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Research Article

First Detection of *Methanobrevibacter oralis* in Dairy Products

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Abstract

Methanogens are detected in human gut from the first moments of life and there is a diversification of methanogens during infancy. However, the sources of acquisition of methanogens are not elucidated. We therefore investigated 49 dairy products as potential sources by applying molecular biology search for methanogens. In the presence of negative controls, we obtained an overall prevalence of 85.7% (42/49) of methanogens by real-time PCR. Further PCR-sequencing identified *Methanobrevibacter smithii* (*M. smithii*) in 41 cases (83.6%) and *Methanobrevibacter oralis* (*M. oralis*) in one case (2%); with sequences exhibiting a 100 % and 99.82% identity with respective reference sequences for *M. smithii* and *M. oralis*. We observed a significant presence (p-value=0.002) of methanogens in unfermented dairy products compared to fermented dairy products. This study demonstrates, for the first time, the presence of *M. oralis* in dairy products and thus gives credit to the fact that dairy products could be a source of methanogens in children.

Keywords: Diary Products; Methanogens; *Methanobrevibacter smithii*; *Methanobrevibacter oralis*; Children

Introduction

Methanogens represent the archaea most present in the mammalian microbiota, especially in the human digestive microbiota where they account for 10% digestive tract of the anaerobic microorganisms [1,2]. Methanogens are detected in humans from birth [3] and there is a diversification of methanogens in humans over the years: while only *Methanobrevibacter smithii* (*M. smithii*) has been detected and cultured in the neonates [3-7], *M. smithii, Methanosphaera stadtmanae* (*M. stadtmanae*), *Methanomassiliicoccus luminiyensis, Methanobrevibacter arboriphilicus, Methanobrevibacter oralis* (*M. oralis*), *Ca.* Methanomethylophilus alvus *and Ca.* Methanomassiliicoccus intestinalis have been isolated the stools of adults [8,9]. The various sources for each one of these different species remain unknown. Accordingly, a recent study demonstrated that the presence of methanogens in human stools is linked to the consumption of dairy products [10]; but this study targeted only two of the seven strains of methanogens present in the human digestive tract, namely *M. smithii* and *M. stadtmanae*. Therefore, we further explored the presence of methanogens in dairy products using molecular biology techniques to target all methanogens currently known in the human digestive tract.

Materials and Methods

Sampling of dairy products

We have investigated the presence of methanogens in different types of dairy products including unfermented dairy products (formula milk, fresh milk, and fresh cheese) and fermented dairy products (yogurt and fermented milk) (Table 1). All these dairy products were purchased in randomly selected supermarkets in Marseille, France in November 2019.

	Dairy products	Samples analyzed	Real-time PCR	PCR-sequencing	Methanobrevibacter species	p-value
Unfermented dairy products	Fresh cheese	5	5	5	*M. smithii*	0.002**
	Fresh milk	8	8	8	*M. smithii*	
	Formula milk	16	16	16	*M. smithii* and *M. oralis*	
Fermented dairy products	Yogurt	10	7	7	*M. smithii*	
	Fermented milk	10	6	6	*M. smithii*	

Table 1: Distribution of dairy products according to the number and results of real-time PCR and PCR-sequencing.

**: High significant.

DNA extraction and PCR assays

DNA extraction was performed as previously described [9]. Briefly, for cheeses, yogurt and formula milk, 0.2 g was suspended in 200 µL of ultrapure water (Fisher Scientific, Illkirch, France), and a sonication step was performed for 30 minutes. DNA was then extracted with the EZ1 Advanced XL Extraction Kit (QIAGEN, Hilden, Germany) using 200 µL as sample volume and 200 µL as the elution volume. For fresh milk and fermented milk, 200 µL were taken and a sonication step was performed for 30 minutes as above. DNA was then extracted with the EZ1 Advanced XL Extraction Kit (QIAGEN) using 200 µL as the sample volume and 200 µL as the elution volume. The PCR assays targeting the 16S rRNA gene of methanogens, including real-time PCR and PCR-sequencing were performed to investigate the presence of methanogens in dairy products using primer pairs and PCR conditions described previously [11,12]. Sterile phosphate buffered saline (PBS) (Fisher Scientific, Illkirch, France) was used as a negative control in each DNA amplifications steps.

Phylogenetic analyses

Sequences were edited using ChromasPro software (ChromasPro 1.7, Technelysium Pty Ltd., Tewantin, Australia). Molecular phylogenetic and evolutionary analyses were conducted in MEGA7 as previously described [13].

Statistical analyses

Data were analyzed with RStudio (https://www.R-project.org/) by chi-square test (** $p < 0.01$, * $p < 0.05$, ns: non-significant). We used the former to compare the proportion of methanogen detection by real-time PCR in fermented dairy products compared to unfermented dairy products.

Results and Discussion

We investigated the presence of methanogens in 49 dairy products including 29 unfermented dairy products, and 20 fermented dairy products (Table 1). The overall prevalence of methanogens in dairy products was 85.7% (42/49) by real-time PCR. The prevalence of methanogens in fermented dairy products was 65% (13/20) versus 100% (29/29) in unfermented products (p-value=0.002). The results here reported were authentified by the fact that negative controls introduced in all experiments, remained negative. PCR-sequencing yielded *M. smithii* in 41 cases (83.6%) exhibiting a 100% sequence similarity with the reference 16S rRNA gene sequence of *M. smithii* ATCC 35061 (accession NCBI: NR_074235) isolated from human stool. Our results are consistent with those in the literature where *M. smithii* was found in dairy products [10]. However, we also found for the first time *M. oralis* in one case in one formula milk and this sequence had 99.82% similarity with the sequence of the reference 16S rRNA gene of *M. oralis* CSUR P5920 (NCBI accession: LR590665.1) isolated from Breast

milk of healthy breast-feeding mother. The phylogenetic tree produced confirmed this similarity with a clustering of the formula milk sequence of *M. oralis* to the reference sequence of *M. oralis* (Figure 1). In addition, statistical analysis has shown that there is no significant correlation between the presence of methanogens in fermented and unfermented dairy products, suggesting that fermentation processes have no impact on the DNA of methanogens present in dairy products.

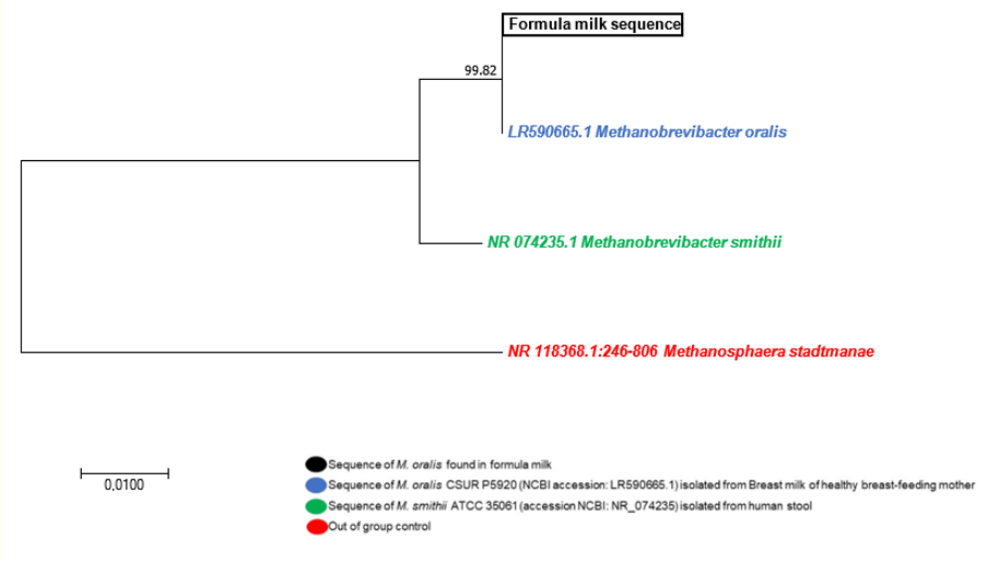

Figure 1: Molecular phylogenetic analysis, based on 16S rRNA partial gene, showed the position of M. oralis sequence detected in formula milk. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.11730134 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1. 000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 4 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 549 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

Conclusion

The presence of *M. oralis* in formula milk suggests another mode of acquisition of this methanogen in children through artificial breastfeeding in addition to that already known through breastfeeding [14]. These data add to previous reports of the detection of methanogens in dairy products [10] and thus giving credit that dairy products could be sources for methanogens for children and suggest that the dairy products may be essential to seed the infant's microbiota with these neglected critical commensals from the first hours of life.

Author Contributions

- Cheick Oumar GUINDO: Conception, draft writing, data collection, data analysis, samples collection.
- Lanceï KABA: Data analysis
- Michel DRANCOURT: Supervision, draft writing, funding acquisition, final validation.
- Ghiles GRINE: Supervision, draft writing, final validation.

Conflicts of Interest

All the authors declare that there is no conflict of interest.

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