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Comparison of automatic methods MALDI-TOF, VITEK2 and manual methods for the identification of intestinal microbial communities on the example of samples from alpacas *(Vicugna pacos)*

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Abstract

Introduction: Universally, in microbiological diagnostics the detection of live bacteria is essential. Rapid identification of pathogens enables appropriate remedial measures to be taken. The identification of many bacteria simultaneously facilitates the determination of the characteristics of the accompanying microbiota and/or the microbiological complexity of a given environment. **Material and Methods:** The effectiveness of the VITEK2 Compact automated microbial identification system and matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI-TOF MS), analytical profile index (API) and Remel RapID tests were compared in identification of bacteria isolated from the alpaca gastrointestinal tract. **Results:** Most isolates were Gram-positive, such as *Bacillus cereus*, *Bacillus flexus*, *Bacillus licheniformis*, *Bacillus pumilus* and *Bacillus subtilis*; *Enterococcus faecium*, *Enterococcus gallinarum*, *Enterococcus hirae* and *Enterococcus casseliflavus*; *Staphylococcus aureus*, *Staphylococcus equorum*, *Staphylococcus lentus*, *Staphylococcus pseudintermedius* and *Staphylococcus sciuri*; *Paenibacillus amylolyticus*; *Cellulosimicrobium cellulans*; *Leuconostoc mesenteroides*; *Clostridium perfringens*; *Corynebacterium stationis*, *Corynebacterium xerosis*, and *Corynebacterium diphtheriae* (the last only isolated manually by API Coryne and the VITEK2 system and *Corynebacteria* (CBC) card). *Corynebacterium diphtheriae* was misidentified by MALDI-TOF MS as *Candida lipolytica* (currently *Yarrowia lipolytica*). Gram-positive and Gram-variable *Micrococcus luteus* were also isolated. Gram-negative *Enterobacter cloacae*, *Enterobacter gergoviae*, *Enterobacter hormaechei* and *Enterobacter ludwigii*; *E. coli*; *Klebsiella pneumoniae* subsp. *pneumoniae*; *Citrobacter braakii* and *Citrobacter freundii*; *Serratia liquefaciens*, *Serratia odorifera* and *Serratia marcescens*; *Morganella morganii* subsp. *morganii*; *Providencia alcalifaciens*; *Pseudomonas aeruginosa*; *Stenotrophomonas maltophilia*; *Moraxella osloensis*; and *Ochrobactrum intermedium* were also found. The yeasts *Candida albicans*, *Candida haemulonii* and *Candida ciferrii* were also present. **Conclusion:** MALDI-TOF MS enabled the identification of pathogens and opportunistic pathogens from the alpaca gut which may represent a high risk to human and animal health.

Keywords: MALDI-TOF MS, *Vicugna pacos*, intestinal microbiota, opportunistic pathogens, microbial identification.

Introduction

Matrix-assisted laser desorption/ionisation–time-of-flight mass spectrometry (MALDI-TOF MS) technique is the result of a combination of mass spectrometry, matrix-assisted laser desorption/ ionisation and the technology of time of flight, a method in which charged ions are forced to fly from a source to a detector and are identified based on the time of their flight to the ion detector (13). This technique can be used for the detection of specific species of bacteria and yeast from human and animal samples (6). Thanks to its capability of detecting many species of bacteria or yeast at the same time, the technique is useful in diagnosing both pathogens and accompanying microbiota. The companion microbiota in human, animal or environmental materials for testing was treated by some researchers in the past as clinically insignificant contamination (15). Nowadays it is known that the digestive tract microbiota, as only one example, is a very important element in maintaining the health of humans and animals. Each unfavourable change in its composition might have an impact on the synthesis of B vitamins or even susceptibility to various infections and the course of the disease (20). The MALDI-TOF technique allows many microbial isolates to be identified in a short time because the throughput of testing can be high, which can significantly facilitate the characterisation of a given microbiota without the need to use advanced genetic techniques such as next-generation sequencing (NGS).

The MALDI-TOF development in mass spectrometry makes characterising the gastrointestinal tract microbiota a simpler proposition. Its utility can be exploited to elucidate the gut microbiota of the alpaca, which is a knowledge gap more noticeable in recent times. Alpacas are among the camelids of the New World, the natural habitat of which is mountainous regions of South America. Interest in this species has been increasing in various countries over the past three decades. Alpacas have gained popularity because of their unique fibre, mild temper, and the possibility of using them in psychotherapy. Also in Poland, alpacas are being bred on an larger scale (15). The meat of New World camelids may be also used for human consumption (28).

Although alpacas are becoming more and more popular, there is much that is not known about them. Knowledge of the pathogens present in alpacas is particularly important, from the public health hazard point of view as well as in making the right decision about treatment, particularly the use of antibiotics. The zoonotic diseases in alpacas are known to be tuberculosis, cryptosporidiosis, colibacteriosis (caused by *E. coli* VTEC O157), salmonellosis, campylobacteriosis, dermatophytosis, leptospirosis, listeriosis, streptococcosis (caused by *Streptococcus equi* subsp. *zooepidemicus* and *Streptococcus suis*), yersiniosis, brucellosis, sarcoptic mange and rabies (2, 12, 30).

Gut associated microbes may influence the alpaca health, but still little is known about the species' microbiota (3, 5, 11). In research conducted by Guerrero-Olmos *et al.* (11) the most prevalent bacterial genus in the alpaca gut microbiome was *Enterococcus* and the dominant species was *Enterococcus hirae* (82%), followed by other members of the genus which were neither *Enterococcus faecalis* nor *Enterococcus faecium* (11). The latest reports concern the profile of faecal bacterial microbiota, characterised by sequencing of 16S rRNA amplicons or the isolation of specific pathogenic microorganisms (5, 29). However, there is a lack of research based on the isolation of the components of a living gut bacterial community in alpacas, which is highly variable impacted by the nutrition and health status of the animal (4, 28). The aims of this study were firstly to compare two automatic methods, MALDI-TOF MS and the VITEK2 fluorogenic system, and two manual biochemical methods, the analytical profile index (API) and RapID tests, for identification of isolated microorganisms from three fallen alpacas guts and faeces, and secondly to describe their gut microbiota.

Material and Methods

Sampling. The materials for the research were sterile Amies swabs (Deltalab, Rubí, Spain) taken from the small intestine, large intestine, rectum and faeces of three alpacas (*Vicugna pacos*). The research material was collected during necropsies conducted at the Department of Pathology and Veterinary Diagnostics of the Faculty of Veterinary Medicine of Warsaw University of Life Sciences in Poland.

Animals. The first alpaca (1A), a male aged about 10 months, was taken in a veterinary animal welfare intervention due to extreme neglect. The animal was in very poor health and had diarrhoea and severe extensive mange lesions over almost all of its skin. Marked hyperkeratosis and poorly embedded hair were visible. The animal had been fed special granules for alpacas and hay. This individual died during transport. The second alpaca (2A), a 12-month-old female, came from a herd without veterinary care. It was sent to the veterinary clinic because of sudden weakness but died also during transport. Similarly to the first case, the animal was emaciated and had severe extensive mange lesions on the entire surface of the skin. The third alpaca (AH) was a healthy 4-year-old male which had died suddenly as a result of choking on a carrot. The animal had been fed special granules for alpacas, carrots and hay.

Table 1. Conditions for bacteria incubation on individual solid media

Type of solid media	Incubation atmosphere	Temperature/time	Comment
Baird-Parker	aerobic	37ºC for 24 ± 2 h	The reading was made after 24h and after 48 h
OCLA	aerobic	37ºC for 24 ± 2 h	Negative plates were read again after 48 h
Yersinia CIN agar	aerobic	30°C/24h	The reading was made after 24h and after 48 h
Columbia agar with 5% sheep blood	aerobic	37°C/24h	+/− 2h
Columbia agar with 5% sheep blood	anaerobic	37°C/24h	+/− 2h
mCCD agar	anaerobic	42°C/48h	Negative plates was read again after 48 h
CHROMagar Campylobacter	anaerobic	42°C/48h	Negative plates was read again after 72 h
Chapman	aerobic	37°C/24h	+/− 2h
MYP Agar	aerobic	37°C/24h	Negative plates was read again after 48 h
MacConkey's agar	aerobic	37°C/24h	+/− 2h
CNA	aerobic	37°C/24h	+/− 2h

OCLA – Oxoid chromogenic *Listeria* agar; mCCD agar – *Campylobacter* blood-free selective; MYP Agar – Mannitol egg yolk polymyxin agar; CNA – Columbia CNA agar with 5% sheep blood

Isolation and identification of microbiota bacteria. All swabs were inoculated directly onto solid media during a six-hour period: Baird-Parker agar (BTL, Łódź, Poland) and Oxoid Chromogenic *Listeria* Agar (OCLA; Oxoid Ltd., Basingstoke, UK) were prepared according to the manufacturers' instructions, as were the selective *Yersinia* CIN agar for the isolation of *Yersinia* spp., Columbia Agar with 5% sheep blood, mCCD Agar (*Campylobacter* Blood-Free Selective), CHROMagar *Campylobacter*, Mannitol Salt Agar (Chapman), MYP (mannitol egg yolk polymyxin) Agar, Columbia CNA Agar with 5% sheep blood and MacConkey Agar (all products of Graso, Gdańsk, Poland). The manufacturers' recommendations were followed for incubation conditions, and are presented in Table 1. Twenty-four-hour cultures on nutrient agar or Columbia agar with 5% sheep blood were used for further identification of bacteria in the bacterial identification tests.

Matrix Assisted Laser Desorption/Ionisation Time-of-Flight Mass Spectrometry (MALDI-TOF MS) sample preparation. Isolations were performed according to a procedure published by the Clinical Laboratory and Standards Institute (CLSI), which was in line with the manufacturer's instructions. A single colony was smeared onto the target plate to obtain even distribution and homogeneous deposition. Each colony was overlaid with 1 μL of matrix solution (α-cyano-4-hydroxycinnamic acid 50/50 with acetonitrile/H_2O containing 2.5% trifluoroacetic acid (Autobio Diagnostics, Zhengzhou, China) and dried at room temperature.

Identification of the isolates. All the isolates were identified with an Autof MS1000 MALDI-TOF system (Chirus, Watford, UK) in positive ion, linear mode, with delayed extraction time of 230 ns, total accumulation of 240, step accumulation of 40, detector voltage of 2.65kV, repulsion voltage of 20 kV, elicitation 1.9 of kV, focus 6.99 of kV, and detection mass range of 2,000–20,000 Da, according to the manufacturer's instructions. Calibration was achieved using the Autobio calibrating agent (Autobio Diagnostics). The database of microorganisms on the basis of which the MALDI TOF identification was carried out was the manufacturer's built-in database in version V1.1.18, containing 5,053 species and 16,892 strains of bacteria. Data concerning spectrum were collected by Autof Acquirer and analysed by Autof Analyzer version V2.0.78 software (Autobio Diagnostics). Identification scores were graded based on the manufacturer's range breakpoints: 9.5–10 indicated that species identification was reliable and subspecies identification possible, 9.0–9.5 showed the species identification to be reliable, 6.0–9.0 meant that only the genus identification was reliable, and 0–6.0 represented an unreliable result.

Biochemical strain identification. For identification of the strains, commercially available biochemical tests were used: API 20 *Enterobacteriaceae* (E), API 20 non-*Enterobacteriaceae* (NE), API Coryne and API Staph; rapID ONE (Gram-negative bacilli), RapID NF Plus (oxidase-positive, Gram-negative bacilli); VITEK2 Gram-negative (GN), Gram-positive (GP), *Bacillus* (BCL), *Corynebacteria* (CBC), Anaerobic and *Corynebacteria* (ANC) and yeast (YST) cards (BioMérieux, Marcy-l'Étoile, France); and RapID ONE and RapID NF Plus (Remel, Lenexa, KS, USA), according to the manufacturers' instructions.

Results

In total, over 121 microorganisms belonging to 54 genera of bacteria and yeast were isolated in this study. Of these, 5 isolates could not be identified by MALDI-TOF MS or any other instrumental and manual method. Most of the isolates were Gram-positive, such as *Bacillus* spp., *Enterococcus* spp. (which were dominant) and *Staphylococcus sciuri*. Other Gram-positive bacteria isolated were *Paenibacillus amylolyticus* and *Corynebacterium stationis*. In addition, Gram-positive and Gram-variable *Micrococcus* cocci were isolated. There were also Gram-negative bacteria isolated: *Enterobacter cloacae*, *Enterobacter hormaechei*,

Enterobacter ludwigii and *Enterobacter gergoviae* (currently *Pluralibacter gergoviae*); *Citrobacter* spp.; *E. coli*; *Stenotrophomonas maltophilia*; *Serratia liquefaciens* and *Serratia odorifera* were also found.

The bacterial species isolated from the individual sections of the intestine from which the swabs were taken differed. The following microorganisms were isolated from the small intestines of the alpacas: *Candida albicans*, *Ochrobactrum intermedium*, *Enterobacter gergoviae*, *Serratia odorifera*, *Moraxella osloensis*, *Staphylococcus sciuri*, *Bacillus licheniformis*, *Bacilus altitudinis* and *Arcticiflavibacter luteus*. The following microorganisms were isolated from the animals' large intestines: *Candida metapsilosis*, *Candida lipolytica* (currently *Yarrowia lipolytica*) and *Candida haemulonii*; *Enterobacter cloacae* and *Enterobacter hormaechei*; *E. coli*; *Serratia liquefaciens*; *Bacillus cecembensis*, *Bacillus pumilus*, *Bacillus cereus*, *Bacillus flexus*, *Bacillus licheniformis* and *Bacillus subtilis*; *Enterococcus hirae* and *Enterococcus casseliflavus*; *Staphylococcus sciuri*; *Brachybacterium conglomeratum*; *Corynebacterium stationis*; *Stenotrophomonas maltophilia*; *Sphingobacterium anhuiense*; *Thallasolituus oleivorans*; *Leeuwenhoekiella nanhaiensis*; and *Paenibacillus amylolyticus*. The rectum and faeces yielded *Citrobacter braakii* and *Citrobacter freundii*; *Serratia odorifera*; *Enterobacter ludwigii*, *Enterobacter cloacae*, *Enterococcus hirae* and *Enterococcus casseiflavus*; *Bacillus aeolinus* and *Bacillus licheniformis*; *Staphylococcus pseudintermedius*; *Micrococcus luteus*; *Leuconostoc citreum*; and *Cellulosimicrobium cellulans.*

Table 2. Results of identification using matrix-assisted laser desorption/ionisation–time-of-flight mass spectrometry (MALDI-TOF MS) and VITEK2 Gram-negative (GN) fluorogenic, analytical profile index for 20 *Enterobacteriaceae* (API 20E), and RapID ONE tests for Gram-negative *Enterobacteriaceae* isolated from three alpacas

Sample source	MALDI-TOF MS (score)	VITEK2 GN (% probability)	API 20E (% probability)	RapID ONE (% probability)
small intestine (1A)	*Citrobacter braakii* (9.003)	*Citrobacter braakii* (97)	*Citrobacter freundii* (94)	*Citrobacter freundii* (94)
colon (1A)	*Citrobacter braakii* (8.770)	*Citrobacter braakii* (89)	*Citrobacter freundii* (92)	*Citrobacter freundii* (90)
rectum (1A)	No spectrum	*Citrobacter freundii* (95)	*Citrobacter freundii* (98)	*Citrobacter freundii* (96)
rectum (1A)	*Citrobacter freundii* (9.617)	*Citrobacter freundii* (95)	*Citrobacter freundii* (92)	*Citrobacter freundii* (95)
faeces (1A)	*Citrobacter freundii* (9.533)	*Citrobacter braakii* (89)	*Citrobacter braakii* (92)	*Citrobacter freundii* (90)
faeces (2A)	*Citrobacter freundii* (8.771)	*Citrobacter freundii* (95)	*Citrobacter freundii* (95)	*Citrobacter freundii* (95)
colon (1A)	*Enterobacter cloacae* (9.440)	*Enterobacter cloacae* complex (95)	*Enterobacter cloacae* (97)	*Enterobacter cloacae* (92)
colon (1A)	*Enterobacter cloacae* (9.607)	*Enterobacter cloacae* complex (97)	*Enterobacter cloacae* (96)	*Enterobacter cloacae* (93)
colon (1A)	*Enterobacter cloacae* (9.543)	*Enterobacter cloacae* complex (96)	*Enterobacter cloacae* (97)	*Enterobacter cloacae* (96)
colon (1A)	*Enterobacter cloacae* (9.010)	*Enterobacter cloacae* complex (95)	*Enterobacter cloacae* (96)	*Enterobacter cloacae* (95)
rectum (2A)	*Enterobacter cloacae* (9.539)	*Enterobacter cloacae* complex (96)	*Enterobacter cloacae* (98)	*Enterobacter cloacae* (95)
faeces (2A)	*Enterobacter cloacae* (9.573)	*Enterobacter cloacae* complex (97)	*Enterobacter cloacae* (96)	*Enterobacter cloacae* (97)
small intestine (2A)	*Enterobacter gergoviae* (9.170)	*Enterobacter cloacae* complex (94)	*Enterobacter gergoviae* (99)	*Enterobacter gergoviae* (99)
colon (2A)	*Enterobacter hormaechei* (9.617)	*Enterobacter cloacae* complex (94)	*Enterobacter cloacae* (91)	*Enterobacter cloacae* (89)
rectum (1A)	*Enterobacter ludwigii* (9.042)	*Enterobacter cloacae* complex (94)	*Enterobacter cloacae* (94)	*Enterobacter cloacae* (86)
small intestine (1A)	*Escherichia coli* (9.691)	*Escherichia coli* (99)	*Escherichia coli* (99)	*Escherichia coli* (99)
small intestine (1A)	*Escherichia coli* (9.679)	*Escherichia coli* (99)	*Escherichia coli* (99)	*Escherichia coli* (99)
colon (1A)	*Escherichia coli* (9.646)	*Escherichia coli* (99)	*Escherichia coli* (99)	*Escherichia coli* (99)
colon (1A)	*Escherichia coli* (9.088)	*Escherichia coli* (99)	*Escherichia coli* (99)	*Escherichia coli* (99)
rectum (1A)	*Escherichia coli* (9.613)	*Escherichia coli* (99)	*Escherichia coli* (99)	*Escherichia coli* (99)
rectum (1A)	*Escherichia coli* (9.550)	*Escherichia coli* (99)	*Escherichia coli* (99)	*Escherichia coli* (99)
small intestine (AH)	*Escherichia coli* (9.616)	*Escherichia coli* (99)	*Escherichia coli* (99)	*Escherichia coli* (99)
small intestine (AH)	*Escherichia coli* (9.509)	*Escherichia coli* (99)	*Escherichia coli* (99)	*Escherichia coli* (99)
small intestine (AH)	*Escherichia coli* (9.587)	*Escherichia coli* (99)	*Escherichia coli* (99)	*Escherichia coli* (99)
small intestine (AH)	*Escherichia coli* (9.594)	*Escherichia coli* (99)	*Escherichia coli* (99)	*Escherichia coli* (99)
small intestine (AH)	*Escherichia coli* (9.578)	*Escherichia coli* (99)	*Escherichia coli* (99)	*Escherichia coli* (99)
small intestine (AH)	*Escherichia coli* (9.649)	*Escherichia coli* (99)	*Escherichia coli* (99)	*Escherichia coli* (99)
small intestine (AH)	*Escherichia coli* (9.603)	*Escherichia coli* (99)	*Escherichia coli* (99)	*Escherichia coli* (99)
small intestine (AH)	*Escherichia coli* (9.568)	*Escherichia coli* (99)	*Escherichia coli* (99)	*Escherichia coli* (99)
colon (AH)	*Escherichia coli* (9.568)	*Escherichia coli* (99)	*Escherichia coli* (99)	*Escherichia coli* (99)
colon (AH)	*Klebsiella pneumoniae* (9.132)	*Klebsiella pneumoniae* subsp. *pneumoniae* (96)	*Klebsiella pneumoniae* subsp. *pneumoniae* (97)	*Klebsiella pneumoniae* (98)
colon (AH)	*Klebsiella pneumoniae* subsp. *pneumoniae* (9.063)	*Klebsiella pneumoniae* subsp. *pneumoniae* (99)	*Klebsiella pneumoniae* subsp. *pneumoniae* (96)	*Klebsiella pneumoniae* (98)

1A –first (male) neglected alpaca; 2A –second (female) neglected alpaca; AH – third (male) healthy alpaca

Enterobacter strains (61.5%) were the most numerous group in the *Enterobacteriaceae* family, followed by *Citrobacter* spp. (30.7%) and *E. coli* (7.7%). The largest group of *Enterobacter* were *Enterobacter cloacae* and single instances of *Enterobacter gergoviae*, *Enterobacter hormaechei* and *Enterobacter ludwigii*. Three of the four isolated *Citrobacter* spp. were confirmed as *Citrobacter freundii* by all identification methods, whereas one of these isolates was determined by VITEK2 GN and API 20E as *Citrobacter braakii* with 89% and 92% probabilities, respectively, and as *Citrobacter freundii* by MALDI-TOF MS. The spectrum which was not defined by MALDI-TOF MS was identified by the other three methods as *Citrobacter freundii* with >95% probabilities (Table 2). The biochemical identification results of *E. coli* isolates by the VITEK2, API and RapID systems were consistent with those from MALDI-TOF MS.

The Gram-negative *Yersiniaceae* strains in 60% of both discussed bacterial identification techniques indicated the same species, *Serratia odorifera*; however, in one case MALDI-TOF MS indicated *Serratia liquefaciens* and VITEK2 *Serratia odorifera* and in another case, MALDI-TOF MS did not indicate any bacteria or genus and VITEK2 found *Serratia marcescens* (Table 3). None of the *Serratia* species produced the red pigment prodigiosin. Colonies of *Serratia odorifera* and *Serratia liquefaciens* had a musty odour.

All Gram-negative *Morganellaceae* were isolated from the alpaca which was healthy until its death (AH). Of these isolates, 40% were *Morganella morganii* and 60% *Providencia alcalifaciens*. *Morganella morganii*, *Providencia alcalifaciens* and *Pseudomonas aeruginosa* were all correctly identified to the species level by the API, RapID, VITEK2 and MALDI-TOF MS techniques.

Among Gram-positive cocci in the MALDI-TOF method, 100% of the results with determination of the bacterial species were obtained, with the VITEK2 GP device 83.3% and the API Staph test, three *Staphylococcus* sp. strains and one *Micrococcus* sp.

Of all Gram-positive cocci, 58.3% were *Enterococcus* spp. of which five strains were *Enterococcus hirae* and two strains of *Enterococcus casseliflavus*, 25% were *Staphylococcus* spp., two of which belonged to *Staphylococcus sciuri* and one *Staphylococcus pseudintermedius*. Other strains were *Brachybacterium conglomeratum* and *Micrococcus luteus*. All details regarding the identification of Gram-positive *cocci* can be found in Tables 4 and 5.

Gram-positive *cocci Brachybacterium conglomeratum* were identified by MALDI-TOF, but neither by VITEK2 nor API Staph. *Brachybacterium conglomeratum* belongs to the *Dermabacteraceae* family and as originally classified as *Micrococcus conglomeratus*. *Micrococcus luteus* (family *Micrococcaceae*) was identified by MALDI-TOF and API Staph as *Micrococcus* sp. but not by VITEK2 GP. *Leuconostoc citreum* (family *Lactobacillaceae*) was identified by MALDI-TOF and VITEK2 as *Leuconostoc mesenteroides* but not by API Staph. VITEK2 identified Gram-positive bacteria (*cocci* and rods) but API Staph identified only *Staphylococcus*, *Kocuria* and *Micrococcus*.

Bacillus spp. has comparable results between MALDI-TOF and VITEK2 BCL with 78.8% identical results. 21.2% of the strains agreed on the genus but not the species (Table 6).

Table 3. Results of identification using matrix-assisted laser desorption/ionisation–time-of-flight mass spectrometry (MALDI-TOF MS) and VITEK2 Gram-negative (GN) fluorogenic, analytical profile index for 20 *Enterobacteriaceae* (API 20E), and RapID ONE tests for Gram-negative *Yersiniaceae*, *Morganellaceae* and *Pseudomonadaceae* isolated from three alpacas

Sample source	MALDI-TOF MS (score)	VITEK2 GN (% probability)	API 20E (% probability)	rapID ONE (% probability)
		Yersiniaceae		
small intestine (2A)	*Serratia odorifera* (9.285)	*Serratia odorifera* (99)	*Serratia odorifera* 1 (97)	*Serratia odorifera* 1,2 (97)
colon (2A)	*Serratia liquefaciens* (9.098)	*Serratia odorifera* (97)	*Serratia liquefaciens* (99)	*Serratia liquefaciens* (98)
rectum (2A)	*Serratia odorifera* (9.606)	*Serratia odorifera* (99)	*Serratia odorifera* 1 (99)	*Serratia odorifera* 1,2 (97)
faeces (2A)	*Serratia odorifera* (9.654)	*Serratia odorifera* (98)	*Serratia odorifera* 1 (96)	*Serratia odorifera* 1,2 (97)
faeces (1A)	no spectrum	*Serratia marcescens* (99)	*Serratia marcescens* (97)	*Serratia marcescens* (98)
		Morganellaceae		
rectum (AH)	*Morganella morganii* (9.362)	*Morganella morganii* subsp. *morganii* (99)	*Morganella morganii* (99)	*Morganella morganii* (99)
rectum (AH)	*Morganella morganii* (9.480)	*Morganella morganii* subsp. *morganii* (99)	*Morganella morganii* (98)	*Morganella morganii* (98)
small intestine (AH)	*Providencia alcalifaciens* (9.589)	*Providencia alcalifaciens* (97)	*Providencia alcalifaciens/rustigianii* (98)	*Providencia alcalifaciens* (98)
rectum (AH)	*Providencia alcalifaciens* (9.067)	*Providencia alcalifaciens* (98)	*Providencia alcalifaciens/rustigianii* (98)	*Providencia alcalifaciens* (99)
rectum (AH)	*Providencia alcalifaciens* (9.611)	*Providencia alcalifaciens* (98)	*Providencia alcalifaciens/rustigianii* (99)	*Providencia alcalifaciens* (96)
		Pseudomonadaceae		
small intestine (1A)	*Pseudomonas aeruginosa* (8.239)	*Pseudomonas aeruginosa* (99)	*Pseudomonas aeruginosa* (99)	*Pseudomonas aeruginosa* (99)
rectum (1A)	*Pseudomonas aeruginosa* (8.856)	*Pseudomonas aeruginosa* (98)	*Pseudomonas aeruginosa* (98)	*Pseudomonas aeruginosa* (98)

1A –first (male) neglected alpaca; 2A –second (female) neglected alpaca; AH – third (male) healthy alpaca

Table 4. Results of identification using matrix-assisted laser desorption/ionisation–time-of-flight mass spectrometry (MALDI-TOF MS), VITEK2 Gram-positive (GP) for Gram-positive *Enterococcaceae* isolated from three alpacas

Sample source	MALDI-TOF (score)	VITEK2 GP (% probability)
small intestine (1A)	*Enterococcus faecium* (9.721)	*Enterococcus faecium* (99)
rectum (1A)	*Enterococcus gallinarum* (9.705)	*Enterococcus gallinarum* (99)
small intestine (1A)	*Enterococcus hirae* (9.686)	*Enterococcus hirae* (99)
colon (2A)	*Enterococcus hirae* (9.618)	*Enterococcus hirae* (99)
colon (2A)	*Enterococcus hirae* (9.697)	*Enterococcus hirae* (99)
colon (2A)	*Enterococcus hirae* (9.670)	*Enterococcus hirae* (99)
rectum (2A)	*Enterococcus hirae* (9.591)	*Enterococcus hirae* (99)
rectum (2A)	*Enterococcus hirae* (9.694)	*Enterococcus hirae* (99)
small intestine (AH)	*Enterococcus casseliflavus* (9.616)	*Enterococcus casseliflavus* (98)
colon (2A)	*Enterococcus casseliflavus* (9.615)	*Enterococcus casseliflavus* (98)
rectum (2A)	*Enterococcus casseliflavus* (8.198)	*Enterococcus casseliflavus* (99)
rectum (AH)	*Enterococcus casseliflavus* (9.637)	*Enterococcus casseliflavus* (99)
rectum (AH)	*Enterococcus casseliflavus* (9.539)	*Enterococcus casseliflavus* (99)

1A – first (male) neglected alpaca; 2A – second (female) neglected alpaca; AH – third (male) healthy alpaca

Table 5. Results of identification using matrix-assisted laser desorption/ionisation–time-of-flight mass spectrometry (MALDI-TOF MS) and VITEK2 Gram-positive (GP) and analytical profile index for Staphylococcus (API Staph) tests for Gram-positive *cocci* isolated from three alpacas

Sample source	MALDI-TOF (score)	VITEK2 GP (% probability)	API Staph (% probability)
colon (1A)	*Staphylococcus aureus* (9.380)	*Staphylococcus aureus* (97)	*Staphylococcus aureus* (94)
rectum (1A)	*Staphylococcus aureus* (9.410)	*Staphylococcus aureus* (99)	*Staphylococcus aureus* (96)
colon (1A)	*Staphylococcus equorum* (9.001)	*Staphylococcus equorum* (92)	*Staphylococcus intermedius* (90)
colon (1A)	*Staphylococcus lentus* (9.457)	*Staphylococcus lentus* (96)	*Staphylococcus lentus* (96)
rectum (2A)	*Staphylococcus pseudintermedius* (7.247)	*Staphylococcus pseudintermedius* (98)	*Staphylococcus simulans* (82)
small intestine (2A)	*Staphylococcus sciuri* (9.111)	*Staphylococcus sciuri* (86)	*Staphylococcus sciuri* (92)
small intestine (2A)	*Staphylococcus sciuri* (9,428)	*Staphylococcus sciuri* (95)	*Staphylococcus sciuri* (92)
small intestine (2A)	*Staphylococcus sciuri* (9.471)	*Staphylococcus sciuri* (95)	*Staphylococcus sciuri* (96)
colon (1A)	*Staphylococcus sciuri* (8.228)	*Staphylococcus sciuri* (95)	*Staphylococcus sciuri* (97)
colon (1A)	*Staphylococcus sciuri* (9.120)	*Staphylococcus sciuri* (95)	*Staphylococcus sciuri* (97)
colon (1A)	*Staphylococcus sciuri* (9.345)	*Staphylococcus sciuri* (95)	*Staphylococcus sciuri* (97)
colon (1A)	*Staphylococcus sciuri* (9.528)	*Staphylococcus sciuri* (97)	*Staphylococcus sciuri* (97)
colon (1A)	*Staphylococcus sciuri* (9.513)	*Staphylococcus sciuri* (95)	*Staphylococcus sciuri* (97)
colon (1A)	*Staphylococcus sciuri* (9.405)	*Staphylococcus sciuri* (95)	*Staphylococcus sciuri* (94)
colon (1A)	*Staphylococcus sciuri* (9.571)	*Staphylococcus sciuri* (87)	*Staphylococcus sciuri* (89)
colon (1A)	*Staphylococcus sciuri* (9.385)	*Staphylococcus sciuri* (88)	*Staphylococcus sciuri* (89)
rectum (2A)	*Micrococcus luteus* (9.169)	unidentified	*Micrococcus* sp. (77)
rectum (AH)	*Lactococcus lactis* subsp. *cremoris* (9.041)	*Lactococcus lactis* subsp. *cremoris* (9.041)	unidentified

1A – first (male) neglected alpaca; 2A – second (female) neglected alpaca; AH – third (male) healthy alpaca

Table 6. Results of identification using matrix-assisted laser desorption/ionisation–time-of-flight mass spectrometry (MALDI-TOF MS) and VITEK2 BCL test for *Bacillus* isolated from two alpacas

Sample source	MALDI-TOF (score)	VITEK2 BCL (% probability)
rectum (2A)	*Bacillus aeolinus* (5.139)	*Bacillus subtilis*/*Bacillus amyloliquefaciens*/*Bacillus atrophaeus* (97)
small intestine (2A)	*Bacillus altitudinis* (8.713)	*Bacillus subtilis*/*Bacillus amyloliquefaciens*/*Bacillus atrophaeus* (95)
rectum (2A)	*Bacillus anthraci* 6.295)	*Bacillus cereus* (98)
colon (2A)	*Bacillus cereus* (9.279)	*Bacillus cereus* (98)
colon (2A)	*Bacillus* sp. (7.835)	*Bacillus cereus* (99)
colon (2A)	*Bacillus cecembensis* (5.993)	*Bacillus pumilus* (93)
colon (2A)	*Bacillus flexus* (5.375)	unidentified
small intestine (2A)	*Bacillus licheniformis* (9.476)	*Bacillus licheniformis* (99)
small intestine (2A)	*Bacillus licheniformis* (8.525)	*Bacillus licheniformis* (99)
colon (2A)	*Bacillus licheniformis* (4.744)	*Bacillus licheniformis* (91)
colon (2A)	*Bacillus licheniformis* (9.253)	*Bacillus licheniformis* (99)
rectum (2A)	*Bacillus licheniformis* (7.233)	*Bacillus licheniformis* (99)
small intestine (AH)	*Bacillus pumilus* (9.157)	*Bacillus pumilus* (92)
colon (2A)	*Bacillus pumilus* (9.547)	*Bacillus pumilus* (98)
colon (2A)	*Bacillus pumilus* (6.156)	*Bacillus pumilus* (96)
colon (2A)	*Bacillus pumilus* (8.884)	*Bacillus pumilus* (98)
colon (2A)	*Bacillus subtilis* (7.181)	*Bacillus subtilis* (98)
colon (2A)	*Bacillus subtilis* (6.428)	*Bacillus subtilis* (95)

1A – first (male) neglected alpaca; 2A – second (female) neglected alpaca; AH – third (male) healthy alpaca

In one case, *Bacillus "*anthraci" (named by MALDI-TOF) was detected and VITEK2 BCL indicated as *Bacillus cereus*. These discrepancies required additional microbiological determinations. The tested *Bacillus* strain formed large, dull, opaque grey colonies with rough edges with circular zones of β-haemolysis surrounding the colonies on blood agar (Fig. 1a) and rough and dry with light pink colonies with

halo egg yolk precipitation on MYP agar (Fig. 1b). Repeated biochemical results allowed its designation as *Bacillus cereus*.

Fig. 1a. *Bacillus* "anthraci" presented aerobic growth of dull grey colonies with a raw matted surface with surrounding β-haemolysis on Columbia agar with 5% sheep blood as classical *Bacillus cereus*

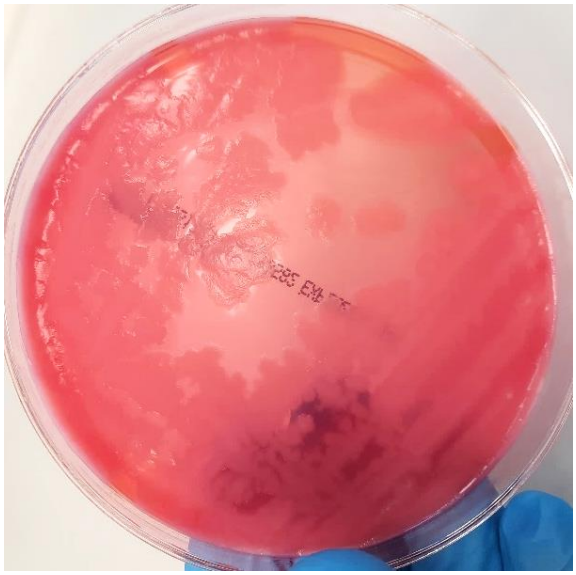


Fig. 1b. *Bacillus cereus* created classical rough and dry with a bright pink colonies with egg yolk precipitation halo on MYP agar

For *Clostridium perfringens* identification both VITEK2 ANC card and MALDI-TOF MS provided identical results at species level for the four isolated strains. Thus both methods can be used for accurate routine anaerobe identification (Table 7).

Some of the isolated bacterial strains (*Arcticiflavibacter luteus*, *Leeuwenhoekiella nanhaiensis*, *Sphingobacterium anhuiense*, *Thalassolituus oleivorans*, *Cellulosimicrobium cellulans*, *Brachybacterium conglomeratum*) caused major diagnostic difficulties due to rare isolation and unspecific colony and cells morphology. These group of bacteria are presented in Table 8. In the MALDI-TOF study, 33.3% were reliable for species, 25% for reliable types and 41.6% for unreliable results. The results obtained in the MALDI-TOF for *Stenotrophomonas* sp. have been confirmed as *Stenotrophomonas maltophilia* by VITEK2 and API 20NE >96%, *Moraxella osloensis* as group level identification (*Moraxella* spp.) and RapID NF Plus as *Moraxella osloensis*. *Ochrobactrum intermedium* was classified as *Ochrobactrum anthropi* in biochemical tests (Table 8).

Leuconostoc citreum in the MALDI-TOF study was confirmed with VITEK2 GP as *Leuconostoc citreum* and it was similarly for *Paenibacillus amylolyticus*. Other bacteria *Cellulosimicrobium cellulans*, formerly known as *Oerskovia xanthineolytica*, grown as colonies less than 2 mm in size, glistening and yellow.

Corynebacterium stationis in the MALDI-TOF study was identified with VITEK2 CBC as *Corynebacterium minutissimum* (Table 9), but *Corynebacterium xerosis* was identified both by MALDI-TOF MS and VITEK2 CBC. Surprisingly, in *Corynebacterium* spp., *Corynebacterium diphtheriae* was identified by biochemical methods: manual API CORYNE and automated VITEK2 CBC card, but as *Candida lipolytica* by MALDI-TOF MS. Gram stain test results reveal presence of slightly curved rods visible in magnification 100 × instead of yeast identified as *Candida lipolytica* by MALDI-TOF MS.

A total of 8 yeast culture were isolated from two alpacas, mainly from 1A. Overall distribution of *Candida* yeast species was as follows: *C. albicans*, *C. haemulonii*, *C. metapsilosis* (*C. ciferrii* in VITEK2) and *C. lipolytica*. For *Candida lipolytica* MALDI-TOF score was 4.495, result unreliable (Table 10).

The smear prepare from putative *Candida lipolytica* shows typical Gram stain bacteria appearing as V-in Y-shaped arrangements or in clumps that resemble Chinese letters. VITEK2 CBC cards identified microorganism as *Corynebacterium diphtheriae* (Table 10).

Table 7. Results of identification using matrix-assisted laser desorption/ionisation–time-of-flight mass spectrometry (MALDI-TOF MS) and VITEK2 Anaerobic and *Corynebacterium* (ANC) fluorogenic test for *Clostridium perfringens* isolated from an alpaca which was healthy until its death (AH)

Sample source	MALDI-TOF MS (score)	VITEK2 ANC (% probability)
small intestine (AH)	*Clostridium perfringens* (9.635)	*Clostridium perfringens* (95)
small intestine (AH)	*Clostridium perfringens* (9.665)	*Clostridium perfringens* (92)
colon (AH)	*Clostridium perfringens* (9.619)	*Clostridium perfringens* (95)
colon (AH)	*Clostridium perfringens* (9.664)	*Clostridium perfringens* (98)

Table 8. Results of identification using matrix-assisted laser desorption/ionisation–time-of-flight mass spectrometry (MALDI-TOF MS), VITEK2 Gram-negative (GN) fluorogenic, VITEK2 Gram-positive (GP), analytical profile index for 20 *Enterobacteriaceae* (API 20E), analytical profile index for 20 non-*Enterobacteriaceae* (API 20NE) and RapID NF Plus tests for different bacteria isolated from two alpacas

Sample source Gram-negative	MALDI-TOF MS (score)	VITEK2 GN (% probability)	API 20E/API 20NE	RapID NF Plus
colon (1A)	*Stenotrophomonas* spp. (*Xanthomonadaceae*), (9.158)	*Stenotrophomonas maltophilia* (96%)	*Stenotrophomonas maltophilia* (99%)	unidentified
small intestine (2A)	*Moraxella osloensis* (*Moraxellaceae*), (9.365)	*Moraxella* group, (94) GN	*Moraxella* spp. (94)	*Moraxella osloensis* (97)
small intestine (2A)	*Arcticiflavibacter luteus* (*Flavobacteriaceae*), (5.436)	unidentified	unidentified	unidentified
colon (2A)	*Leeuwenhoekiella nanhaiensis* (*Flavobacteriaceae*), (5.259)	unidentified	unidentified	unidentified
small intestine (1A)	*Ochrobactrum intermedium* (9.574) (*Brucellaceae*)	*Ochrobactrum anthropi* (82)	*Ochrobactrum anthropi* (97)	*Ochrobactrum anthropi* (97)
colon (2A)	*Sphingobacterium anhuiense* (*Sphingobacteriaceae*) (9.158)	unidentified	unidentified	unidentified
colon (2A)	*Thalassolituus oleivorans* (5.545), (*Oceanospirillaceae*)	unidentified	unidentified	unidentified
Sample source Gram-positive	MALDI-TOF (score)	VITEK2 GP (probability %)	API STAPH	
rectum (2A)	*Cellulosimicrobium cellulans* (8.475) (*Promicromonosporaceae*)	unidentified	unidentified	
colon (2A)	*Paenibacillus amylolyticus* (6.065) (*Paenibacillaceae*)	*Paenibacillus amylolyticus*, BCL (93)	unidentified	
colon (1A)	*Brachybacterium conglomeratum* (7.247)	unidentified	unidentified	
rectum (2A)	*Leuconostoc citreum* (4.426)	*Leuconostoc mesenteroides* (97)	unidentified	

1A –first (male) neglected alpaca; 2A – second (female) neglected alpaca

Table 9. Results of identification using matrix-assisted laser desorption/ionisation–time-of-flight mass spectrometry (MALDI-TOF MS) and VITEK2 *Corynebacterium* (CBC) fluorogenic and analytical profile index for Coryneform bacteria (API CORYNE) tests for *Corynebacterium* spp. isolated from one fallen alpaca

Sample source	MALDI-TOF MS (score)	VITEK2 CBC (% probability)	API Coryne
colon (1A)	*Candida lipolytica*	*Corynebacterium diphtheriae* (91)	*Corynebacterium diphtheriae* (95)
small intestine (1A)	*Corynebacterium stationis* (9.560)	*Corynebacterium* sp. (97)	*Corynebacterium diphtheriae* (97)
small intestine (1A)	*Corynebacterium stationis* (9.465)	*Corynebacterium minutissimum* (96)	*Corynebacterium* sp. (89)
colon (1A)	*Corynebacterium stationis* (9.376)	*Corynebacterium minutissimum* (95)	*Corynebacterium* sp. (82)
colon (1A)	*Corynebacterium stationis* (8.985)	*Corynebacterium minutissimum* (93)	*Corynebacterium* sp. (91)
colon (1A)	*Corynebacterium stationis* (9.167)	*Corynebacterium minutissimum* (97)	*Corynebacterium* sp. (89)
small intestine (1A)	*Corynebacterium xerosis* (9.440)	*Corynebacterium xerosis* (98)	*Corynebacterium* sp. (85)

1A –first (male) neglected alpaca; 2A – second (female) neglected alpaca

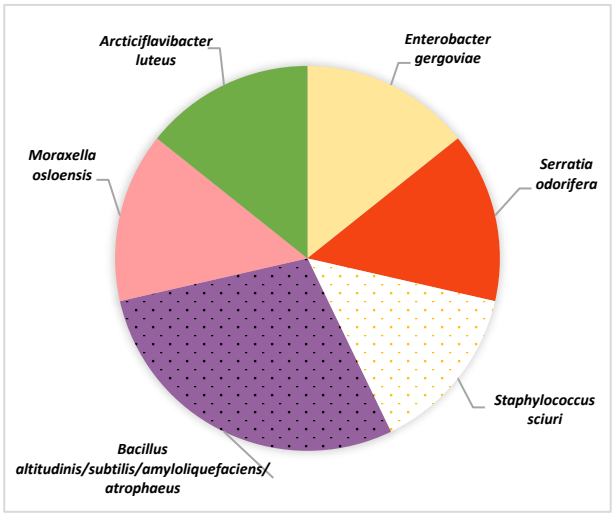
Table 10. Results of identification using matrix-assisted laser desorption/ionisation–time-of-flight mass spectrometry (MALDI-TOF MS) and VITEK2 yeast (YST) fluorogenic test for *Candida* isolated from two alpacas

Sample source	MALDI-TOF MS (score)	VITEK2 YST (% probability)
small intestine (1A)	*Candida albicans* (8.982)	*Candida albicans* (96)
small intestine (1A)	*Candida albicans* (6.608)	*Candida albicans* (98)
small intestine (1A)	*Candida albicans* (7.777)	*Candida albicans* (97)
small intestine (1A)	*Candida albicans* (5.434)	*Candida albicans* (91)
colon (1A)	*Candida albicans* (7.651)	*Candida albicans* (91)
colon (2A)	*Candida haemulonii* (4.777)	*Candida haemulonii* (95)
colon (1A)	*Candida lipolytica* (4.495)	*Corynebacterium diphtheriae* (91)
colon (1A)	*Candida metapsilosis* (4.683)	*Candida ciferrii* (89)

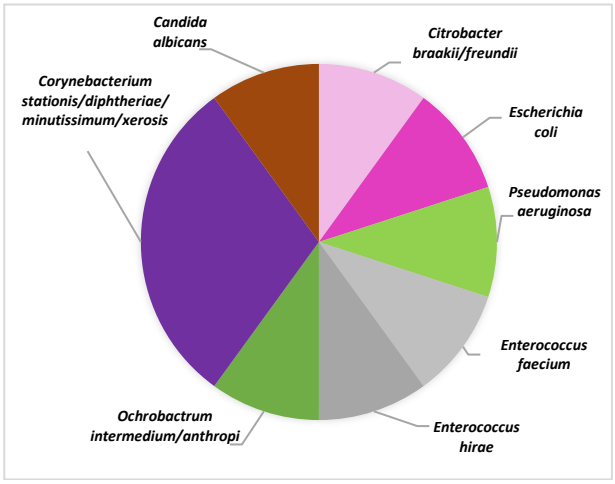
1A –first (male) neglected alpaca; 2A – second (female) neglected alpaca

For each alpaca, significant differences in microbial community compositions were observed. The small intestine, colon and rectum of alpaca 1A and 2A had the richest diversity of microorganisms than healthy alpaca (AH). In small intestine 1A alpaca 7 bacteria (Gram-negative rods, Gram-positive *Enterococcus*, and *Corynebacterium*) and 1 yeast *Candida albicans*, in 2A alpaca 6 bacteria (Gram-negative rods, Gram-positive *Staphylococcus*, and *Bacillus*) were found. In AH alpaca 5 bacteria (Gram-negative rods, Gram-positive *Enterococcus*, *Bacillus* and *Clostridium*) were found (Fig. 2). In colon in 1A alpaca 10 bacteria (Gram-negative rods, Gram-positive *Staphylococcus*, and *Corynebacterium*) and yeast *Candida albicans*, *Candida. metapsilosis*/*ciferrii*, in 2A alpaca 12 bacteria (Gram-negative rods, Gram-positive *Enterococcus*, and *Bacillus*) and *Candida. haemulonii* were found. In AH alpaca 3 bacteria (Gram-negative rods and Gram-positive Clostridium) were found (Fig. 3).

1A



2A



AH

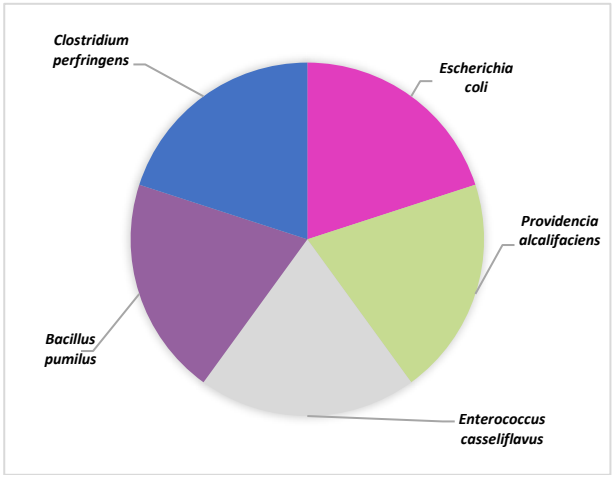
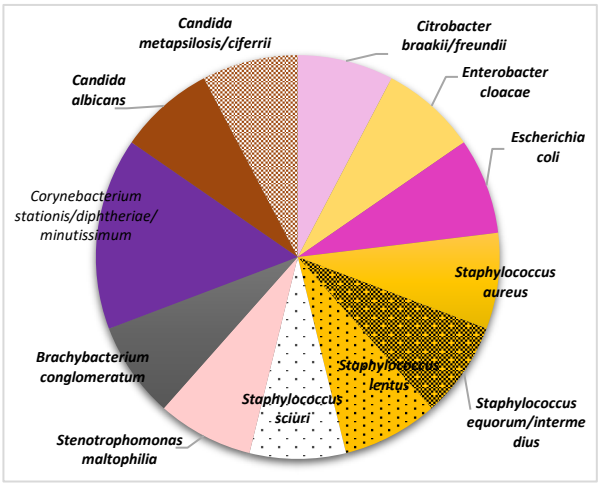
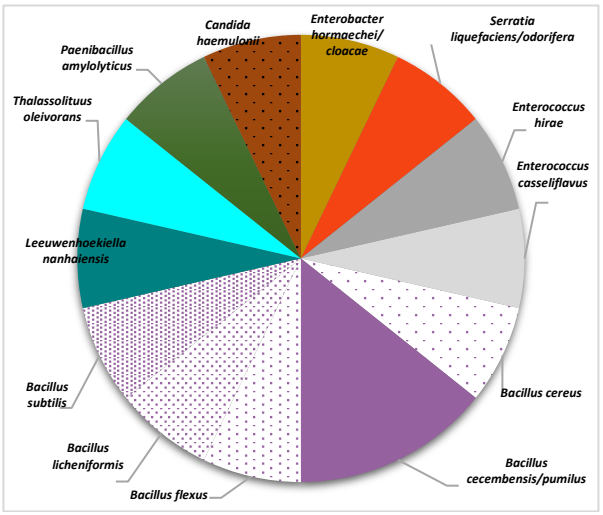


Fig. 2. Complexity of microbial communities in small intestine
1A – the first alpaca; 2A – the second alpaca; AH – healthy alpaca

1A



2A



AH

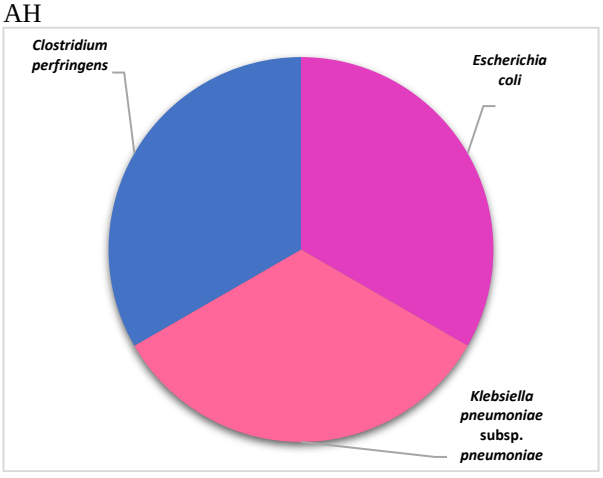
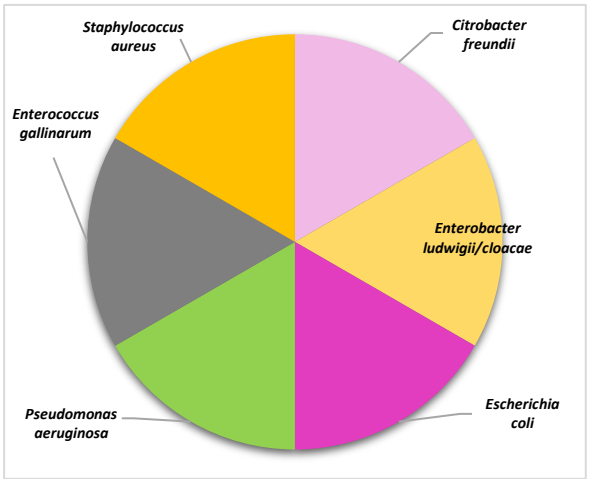
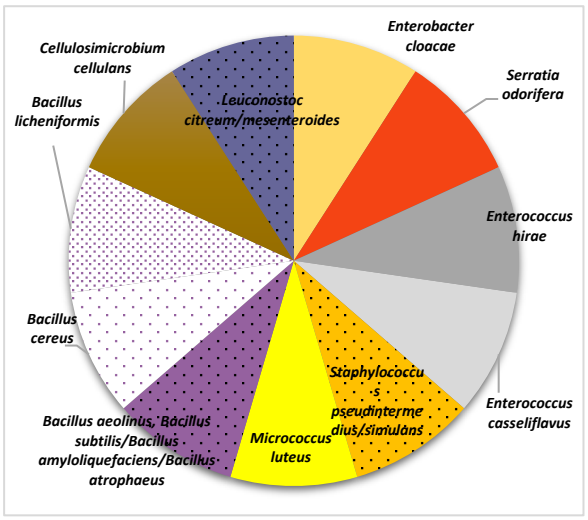


Fig. 3. Complexity of microbial communities in colon
1A – first (male) neglected alpaca; 2A – second (male) neglected alpaca; AH – third (male) healthy alpaca

1A



2A



AH

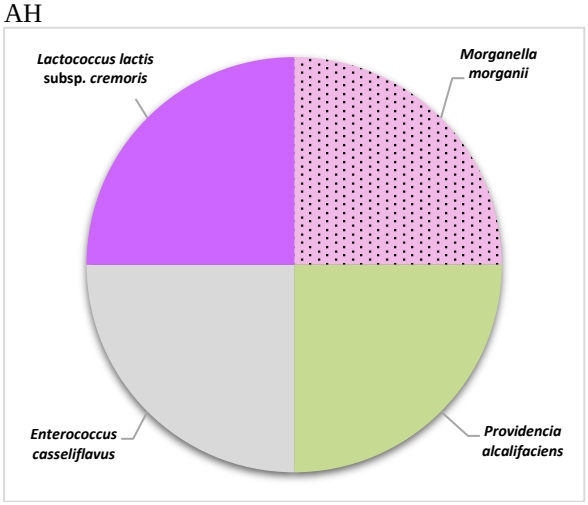


Fig. 4. Complexity of microbial communities in rectum
1A – first (male) neglected alpaca; 2A – second (female) neglected alpaca; AH – third (male) healthy alpaca

In rectum in 1A alpaca 6 bacteria (Gram-negative rods, *Gram*-positive *Staphylococcus*, *Enterococcus*), in 2A alpaca 11 bacteria (Gram-negative rods, Gram-positive Enterococcus, *Staphylococcus* and *Bacillus*) were found. In AH alpaca 4 bacteria (Gram-negative rods and Gram-positive *Enterococcus*) were found (Fig. 4).

Staphylococcus aureus, *Staphylococcus* CNS and *Candida* spp. were found in alpacas with clinical symptoms (alpaca 1A, 2A), whilst *E. coli* is primarily found as gut commensal in healthy animals (lack in 2A).

Discussion

Bacterial identification was performed by MALDI-TOF MS, VITEK2, API 20E, 20NE, STAPH and RapID ONE and RapID NF Plus. Automatic and manual methods were compared (Tables 2–10).

All investigated automatic and manual methods turned out to be powerful tools for fast, accurate identification of microorganisms isolated from alpacas. However, several rare and poorly known bacteria were identified only by MALDI-TOF MS during the research: *Ochrobactrum intermedium*, *Cellulosimicrobium cellulans*, *Brachybacterium conglomeratum*, *Thallasolituus oleivorans* and *Leeuwenhoekiella nanhaiensis.*

Ochrobactrum intermedium is currently referred to as an emerging opportunistic pathogen of humans (16). It is Gram-negative, aerobic, short straight or slightly curved bacilli, belongs to the genus *Ochrobactrum* and the family *Brucellaceae* (1). *Ochrobactrum intermedium* is difficult to distinguish from other *Ochrobactrum* species by classical microbiological methods. More and more often identified with the use of MALDI-TOF (14, 22).

Cellulosimicrobium cellulans is Gram-positive bacilli belonging to the order *Actinomycetales*, widely distributed in the environment and have been isolated mainly from soil and grass. The bacterium was described as vancomycin resistant, opportunistic pathogen. In humans, specially, children up 5 years of age and adults with immunosuppression, such as HIV or over 70 years of age, the bacterium causes bacteremia, endophthalmitis, tenosynovitis and peritonitis. Many of *Actinomycetales* infections are characterised by a relatively long history with minimal clinical signs of infection at initial presentation, which often leads to a delay in diagnosis (26).

Brachybacterium conglomeratum is Gram-positive, nonmotile and nonsporing aerobic or facultatively anaerobic bacteria. Although *Brachybacterium* spp. was isolated from, among others, plants, salt-fermented seafood, and the surface of French Gruyère and Beaufort cheeses and even *Brachybacterium. paraconglomeratum* from human eye infection (10, 24, 25). However, there is no information available on the pathogenicity of *Brachybacterium conglomeratum* in humans and animals.

Thallasolituus oleivorans was isolated from sea water/sediment samples collected in the harbour of Milazzo, Sicily, Italy. A Gram-negative cell curved, vibrioid, occasionally screw-like morphology with characteristic monopolar, monotrichous flagella and was described that has been shown to play an important role in the biodegradation of crude oil (9). Until now, no isolation from animals has been described. In this study

Thallasolituus oleivorans was isolated from the large intestine of alpacas.

Leeuwenhoekiella nanhaiensis pertaining to Nanhai from where the type strain was isolated (the Chinese name for the South China Sea). It is a Gram-negative, rod-shaped and yellow bacterium, motile by gliding and it was isolated from a water sample collected from the deep South China Sea (21). Until now, no isolation from animals has been described.

The MALDI-TOF identification is based on the spectral fingerprints which vary between microorganisms (compounds detected in the spectrum and specific molecular masses). The same species can give different mass spectra depending on growth conditions and extraction method used. Bacteria preparation for the study makes a huge impact on specificity and sensitivity. Most common systems of identifications based on biochemical reactions (VITEK2, API, RapID) has limitations due to focus on bacteria and yeasts presents in human medicine.

Moreover, if organisms are encountered frequently, MALDI-TOF can accurately identify most closely related species. However, MALDI-TOF is currently unable to differentiate *E. coli* from *Shigella*, *Burkholderia mallei/pseudomallei* and *Burkholderia cepacia* complex, *Enterobacter cloacae* complex, as well as the *Mycobacterium tuberculosis* complex (7, 8, 17, 18, 19).

Our research confirmed the results indicating that some MALDI-TOF devices are unable to distinguish *Bacillus cereus* from *B acillus anthracis*, however Manzulli *et al.* (23), showed slight differences in the pattern of the spectra of the *B acillus cereus* group, which in the future may contribute to the creation of an algorithm distinguishing these bacteria (21).

MALDI-TOF is useful tools for anaerobic bacteria identification (6). Conventional identification of anaerobes is mainly based on the detection of phenotypic characteristics, such as Gram staining, colony morphology, microscopic examination, differential growth on selective media and spore forming. Most of these conventional methods are arduous and time-consuming processes.

Another limitation may derive from lack of spectra in the database or when some members of a species complex are in the database, but others are not (8, 23). Our study found that there are still many bacteria not included in the databases currently available for VITEK2, API, RapID and MALDI-TOF systems. Manufacturers try to add newly detected spectra to database, however it is an ongoing process which needs further development (6). Developments of the databases to include an expanded number of new species and more robust mass spectra and biochemical profiles for new species will greatly improve the performance and utility of these systems for bacterial and yeast identification.

Therefore, using back up methods such as sequencing or VITEK2 can be highly effective for MALDI-TOF results confirmation. NGS analysis is most popular tool for microbiome identification and microorganism classification while the MALDI-TOF MS and biochemical systems are a popular commercial methods commonly used in clinical microbiology laboratories (6, 17). Gene sequence analysis has important limitation, detecting only genetic material, without information about liveness of identified microorganisms. Culture and biochemical identification are the "gold standard" for living bacterial and yeast. MALDI-TOF MS is showed to be a simple, rapid, accurate tool for identification of common and rare observed microorganisms (6). Identification of veterinary-specific microorganisms is limited and not common on all systems and restricts their usefulness in analyses of animals (23). MALDI-TOF is a quick method, which accurately identified most veterinary isolates of bacteria and yeast to the species level. Thus, MALDI-TOF constitutes a valuable diagnostic tool in the veterinary clinical laboratory (6).

The number of animals tested may seem to be a limitation in these study. However, obtaining autopsy material for microbiological tests from dozens of alpacas is impossible without deliberate slaughtering. Few animals go to the section in a fresh state, which allows the collection of intestinal material for microbiological tests. On the basis of these three cases, we showed the usefulness of the MALDI-TOF method in the study of the live intestinal microbiota of alpacas. This method can be used in the future to research the rapid identification of microbiota, which may be the basis for the diagnosis and treatment of diseases of the digestive system. This study demonstrates that both MALDI-TOF MS and popular systems based on biochemical reactions are powerful tools for fast, accurate identification of microorganisms isolated from alpacas. Moreover it indicated that the VITEK2 and API or RapID tests are accurate and inexpensive identification systems in comparison with MALDI -TOF. Especially in case of common bacteria such as *E. coli*, *Staphylococcus*, *Enterococcus* (23).

The microbiological methods for proper diagnosis and bacterial identification including underestimated opportunistic or rare pathogens, is still poorly studied in domestic animals. As these bacteria and fungi may represent a potential risk for human health further improvement of diagnostic methods and identification is required.

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