

Background

Bovine mastitis represents a long-lasting problem in dairy cattle farms worldwide. It is the most critical production disease of cattle, which causes high economic losses. The causative agents of bovine mastitis are mainly bacteria. However, there has been a recent increase in the importance of non-bacterial agents such as molds, yeasts, or microalgae *Prototheca* spp.

A colorless, achlorophyllous, unicellular algae of the genus *Prototheca* (family *Chlorellaceae*) is the known plant pathogen in human and animal populations (Figure 1). The incidence of mastitis caused by *Prototheca* spp. is increasing steeply but the epidemiology of the spread of this pathogen remains neglected. Several *Prototheca* species, namely *P. ciferrii* (formerly *P. zopfii* genotype 1), *P. bovis* (formerly *P. zopfii* genotype 2), *P. wickerhamii*, *P. blaschkeae*, *P. cutis*, and *P. miyajii* cause infections both in humans and in animals [1, 2].

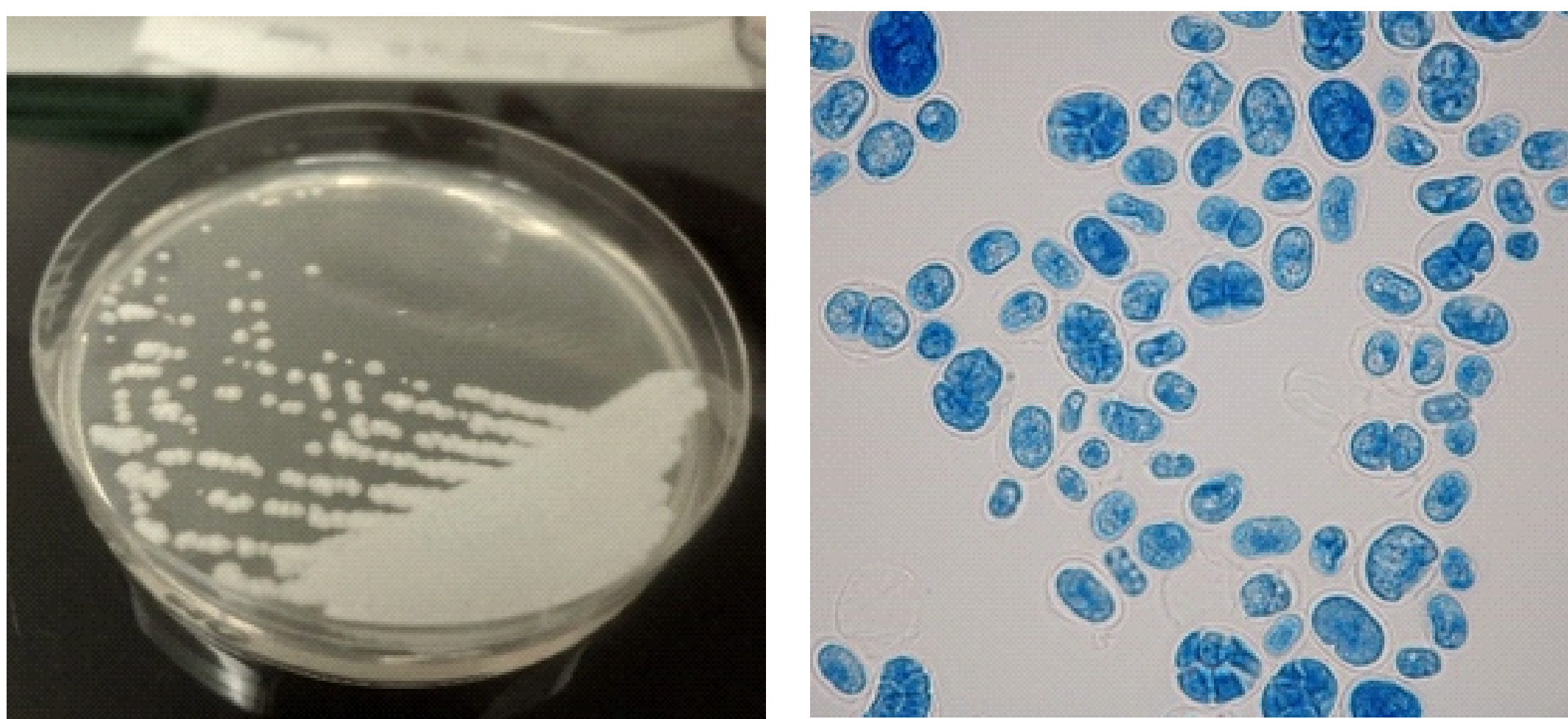


Figure 1. *Prototheca ciferrii* a) culture on a Sabouraud Dextrose Agar plate, b) lactofenol blue colored cells in 1000x magnification.

The causes of mastitis spread are related to the economically limited hygiene standards in livestock farms (Figure 2), the increased incidence of metabolic disorders caused, for example, by mycotoxins contaminated feed, and also by the unnecessary application of antibiotics. Paradoxically, mastitis caused by non-bacterial pathogens is often treated with antibiotics, which not only do not eliminate the infectious agent but often worsen the course of the disease. The only defense against non-bacterial mastitis agents is consistent prevention in dairy farms.

Non-bacterial mastitis agents are not routinely diagnosed, so there is a lack of experience in their eradication. Therefore, this work has focused on mapping the occurrence of non-bacterial mastitis caused by yeasts, molds, and especially microalgae *Prototheca* spp. in dairies in the Czech Republic.



Figure 2. Inadequate hygiene measures are a common reason for the spread of mastitis in dairy cattle farms.

Methods

A total of 1051 bulk tank milk samples from 21 dairies in the Czech Republic were tested for the presence of non-bacterial mastitis agents. The presence and number of bacteria, fungi, yeasts, and *Prototheca* algae were determined using standard culture methods. Identification of individual yeast and fungal isolates was performed using the MALDI-TOF method. Samples suspicious of the presence of *Prototheca* were

identified using a two-stage real-time PCR system [3]. The bacterial and fungal microbiota composition was subsequently determined and compared in 10 *Prototheca*-positive and 10 *Prototheca*-negative samples using a next-generation sequencing based on the analysis of bacterial V3/V4 or fungal ITS variable regions.

Results

The average number of microorganisms of all samples detected by cultivation methods was $(129 \pm 203) \times 10^3$ per ml. A total of 70 (6.66%) of all bulk tank milk samples tested were found to contain at least one species of mold. At least one yeast species was present in 1013 bulk tank milk samples out of 1051, i.e., in 96.4% of cases. It means that only about 3% of all milk samples tested showed no yeast contamination. In 46 samples (4.38%), one of the *Prototheca* species was detected, and the most prevalent of them was *Prototheca bovis*, which was present in 37 bulk tank milk samples (Figure 3).

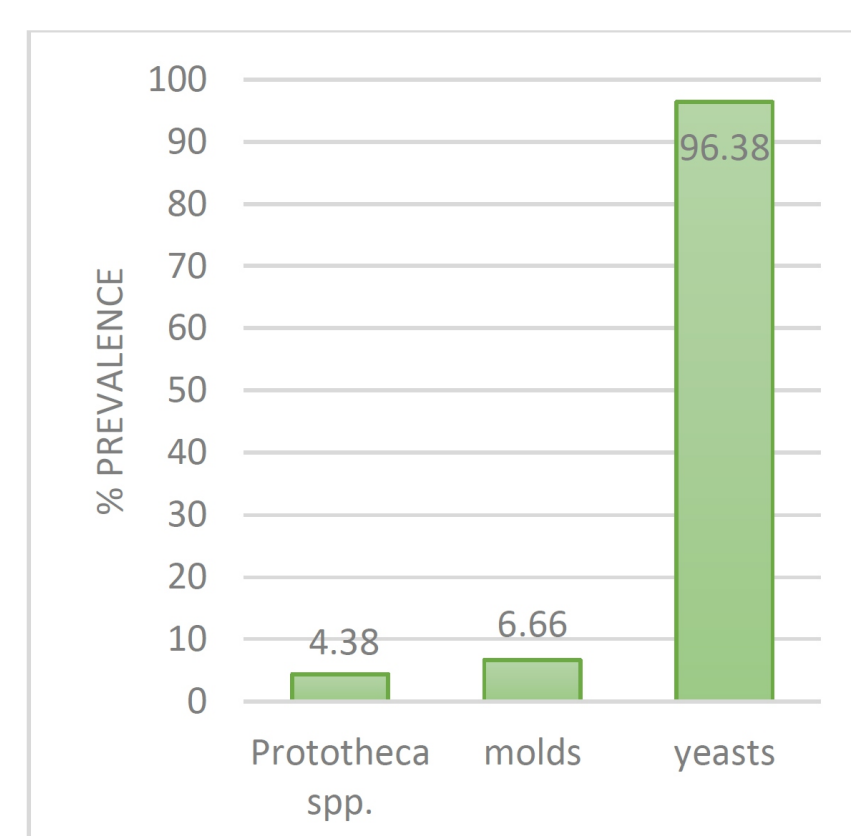


Figure 3. Non-bacterial mastitis agents prevalence in bulk tank milk samples.

A total of 28 different mold species, 64 yeast species, and four species of the microalgae *Prototheca* were identified using standard cultivation methods in bulk tank milk samples from dairies in the Czech Republic. The most commonly represented species of molds, yeasts, and *Prototheca* algae are listed in Table 1.

Table 1. The most prevalent *Prototheca*, fungi, and yeast species detected in bulk-tank milk samples

algae <i>Prototheca</i>		Yeasts		Fungi	
species	% prevalence	species	% prevalence	species	% prevalence
<i>Prototheca bovis</i>	80,4	<i>Kluyveromyces marxianus</i>	26,1	<i>Phoma herbarum</i>	14,1
<i>Prototheca blaschkeae</i>	10,9	<i>Pichia kudriavzevii</i>	12,6	<i>Aspergillus fumigatus</i>	7,8
<i>Prototheca ciferrii</i>	4,3	<i>Pichia cactophila</i>	10,8	<i>Penicillium commune / italicum</i>	7,8
<i>Prototheca</i> spp.	4,3	<i>Pichia ferementans</i>	6,5	<i>Penicillium chrysogenum</i>	6,3
		<i>Candida parapsilosis</i>	6,5	<i>Lichtheimia corymbifera</i>	6,3

The presence of *Prototheca* spp. in bulk tank milk samples affected the average number of bacterial and fungal species detected using next-generation sequencing. *Prototheca*-positive samples were identified with an average number of bacterial and fungal species more than 10% lower than *Prototheca*-negative milk samples (Figure 4).

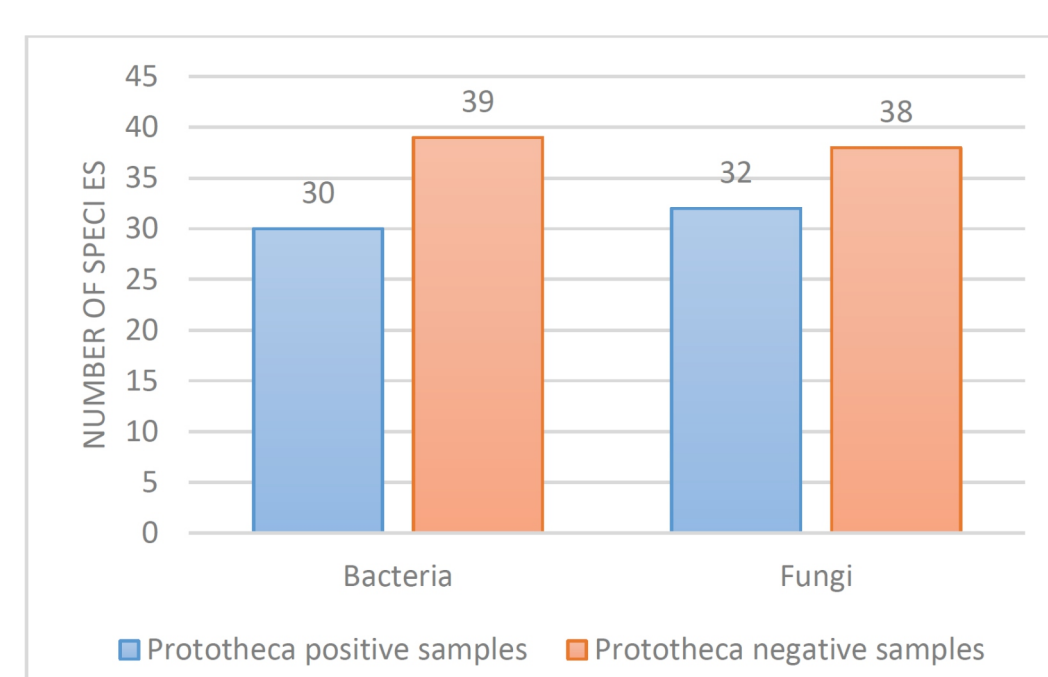


Figure 4. Average number of detected bacterial and fungal species in bulk tank milk samples depending on the presence of *Prototheca* spp.

A different profile of the average representation of bacterial and fungal species was observed in the *Prototheca*-positive bulk tank milk samples compared to the *Prototheca*-negative samples (Figure 5).

The average population composition at the bacterial genus level, *Acinetobacter*, *Moraxella*, *Psychrobacter*, *Aerococcus*, *Erwinia*, and *Streptococcus*, were more abundantly represented in the *Prototheca*-positive samples than in the *Prototheca*-negative samples. On the contrary, the genera *Rothia*, *Flavobacterium*, *Lactococcus*, *Escherichia*, *Aeromonas*, and *Castellania* were, on average, represented with higher frequency in *Prototheca*-negative samples. Bacterial genera *Psychrobacter* and *Lactococcus* were represented in the same percentage in both *Prototheca*-positive and -negative bulk tank milk samples. Fungal genera *Candida*, *Pichia*, *Wickerhamiella*, *Prillingera*, and *Babjeviella* were, on average, more frequently represented in *Prototheca*-negative milk samples. In contrast, the fungal genera *Kluyveromyces*, *Debaromyces*, *Trichosporon*, *Cladosporium*, *Geotrichum*, and *Galactomyces* were more abundantly represented in *Prototheca*-positive samples. The only fungal genera *Cutaneosporon* was represented in the same average percentage in both *Prototheca*-positive and *Prototheca*-negative bulk tank milk samples.

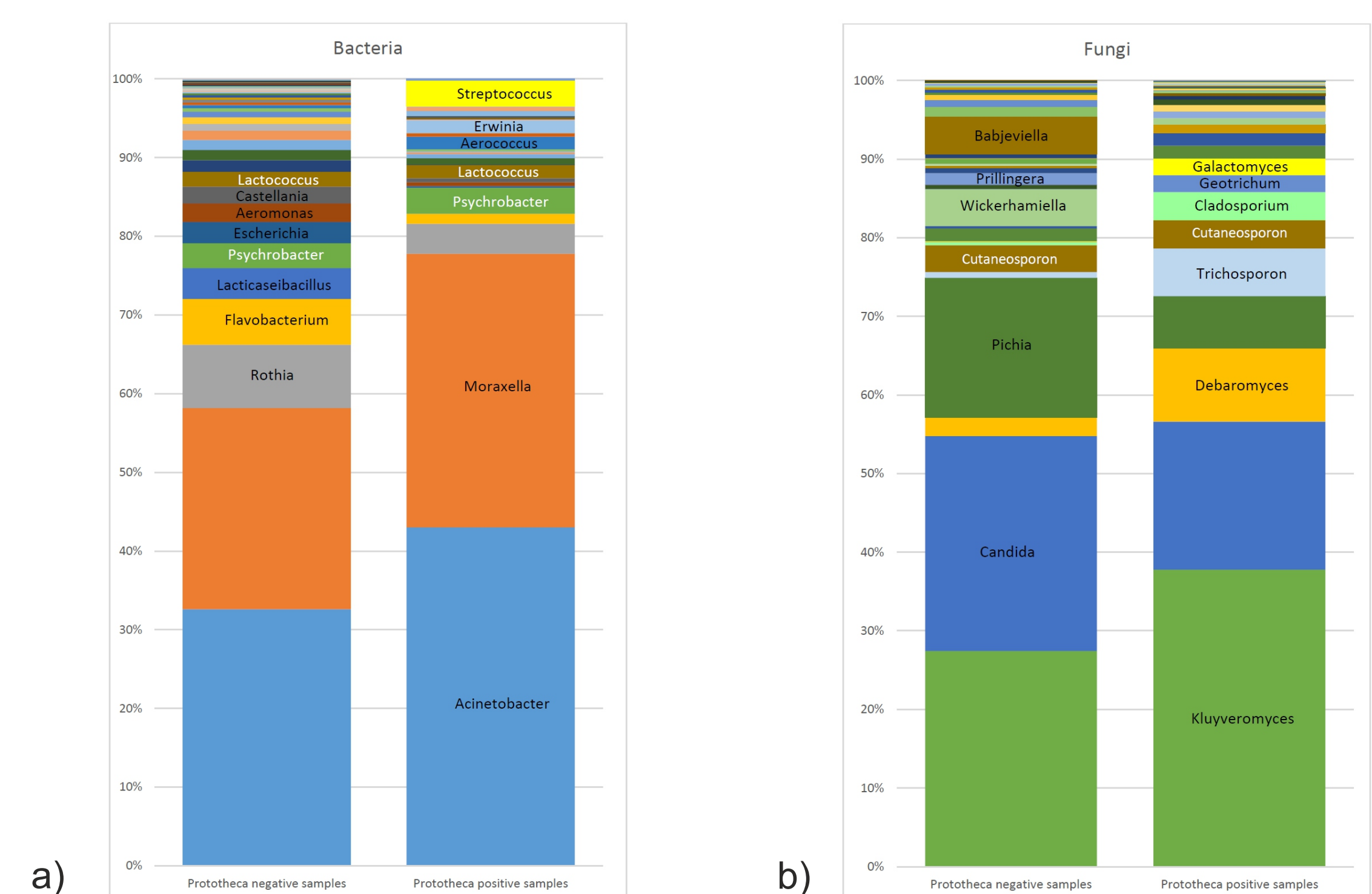


Figure 5. Average a) bacterial and b) fungal microbiota composition of *Prototheca*-negative and *Prototheca*-positive bulk tank milk samples at the genus level.

Methods

The incidence of non-bacterial mastitis in dairy cattle is increasing worldwide. Significant numbers of these non-bacterial mastitis agents, such as molds, yeasts, or microalgae *Prototheca*, have been recorded in milk samples from various dairy farms in the Czech Republic. In particular, the frequency of yeasts in milk samples detected by culture methods was alarming. The *Prototheca* contamination in bulk tank milk samples impacted the microbiota's composition at both bacterial and fungal levels. This fact may lead to changes in the characteristics and quality of milk and consequently causes significant economic losses for farmers and milk producers.

References:

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