Activities completed by Dr.Silvio De Luca at Vetsuisse Fakültat: an integrative report

Abstract

Aim of my project at Vetsuisse Fakültat in Bern (CH) during a two months-length time frame (01.02.2016-01.04.2016) was to recognize the MALDI-TOF MS as a reliable tool for the identification of *Brachyspira* specie, in particular the *B.hyodysenteriae*, agent of swine dysentery, rather than the classic biochemical and genomic-based methods. A specific 'spirochaetal database' of Main spectra profile (MSP) has been improved and used for the identification of species. Two different method available for the preparations of samples were used. A collection of *Brachyspira* strains from Switzerland and Italy was used to test the effective reliability of MALDI-TOF giving a result of 93% of accurate identification, giving a strong base for future studies on this field.

Introduction

Bacteria belonging to *Brachyspira* genus are responsible of enteric disease in animals and human (Fellström et al., 1995; Lee et al., 1994). Among them, *B.hyodysenteriae* is a significant health burden in pig farms, causing Swine Dysentery (SD), a disease that determines important losses due to mortality and poor performance of pigs (Hampson, 2012). SD affects primarly growers and fatteners, clinically ranging from mild, mucous diarrhea to severe haemorrhagic enteritis with a mortality rate of 50-90%. Standard methods for the identification of *B.hyodysenteriae* based on biochemical features are often inconclusive as well as PCR-based method are time-consuming (Calderaro et al., 2012). The matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a rapid, accurate, and cost effective method of microbial characterization and identification. This technology generates characteristic mass fingerprints, that are unique signature for each microorganism and are thus ideal for an accurate microbial identification at the genus and species level (Croxatto et al., 2012). Moreover, MALDI-TOF has a potential to be used for strain typing and identification. A database of reference spectra or main spectra profile (MSP) is needed for the comparison of unknown spectra with reference spectra in order to assess a genus or species-level identification through giving a score. The aim of this study was to assess MALDI-TOF as a reliable tool for the identification of *B.hyodysenteriae* and to differentiate it from the other *Brachyspira* species. The 'spirochaetal database' already available in Bruker Daltonic MSP database has been

improved , adding a group of *Brachyspira hyodysenteriae* MSP formed by four different strains. Moreover, a MSP of the recently designed *Brachyspira pulli* has been recognized and added to the database.

2. Material and method

2.1 Bacterial strains and growth conditions

Overall, 68 different strains were included in this study. On this, forty-eight belonged from pigs reared in Switzerland and included in a surveillance program for the control of swine dysentery. These strains have been cultivated for 5 days on a modified-BJ medium, containing colistin (6,25 mg/L), vancomicin (6,25 mg/L), spectinomycin (200 mg/L) and spiramycin (25 mg/L). Suspected colonies have been transferred on a blood agar for three days as described by Olson et al. (1992) and finally transferred on a Fastidious Anaerobic Agar (FAA) added with colistin (10 mg/L) for three days. Culturing has been even performed in an anaerobic chamber at 42 C° .Moreover, 16 strains were added from a collection provided by the Istituto Zooprofilattico of Umbria and Marche (Italy). On this, 14 had been identified by nox-RFLP (Rhode et al., 2002) as *B.hyodysenteriae* , one as *B.intermedia* and one as *Brachyspira* spp.

2.2 Nox-sequencing

Three *B.hyodysenteriae* (B541, B718, B534) from the Italian collection, one reference strain of *B.hyodysenteriae* (CCUG 46668), one strain of *B.pilosicoli* (JF_4757), one strain of *B.pulli* (B514) and one strain of *B.murdochii* (JF_4755) were selected in order to add their mass spectra profiles (MSP) on the database already present in the Bruker library. Prior to this, a sequencing of *nox*-gene has been performed in order to ensure a correct identification. A forward primer [Bnoxf; 5′-TAG C(CT)T GCG GTA T(CT)G C(AT)C TTT GG-3′] and a reverse primer [Bnoxr; 5′-CTT CAG ACC A(CT)C CAG TAG AAG CC-3′] specific for the *Brachyspira nox* gene were used to amplify a 939-bp fragment as suggested by Rohde et al. (2002). Bacterial growth was removed from a 3-day-old pure culture on FAA and DNA extraction was performed using a DNeasy Blood & Tissue Kit following manufacturer's instructions. Two µl of DNA was added to 28 µl of PCR premixture to yield final concentrations of 1.5 mM $MgCl_2$, PCR buffer B, a 0.2 mM concentration of each deoxynucleoside triphosphate (Solis Biodyne, Estonia), 0.5 µM Bnoxf, 0.5 µM Bnoxr, and 2.5 U of *Taq* polymerase (FIREPol® DNA Polymerase, Solis Biodyne, Estonia). After initial denaturation at 94°C for 3 min, the mixture was subjected

to 35 cycles each cycle involved denaturation at 94°C for 30 s, annealing at 60°C for 45 s, and extension at 72°C for 60 sec. The amplification was finished after a final extension step at 72°C for 10 min. An aliquot of 6 µl of the PCR product was visualized after separation by electrophoresis in a 1% agarose gel and staining with ethidium bromide. PCR products were purified using a High Pure PCR Product Purification Kit (Roche, Switzerland) following manufacturer's instructions. 0.5 µM of BnoxF and 0.5 µM BnoxR were also used for the sequencing in 10 total µl of a reaction composed by 1 µl of (5X) BigDye (Applied Biosystems, California, US), 2 µl of (10X) Buffer sequencing (Applied Biosystems, California, US), 3 µl of PCR purified products and UltraPure water. A 3130 xl Genetic Analyzer with a 16-capillary system (Applied Biosystems, California, US) was used for the all the sequencing assays.

2.3 MALDI-TOF MS database

Sequenced strains were used for the user-defined library achieving new main spectra profile. Spectra were obtained with MALDI Biotyper (Bruker Daltonics, Germany) from each isolate after the 'protein extraction method' or 'ethanol-formic acid method' (Calderaro et al., 2012; Goldstein et al., 2013). A suspension of approximately 10^5 bacteria/ml was obtained in 300 µl of ultrapure water and 900 µl of pure ethanol was added to the suspension. After mixing, the suspension was centrifuged at 13000 *g* for 2 minutes. Supernatant was discarded and the pellet was dried at room temperature for 5 minutes. the pellet was resuspended in 50 µl of 70% formic acid and 50 µl of 100% acetonitrile to the pellet. After mixing, the suspension was centrifuged again at 13000 *g* for 2 minutes. For each strains, 8 spots onto a MSP 96 polished steel target plate were filled (Bruker Daltonics, Germany) using 1 µl of supernatant. After drying, all spots were overlaid with 1 µl of matrix, composed by a satured solution of α-cyano4-hydroxycinnamic acid (CHCA). Each spot was measured 3 times, giving 24 different spectra. A Microflex LT mass spectrometer were used to perform MALDI-TOF MS measurements, using a 20 hz nitrogen laser and FlexControl software. All the spectra were obtained in linear mode with an accelerating voltage of 20kV and analyzed within a mass range of 2000-20000 Da. At least 20 spectra on 24 obtained for each strain were analyzed by FlexAnalysis 3.1 and downloaded in MALDI Byotiper 3.0 to create a MSP. The main spectra were assembled on the basis of raw spectra after smoothing, baseline subtraction, normalization and peak picking. A cluster analysis was performed using statistical tool already integrated in MALDI

Biotyper , using as parameter settings “Distance Measure Euclidian” and “Linkage Complete”.

2.5 Intact cell method (ICM) versus Protein Extraction method (PEM)

Twenty-two strains were submitted to MALDI-TOF analysis using two different preparation method. The Intact Cell Method has provided use of a sterile device to transfer one or few colonies to a single spot of a MSP 96 polished steel target plate. After, 1 µl of 70% formic acid and, after drying, 1 µl of matrix were added to the spot. The protein extraction method is already described in the 2.3 chapter.

2.4 MALDI-TOF MS identification

Sixty-eight strains collected during an g monitoring program on swine dysentery in Switzerland and 16 strains belonging to a strain collection provided by the Istituto Zooprofilattico of Umbria and Marche (Italy) were submitted to the MALDI-TOF MS identification. For each analysis, a negative control (2 µl of matrix) has been used. A cluster analysis using BioNumerics 7.1 software (AppliedMath) was performed to assess the presence of species-specific clusters.

3. Results

The ‘spirochetal database’ recognized in this study provides MSP belonging to six different species of *Brachyspira* (fig.1).

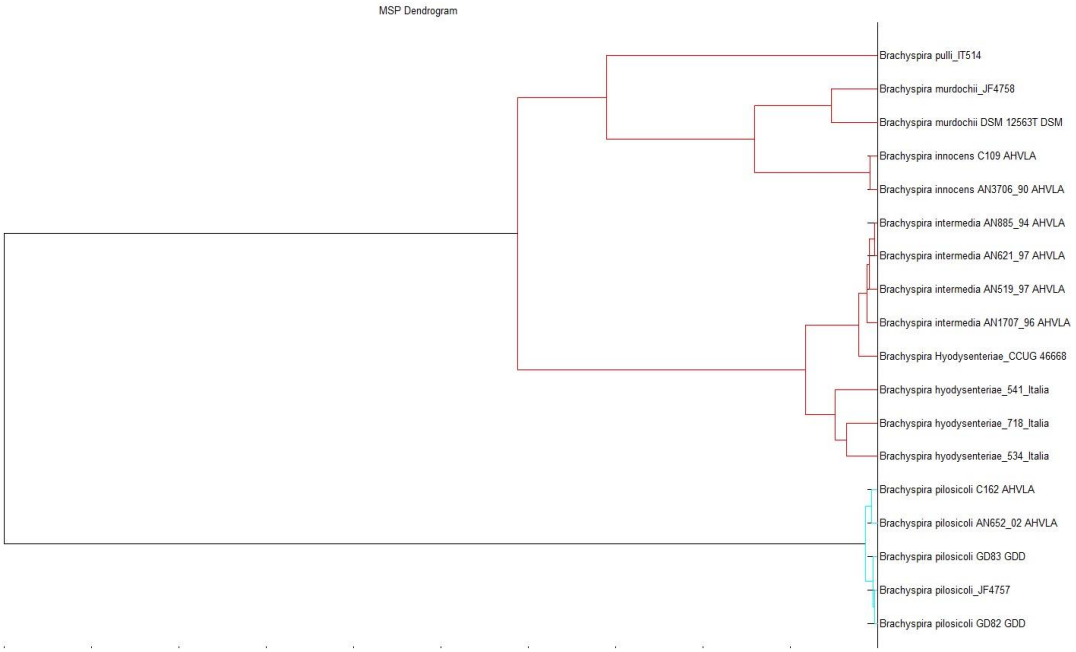

Figure 1: Main spectra profile based dendrogram of 18 strains representing six different species of *Brachyspira* (*B.pulli, B.murdochii, B.innocens, B.intermedia, B.hyodysenteriae, B.pilosicoli*).

On this, 11 MSP were already provided by Bruker Daktonics Corporation (*B.murdochii* DSM 12563T, *B.innocens* C109 AHVLA, *B.innocens* AN3706_90 AHVLA, *B.intermedia* AN885_95 AHVLA, *B.intermedia* AN621_97 AHVLA, *B.intermedia* AN519_97 AHVLA, *B.intermedia* AN1707_97 AHVLA, *B.pilosicoli* C162 AHVLA, *B.pilosicoli* AN652_02 AHVLA, , *B.pilosicoli* GD82 GDD *B.pilosicoli* GD83 GDD). Seven more MSP profiles were added to them, generating six well-distinguished groups, expect for the case of *B.hyodysenteriae* CCUG 46668 that was close to *B.intermedia* group. The other three *B.hyodysenteriae* MSP (B534, B541,B718) were close to each other, providing a newly designed MSP cluster of this species. *B.pilosicoli* group was instead homogenous and clearly separated from the other groups of MSP. In the end, *B.pulli* was very close to *B.murdochii* and *B.innocens* group, as well that this recently designed species is a nonpathogenic bacteria, such as the other two. In figure 2 are shown four representative spectra of two strains (151090922 and 151091035) of 22 submitted to the parallel analysis using the Intact Cell Method (ICM) and the Protein Extraction Method (PEM). A statistical

analysis using GraphPad Prism were performed using scores provided after MALDI-TOF analysis (fig.3)

On 84 strains analyzed with the MALDI-TOF, 34 were *B.innocens*, 30 *B.hyodysenteriae*, 9 *B.murdochii*, 8 *B.pilosicoli*, *2 B.intermedia* and 1 *B.pulli*. A UPGMA-based dendrogram were assembled through the BioNumerics 7.1 software using spectrum data for the fingerprintings (figure 4). Nine clades were identified, belonging to all the six species present in the MSP database. Two groups not-strictly closed for three different species were recognized (*B.murdochii, B.innocens, B.hyodysenteriae*) and one group for each of other species (*B.pilosicoli, B.intermedia, B.pulli*). Six spectra were not well-identified as well as they were in a different cluster than expected (e.g *B.hyodsenteriae* in a *B.murdochii*_cluster), suggesting a difficulty for the software to clarify the identification of these strains.

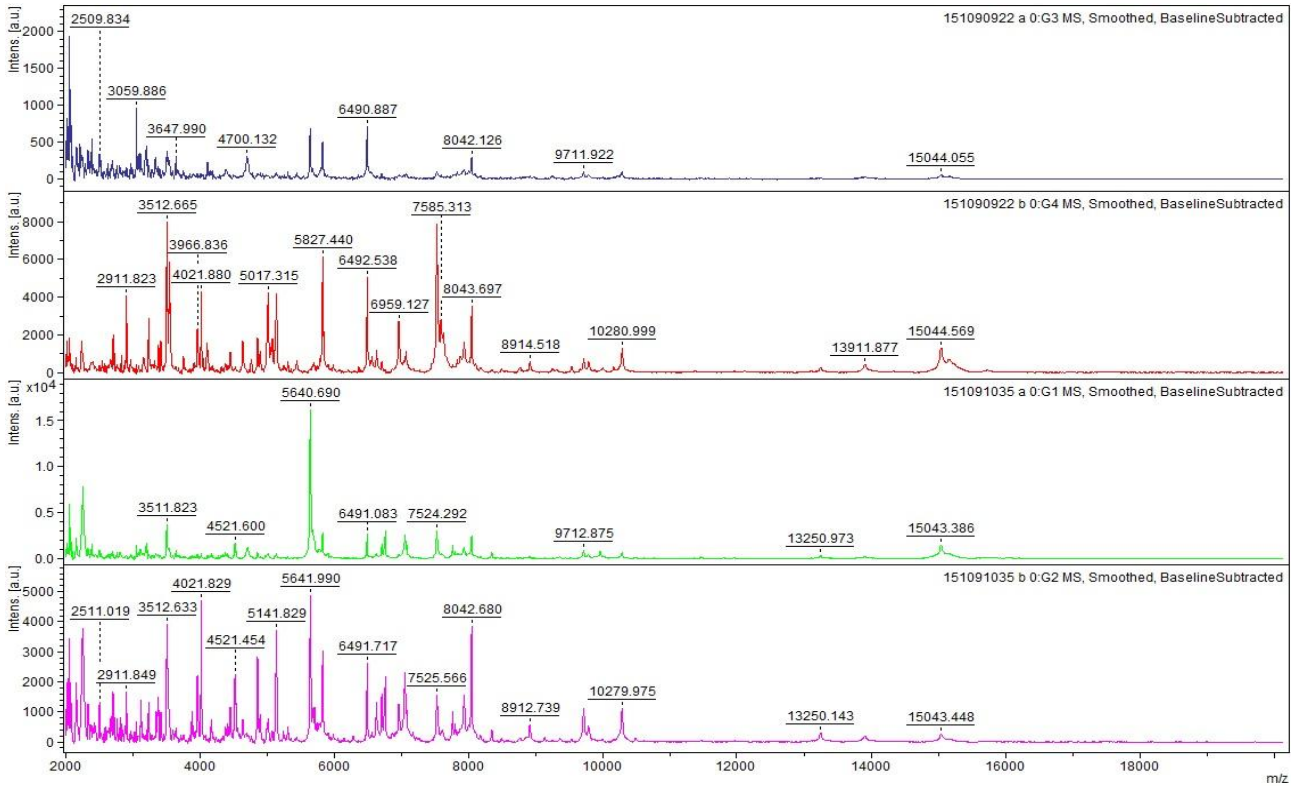

Figure 2: Representative spectrum of 2 strains of *Brachyspira* spp.; a:ICM, b:PEM

a

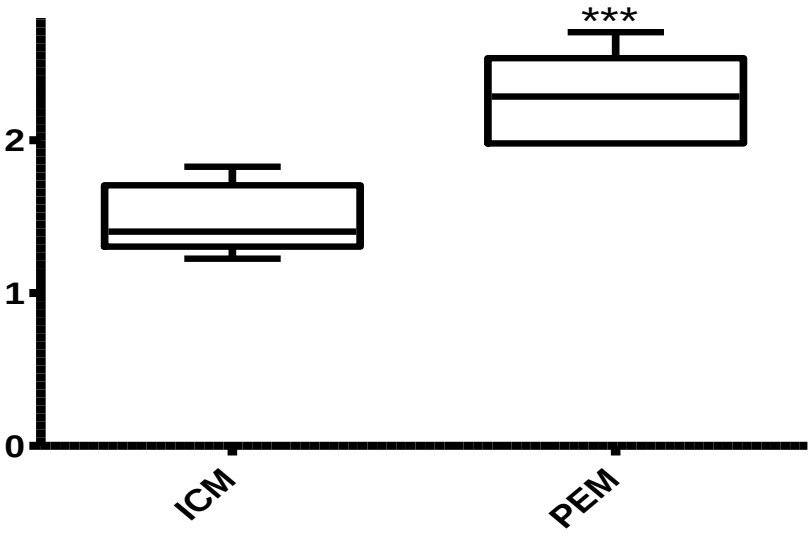

Figure 3: Intact Cell Method vs Protein Extraction Method

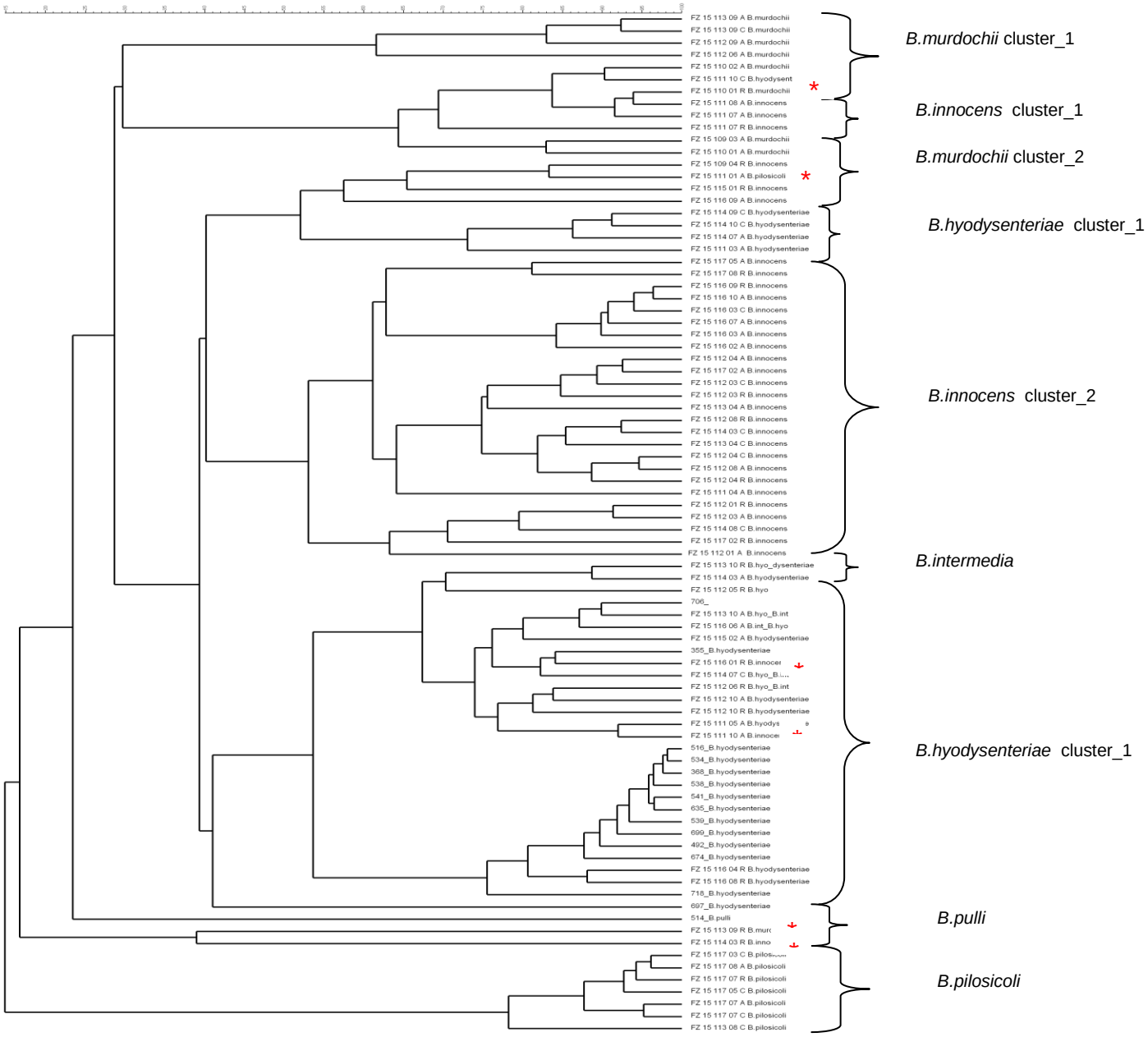

Figure 4: UPMGA-based dendrogram using BioNumerics 7.1 software: unclear identifications are marked (*)

4. Discussions and Conclusions

The aim of this study was to recognize the MALDI-TOF MS ,an innovative technique which is able to identify bacteria through the recognition of protein peak patterns, as a reliable tool for the identification of *Brachyspira* spp, in particular *B.hyodysenteriae*. Database provided by Bruker Daltonics contains main spectra profiles of some species of *Brachyspira* but reference spectra needed for the identification of *B.hyodysenteriae,* agent of Swine Dysentery, are still lacking. Main spectra profile of 3 on-field strains of *B.hyodysenteriae*, one of them was a reference strain (CCUG 46668), as well as 1 strain of *B.pulli*, 1 strain of *B.murdochii* and 1 strain of *B.pilosicoli* were added to the database. Sequencing of nox-gene allowed to correctly identified these strains. The result of cluster analysis using MSP allowed to have MSP clusters for 6 different species. *B.intermedia* and *B.hyodysenteriae* MSPs were close; in particular, *B.hyodysenteriae CCUG 46668* and *B.intermedia AN1707_96* were not-well divided in the respective clusters, providing in some cases problems for a correct identification. Interestingly, *B.pulli*, *B.murdochii* and *B.innocens* were close as well as all of them are considered to be commensal species of *Brachyspira* with any pathological and clinical significance (Mirajkar et al, 2015). *B.pilosicoli* group was clearly far from the other *Brachyspira* species: this finding could depend on the fact that this species is not strictly animal-adapted showing a different protein peak patterns than the others, as reported also by Calderaro et al.(2012). Parallel analysis of two different methods for the loading of MSP 96 polished steel target plate, the Intact Cell Method (ICM) and Protein Extraction Method (PEM) were also performed on 22 samples. The quality of spectrum provided by PEM, considering number of peaks and distance between them, was greater than the spectrum provided by ICM. Furthermore, the scores acquired after MALDI-TOF analysis, were higher for the PEM ($p<0,005$). In other studies, PEM derived spectra were greater than spectra provided by ICM method, even using culture derived from agar plate as well from broth, giving better base peak resolutions and higher number of peaks (Goldstein et al., 2012). The presence of proteins derived from agar plates can potentially reduce the number of peaks, interfering with the analysis of spectrum (Croxatto et al, 2011). This can depend on the typical growth of *Brachyspira* species of agar plates, strictly attached to the agar surface, making difficult the harvesting of colonies using a sterile tip before to load spots on steel plate. Based on the cluster analysis using the BioNumerics 7.1 software (AppliedMath), considering clusters composed by a single species, 9 clusters were recognized for a total of 84 strains. On this, two big clusters, belonging to *B.innocens* and *B.hyodysenteriae* species were

present. As for the MSP base dendrogram, the *B.pilosicoli* group was far from the others. Six identification were unclear, probably due to a failure of software to compare the unknown spectra with the reference spectra. Based on cluster analysis, 93% of the total number of strains were well identified. Our results are similar to other studies involving MALDI-TOF and strains of *Brachyspira* (Calderaro et al., 2012, Werneke et al., 2013, Prohaska et al., 2014) suggesting that the MALDI-TOF MS is a reliable tool for the identification of *Brachyspira* species. However, the "*Brachyspira* database" has to be improved, with additional isolates of newly designed species, as *B.suanatina* or *B.hampsonii*.

5. Acknowledgements

I would like to thank Dr. Pamela Nicholson for her technical support in the cultivation *Brachyspira* isolates and sequencing. I especially thank Professor Joachim Frey and Professor Vincent Perreten for their scientific assistance and for the opportunity given to me to work in the Institut für Veterinärbakteriologie, Bern (CH). Furthermore, I want to thank Dr. Chiara Magistrali , Dr. Lucilla Cucco and Dr. Francesca Romana Massacci from the Istituto Zooprofilattico dell'Umbria e delle Marche (Italy) for the provision of on field strains of *Brachyspira*, and Dr. Friederike Zeeh and Dr.Niels Grützner from the Swine animal clinic, Vetsuisse-Faculty, Bern (CH) for the provision of fecal samples and their kindly assistance during my period in Bern.

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