactobacillus* dominated all samples, demonstrating that *chicha* is a source of organisms related to known probiotics. Significantly greater taxonomic similarity was observed between communities in *chicha* samples taken within a village than those from different villages. Community composition varied among *chicha* samples, even those separated by short geographic distances, suggesting that ecological and/or evolutionary processes, including human preference, may be responsible for creating locally adapted and regionally resilient ferments. Our results suggest that traditional fermentation may be a form of domestication that provides endemic beneficial inocula for consumers.

INTRODUCTION

Fermentation converts simple carbohydrates into secondary compounds, including alcohols and lactic acid, and it is promoted by human societies worldwide as a means to improve the flavor, nutritional value, and preservation of food and drinks. Fermentation is mediated by a variety of microorganisms; for example, yeasts convert carbohydrates into carbon dioxide and alcohol to produce alcoholic beverages, while bacteria create lactic acid, the tangy flavor characteristic in food such as sauerkraut and yogurt. Conventional fermentation utilizes commercially available bacteria or yeast for fermentation, often from a single laboratory-isolated strain. In contrast, traditional fermentation, colloquially termed wild fermentation, harnesses diverse bacteria and yeast resident in the environment to cultivate a ferment over many generations (Katz,

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2003; Scott & Sullivan, 2008; McGee, 2013). Wild fermentation utilizes diverse communities of bacteria and yeast that undergo ecological succession in the fermentation vessel as the community structure changes in response to conditions created by preceding species.

In a typical wild ferment, pioneer species such as *Leuconostoc* spp. bacteria begin the successional process by consuming raw material and creating acidic conditions. This facilitates the emergence and proliferation of *Lactobacillus* spp. and yeasts that dominate the finished product (J. Cho et al., 2006). Repeated lacto-fermentation of a single food product type over long periods of time can be considered a form of microbial domestication, where human choice and adaptation to unique environmental fluctuations (*e.g.* temperature, pH, and disturbance) result in a resilient, microbial ecosystem (Swenson, Wilson, & Elias, 2000; Diamond, 2002; Libkind et al., 2011). Thus, ecological processes inherent in wild fermentation, including human-mediated selection, result in artisanal products unique to a particular region and cultural practice (*e.g.* Lambic ales, Old World wines, cheeses, and sourdough breads).

Chicha is a traditional fermented beverage still produced by indigenous groups throughout the Amazon basin. Archaeologists have identified traces of 1600-year-old sprouted maize *chicha* in 150-liter clay vats in the remains of a pre-Incan civilization in Cerro-Baul, Peru, making it one of the oldest known ferments (Moseley et al., 2005). Today, indigenous groups brew *chicha* from sweet cassava (*Manihot esculenta*), or *yuca*, a staple tuber cultivar in tropical climates. It is typically a low alcohol beverage (2-5%), with a milky consistency and somewhat sour flavor. *Chicha* is typically prepared over a 2-3 day period. First, the roots of *yuca* are peeled, washed, and boiled until soft. Water is drained off and the root is mashed with a dedicated pestle, while the brewer masticates pieces of the mash and periodically spits into the mash. Recipes vary according to the brewer's taste. For example, different types of yuca can be mixed together, or raw yam (*Dioscorea* sp.) is sometimes masticated and added. Each new batch is added to a vessel containing remains of the previous brew, thus inoculating the fresh mash with a starter culture that is maintained over long periods of time and multiple generations.

Many forager-horticulturalist groups in the Amazon region subsist on cassava tuber and plantain, supplemented by animals, fish and fruits collected from the surrounding forest (Hill et al., 1984; B. Piperata & Dufour, 2007). Fermented foods such as *chicha* are a key component of the diet for some, since fermentation improves bioavailability and synthesis of essential vitamins and minerals that may otherwise be lacking (Boonnop, Wanapat, Nontaso, & Wanapat, 2009; Ahaotu, Ogueke, & Owuamanam, 2011). This is particularly important since chronic nutritional stress among indigenous groups can stunt growth (Blackwell et al., 2009; B. A. Piperata et al., 2011). Furthermore, fermentation facilitates decomposition of organic toxins such as naturally-occurring cyanides in *yuca* that cause weakness, hypothyroidism, and paralysis (Lei, Amoa-Awua, & Brimer, 1999). Living ferments also contain viable probiotics (microbes beneficial to human health) and prebiotics (nutrients required by these beneficial microbes) (Saulnier et al., 2009).

Despite the importance and widespread consumption of *chicha,* no studies to date have characterized the microbial community present in *chicha* using modern culture-independent techniques. Several groups of lactic-acid bacteria including *Lactobacillus* spp., have been detected using culture-dependent methods (Axelsson, 2009; C. C. A. D. A. Santos et al., 2012). Some species of *Lactobacillus* are considered to be beneficial to human health given their ability to bind to the lining of the intestinal tract, compete with pathogens and stimulate mucus production (Kravtsov et al., 2008). They also improve the uptake of nutrients by enhancing mineral absorption, degrading antinutrients (*e.g.* digestion inhibitors synthesized as a plant's self-defense against herbivores), and promoting host growth factors (Turpin et al., 2010). Commercially-isolated *Lactobacillus* strains are commonly added to pasteurized dairy products such as yogurt or sold in capsule form as an increasingly popular solution for an array of common health problems, including irritable bowel syndrome and other conditions related to chronic inflammation of the intestinal tract (Allgeyer, Miller, & Lee, 2010; Ranadheera, Baines, & Adams, 2010; Yang & Sheu, 2012). However, traditional amylaceous (starch-based) ferments such as *chicha* are thought to contain novel strains of *Lactobacillus* that have higher rates of cell adhesion compared to commercially available strains (Turpin et al., 2012), and are therefore likely understudied sources of beneficial microbes in indigenous populations.

Just as in any ecosystem, bacterial communities in fermented foods are shaped by a variety of ecological processes, including environmental selection and dispersal, that select for a subset of potential inhabitants from a metacommunity (a set of communities linked by dispersal of multiple, interacting species). Lactic-acid bacteria in wild ferments have the unique ability to survive nutrient saturation and starvation, suggesting that *Lactobacillus* is adapted to fermentation processes utilized by humans and other animals (Ganesan, Dobrowolski, & Weimer, 2006; Suzzi, 2011). Other microorganisms such as *Saccharomyces cerevisiae,* the yeast species that ferments the majority of conventional beer and wine, are broadly considered domesticated because they are only found in human-controlled environments (Fay & Benavides, 2005). Recent research shows geographic divergence in artisan cheese cultures that correlates with microbes found on surfaces in the processing facility (Bokulich & Mills, 2013), hinting at the possibility that diverse microbial communities undergo geographic divergence in human-mediated ecosystems. However, it is unclear how microbial community composition in a small-batch wild ferment varies over time or as a factor of the ambient environment (*i.e.* processing surfaces or the human cultivator).

To address this question, we collaborated with an indigenous group of Shuar (as part of the Shuar Health and Life History Project [http://www.bonesandbehavior.org/shuar/]) engaged in a forager-horticulturalist lifestyle in the remote Cross-Cutucú region of the Ecuadorian Amazon. We assessed taxonomic similarity of bacterial communities in *chicha* batches across two different villages to determine whether they were more similar over time within the same household than they were to batches from neighboring houses. Then we compared bacterial communities across different villages to determine whether *chicha* communities were more similar within a village than across villages.

MATERIALS & METHODS

Population and Location

All samples were taken within the Cross-Cutucú region of Amazonian Ecuador, which lies east of the Cutucú Mountains in the tropical Amazon rainforest. This region has an annual rainfall of more than 4,000 mm (158 inches) and average daytime temperatures of 29C (85F) (Kricher, 1999). The Shuar are a forager-horticulturalist group indigenous to the Ecuadorian Amazon rainforest and live primarily in small riverine villages. They are a natural fertility population and commonly live with extended family units in traditional thatch-roof, earthen-floor houses. Their present day economy is based on horticulture, hunting and gathering, yet they are currently experiencing increasingly rapid infrastructure development and market integration as a result of regional economic development (Karsten, 1935; Liebert et al 2013). However the villages in the present study subsist with limited daily access to markets or exposure to economic development, and adequate nutrition remains a concern in the area. Rates of infectious disease and parasite loads remain high throughout this population, accounting for 15% adult mortality in 2008 (World Health Organization, 2011; McDade et al., 2012;). For example, Cepon-Robins and colleagues (Cepon-Robins et al., 2013) reported that 65% of the population in this particular region is infected with parasitic worms, with even higher prevalence among children. Stunting among children is a common public health concern, and is relevant to ongoing studies investigating metabolic health in the context of economic transitioning populations (R. V. Santos, Coimbra Jr, Coimbra Jr, Santos, & Escobar, 2003; Foster et al., 2005; Orellana, Santos, Coimbra Jr, & Leite, 2009; Blackwell et al., 2009; Liebert et al., 2013) making nutrition-related health research a high priority in this region. Further, documentation of nutritional benefits of chicha consumption are locally useful in health education and cultural preservation.

Sample Collection

We collected samples in two villages in the Cross-Cutucú region of Morona Santiago, Ecuador. Village 1 (V1; pop. 50) is located approximately two-four hours by motorized canoe (depending on water levels) from the nearest port with road access. A nearby spring located upstream from the village provides water for bathing and cooking. Village 2 (V2; pop. 400) is located twenty-minutes by foot from V1 (including a bridgeless river crossing). Water is drawn from a spring to a reserve that flows through pipes to some houses, others get water from small streams or seeps. In both communities each household has their own *chicha* ferment, containing brews that are produced by the resident women. New batches are produced every 3-5 days or as needed.

We collected 2 mL of mature *chicha* from five households in V1 and two households in V2, during August 2012 (sample volume was limited due to limited portable freezer space on site). Over a period of two weeks, we collected samples from each of these seven ferments up to three times, each representing independent batches (with a shared starter culture). We sampled 300mL of spring water (concentrated on a 0.45 micron pore, cellulose acetate filter) that residents in V1 use to prepare *chicha*. We were unable to collect water from V2 due to equipment malfunction. All samples were immediately frozen (-20C) before being transported and stored at the University of Oregon until they were processed. All samples were examined under a light microscope for evidence of helminth eggs or macrophages.

Ethics Statement

This study was conducted in Shuar communities located within Canton Tiwintza, Morona Santiago, Ecuador. Authorization for the Shuar Health and Life History project research was provided by the Federación Interprovincial de Centros Shuar (FICSH). No human data was gathered as part of this project, and the bacterial data gathered was purged of any human mitochondrial sequences by removing all sequences classified within the Order Rickettsiales before archiving. Genetic material resulting from this research will never be sold for use on human DNA research or commercial cell-line patenting.

Microbial DNA Extraction and Sequencing

Fermentation maturity of samples was confirmed with litmus paper ensuring a pH range between 4.0-4.5 (Luedeking & Piret, 1959; C. C. A. D. A. Santos et al., 2012). Whole genomic DNA was extracted from all samples using MO BIO Power Plant Pro kit including phenol separation solution step (MO BIO Laboratories, Carlsbad, CA) and amplified on the V4 region of the 16S rRNA (F515/R806 primer combination: 5′-GTGCCAGCMGCCGCGGTAA-3′, 5′-TACNVGGGTATCTAATCC-3′) (J. G. Caporaso et al., 2010; Meadow et al., 2013). DNA amplifications were performed in triplicate and pooled prior to sequencing. The reverse primer included a 12 bp Golay barcode for demultiplexing in downstream analysis. PCR conditions followed Caporaso et al. (J. G. Caporaso et al., 2010). Amplicons were purified using gel electrophoresis and the MO BIO UltraClean GelSpin DNA extraction kit. Equal amounts of purified amplicons from each sample were pooled and sent to the Dana Farber Cancer Institute Molecular Biology Core Facility (http://www.dana-farber.org), to be sequenced on the Illumina MiSeq platform using a paired-end 250 bp protocol. All sequences have been deposited in the MG-RAST archive under accession numbers 4545634.3-4545652.3.

Sequence Processing and Statistical Analysis

Sequence processing was conducted in QIIME (J. Caporaso, Kuczynski, & Stombaugh, 2010) using MacQiime (version 1.6.0, http://www.wernerlab.org/software/macqiime). Quality filtered forward reads (Phred score>20; 250bp) were binned with barcodes corresponding to the respective sample IDs. Operational taxonomic units (OTUs) were assigned at 99% genetic similarity. Representative OTU sequences were aligned to the Greengenes database (October 2012 version) and assigned taxonomic nomenclature. We rarified all samples to 19,000 sequences for even sampling depth; two samples significantly below that threshold were omitted from further analysis.

Community similarity was calculated in two different ways: with the taxonomy-based Bray-Curtis metric, and by calculating the number of OTUs shared between samples. Bray-Curtis quantifies the taxonomic dissimilarity between two communities using a constrained scale between 0 and 1 without regard to species abundance. We determined if differences were significant using PERMANOVA (Adonis method). We then used one-way ANOVA (SPSS Statistics version 20.0.0) to investigate differences in the number of shared OTUs across households and across villages.

RESULTS

We generated a total of 1,055,214 barcoded sequences 249 base pairs in length. Sequences were quality filtered and rarefied to 19,000 OTUs per sample. The nineteen samples used for analysis represent one to three *chicha* batches from 7 different households (five from Village 1 and two from Village 2). The bacterial communities in all samples were dominated by members of the genus *Lactobacillus*. Of the ten most abundant OTUs across samples, nine were *Lactobacillus;* the other was an Acetobacter. These 10 OTUs each represented >1% of each sample, collectively accounting for 71% of the sequences in all samples (Figure 1). The top two most abundant species, *L. acidophilus* and *L. reuteri*, account for 51% of the entire dataset. Two of the most abundant *Lactobacillus* OTUs were only 96% and 98% similar to existing isolates in the NCBI database, suggesting the presence of previously undescribed taxa.

The bacterial communities detected in water samples had higher phylum level diversity than *chicha* (127,558 OTUs per sample). Whereas Lactobacillaceae dominated *chicha, Delftia acidovorans* (NC_010002), a member of the Comamonadaceae first isolated from a sewage treatment plant in Germany (Schleheck et al., 2004), was the most abundant OTU encountered in water (18.4% of the total)., The bacteria most commonly shared between water and *chicha* were species within the genus *Acetobacter.* This clade oxidizes alcohol and sugar to create acetic acid and are found in traditional balsamic vinegar production (Gullo, De Vero, & Giudici, 2009). Overall, community composition of *chicha* is very different from water, indicating the microbial population is driven by more than just the water source.

Bacterial communities in *chicha* were significantly different across the two villages ($F_{1,12} = 1.11$, $p = 0.038$; from PERMANOVA on Bray-Curtis dissimilarity matrix), but they were not significantly different across households within a village ($F_{5,8} = 0.38$, $p = 0.73$). Water samples were significantly different from the *chicha* samples ($F_{1,17} = 8.25$, $p = 0.005$). More OTUs were shared between households within a village than across villages (Figure 2: $M_{DifferentVillage} = 7.44$, $M_{SameVillage} = 8.31$, $F_{1,89} = 4.11$, $p = 0.046$), but batches from the same household did not have more OTUs in common than they did with batches from the same village ($M_{DifferentHouse} = 7.93$, $M_{SameHouse} = 8.27$, $F_{1,89} = 0.28$, $p = 0.60$).

No helminth eggs or macrophages were detected in the samples. However, the samples underwent two freeze-thaw cycles before microscopic examination, which is known to reduce visibility of parasites. Additional research is needed before we can present any evidence that fermentation affects the presence or absence of parasites.

DISCUSSION

Humans continuously and intimately interact with microorganisms. In the case of *chicha*, the microbial community present throughout the fermentation process is directly linked to the human microbiome; microbes from saliva combine with a starter culture to inoculate each new mash. In turn, mature lacto-ferments are consumed and become a potential source of beneficial microbes for the human microbiome (Figure 3) (Dethlefsen, McFall-Ngai, & Relman, 2007; Costello et al., 2009; Spor, Koren, & Ley, 2011; The Human Microbiome Project Consortium, 2012; Linnenbrink et al., 2013;). Intriguingly, all of the numerically dominant species of *Lactobacillus* we detected in *chicha* have also been reported in the human oral and fecal microbiome (Dewhirst et al., 2010).

To better understand the relationship between human cultivators and their ferments, we were interested in knowing if microbial composition showed taxonomic divergence over geographic space. We observed that the microbial communities in *chicha* were more similar within a village than between villages ($p<0.05$). This variation could result from a combination of mechanisms, including distance-limited dispersal, stochastic succession, and human-mediated selection. Dispersal between *chicha* ferments could occur if starter cultures are mixed or if a brew mistress contributes saliva to her neighbor's *chicha*. Distance and geographic barriers (*e.g.* a bridgeless river in our case) could limit the opportunity for dispersal between the two villages and may partially explain the observed variation in microbial community composition (Bokulich & Mills, 2013; Linnenbrink et al., 2013). Given that *chicha* is typically generously shared with neighbors within a village, it is not surprising that we did not see significant taxonomic dissimilarity at the household level.

Each new *chicha* batch represents a unique opportunity for succession, which could be contingent on the order and frequency of species arrival. In addition, competition between microbes, abiotic conditions, and random chance could all shape the communities within each *chicha* vessel. The water source used in the fermentation vessel (V1: hauled in vessels from a spring; V2: piped to houses from a reservoir) may represent a source of either facilitative or competing microbes that could influence the final composition of the ferment. Since households within a village rely primarily on the same water source, this could help explain why *chicha* is more similar within a village but not within an individual household. Differences in cultivation practice between the two villages may also contribute to variation (human-mediated selection)*.* Expressions among the Shuar such as “the prettiest girl makes the sweetest *chicha*” suggest individuality and personal preferences play a role in the cultivation of this lacto-ferment, as do commonly heard conversations regarding individual preferences for “sweeter” or for “stronger” (i.e., longer ferment, more acidic flavor) *chicha*.

These three processes help explain the microbe community variation we observed at a single time point. In this system, the leftover *chicha* in the fermentation vessel acts as a starter culture for each new batch, allowing the possibility for domestication (adaptation by organisms to an intimate association with human beings). It is impossible to know how long microbial communities in *chicha* have been undergoing domestication. If we assume some level of methodological continuity over time—and we know that archaeologists have found remains of the ferment brewed 16 centuries ago (Moseley et al., 2005)—it is likely that the inoculum has been inherited over many generations. If this were the case, a co-evolutionary relationship could emerge from such long-term ecological interactions between brew and brewer (Figure 3). Future work is necessary to explore these ideas in greater depth.

This study demonstrates that this traditional, or wild, fermentation, promotes a diversity of microorganisms, including *Lactobacillus* strains that might provide a broad range of health benefits (Costello et al., 2012). Lactic-acid bacteria have high rates of cell adhesion allowing for direct interface with the human intestine, and have been shown to protect against pathogens, modulate immune response, and promote mucus secretions to soothe the intestinal lining. In addition, lactic-acid bacteria provide digestion assistance, improving vitamin and mineral bioavailability while degrading antinutrients and other phytotoxins such as cyanide.

Beneficial microbes are now considered an essential component of human health (Dethlefsen et al., 2007; Blaser, 2011; I. Cho & Blaser, 2012), and local sources of such microbes could increase nutritional security and sovereignty among the Shuar consumers. In the tropics, cultivated food is vulnerable to spoilage due to high heat and humidity. The living, lacto-ferment is a diverse, locally adapted culture whereas a commercial probiotic may not be resilient to local conditions, thereby requiring dependence on continued purchase of the product. Geographically distinct *chicha* assemblages provide evidence for microbial domestication, and may indicate a coevolutionary relationship with human cultivators.

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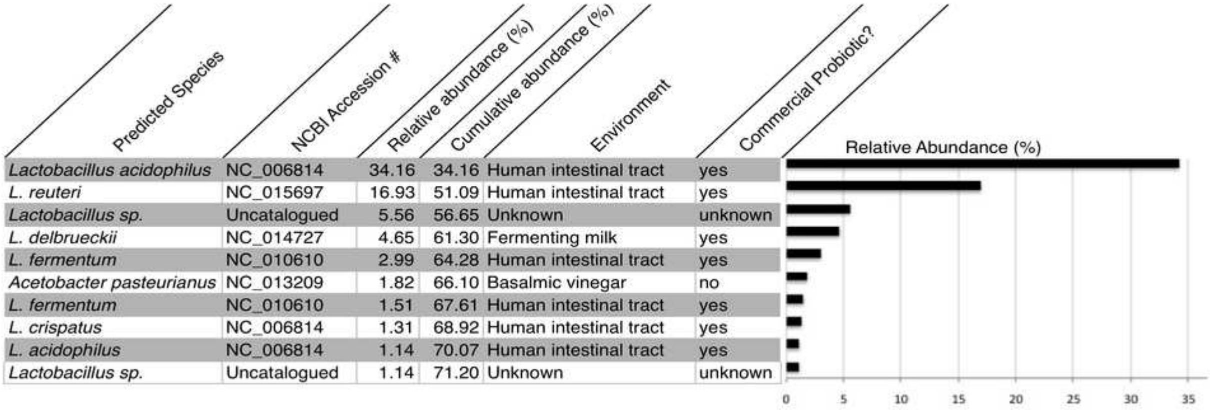
Figure 1

Ten most abundant microbes in *chicha* dominated by *Lactobacillus* bacteria

Fourteen *chicha* samples from seven independent ferments rarified to 19,000 OTUs per sample yields 266,000 evenly distributed sequences. OTU identities are from an NCBI BLAST search. We found ten OTUs with greater than 1% relative abundance. Collectively, these ten OTUs account for 71% cumulative abundance of the entire rarified dataset.



Predicted Species	NCBI Accession #	Relative abundance (%)	Cumulative abundance (%)	Environment	Commercial Probiotic?
Lactobacillus acidophilus	NC_006814	34.16	34.16	Human intestinal tract	yes
L. reuteri	NC_015697	16.93	51.09	Human intestinal tract	yes
Lactobacillus sp.	Uncatalogued	5.56	56.65	Unknown	unknown
L. delbrueckii	NC_014727	4.65	61.30	Fermenting milk	yes
L. fermentum	NC_010610	2.99	64.28	Human intestinal tract	yes
Acetobacter pasteurianus	NC_013209	1.82	66.10	Basalmic vinegar	no
L. fermentum	NC_010610	1.51	67.61	Human intestinal tract	yes
L. crispatus	NC_006814	1.31	68.92	Human intestinal tract	yes
L. acidophilus	NC_006814	1.14	70.07	Human intestinal tract	yes
Lactobacillus sp.	Uncatalogued	1.14	71.20	Unknown	unknown

Figure 2

Chicha from the same village contain more shared OTUs

We counted shared OTUs between every possible combination of *chicha* samples. The average number of matching OTUs between samples that are paired within the same village was significantly higher than those paired from different villages. We considered *chicha* samples from the same house as independent since there was no significance by house but they are grouped together in this figure for visualization purposes.

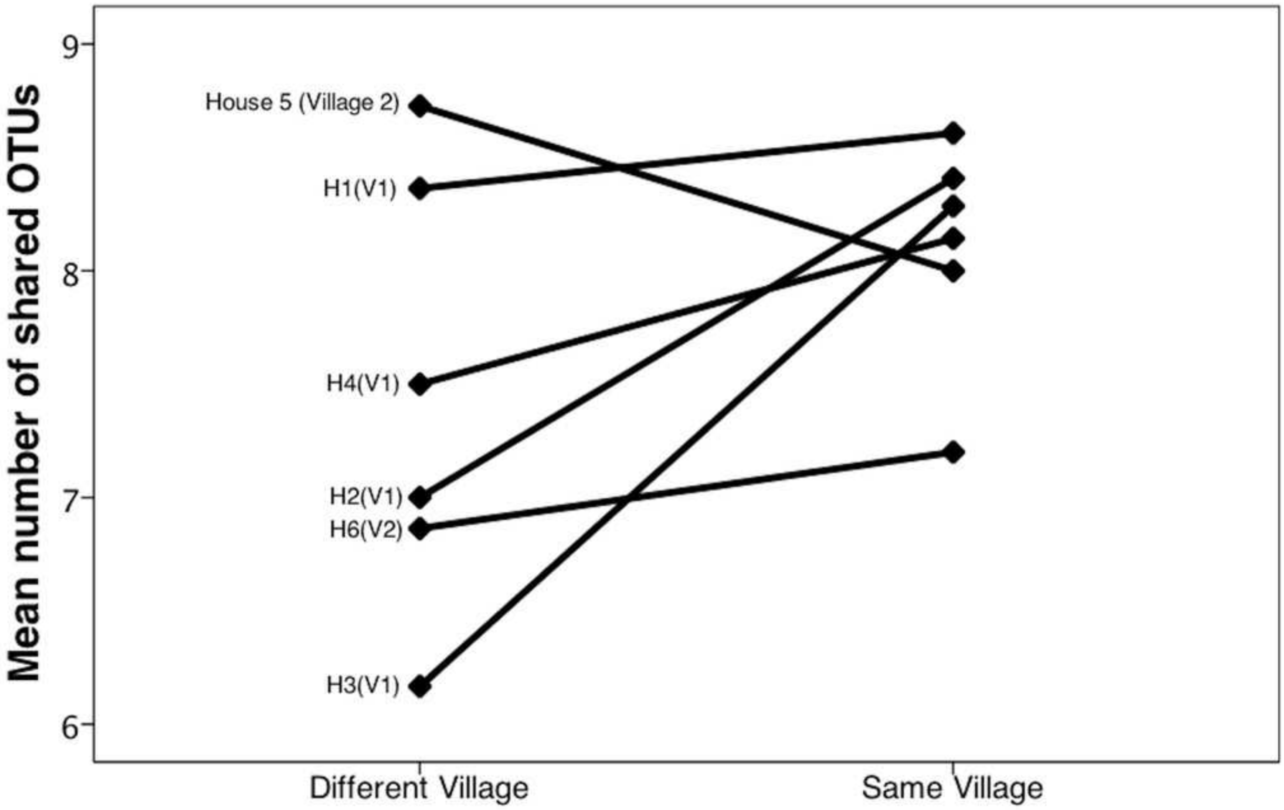


Figure 3

Theoretical model of domestication and coevolution between human cultivators and a locally adapted ferment

Wild fermentation is an ecological phenomenon driven by distance-limited dispersal, human-mediated selection, and stochastic succession that lead to geographically diversified lacto-ferment cultures. Domestication arising from behavior patterns and local abiotic factors has been linked to coevolutionary relationships (*e.g.* leaf-cutter ants and their fungal cultivar) [54]. This process can be considered a process of microbial domestication and may indicate coevolution with the human cultivator over many generations.

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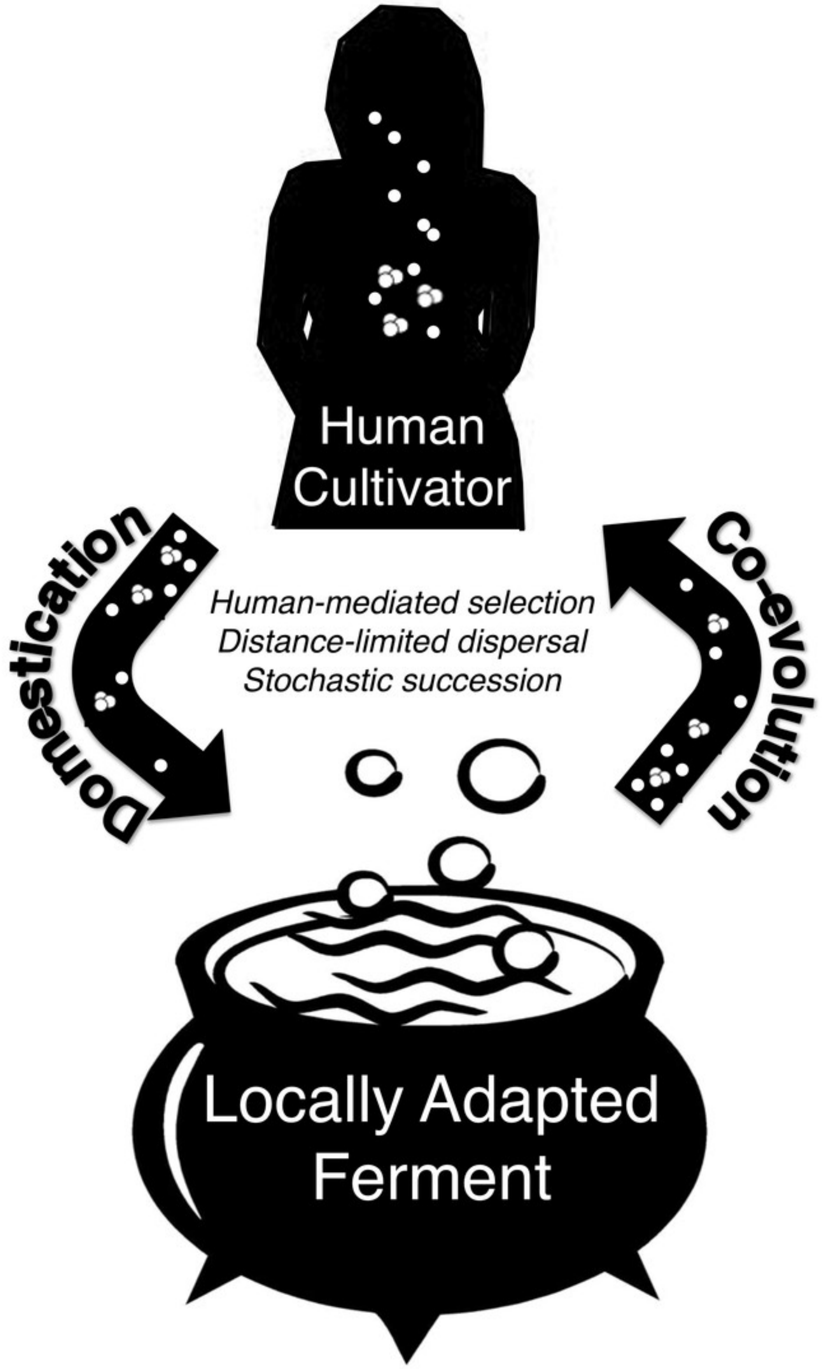

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