TURVAL Biotechnologies, Ltd.

SCIENTIFIC EXPERTISE: "THE SCIENCE UNDERLYING TRADITIONAL HUMAN ACTIVITY FOR INDUSTRY EXPLOITATION OF *KLUYVEROMYCES MARXIANUS* – improved lactose utilisation as a footprint of domestication in the *K. marxianus* B0399 "*fragilis*" lineage

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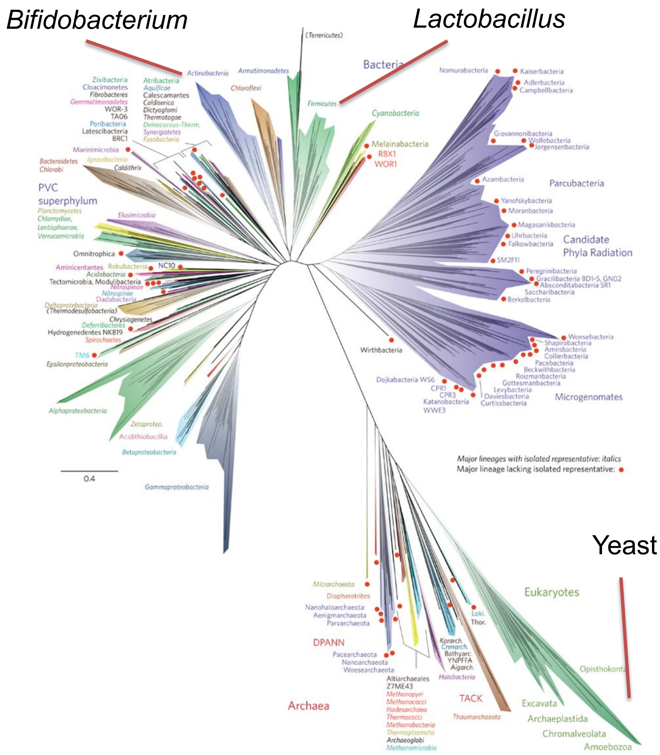
Introduction.

Yeasts (which are about 2000 species in total) have a huge importance in our daily life. They are widely used both traditional and modern biotechnology for the production of foods, beverages, enzymes, chemicals (e.g. fragrances and plastics), but also pharmaceutical products such as probiotics - the focus of this expertise. As represented on the "tree of life" there is a great "evolutionary distance" of many billions of years between yeasts as eukaryotic organisms and bacteria as prokaryotic ones, which also closely connects with a relevant biological difference between yeast probiotic species and bacterial probiotics as well as the difference in their interaction with the host.

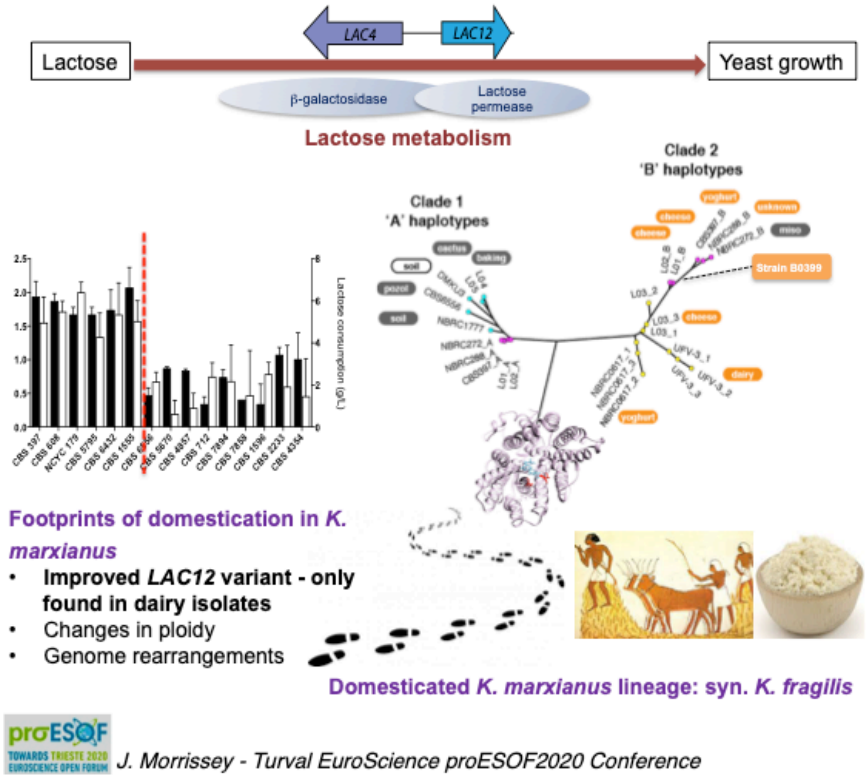


Hug, L., Baker, B., Anantharaman, K. et al. A new view of the tree of life. Nat Microbiol 1, 16048 (2016). https://doi.org/10.1038/nmicrobiol.2016.48

***Kluyveromyces marxianus:* "traditional" but ''non-conventional'' dairy yeast.**

The majority of industrially beneficial yeasts are budding yeasts of the subphylum Saccharomycotina comprising the dominant *Saccharomyces cerevisiae* and other species known in the fermented beverage (beer, wine) and food (meat, coffee, cocoa beans) industries, while the *Kluyveromyces* genus has been the most relevant one in the dairy industry. Along with an increase in fundamental research with *Kluyveromyces marxianus* in the last two decades, this dairy yeast has gained a particular industrial interest and emerged from the shadow of its cousin species, *Kluyveromyces lactis.* This is mainly because of its traits that render it specifically suitable for industrial application, such as thermotolerance, the capacity to assimilate a wide range of sugars (lactose in particular), secretion of lytic enzymes etc. In the last years, the taxonomy of these yeasts has been revised so *K. marxianus* and *K. fragilis* are actually synonyms for the same species. The QPS (EU – European Food Safety Authority) and GRAS (USA – Food and Drug Administration) certified, regulatory status of the aforementioned species confirm that they are safe to use in food and beverages without additional regulatory requirements. This yeast has been frequently isolated from dairy sources, such as fermented milks, yoghurt, French and Italian cheeses. For example, the strain *K. marxianus fragilis* B0399 was isolated from an ancient, fermented milk drink, kefir, known for its numerous pro-health effects for quite a long time, one of the reasons why potential health benefits have been attributed to this yeast ever since its classification.

K. marxianus fragilis **domestication (evolution crafted by human hand) increased its lactose utilization capacity.**



Human activity and traditional fermentations taking place over thousands of years, such as those of ancient farmers using fermented goat and sheep milk to make tasty and beneficial beverages like kefir, have shaped the evolution of the *K. marxianus* yeast in the direction of what's very useful for us now, in biotechnology, food and health industry.

One of this evolutionary shaping outcomes regards the capacity of *K. marxianus* to use lactose as the sole lactose source based on the existence of the two enzymes, *lactose permease* (encoded by *LAC12*) and β-*galactosidase* (encoded by *LAC4*). Lactose permease enables transfer of lactose molecules from outside to inside of the yeast cell while β-galactosidase enables the digestion of the lactose (disaccharide) to two simple, monosaccharide sugars, glucose and galactose. In the last few years, my research group at UCC (Ireland) studied a collection of different *K. marxianus* isolates and assessed their ability to grow on and use lactose as a carbon source. We discovered that the *K. marxianus* collection could be differentiated into two clusters: the "good utilisers" and "poor utilizers" depending on the ability of the strain to grown on lactose. The group discovered that this difference was related to the existence of polymorphisms in *LAC12* resulting in two variants of lactose permease, one found in the group of "good utilizers", which were much more efficient at lactose assimilation.

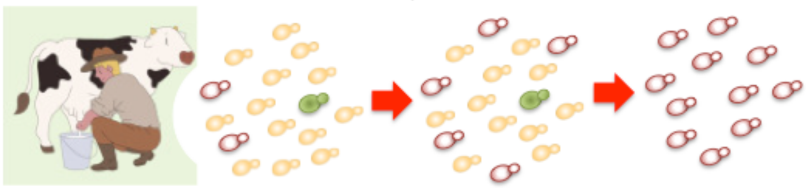
We then looked at the *Kluyveromyces* genus as a whole and found that only two groups of yeast had this "improved" permease gene and those are certain strains of *K. marxianus* designated as *K. marxianus* B (B haplotype) and *K. lactis* var. *lactis*. This improved trait, initially created as one of many spontaneous mutations, was actually selected during the course of the evolution in such an environment where it gave an advantage to cells which carried it – in traditionally fermented milk products having lactose as the main carbon source. This hypothesis of the "molecular domestication" or "evolution directed by human hand", resulting in the creation of the "dairy *K.*

marxianus lineage" (haplotype B) encoding the improved Lac12, continues to be reconfirmed with new genetic sequences of *K. marxianus* genomes, which comprises also the recently sequenced *K. marxianus* B0399 strain. Essentially, the syn. *K. fragilis* B0399 explains the "lactic pedigree" of this strain.

History of the improved *LAC* genes reveals that the human activity combined with the chance encounter between two *Kluyveromyces* cousin species resulted in completely man-made yeast species.

Tracing of the evolutionary footprints of the improved *LAC* genes in the *Kluyveromyces* genus showed two domestication events: the first one happened following introduction of *K. marxianus* yeast in milk, most likely by insects, a natural reservoir of *K. marxianus*, where it contributed to milk fermentation and where the improved variant *K. fragilis* was selected. The second event actually gave the birth to the novel yeast species, *K. lactis*, which was created by mating of the improved *K. marxianus* species ("*K. fragilis*") and another *Kluyveromyces* species, *K. drosophilarum* (syn. *K. lactis* var. *drosophilarum)* – brought into the fermentation milk medium one more time by a fruit fly, Drosophila.

Conclusion: Natural genetic variations combined with human activity may lead to selection of probiotic-type and food-production traits.



The analysis of human tartar found on fossil of human teeth shows that humans were consuming milk, most likely as cheese or other fermented products by 5,500 years ago demonstrating that traditional milk fermentation activities represent a "hotbed of evolution". Since this "domesticated lineage" of *K. marxianus* strains has been shown to contain other genome rearrangements such as mutations, hybrids and gene transfer, or so called "genetic footprints of domestication", it is reasonable to hypothesize that other beneficial properties, such as those promoting health and probiotic-type of traits or those guaranteeing good taste of dairy products, could also have been selected during the long history of use of fermented milks (kefir) as "pro-life" beverages.

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