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Natural Plasmids in a Swiss Fermented Milk and Colostrum Product assessed by Microbiome Array

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Article Info

Abstract

Natural plasmids are present in fermented foods ranging from yogurts to sourdoughs, sausages, kimchi or pickling crabs. In this study, we describe the natural plasmids present in a product obtained by fermentation of bovine milk and colostrum. We used a microarray consisting in a chip covered in short DNA sequences that are specific to target microorganisms for a total of approximately 12,000 species. As far as plasmids are concerned, the array yielded 48 iterations, each one corresponding to a unique plasmid target. We discuss the putative role of some of these plasmids in contributing to the known properties of probiotics in supporting health and longevity.

Keywords: Plasmids; Milk; Colostrum; Microbiome; Gene Transfer; Immunity.

Introduction

Foods and beverages fermented by microbes were among the first processed aliments our human ancestors consumed [1], and it is well accepted, since the observation by Metchnikoff, the "*Father of Natural Immunity*", that microbes of fermented foods contribute to human health and longevity [2]. Today's research on the role of the microbiota confirms the intuition of our ancestors who exploited the health benefits of fermented foods and beverages; we are learning that microbes are essential constituents of our bodies and, possibly, our minds since the brain itself has its own unique microbiota [3]. The role of the microbiota in determining health and longevity is now so embedded in popular culture that Vogue UK recently highlighted this concept with the following words "*The human body contains many trillions of microbes. Their interactions create vitamins, break down our food, fight infections and communicate with our genes: without a microbiome we are royally buggered*" [4]. This perception fits in the developing idea that healthy life expectancy (HALE) is different from life expectancy *tout court*; while global average life expectancy has increased in the past twenty years, HALE has not increased at the same pace. Decrease of mortality in children and adults is responsible for increased life expectancy, but it is a life affected by chronic diseases up to the point that respected Authors write of "*morbidity expansion*" [5]. It is increasingly accepted that a healthy microbiota is the key to healthy aging and life expectancy [6]. The "*trillions of microbes*" of the human microbiota harbor a proportional number of genes in their chromosomes as well as a huge number of plasmids carrying advantageous genetic information that can be transferred to other microbes and, possibly, human cells. Plasmids are made of double-strand DNA that is generally of small size and assumes a closed conformation often referred to as circular, even though the topology of DNA in plasmids may prove highly complex. Plasmids exist in nature in prokaryotes and in some eukaryotes; the genes present in plasmids provide cells with genetic advantages that favor survival and can be transferred to other cells. Since plasmids have been used for decades in laboratories for genetic engineering, it is important to distinguish between natural plasmids and their

genetically-modified counterparts that are generated for research and clinical applications [7]. Natural plasmids are omnipresent in fermented ailments ranging from Bulgarian yogurts [8] to Thai sausages, Kimchi or pickling crabs [9], from Swedish and German sour doughs [10] to Indonesian *dadih* (fermented buffalo milk) [11]. We recently described the microbial composition of a Swiss fermented milk and colostrum product whose versions have been used in the nutritional approach to chronic conditions since 2014; in that study, we focused our attention on the composition in bacteriophages [12]. Our description was based on exhaustive genetic analysis using a novel type of microarray, the Axiom Microbiome Array, a tool for analysis that is *"the most comprehensive microbial detection platform built to date"* [13]. Using this extraordinarily potent array to characterize in fine detail the microbial composition of the fermented product quoted above, we noticed that its biodiversity was much higher than previously thought and comprised a significant number of plasmids. Here we describe the plasmids present in this fermented product and we discuss the putative role of some of these plasmids in conferring health supporting properties with particular reference to their role in HALE and detoxification.

Materials and Methods

The Axiom Microbiome Array was performed by Eurofins Microbiology Laboratories Inc. (New Berlin, Wisconsin, USA) on a commercially available product designated Freeze-Dried Bravo-Colostrum and Probiotic Complex (Silver Spring Sagl, Arzo-Mendrisio, Switzerland). The results presented in this paper refer to the same product whose composition in phages was recently described [12]. After fermentation of bovine milk and colostrum at room temperature for 48 hrs, the resulting fermented product undergoes the process of freeze drying. The characteristics of the product as far as nutrition facts and microbial composition are concerned are described in a recent paper [14]. The Axiom Microbiome array used for this analysis is a chip covered in short DNA sequences that are specific to certain target organisms and a total of approximately 12,000 species are included. For each target organism, the Microbiome Detection Analysis Software (MiDAS) looks at the ratio of probes that successfully bound to their target (Probes Observed) to the total number that could potentially bind (Probes Expected), and uses this to calculate the Initial Score, which is proportional to the likelihood of the target existing in the tested sample. Once the software identifies the target that it deems most likely to be present in the actual sample, the probes that correspond to the chosen target are removed and the process is repeated until adding new species no longer adds explanatory power to the software's model of the composition of the sample. The products of this iterative analysis are called Iterations. Probes are considered positive when presenting signal intensity higher than 99th percentile of the random control probe intensities with a percentage of more than 20% of target-specific probes detected. In other words, a target organism is considered present in the sample when the percentage of observed probes is greater than 20% of the expected probes. In the example provided by the Authors who first described this assay [13], DNA from vaccinia virus was detectable when there were 1,000 or more copies of the viral DNA in the examined sample. The number of probes observed at 1,000 copies was 78 out of 293 expected probes that is about 26%, and this value was deemed positive for the presence of the virus. Although this is not a quantitative assay, some sort of quantification can be deducted; in the example of the vaccinia virus DNA, the number of probes observed at 10,000 copies was higher than that observed at 1,000 copies as it was 148 out of 286 probes expected, or about 52%. The increase of one order of magnitude in the number of copies of vaccinia virus DNA was reflected by the increase from 26 to 52%. Such proportionality between that quantity of DNA and the percentage of observed copies is present in bacteria as well. When *Shigella flexneri* was tested, its DNA was positively detected at 100 genome copies and above, with a lower limit at 10 genome copies. The number of probes observed at 100 copies was 2,185 out of 7,398 probes expected, or about 29%, whereas the number of probes observed at 1,000 copies was 3,781 out of 8,009 probes expected, or about 47% [13]. The Axiom Microbiome Array is capable of accurately detecting members of mock microbial communities used for testing, achieving 100% accuracy in species identification; the specificity of the array yields precious information for diagnosis and surveillance of pathogens in food matrices, at the same time providing strain level detections since the array was designed to detect specific genomic regions of the sequences represented on the chip.

Results

The array highlighted the presence of a very high number of targets corresponding to microbial species that fit into the definition of probiotics and are members of the families of *Streptococcaceae*, *Lactobacillaceae*, *Leuconostocaceae*, *Bifidobacteriaceae*, and *Thermaceae*. Since the product contains a blend of lyophilized kefir grains and yogurt starter with live cultures that are used to ferment milk and colostrum, we hypothesize that fermentation by the complex microflora of the kefir grains contributed to the extreme biodiversity evidenced by the Axiom Microbiome Array. Consistent with this hypothesis, it was recently demonstrated that a typical fermented kefir contains hundreds of different microbial species; such a biodiversity is deemed responsible for the known health-supporting properties of kefirs that range from modulating the immune system to fighting carcinogenesis and allergies, from favoring wound healing to regulating cholesterol metabolism and blood pressure [15].

Table 1 shows the plasmid composition of the product. The array provided 48 iterations, each one corresponding to a unique target. The targets are ordered in descending sequence with those showing the highest percentage of observed probes at the top of the list. Although the assay is not quantitative, it can be deduced that higher percentages correspond to higher number of copies [13].

Table 1. List of targets corresponding to plasmids.

Target description	Conditional Score	Initial Score	Probes Expected	Probes Observed	%
Lc. lactis subsp. *lactis* KLDS 4.0325 plasmid 2	5.99	40.75	13	11	84.61
S. thermophilus LMD-9 plasmid 2	155.25	155.25	57	43	75.43
Lb. paracasei subsp. *paracasei* JCM 8130 plasmid pLBPC-2 DNA	66.02	197.87	73	55	75.34
Lc. lactis lactis bv. *diacetylactis* DPC220 plasmid pAH82	92.13	170.15	63	47	74.6
Le. mesenteroides mesenteroides FR52 plasmid pFR18	63.49	90.57	36	26	72.22
Lc. lactis cremoris HP plasmid pHP003	12.45	103.24	41	29	70.73
Lc. lactis plasmid pSK11B	128.09	163.05	67	47	70.14
S. thermophilus SMQ-173 plasmid pSMQ173b	128.2	128.2	53	37	69.81
Lc. lactis subsp. *cremoris* A76 plasmid pQA504	136.59	136.59	56	39	69.64
S. thermophilus LMD-9 plasmid 1	123.93	123.93	52	36	69.23
Lc. lactis IL964 plasmid pIL105	106.65	126.21	52	36	69.23
Lc. lactis IL964 plasmid pIL105	103.11	126.21	52	36	69.23
Lc. lactis subsp. *cremoris* UC509.9 plasmid pCIS2	46.14	57.96	25	17	68.0
Lc. lactis DCH-4 plasmid pSRQ700	72.45	150.87	65	44	67.69
Lb. plantarum subsp. *plantarum* P-8 plasmid LBPp2	2.57	198.2	88	58	65.9
Lc. lactis subsp. *cremoris* SK111plasmid 5	115.99	147.8	67	44	65.67
Lb. plantarum subsp. *plantarum* P-8 plasmid LBPp5	2.66	153.2	69	45	65.21
Lc. lactis subsp. *cremoris* SK11 plasmid 2	90.17	117.35	54	35	64.81
Lc. lactis cremoris NIZO B40 plasmid pNZ4000	14.74	228.55	106	68	64.15
Le. citreum KM20 plasmid pLCK1	17.86	159.68	76	48	63.15
Lc. lactis subsp. *lactis* bv. *diacetylactis* plasmid pVF18	93.2	132.29	62	39	62.9
Lc. lactis subsp. *cremoris* UC509.9 plasmid pCIS5	81.58	116.52	56	35	62.5
Lb. buchneri CD034 plasmid pCD034-3	94.91	140.4	69	43	62.31
Lc. lactis cremoris Cremoris Wg2 plasmid pWVO2	26.9	61.67	31	19	61.29
Lc. lactis subsp. *lactis* plasmid pIL1	0.83	54.63	32	18	56.25
Lb. casei str. Zhang plasmid plca36	100.3	158.65	96	53	55.2
Lc. lactis subsp. *lactis* strain MJC15 plasmid pCD4	52.85	56.57	35	19	54.28
Lc. lactis plasmid pWC1	38.57	50.32	30	16	53.33
Lc. lactis subsp. *lactis* plasmid pIL3	15.13	110.35	69	36	52.17
Lc. lactis plasmid pND324	0.47	36.11	23	12	52.17
Lb. gasseri plasmid pLgLA39	93.5	149.57	101	52	51.48
Lb. salivarius CECT 5713 plasmid pHN1	73.16	124.25	88	44	50.0
S. macedonicus ACA-DC 198 plasmid pSMA198	31.1	69.82	48	24	50.0
Lb. salivarius CECT 5713 plasmid pHN1	30.61	124.25	88	44	50.0
Lc. lactis subsp. *cremoris* SK11 plasmid 1	75.05	94.81	67	33	49.25
Lb. salivarius UCC118 plasmid pSF118-44	71.3	118.83	90	43	47.77
Lc. lactis subsp. *lactis* CV56 plasmid pCV56C	3.87	86.4	65	31	47.69
S. thermophilus ST135 plasmid pER35	78.45	102.38	84	38	45.23
Lb. helveticus R0052 plasmid pIR52-1	35.13	74.41	62	28	45.16
Lc. lactis subsp. *lactis* KF147 plasmid pKF147A	45.99	93.75	93	38	40.86
Lb. paracasei subsp. *paracasei* plasmid pCD01	12.17	70.63	88	34	38.63
P. damnosus plasmid pF8801	62.73	62.73	86	31	36.04
Lb. buchneri CD034 plasmid pCD034-1	63.05	63.05	100	34	34.0
Lc. lactis plasmid pKL001	6.34	25.25	51	16	31.37
Lb. salivarius CECT 5713 plasmid pHN2	0.16	23.76	69	20	28.98
Lb. paracasei N1115 plasmid	21.36	21.36	70	20	28.57
Le. mesenteroides subsp. *mesenteroides* cryptic plasmid pTXL	7.63	7.63	63	15	23.8
Lb. brevis ATCC 367 plasmid 1	3.44	3.44	61	14	22.95

Table 2 shows the targets corresponding to *Lactobacilli* present in the product. The array identified the following *Lactobacillus* strains: *paracasei, casei, helveticus, kefiranofaciens, plantarum, gallinarum, delbrueckii, rhamnosus, salivarium, acidophilus, farraginis* and *antri*. The substrains are listed in the table.

Table 2. List of targets corresponding to Lactobacilli.

Target Description
Lactobacillus paracasei subsp. *paracasei* Lpp43 draft (205 frags)
Lactobacillus paracasei subsp. *paracasei* Lpp221 draft (265 frags)
Lactobacillus paracasei subsp. *paracasei* Lpp228 draft (188 frags)
Lactobacillus paracasei subsp. *paracasei* Lpp189 draft (295 frags)
Lactobacillus paracasei subsp. *paracasei* Lpp226 draft (131 frags)
Lactobacillus paracasei subsp. *paracasei* Lpp74 draft (190 frags)
Lactobacillus paracasei subsp. *paracasei* Lpp17 draft (131 frags)
Lactobacillus paracasei subsp. *paracasei* Lpp219 draft (238 frags)
Lactobacillus paracasei subsp. *paracasei* Lpp223 draft (168 frags)
Lactobacillus paracasei subsp. *paracasei* Lpp225 draft (74 frags)
Lactobacillus paracasei subsp. *paracasei* Lpp229 draft (317 frags)
Lactobacillus paracasei subsp. *paracasei* Lpp125 draft (192 frags)
Lactobacillus paracasei subsp. *paracasei* Lpp49 draft (109 frags)
Lactobacillus paracasei subsp. *paracasei* Lpp227 draft (596 frags)
Lactobacillus paracasei subsp. *paracasei* CNCM I-4648 draft (852 frags)
Lactobacillus paracasei subsp. *paracasei* CNCM I-4270 draft (461 frags)
Lactobacillus paracasei subsp. *paracasei* CNCM I-2877 draft (481 frags)
Lactobacillus paracasei subsp. *paracasei* Lpp46 draft (90 frags)
Lactobacillus paracasei subsp. *paracasei* Lpp70 draft (474 frags)
Lactobacillus paracasei subsp. *paracasei* Lpp120 draft (201 frags)

Lactobacillus paracasei subsp. *paracasei* Lpp22 draft (270 frags)	*Lactobacillus delbrueckii* subsp. *bulgaricus* ND02 chromosome
Lactobacillus paracasei subsp. *paracasei* 8700:2 draft (90 frags)	*Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842 chromosome
Lactobacillus paracasei subsp. *paracasei* Lpp126 draft (1355 frags)	*Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC BAA-365 chromosome
Lactobacillus paracasei subsp. *paracasei* Lpp7 draft (611 frags)	*Lactobacillus delbrueckii* subsp. *bulgaricus* 2038 chromosome
Lactobacillus paracasei subsp. *paracasei* Lpp37 draft (264 frags)	*Lactobacillus delbrueckii* ZN7a-9 draft (75 frags)
Lactobacillus paracasei COM0101 draft (184 frags)	*Lactobacillus rhamnosus* CRL1505 draft (218 frags)
Lactobacillus casei CRF28 draft (57 frags)	*Lactobacillus rhamnosus* ATCC 8530 chromosome
Lactobacillus casei M36 draft (78 frags)	*Lactobacillus rhamnosus* HMP0056 *Lactobacillus rhamnosus* LMS2-1 draft (162 frags)
Lactobacillus casei A2-362 draft (167 frags)	*Lactobacillus rhamnosus* HMP0056 *Lactobacillus rhamnosus* LMS2-1 draft (116 frags)
Lactobacillus casei Lpc-37 draft (150 frags)	*Lactobacillus rhamnosus* CRL1505 draft (218 frags)
Lactobacillus casei T71499 draft (55 frags)	*Lactobacillus rhamnosus* strain 24 draft (90 frags)
Lactobacillus casei A2-362 draft (164 frags)	*Lactobacillus rhamnosus* 2166 draft (84 frags)
Lactobacillus casei LcA draft (18 frags)	*Lactobacillus rhamnosus* LRHMDP3 draft (47 frags)
Lactobacillus casei LcY draft (14 frags)	*Lactobacillus rhamnosus* LRHMDP2 draft (50 frags)
Lactobacillus casei UCD174 draft (116 frags)	*Lactobacillus rhamnosus* 51B draft (21 frags)
Lactobacillus casei 21/1 draft (75 frags)	*Lactobacillus salivarius* ACS-116-V-Col5a draft (154 frags)
Lactobacillus casei BL23 chromosome	*Lactobacillus salivarius* cp400 draft (89 frags)
Lactobacillus casei LC2W chromosome	*Lactobacillus salivarius* NIAS840 draft (4 frags)
Lactobacillus casei BD-II chromosome	*Lactobacillus salivarius* ATCC 11741 draft (54 frags)
Lactobacillus casei W56	*Lactobacillus salivarius* cp400 draft (89 frags)
Lactobacillus casei Lc-10 draft (76 frags)	*Lactobacillus salivarius* strain JCM1046
Lactobacillus casei 5b draft (91 frags)	*Lactobacillus salivarius* GJ-24 draft (11 frags)
Lactobacillus casei UW1 draft (143 frags)	*Lactobacillus salivarius* CECT 5713 chromosome
Lactobacillus casei 32G draft (42 frags)	*Lactobacillus acidophilus* JCM 1132 draft (26 frags)
Lactobacillus helveticus CNRZ32	*Lactobacillus acidophilus* CIRM-BIA 445 draft (41 frags)
Lactobacillus helveticus MTCC 5463 draft (593 frags)	*Lactobacillus acidophilus* NCFM chromosome
Lactobacillus helveticus CIRM-BIA 104 draft (234 frags)	*Lactobacillus acidophilus* CIP 76.13 draft (55 frags)
Lactobacillus helveticus CIRM-BIA 953 draft (203 frags)	*Lactobacillus acidophilus* DSM 9126 draft (45 frags)
Lactobacillus helveticus CIRM-BIA 103 draft (222 frags)	*Lactobacillus acidophilus* ATCC 4796 draft (69 frags)
Lactobacillus helveticus CIRM-BIA 101 draft (213 frags)	*Lactobacillus acidophilus* La-14
Lactobacillus helveticus CIRM-BIA 951 draft (179 frags)	*Lactobacillus farraginis* JCM 14108 draft (129 frags)
Lactobacillus helveticus DPC 4571	*Lactobacillus antri* DSM 16041 draft (76 frags)
Lactobacillus helveticus R0052 chromosome	
Lactobacillus helveticus H10 chromosome	
Lactobacillus helveticus DSM 20075 draft (235 frags)	
Lactobacillus helveticus H9	
Lactobacillus kefiranofaciens subsp. *kefiranofaciens* JCM 6985 draft (123 frags)	
Lactobacillus kefiranofaciens ZW3 chromosome	
Lactobacillus *plantarum* UCMA 3037 draft (92 frags)	
Lactobacillus *plantarum* strain AG30 draft (48 frags)	
Lactobacillus *plantarum* IPLA88 draft (208 frags)	
Lactobacillus *plantarum* 2025 draft (595 frags)	
Lactobacillus *plantarum* 19L3 draft (241 frags)	
Lactobacillus *plantarum* 2165 draft (192 frags)	
Lactobacillus *plantarum* 16	
Lactobacillus *plantarum* ZJ316	
Lactobacillus *plantarum* 4_3 draft (105 frags)	
Lactobacillus *plantarum* WCFS1	
Lactobacillus *plantarum* WJL draft (102 frags)	
Lactobacillus *plantarum* strain Lp90 draft (33 frags)	
Lactobacillus *plantarum* JDM1	
Lactobacillus *plantarum* LP91 draft (145 frags)	
Lactobacillus *plantarum* strain DmCS_001 draft (83 frags)	
Lactobacillus *plantarum* subsp. *plantarum* P-8	
Lactobacillus *plantarum* subsp. *plantarum* NC8 draft (10 frags)	
Lactobacillus *plantarum* subsp. *plantarum* strain wikim18 draft (323 frags)	
Lactobacillus *plantarum* subsp. *plantarum* ST-III chromosome	
Lactobacillus *plantarum* subsp. *plantarum* ATCC 14917 draft (36 frags)	
Lactobacillus *plantarum* subsp. *plantarum* JCM 1149 draft (39 frags)	
Lactobacillus gallinarum JCM 2011 draft (74 frags)	
Lactobacillus delbrueckii subsp. *lactis* DSM 20072 draft (167 frags)	
Lactobacillus delbrueckii subsp. *lactis* draft (23 frags)	
Lactobacillus delbrueckii subsp. *lactis* draft (75 frags)	
Lactobacillus delbrueckii subsp. *lactis* CRL581 draft (231 frags)	
Lactobacillus delbrueckii subsp. *lactis*	
Lactobacillus delbrueckii subsp. *lactis* draft (75 frags)	
Lactobacillus delbrueckii subsp. *bulgaricus* draft (14 frags)	
Lactobacillus delbrueckii subsp. *bulgaricus* CNCM I-1519 draft (70 frags)	
Lactobacillus delbrueckii subsp. *bulgaricus* CNCM I-1632 draft (69 frags)	

Table 3 shows the targets corresponding to *Lactococcus lactis* strains present in the product. The substrains identified by the array are listed in the table.

Table 3. List of targets corresponding to Lactococci.

Target Description
Lactococcus lactis subsp. *lactis* bv. *diacetylactis* str. LD61 draft (132 frags)
Lactococcus lactis subsp. *lactis* bv. *diacetylactis* str. TIFN4 draft (182 frags)
Lactococcus lactis subsp. *lactis* bv. *diacetylactis* str. TIFN2 draft (143 frags)
Lactococcus lactis subsp. *lactis* bv. *diacetylactis* strain GL2 draft (22 frags)
Lactococcus lactis subsp. *cremoris* CNCM I-1631 draft (131 frags)
Lactococcus lactis subsp. *cremoris* TIFN3 draft (412 frags)
Lactococcus lactis subsp. *cremoris* GE214 draft (243 frags)
Lactococcus lactis subsp. *cremoris* TIFN1 draft (291 frags)
Lactococcus lactis subsp. *cremoris* TIFN7 draft (370 frags)
Lactococcus lactis subsp. *cremoris* TIFN5 draft (646 frags)
Lactococcus lactis subsp. *cremoris* TIFN6 draft (748 frags)
Lactococcus lactis subsp. *cremoris* HP draft (213 frags)
Lactococcus lactis subsp. *cremoris* MG1363 chromosome
Lactococcus lactis subsp. *cremoris* NZ9000 chromosome
Lactococcus lactis subsp. *cremoris* A76 chromosome
Lactococcus lactis subsp. *lactis* Dephy 1 draft (62 frags)
Lactococcus lactis subsp. *lactis* A12 draft (42 frags)
Lactococcus lactis subsp. *lactis* YF11 draft (71 frags)
Lactococcus lactis subsp. *lactis* JCM 5805 = NBRC 100933 draft (88 frags)
Lactococcus lactis subsp. *lactis* CV56 chromosome
Lactococcus lactis subsp. *lactis* KLDS 4.0325
Lactococcus lactis subsp. *lactis* Il1403 chromosome
Lactococcus lactis subsp. *lactis* KF147 chromosome
Lactococcus lactis subsp. *lactis* strain 511
Lactococcus lactis subsp. *lactis* NCDO 2118
Lactococcus lactis subsp. *lactis* NCDO 2118 draft (509 frags)
Lactococcus lactis subsp. *lactis* IO-1 DNA

Discussion

Here, we discuss the main features of some of the plasmids evidenced by the Axiom Microbiome Array in the product object of this study as they potentially relate to human health, longevity and healthy life expectancy.

Lactobacillus (Lb.) casei strain Zhang plasmid plca 36

The probiotic *Lb. casei* Zhang was identified by screening of lactic acid bacteria isolated from samples of *koumiss*, a traditional home-made drink obtained from mare's milk by nomadic populations in China and Inner Mongolia [16,17]. Traditionally, consumption of *koumiss* is deemed beneficial for improving the symptoms of digestive diseases and of a wide range of chronic diseases, ranging from tuberculosis to bronchitis and anemia, thus suggesting that it may be effective in strengthening and modulating the immune system. Consistent with these anecdotic observation, it was demonstrated that *Lb. casei* Zhang has antipathogen, antioxidant, and immunomodulatory properties and is highly resistant to the acidic environment of the stomach as well as to the stress exerted by the components of bile [16,17]. *Lb. casei* Zhang has a 2,861,848-bp circular chromosome and a plasmid of 36-kb exhibiting a lower content of G+C in comparison with the chromosome [18]. *Lb. casei* Zhang has more phosphotransferase system (PTS)-related proteins than other Lactobacillus strains; significant redundancy of PTSs encoded in the chromosome of *Lb. casei* Zhang may be advantageous for the transport and use of a large panel of carbon sources, thus highlighting the flexibility and adaptability to different environments of this peculiar probiotic. Plasmid plca36 contains 44 predicted coding regions, and a precise function is assigned to 23 of them [18]. Of particular interest is a relBE toxin-antitoxin (TA) locus. TA loci or systems were originally described in low-copy-number plasmids; they have the function to maintain the plasmid stability by post-segregational elimination of plasmid-free daughter cells. However, bioinformatics and experimental evidence demonstrated that the TA modules are widely spread not only upon plasmids but also on bacterial chromosomes. The relBE TA module of plca36 may serve the role of allowing survival of the host under extreme nutritional stress as it works as a cell growth modulator which helps *Lb. casei* Zhang to deal with nutritional stress. Also a region encoding a cluster of conjugation genes (tra) is of interest since it shows high similarity and co-linearity with analogous regions of plasmids pWCFS103 and pMRC01 from *Lb. plantarum* and *Lb. lactis*, respectively. Comparative gene analysis revealed that plasmids from the genus Lactobacillus may have contributed to the environmental adaptation by providing carbohydrate and amino acid transporters. In summary, the genes encoded in plca36 appear to confer a significant advantage in surviving the harsh environmental conditions that are presumably encountered in Inner Mongolia. If presence of an advantageous plasmid from a Mongolian strain in a Swiss product were not strange enough, the Axiom Microbiome Array provided another element of microbiological curiosity;

DNA from the chromosome of *Lb. casei* Zhang was not detected in the product. In other words, it appears that there is the plasmid but not the bacterium as if the plasmid *travelled* from Mongolia to Switzerland through innumerable passages in bacterial hosts of kefir grains sharing the genetic advantages originated in Mongolia with bacteria cultivated in Europe.

Lactobacillus (Lb.) buchneri CD034 plasmids pCD034-1 and pCD034-3

Lb. buchneri strains are known to improve stability of fermenting matrices upon oxygen exposure of conserved organic material thanks to their heterofermentative metabolism and the production of high levels of acetate [19]. *Lb. buchneri* CD034 possesses a circular chromosome and three additional plasmids two of which, pCD034-1 and pCD034-3, were detected by the Axiom Microbiome Array. In analogy with the case of *Lb. casei* Zhang, DNA from the chromosome of the bacterium was not observed; in this case, however, exotic transmigration of microbial DNA is probably not involved as *Lb. buchneri* is widely represented in Europe. The largest plasmid, pCD034-3, codes for 57 genes that comprise a putative polysaccharide synthesis gene cluster, whereas the functions of the two smaller plasmids, pCD034-1 and pCD034-2, are not fully elucidated [20]. In the examined product, the percentage of observed probes for pCD034-3 was 62% of expected probes, whereas the percentage was significantly lower for pCD034-1 (34%). Absence of the chromosomal DNA of the bacterium and of plasmid pCD034-2 seems to indicate that the fermented product retained only the advantageous genetic information contained in plasmids pCD034-1 and pCD034-3 that were probably selected by the peculiarities of kefir fermentation. *Lb. buchneri* is characterized by heterofermentative metabolism with high production of acetate and, in modern agriculture, these characteristics are exploited for grass silage as they result in improved stability upon oxygen exposure of the conserved plant material. The process of silage features three phases; at first, aerobic conditions are present during harvesting and preparation of green fodder. Then, proper ensiling begins after storage of the ensiled material under conditions that prevent aeration and lead to anaerobiosis. During the fermentation that follows, water soluble carbohydrates are used by lactic acid bacteria and lactic acid is produced with consequent acidification. When the silage is opened, aerobic conditions are restored as oxygen is available to the ensiled material. Since these aerobic conditions may then favor growth of spoiling microbes such as yeasts, fungi and other bacteria, *Lb. buchneri* is inoculated into the fresh plant biomass before ensiling so to preserve the stability of the material when aerobic conditions are restored at the opening of the silage [19]. Interestingly, similar conditions of aerobiosis followed by anaerobiosis and then by aerobiosis, occur during fermentation of the product described in this study, in particular considering the lower strata of the fermenting product that are not exposed to oxygen until the end of the process. Therefore, it can be assumed that acquisition of *Lb. buchneri* plasmids by other Lactobacilli

occurred as means of exploiting an evolutionary advantage associated with the peculiar conditions of fermentation. In other words, the transferable genetic information of *Lb. buchneri* may contribute to the stability of the product object of this study just as they contribute to the stability of fermented plant biomasses in ensiling. What is peculiar is the fact that, in ensiling, *Lb. buchneri* is purposely added as inoculum, whereas in the product described in this study, its plasmids occur naturally as if natural evolution were well aware of what are now considered sophisticated methods of modern agriculture.

Lactococcus (Lc.) lactis, subsp. *cremoris* SK11 plasmids SK11B, 1, 2, 5

Strains of *Lc. lactis* carry plasmids that encode traits that are important for the dairy industry; a list of the targets corresponding to substrains of *Lc. lactis* evidenced by the Axiom Microbiome Array is reported in table 3. Among these, *Lc. lactis* subsp. *cremoris* SK11 is widely used in manufacture of fermented milk products. The Axiom Microbiome Array evidenced the presence of 4 plasmids of *Lc. lactis* subsp. *cremoris* SK11, namely SK11B, SK11 5, SK11 2, and SK11 1, here listed from the most to the least represented (Table 1). The significant over-representation of pSK11B is consistent with the hypothesis that this is a high-copy-number plasmid in comparison with the other three. It should be noticed, however, that other plasmids of different *Lc. lactis* subsp. *cremoris* strains were more represented than SK11B. Other plasmids pertaining to *Lc. lactis* subsp. *cremoris* strains identified by the array were; pHP003, pQA504, pCIS2, pNZ4000, pCIS5, and pWVO2 (Table 1). Many substrains of *Lc. lactis* subsp. *cremoris* have plasmids that encode traits responsible for catabolism of lactose, utilization of citrate, synthesis of proteases, production of bacteriocin, resistance to phages, synthesis of the exopolysaccharide, and, most important in this context, resistance to heavy metals [21]. Plasmid SK11B carries the gene aldC that codes for a protein of 221 amino acids with more than 90% identity to an alpha-acetolactate decarboxylase of the *Lc. lactis* IL-1403 chromosome. This enzyme leads to production of acetoin, a molecule that plays important roles in the regulation and detoxification of pyruvate metabolism through pyruvate dehydrogenase [21]. The enzyme is also involved in regulation of biosynthesis of branched-chain amino acids, a metabolic step of great importance for protein building. However, possibly more interesting, is the role that the enzyme may have in formation of a deep electron sink. The insertion sequence upstream of the gene aldC encoded by the plasmid could be responsible for over expression of the gene with the resulting creation of additional electron sinks. Creation of electron sinks may be instrumental in the processes of detoxification and it is postulated that electron sinks were at the basis of life evolution on Earth. According to Authors from the Centre National de la Recherche Scientifique of Marseille, France, a deep electron sink available to the last universal common ancestor before the divergence of Bacteria and Archaea was responsible for emergence of bioenergetic pathways that eventually led to aerobic respiration when oxygen became available [22].

Lactobacillus (Lb.) helveticus R0052 plasmid pIR52-1

Lb. helveticus was described for the first time in 1919 by Orla-Jensen and was named Thermo bacterium helveticum, where the prefix *thermos* referred to the high temperature utilized to produce the Emmental cheese from where the bacterium was originally isolated [23]. In addition to dairy products, *Lb. helveticus* is naturally present in the human intestine where it has a number of health-supporting properties. The bacterium successfully colonizes the gut because it has the ability to survive the acidic environment of the stomach and withstand the components of bile [24]. The ability to survive in an acidic environment explains how the bacterium can proliferate rapidly in fermenting milk because of its resistance to acid stress; rapid growth in milk is accompanied by expression of genes coding for proteolytic enzymes that lead to release of a great quantity of newly formed peptides, including bioactive peptides derived from cleavage of milk proteins, in particular casein. *Lb. helveticus* cell-wall proteolytic enzymes lead to generation of tripeptides, Ile-Pro-Pro (IPP) and Val-Pro-Pro (VPP), deriving from the hydrolysis of casein in milk [25]. These tripeptides have a number of health-supporting activities that may explain the role of the probiotic in promoting HALE. They inhibit the angiotensin converting enzyme and, therefore, exert a preventive/therapeutic effect on hypertension. They also improve vascular endothelial function, a key element in prevention of cardiovascular diseases and this effect is independent from their hemodynamic effect. A clinical study concluded that administration of a casein hydrolysate - that is analogous to the hydrolyzed casein naturally present in fermented milk containing *Lb. helveticus* - could help preventing cardiovascular diseases in hypertensive subject [26]. Another study [27], reported that both IPP and VPP stimulated beneficial adipogenic differentiation with effects similar to those induced by insulin with potential benefits in the presence of insulin resistance. Most important for increasing HALE and achieving optimal health, it was demonstrated that IPP and VPP inhibited cytokine-stimulated pro-inflammatory alterations such as reduction in adipokine levels and activation of the nuclear factor kappa B pathway [27]. These observation are of utmost importance if we consider that chronic inflammation is responsible for the discrepancy between the observed increase in life expectancy and the non-corresponding increase in HALE as discussed by Nunn et al. [28]. According to these Authors *"although average global life expectancy has gone up, it does not seem to be due to an increase in HALE, and is certainly not approaching anything like that possible for a human. One key driver for morbidity expansion is lifestyle- induced inflammation"* that leads to accelerate aging. The health-supporting properties of *Lb. helveticus* encompass the ability to detoxify carcinogenic organic compounds and modulate the immune system [29]. As far as detoxification is concerned, one of the mechanisms consists in binding and eliminating carcinogenic compounds such as heterocyclic amines that derive from cooking fish or meat. Another

mechanism consists in the formation of toroidal structures that bind carcinogenic and toxic metals such as cadmium or aluminum as it will be discussed later. As far as immune modulating properties are concerned, *Lb helveticus* strain R0052 works on several mechanisms associated with immune responses. Of particular interest are the effects on macrophages that may explain the efficacy of one version of the fermented product here described [30]. Milk fermented with *Lb. helveticus* or its cell-free supernatant stimulated macrophages in culture and modulated the proliferation of lymphocytes *in vitro* [29]. These observations demonstrate that the immune-modulating properties of *Lb. helveticus* are associated with generation of molecules that are present in the cell-free supernatant. Since 2011 we postulated that also peptides deriving from the activity of glycosidases may contribute to the immune-modulating properties of the product here described [30]. *Lb. helveticus* DSM 20075, well represented in the product object of this paper (Table 2), codes for beta-galactosidase [31], and it was demonstrated that the activity of this enzyme is a common first-stage modification of the three major subtypes of vitamin D binding protein to vitamin D binding protein-Macrophage Activating Factor (DBP-MAF) [32]. The immune-modulating properties of the *Lb. helveticus* strains present in the product here described lead to further intriguing propositions as far as their involvement in HALE is concerned. For example, *Lb. helveticus* strain R0052, efficiently inhibited the invasion of two different strains of *C. jejuni* in human colon cancer cells [33] at the same time protecting healthy cells from alterations of innate immunity [34]. In another study, oral administration of a probiotic containing *Lb. helveticus* reduced the symptoms of depression after myocardial infarction [35]. The same formulation reduced apoptosis in the limbic areas of the brain that are involved in the pathophysiology of depression, thus suggesting that strains of *Lb. helveticus* exert their influence on the gut-brain axis possibly normalizing the function of the central nervous system [36]. *Lb. helveticus* plasmids contribute to these health-supporting properties since they contain information associated with proteolytic activity and lactate production. *Lb. helveticus* R0052 has a plasmid with eight open reading frames (ORFs); four of these codes for proteins whose function is unknown. Non-coding elements control the number of copies of the plasmid and its maintenance [37].

Lactobacillus (Lb.) plantarum subsp. plantarum P-8 plasmid LBPp2

Lb. plantarum strains are able to survive the acidic environment of the human stomach and are resistant to bile, thus being able to successfully colonize the gut and exert probiotic functions such as balancing the microbiota and preventing hyperlipidemia [38]. The strain P-8 was isolated from a traditionally fermented dairy product in China and was described in a publication of 2012 [39]. The bacterium has a circular chromosome of 3.03 Mb and seven plasmids. Among these, LBPp2 is the most abundant and possesses eight genes involved in regulation of metabolism; five of these code for galactosidases, thus further reinforcing the concept of plasmids being responsible for DBP-MAF generation from milk vitamin D binding protein [30,32]. In addition, LBPp2 codes for transposase and topoisomerase. The latter enzyme is responsible, among other functions, for the formation of toroids of DNA that may have particular importance for detoxification. In plasmids where the topology of DNA is in the shape of toroids, metals with electropositive charge such as cadmium, mercury or aluminum bind to the electronegative backbone of DNA with formation of disc-shaped structures where the metal is surrounded by the DNA toroid [40]. This feature of plasmids may contribute to the overall detoxifying effect of one version of the product that we reported in 2017 [41].

Conclusions

Presence of genetic information outside chromosomes in bacterial cells was known well before the observation of Watson and Crick in 1953; researchers in the late 1940s observed how extra chromosomal genetic elements served to transmit traits that were advantageous for microbes to survive in hostile environments and a number of designations for these hereditary elements was proposed; choncriogenes, plasmagenes, pangenes and episomes were among the names used before Joshua Lederberg in 1952 coined the word plasmid to designate "any extra chromosomal hereditary element" [42]. Genetic and molecular characterization led to classify plasmids in five categories; fertility F-plasmids, resistance plasmids, virulence plasmids, degradative plasmids, and Col plasmids. Plasmids, just as microbes in general, were at first considered *villains* because they were identified as elements responsible for transmitting resistance to antibiotics. However, just as the perception of microbes has changed up to the point that fashion magazines praise their health-promoting properties, also the perception of plasmids is changing. These small, highly mobile, genetic elements make possible the horizontal transfer of genetic information that reacts and adapts to the ever-changing environment. This transferable information is essential for survival in the competitive natural environment and, if acquisition and transfer of antibiotic resistance is detrimental for humans infected by pathogenic bacteria, resistance to toxic carcinogenic metals may prove highly appreciated in today's polluted environment [43]. The plasmids detected by the Axiom Microbiome Array in the Swiss fermented product here described are associated with a number of healthy properties that may be responsible for the effects observed in conjunction with the use of the product in the context of complementary nutritional approaches to chronic conditions [14,30,41,44,45]. According to the results presented here and in Pacini and Ruggiero [12], we feel that the product described in this study may be useful for improving hale as well as for synergizing with therapeutic strategies in chronic conditions associated with inflammation and exposure to toxicants. In addition, it is tempting to speculate that plasmids may have other functions in addition to those associated with their role as accessory information for probiotic microbes. For

example, they could transfer genetic information to mammalian cells and this information may prove useful to withstand adverse environmental conditions such as those involving exposure to toxic metals. It is known since 1975 that a fraction of plasmid DNA may integrate in the nucleus of mammalian cells [46] and it was demonstrated that small plasmids can persist for hours in serum of healthy subjects [47]. Genetically-modified plasmids have been proposed as therapy for a number of diseases ranging from cancer [48] to non-treatable neurological disorders, such as ischemic stroke, Parkinson's disease, Alzheimer's disease, and multiple sclerosis [49]. It is therefore conceivable that the vast array of natural plasmids detected in the product here described may contribute to its health-supporting properties that can be envisaged in the broader context of increasing HALE and promoting detoxification.

Disclosures

Marco Ruggiero is CEO of Silver Spring Sagl, the company producing the fermented product described in this paper. Stefania Pacini works as Quality Control responsible person for Silver Spring Sagl. Both had no prior knowledge of the results of the Axiom Microbiome Array that was independently performed by Eurofins Microbiology Laboratories Inc. (New Berlin Wisconsin, USA) on a commercially available lot as part of the company's quality controls.

Advisory

No information in this paper is intended or implied to be a substitute for professional medical advice, diagnosis or treatment.

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