Universitat Autònoma de Barcelona

Proteome characterization of Brachyspira strains. Identification of bacterial antigens

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Doctoral Thesis

Mª Vanessa Casas López

Bellaterra, July 2017

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Doctoral thesis presented by **Mª Vanessa Casas López**

To obtain the **Ph.D. in Immunology**

This work has been carried out in the Proteomics Laboratory CSIC/UAB under the supervision of Dr. Joaquin Abián and Dra. Montserrat Carrascal.

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“At My Most Beautiful”

R.E.M. from the album “Up” (1998)

“And after all, you’re my wonderwall”

Oasis from the album “(What´s the Story?) Morning Glory” (1995)

Agradecimientos

A mis directores de tesis, por su tiempo, sus ideas y consejos. Por participar en esta aventura bacteriana que ha sido complicada en muchos aspectos.

A toda la gente con la que he compartido laboratorio estos años. De todos y cada uno he aprendido algo.

A todas las personas que han contribuido a esta tesis, con su trabajo o con sus ánimos. Por los paseos, llamadas, abrazos, notas en post-its, y eterna paciencia.

A miña familia, por lembrarme sempre o que realmente é importante.

INFINITAS GRACIAS!!!

"…The older I get, the more I appreciate the difference between the results of scientific investigations and the methods by which the results are obtained. The results constitute the body of scientific knowledge- they are science. But, as has been noted by others, the facts of science exist in nature and are waiting to be discovered; if not found by one investigator, they will be found by another. The methods, however, are the creations of individual scientists; they are more art than science. For this reason, and depending on one's mood, they may be even more admirable than the science they make possible…"

NORMAN H. HOROWITZ

One-gene-one-enzyme: Remembering biochemical genetics.
Protein Sci. 4, 1017–1019 (1995).

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Abbreviations

2D	Two-dimensional
ACP	Acetyl-Phosphate
ELISA	Enzyme-linked immunosorbent assay
FASP	Filter-aided sample preparation
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GTA	Gene transfer agent
HIS	Human intestinal spirochetosis
HPLC	High-performance liquid chromatography
HSP	Heat shock protein
HSPC	Heat shock protein complex
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC-MS/MS	Liquid chromatography coupled to tandem mass spectrometry
LOS	Lipooligosaccharides
LPS	Lipopolysaccharide
MALDI-TOF	Matrix-assisted laser desorption/ionization-time of flight
MGF	Mascot generic format
OMP	Outer membrane protein
OMV	Outer membrane vesicle
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PEPCK	Phosphoenolpyruvate carboxykinase
PIS	Pig intestinal spirochetosis
PSM	Peptide spectrum match
PTM	Post-translational modification
SAS	Single aminoacid substitution
SERPA	Serological proteome analysis
Vsp	Variable surface protein

1. Introduction

1.1 *Brachyspira*

1.1.1 Spirochaetes

The species of the genus *Brachyspira* (formerly Treponema or Serpulina) are anaerobic bacteria classified within the phylum Spirochaetes (Stanton, 2006). Spirochaetes (aka spirochetes) present a very characteristic structure (Figure 1.1), composed of an outer membrane, an internal protoplasmic cell cylinder and, attached at each end of the protoplasmic cylinder, the periplasmic flagella (located in the periplasmic space). This conformation determines the final shape of spirochetes, either a helix or flat wave depending on the species, and how they swim and respond to chemical stimuli (Charon et al., 2012).

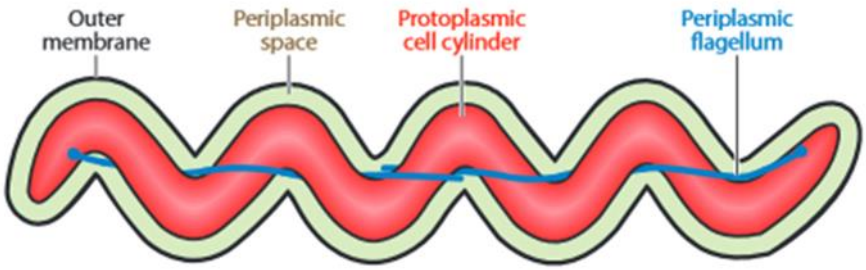

Figure 1.1. Spirochete structure. Image from Charon et al., 2012.

Spirochetes have some advantageous characteristics compared with other species, such as the ability to swim in highly viscous media, which determines their capacity to survive and invade ecological niches from where other species are expelled. In addition, the location of the flagella in the periplasmic space, shields them from the interaction with specific antibodies and protects them from hostile environmental conditions (pH or salt concentration) (Charon and Goldstein, 2002).

These characteristics of spirochetes are critical for the adaptation of *Brachyspiras* to media. Probably, the most notorious *Brachyspira* species, in terms of its

relevance in disease are *Brachyspira hyodysenteriae* and *Brachyspira pilosicoli*. These species are intestinal pathogens for which the ability to move through mucous gels and to be attracted by mucus components are very important (Stanton, 2006).

1.1.1.1 *Brachyspira hyodysenteriae*

Brachyspira hyodysenteriae is the causative agent of swine dysentery. This species affects swine and birds with swine as the main target. The colonization starts along the intestinal epithelium of the caecum and colon and then proceeds into epithelial and goblet cells. *Brachyspira* cells do not attach to the intestinal cells, ,and they show a high attraction to mucin during chemotaxis assays (Stanton, 2006).

Brachyspira hyodysenteriae is 6-8.5 μm long and 0.32-0.38 μm wide (Figure 1.2). The number of periplasmic flagella varies between 7 and 14 (Hampson, 2012).

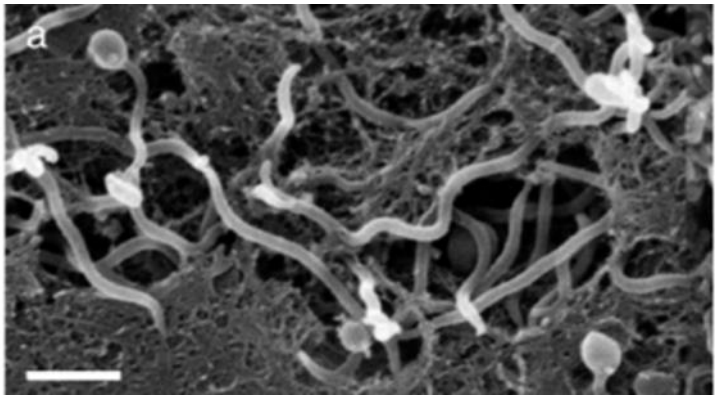

Figure 1.2. *B. hyodysenteriae* grown on sheep blood agar for 3 days.
Image from (Wood et al., 2006)

The genome of this species has been sequenced (Bellgard et al., 2009); it contains 3,000,694 bp and a plasmid of 35,940 bp. Compared to other *Spirochete* species, such as *Borrelia* or *Leptospira* that contain numerous plasmids or replicons, the *B. hyodysenteriae* genome organization is simpler, but the genome size is larger than those of *Borrelia* or *Treponema* spp. *Brachyspira*

hyodysenteriae genome size could be related to the versatility needed by *Spirochetes* to survive in a complex and changing environment like the large intestine (Bellgard et al., 2009).

The initial report on the genome of the reference strain WA1 identified 2122 protein coding genes (Bellgard et al., 2009). This number increased up to 2613 in subsequent analyses (Mappley et al., 2012; Wanchanthuek et al., 2010). In a recent study, where the genomes of nineteen *B. hyodysenteriae* strains were sequenced (Black et al., 2015), an average of 2654 protein coding genes were identified.

Comparative genomic analysis of 19 *B. hyodysenteriae* strains showed that the average percentage identity of aligned sequences was greater than 94.96% and protein homology ranged from 75.6 to 88.5% (Black et al., 2015). Thus, this study concluded that the genomes of these isolates were clonal, i.e. bacterial isolates were indistinguishable in genotype

The genome of *B. hyodysenteriae* strain WA1 contains a 15 kb region that encodes a prophage-like gene transfer agent (GTA). This GTA corresponds to the VSH-1 particle (virus of *Serpulina hyodysenteriae*) first described in 2005 for the *B. hyodysenteriae* strain B204. The VSH-1 assembles and transfers random fragments of 7.5 kb of the host DNA between *B. hyodysenteriae* cells (Bellgard et al., 2009; Matson et al., 2005; Motro et al., 2009).

GTAs expression was induced by treatment with mitomycin C and antimicrobials and it has been shown that they transfer chloramphenicol resistance to *B. hyodysenteriae* (Matson et al., 2005; Stanton, 2006). GTA particles may take part in the “microevolution” observed in farms, when the analysis of isolates shows new variants of this species. These new variants display alterations in phenotype, such as antimicrobial susceptibility or virulence (Hampson, 2012).

Among the virulence factors of *B. hyodysenteriae* are lipooligosaccharides (LOS), haemolysin/haemolytic activity, chemotaxis/motility, oxygen metabolism/NADH oxidase activity, and membrane proteins such as VspH and BlpA (Stanton, 2006). The relationship between virulence and NADH oxidase levels, motility and

haemolytic activity was established from the study of mutated strains. Thus, it was shown that strains lacking *nox* (NADH oxidase)*, flaA* and *flaB* (flagella)*, tlyA* (hemolysin) genes were avirulent (Hyatt et al., 1994; Kennedy et al., 1997; Stanton, 2006; Stanton et al., 1999).

1.1.1.2 *Brachyspira pilosicoli*

Brachyspira pilosicoli is the causative agent of intestinal spirochetosis (IS). This species has been isolated from a wider number of host than *B. hyodysenteriae* including pigs, humans, birds and dogs (Neo et al., 2013b). In pig it produces and intestinal spirochetosis (PIS) of milder characteristics. As reported for *B. hyodysenteriae*, diet has an influence on the colonization and disease development of B. *pilosicoli* (Hampson, 2012). The corresponding human disease (HIS) affects mainly immunodepressed individuals.

B. pilosicoli is characterized by its one cell end attachment to the intestinal mucosal surface: one extreme of the bacteria attaches to the apical cell membrane and the other is extended away from the intestinal surface (Figure 1.3). Microscopically, this is observed as a parallel array of cells creates the appearance of a “false brush border” or “fringe” on the mucosal epithelial cells (Figure 1.4). This disposition of spirochetes is considered by histologists as a proof for intestinal spirochetosis (Stanton, 2006).

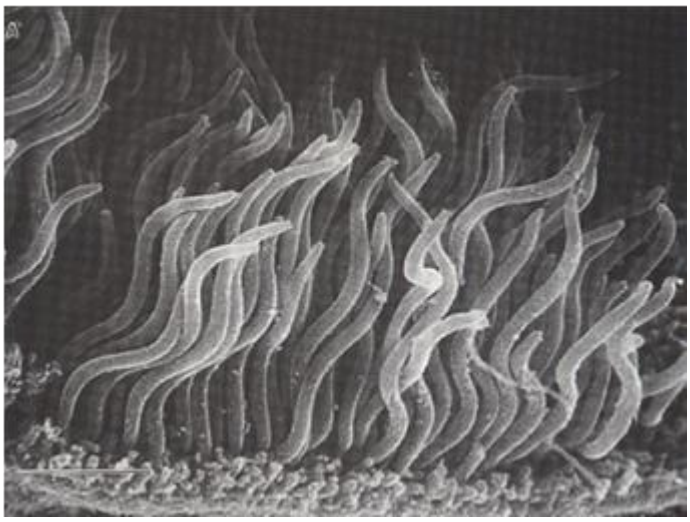

Figure 1.3. *B. pilosicoli* one cell end attachment to the host tissue. Image from Stanton, 2006.

B. pilosicoli is 6-10 μm long and 0.25-0.30 μm wide. It contains four to seven periplasmic flagella, fewer than *B. hyodysenteriae* (between 7 and 14) (Hampson, 2012).

The *B. pilosicoli* genome was sequenced by Wanchanthuek et al. in 2010. This species contains a single circular chromosome of 2,586,443 bp, and unlike *B. hyodysenteriae*, it does not contain plasmids but does contain a bacteriophage region in its chromosome. For the strain 95/1000, a total of 1987 protein-coding genes were initially described (Wanchanthuek et al., 2010). This figure increased in a posterior revision to 2301 protein coding genes (Mappley et al., 2012). The genomes of two more *B. pilosicol*i strains, B2904 and P43/6/78, have been sequenced, with 2658 and 2208 protein coding genes, respectively (Lin et al., 2013; Mappley et al., 2012).

Compared to the *B. hyodysenteriae* genome, *B. pilosicoli* has specific genes, involved in different processes such as energy production, metabolism of carbohydrates and amino acids, and capsule biosynthesis. On the other hand, *B. pilosicoli* lacks of elements present in *B. hyodysenteriae* genome, such as genes related to beta-glucosidase activity, histidine metabolism, interconversion of PEP and pyruvate, and *rfbBADC* genes. This last set of genes is located on the *B. hyodysenteriae* plasmid and predicted to be involved in the O-antigen

biosynthesis, i.e. a polysaccharide that consists of a number of repeats of the oligosaccharide O-unit (Wanchanthuek et al., 2010).

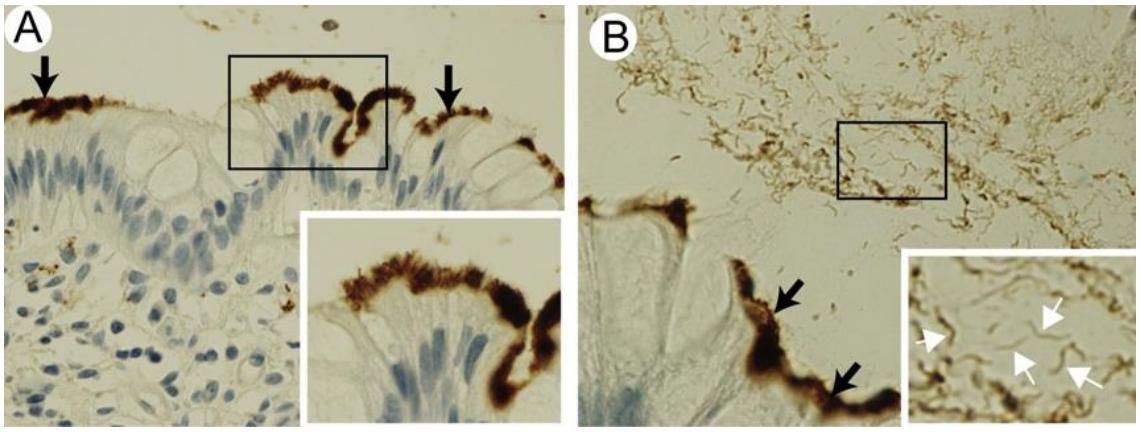

Figure 1.4. *B. pilosicoli* false intestinal brush border (black arrows) and spiral organisms within mucus (white arrows). Image from (Ogata et al., 2016)

In 2012, Mappley et al. selected three *B. pilosicoli* strains (B2904, 95/1000 and WesB) isolated from different organisms (chicken, pig and human, respectively). These strains showed different genome sizes and 54.9-68.4% of proteins were conserved among strains (Mappley et al., 2012). Thus, in contrast to *B. hyodysenteriae* genomes, the genomes of *B. pilosicoli* strains were considered recombinant, i.e. isolates of *B. pilosicoli* shows an important genetic variation among them (Neo et al., 2013b). This adaptability could be related to the ability to colonize a wider range of hosts than other *Brachyspira* species (Neo et al., 2013a).

Unlike *B. hyodysenteriae*, *B. pilosicoli* does not present plasmids. Still, a region encoding a VSH-1 prophage-like GTA has been identified in the genome of *B. pilosicoli* strains B2904, 95/1000 and WesB (Mappley et al., 2012). The functionality of these GTA particles and their ability to transfer genetic material between strains is not known. (Wanchanthuek et al., 2010).

Due to its genomic content, *B. pilosicoli* is predicted to be more resistant to oxidative stress than *B. hyodysenteriae* and has fewer genes related to chemotaxis; thus, these species should have different chemotactic behaviour. This consideration could explain the different host range and colonization between the species (Hampson, 2012).

1.1.2 Diseases

1.1.2.1 Swine dysentery

Swine dysentery is a severe mucohaemorrhagic colitis that affects the large intestine. This disease affects piglets; up to 90-100% of a herd can be infected. Without adequate treatment, mortality can reach 30% (Stanton, 2006).

Regarding immune response against the disease, animals that recover from swine dysentery are protected against reinfection for 17 weeks. In suckling pigs, the disease is uncommon, likely due to some acquired protection from the mother (Hampson, 2012).

This pathology is distributed worldwide, although its evolution has been dissimilar, mainly due to differing disease management established in each country. For instance, in the USA, the incidence of disease diminished in the nineties because of constant medication with carbadox and through control and elimination methods (Burrough, 2016; Hampson, 2012).

The mechanisms responsible for tissue destruction during swine dysentery are not totally understood; haemolysins and LOS are thought to be involved in this process (Hampson, 2012). Serine protease activity has been observed in outer membrane preparations from different strains of *B. hyodysenteriae* and *B. pilosicoli* (Muniappa and Duhamel, 1997)(Muniappa and Duhamel, 1997). This activity was confirmed due to the cleavage patterns identified by using these preparations. It has been hypothesized that the serine protease could be associated with binding and damage to the intestinal surface. Moreover, other bacterial species could act in synergy with *B. hyodysenteriae* to develop the disease. It has been observed that when gnotobiotic pigs (pigs reared in absence of microorganisms) were inoculated with *B. hyodysenteriae*, the clinical disease was not manifested. In contrast, when inoculated with colonic scrapings obtained from infected pigs or with *B. hyodysenteriae* together with other anaerobe bacteria typical of the intestinal pig microbiota, the animals developed the disease. This fact points to a necessary “environment” for the disease to prosper. Among the microorganisms that have been demonstrated to create the right

conditions for disease development are anaerobes such as *Bacteroides vulgatus* and *Fusobacterium necrophorum* (Burrough, 2016).

Dietary factors are associated with the concept of “environment” for disease development. Colonization can be inhibited by diets with a high content in inulin or highly digestible fibre (Hampson, 2012). It is thought that diet is associated to changes in the colonic microbiota (Burrough, 2016).

1.1.2.2 Human Intestinal spirochetosis

HIS is a mild to moderate diarrhoeal disease that appears to be more widespread in developing countries and rural areas; it was detected in 32.6% of stool cultures in North West Australia compared to 1.5% in United Kingdom (Walker et al., 2015). In a study in Sweden, HIS was detected with a prevalence of 2%; and it was thought that could be associated with irritable bowel syndrome (Walker et al., 2015). Similar results have been published in Japan, where in a study of 4254 patients, 1.7% were diagnosed in intestinal spirochetosis. This percentage increased to 5.5% when the population studied was patients with Human Immunodeficiency Virus infection (Tateishi et al., 2015). A closely-related species, *Brachyspira aalborgii*, can also be isolated from HIS cases and is considered also a causative agent of the disease.

HIS is commonly diagnosed with a histopathologic analysis although this analysis cannot discern the species involved. Characterization of the specific bacteria involved in the disease used to be performed by PCR. Using this technique, Trivett-Moore et al. found that *B. pilosicoli* was the predominant species in cases of HIS from Australia (Trivett-Moore et al., 1998). In contrast other studies performed in Northern Japan and the Netherlands , the predominant species was *B. aalborgii* (Sato et al., 2010; Westerman et al., 2013). The different origin of samples included in these studies could point to an influence in the geographic location for the predominant species causing the disease.

Despite the importance of these two species in HIS, *B. pilosicoli* is the species that have been received most attention in research and, in fact, the genome of *B.*

aalborgii has not been sequenced yet. This possibly results from the role that *B. pilosicoli* plays in farm animals disease (PIS).

1.1.3 Treatment and control

1.1.3.1 Antibiotics

Due to resistance to tylosin and lincomycin, pleuromutilins (tiamulin and valnemulin) are currently the main treatments against swine dysentery. In recent years, resistance to pleuromutilins has been detected, and thus, the proper use of antimicrobials is recommended in the treatment of swine dysentery (Burrough, 2016).

For the treatment of intestinal spirochetosis, the chemicals used are based on those developed for swine dysentery (Hampson, 2012).

1.1.3.2 Vaccines

It has been observed that animals that develop the disease show some degree of protection against reinfection. Thus, the development of a vaccine against swine dysentery began at almost the same time that the causative agent of the disease was identified.

Early experiments were directed to characterize the pig immune response against *B hyodysenteriae* extracts and the protection acquired after an infection. In one of these studies pigs were inoculated with *B. hyodysenteriae* isolates bearing different serotypes. After recovery the animals were reinfected with homologous or heterologous serotypes. It was observed that response was related to the serotype of the *B. hyodysenteriae* strain and that colonic gross lesions were not observed when the animals were reexposed to an homologous serotype (Joens et al., 1983). They suggested that an efficent vaccine against swine dysentery would require a pool of serotypes.

Several known proteins and virulence factors were also tested in order to generate vaccines including flagellar proteins and outer membrane proteins (OMPs). The immunogenicity of endoflagellar proteins and its protective effect in

mice was studied by Boyden (Boyden et al., 1989). After oral challenge with strains of two different serotypes they found that endoflagellar proteins conferred protection to mice. However, subsequent experiments with the endoflagellar protein FlaB1 showed that this protein was unable to reduce the incidence or severity of the disease in swine (Gabe et al., 1995). Similarly, outer membrane proteins such as BmpB (La et al., 2004) and SmpB (Holden et al., 2008) were also tested as vaccine targets against swine dysentery but none reached full protection against the disease.

Reverse vaccinology is also being used to develop vaccines against swine dysentery. In this approach, genome ORFs are selected in silico on the basis of their homology with known virulence factors and their expression products tested afterwards in animals for its capacity to produce an efficient vaccine against the disease. In the first study of this kind with *Brachyspira*, eight pigs were vaccinated using the product of six ORFs which were selected from a group of 19 proposed ORFs from *B. hyodysenteriae*. After treatment, only one of the animals developed clinical signs of swine dysentery after infection. However, the post-mortem culture of faeces, caecum and colon was positive for *B. hyodysenteriae* in 7 of 8 vaccinated pigs, although the lesions were less severe than the observed in non-vaccinated pigs (Song et al., 2009). In a similar approach, 33 ORFs were selected as potential vaccine targets against *B. hyodysenteriae*. This list of candidates is part of a patent and no information is available about the results and the conferred protection (Bellgard et al., 2015).

Compared with swine dysentery, there have been fewer attempts to develop a vaccine for intestinal spirochetosis. A bacterin was tested, but it did not produce any reduction on intestinal colonization relative to control animals (Hampson, 2000). Other attempts include the outer membrane protein Bpmp72, which was patented as a candidate for vaccine and therapeutic treatment (Hampson and La, 2009), and oligopeptide binding proteins that showed a reduction in the colonization of mice (Movahedi and Hampson, 2010).

Despite these efforts, no vaccination is currently available against swine dysentery or intestinal spirochetosis.

1.1.4 Antimicrobial resistance

Strains resistant to antibiotics have emerged since the beginning of the antibiotic era, but an alarming number of resistant strains have appeared in the last twenty years (Fair and Tor, 2014). The improper use of antibiotics has become a significant problem worldwide, involving economic expense and dramatic health consequences. In the USA, approximately 2 million people are infected each year with antibiotic-resistant bacteria (and 23 000 die). In the EU, 400 000 patients present resistant bacteria yearly (and 25 000 of those patients die) (Cohen et al., 2015). In terms of economic losses, $20 billion have been invested yearly in excess healthcare costs because of resistant infections in the USA and €1.6 billion in the EU (Fair and Tor, 2014).

In addition to the human mismanagement of antibiotics, antimicrobials have been used in food animals in North America and Europe for almost 50 years. These products are given to animals as therapy but also in subtherapeutic doses to promote growth. It was estimated that during the early 1990s, 11.2 kilotons of antimicrobials were given to animals for nontherapeutic purposes, and 900 tons were administered as therapy. This use of antimicrobials promotes the emergence of resistant strains and their persistence in the environment (Gorbach, 2001).

In fact, antimicrobial resistance is one of the main health problems of the 21st century, according to Centers for Disease Control and Prevention (Centers for Disease Control and Prevention; Cohen et al., 2015).

All the antimicrobials used for treatment of *B. hyodysenteriae* and *B. pilosicoli* (tylosin, lincomycin and pleuromutilins) target the ribosome. The reduced susceptibility to these antimicrobials comes from mutations in the target region (the 23S rRNA gene and/or the ribosomal protein L3) (Hidalgo et al., 2011; Klitgaard et al., 2015; Pringle et al., 2004; Rugna et al., 2015).

Strains of *B. hyodysenteriae* and *B. pilosicoli* that are resistant to antimicrobials have been reported in different countries. For instance, in 2001 in Sweden, 65% of *B. hyodysenteriae* isolates were resistant to tylosin, and thus tiamulin was

recommended as an alternative (Karlsson, M, Gunnarsson A, 2001). In the same country, treatment of *B. pilosicoli* with tiamulin failed, as 14% of the isolates were resistant to this antibiotic (Pringle et al., 2006). In 2012, in a study of the evolution of antimicrobial resistance between 1990 and 2010, for *B. hyodysenteriae* tiamulin resistance increased from 1990-2003, but after this period the levels of resistance stabilized. For *B. pilosicoli,* the levels were constant (10-15%) during this period. These values may be due to control management in that country (Pringle et al., 2012). *B. hyodysenteriae* strains resistant to pleuromutilins have been detected over the last ten years in Japan (Ohya and Sueyoshi, 2010), Spain (Hidalgo et al., 2011), Italy (Rugna et al., 2015) and the USA (Mirajkar et al., 2016).

In consequence, legislation regarding the use of antimicrobials in animals has changed in recent years. All antimicrobials have been banned for growth purposes in the EU since 2006. Since 1 January 2017 in the USA, the use of medically important antimicrobials for growth purposes is prohibited, and therapeutic use will require veterinary supervision according the U.S. Food and Drug Administration guidance (FDA) (Mirajkar et al., 2016). However, some studies have shown that resistance to antibiotics would have appeared even if antimicrobials were correctly used (Fair and Tor, 2014). Thus, discovery of new antibiotics, although necessary, could result in temporary solutions. Development of new, effective therapies assuring long term protection would require a much deeper knowledge on pathogen biology and host immune response (Cohen et al., 2015).

1.2 Bacterial proteomics

1.2.1 Proteomes and subproteomes

The small genome size of bacteria and advances in mass spectrometry techniques have recently enabled total proteome characterization of several prokaryotic species. Microbial species such as *Streptococcus pneumoniae* (Sun et al., 2011), *Neisseria meningitidis* (Bernardini et al., 2004), *E. coli* (Iwasaki et al., 2010; Lopez-Campistrous, 2005), *Bifidobacterium longum* (Yuan, 2006), *Herbaspirillum seropedicae* (Chaves et al., 2007), *Borrelia burgdorferi* (Barbour et al., 2008), *Lepstospira interrogans* (Cao et al., 2010; Malmström et al., 2009), *Treponema pallidum* (McGill et al., 2010), *Bartonella quintana* (Fabietti et al., 2013) and *Bordetella pertussis* (Alvarez Hayes et al., 2015) have been the subject of deep proteome analyses.

Different technical approaches have been used to characterize these proteomes. In general, works published before 2010 rely mainly on polyacrylamide gel electrophoresis (mono-dimensional or two-dimensional) to separate proteins. This technique was combined with MALDI-TOF (matrix-assisted laser desorption/ionization-time of flight) mass spectrometry for identification purposes (Rabilloud and Lelong, 2011). The two-dimensional gel approach (2-DE) has proved to be very useful for the proteomic study of bacterial proteomes, due to its suitability to compare different physiological states or readily visualize post-translational modifications (PTMs) in proteins. However, this approach can only depict a fraction of the proteome (Hecker et al., 2008).

More recent studies apply shotgun proteomics, i.e. the identification of proteins present in a mixture through the analysis of peptides obtained from proteolytic digestion (Zhang et al., 2013), which takes advantage of the development of technologies coupling liquid chromatography to tandem mass spectrometry (LC-MS/MS). Due to its greater proteome coverage and efficiency, shotgun strategies have superseded the use of gel-based approaches in the analysis of very complex samples. Still, full proteome analysis by shotgun proteomics is frequently carried out after sample fractionation in order to reduce sample

complexity and optimize sensitivity and coverage. Examples of fractionation methods that have been used for deeper characterization of bacterial proteomes are Offgel electrophoresis (protein/peptide fractionation in solution based on isoelectric point) (Michel et al., 2003) and multidimensional chromatography (coupling of two or more independent chromatographic systems) (Zhang et al., 2010).

More than 60% of proteome coverage using a shotgun approach has been reported for species, such as *E. coli* (63% of proteome coverage (Iwasaki et al., 2010)), *Leptospira interrogans* (61% (Cao et al., 2010)) or *Streptococcus pneumoniae* (63% (Sun et al., 2011)).

Proteomics has not only been used to study the total proteome of bacteria but also to characterize bacterial compartments and subproteomes that contain proteins directly involved in the interaction with the host. Surface, extracellular and immunoreactive proteins have become increasingly important for these interactions which are related to pathogenicity and the immune response.

1.2.1.1 Surface proteome

Subcellular fractionation has been widely used to selectively isolate cell walls and membranes. This is achieved by means of sequential extractions and centrifugations with various chemical reagents (Bittaye and Cash, 2015; Wright et al., 2005).

Another way to characterize proteins located on the bacterial surface is by biotinylation with chemical reagents. Biotinylated proteins are purified by affinity enrichment and analysed by mass spectrometry (Biller et al., 2014; Cao et al., 2010).

Cell shaving is a novel method that has proved to be successful in the study of the surface of Gram-positive bacteria and to propose vaccine candidates (Doro et al., 2009; Solis et al., 2010). This method consists of the selective digestion of surface proteins by the action of proteases under conditions that preserve the integrity of the cell (Figure 1.5). In Gram-negative bacteria this approach is more

difficult due to the lability of the membrane compared to the cell wall (Bittaye and Cash, 2015).

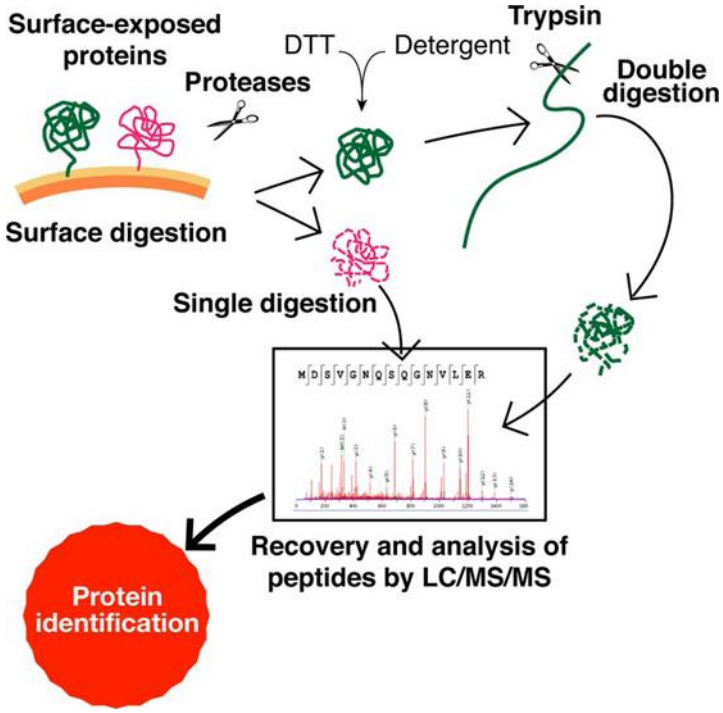

Figure 1.5. *Cell shaving* method workflow. Image from (Doro et al., 2009)

Cell treatments when using these methods can produce some cell disruption, so that contamination with proteins from the cytosolic compartment can be a major problem. This implies further result data analysis and filtering to remove interfering false positives.

1.2.1.2 Extracellular proteome

Bacteria release selectively proteins into the extracellular space, and these proteins may act as virulence factors in pathogenic species. Released peptides and proteins are interesting from the immunogenic point of view because rapid T-cell response is better triggered by secreted antigens (Kaufmann and Hess, 1999).

Numerous works on the characterization of secreted bacterial proteins have been published. For instance, it was reported that the secretome (the secreted proteome) of *Streptococcus pneumoniae*, contained a number of known virulence factors , and some proved to be highly immunogenic in Western blots (Choi et al.,

2012). In the case of *Leptospira interrogans,* known virulence factors were also detected together with lipoproteins, proteins involved in cell-host interaction and in cellular processes related with cell defence/survival (Zeng et al., 2013).

In some cases, pathogenicity is directly derived from secreted proteins; for instance, enterotoxigenic *E. coli* is pathogenic because of toxins secreted to the host cell. A list of vaccine candidates selected from the proteins identified in the secreted proteome of this species has been published (Boysen et al., 2015).

Gram-negative bacteria are also capable of releasing vesicles to the media in normal conditions, i.e. not related with cell lysis or apoptosis. These vesicles are originated from the outer membrane and contain various components such as lipopolysaccharides (LPS), glycerophospholipids, and proteins from the outer membrane, the periplasm and even from the cytoplasm (Figure 1.6) (Bai et al., 2014; Kuehn and Kesty, 2005; Lusta, 2015). The biogenesis of these outer membrane vesicles (OMVs) is not fully understood. Initially it was thought that OMVs production was a stochastic process but recent investigations have shown that it is a deliberate process due to the selectivity of OMVs protein cargo (Haurat et al., 2010; Schwechheimer and Kuehn, 2015). Pathogens can manipulate the composition of these OMVs to cope with stress situations and its production occurs during infection (Bai et al., 2014; Ellis and Kuehn, 2010). These outer membrane vesicles (OMVs) are considered potential virulence factors and are important for bacterial pathogenicity from an offensive and defensive point of view, (Bai et al., 2014). In this context, OMVs have been proposed as vaccine candidates against the pathogenic bacteria *Haemophilus influenza* (Roier et al., 2015).

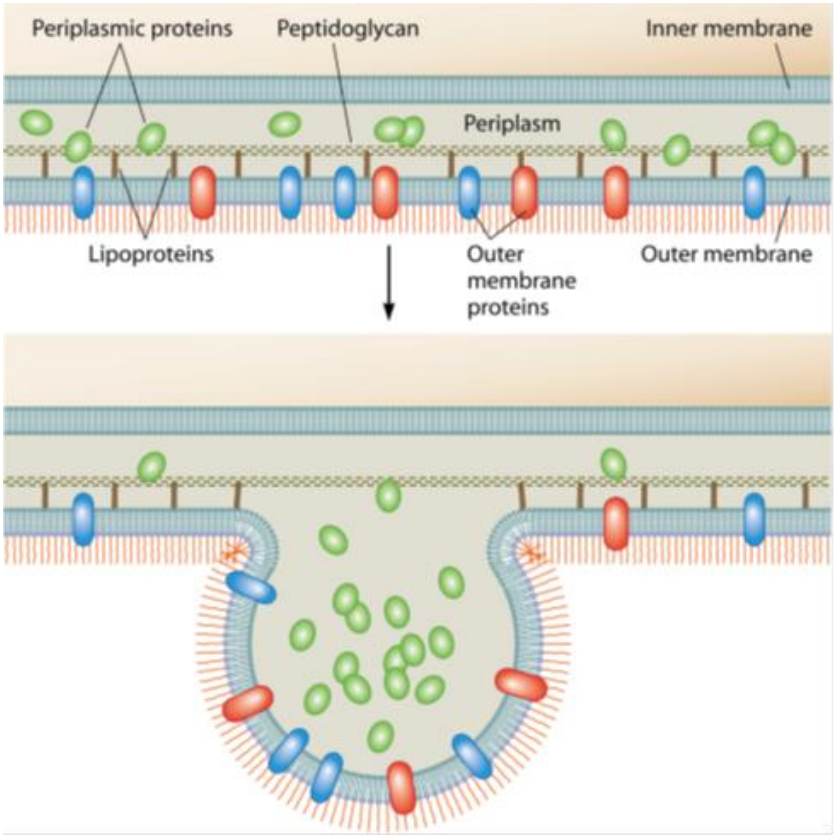

Figure 1.6. Model of OMV production. Image from (Ellis and Kuehn, 2010)

Moonlighting proteins are a subset of multifunctional proteins, in which the multiple functions are not due to protein homology, promiscuous enzyme activity, gene fusion or pleiotropic effects, i.e. one gene influencing two or more unrelated phenotypic traits.

Intracellular proteins that perform another function on the cell surface are intracellular/surface moonlighting proteins (Wang and Jeffery, 2016). This is the case of enolase, a typical cytoplasmic enzyme which can be found at the cell surface playing a role in cell-host interaction by its binding to host plasminogen. Due to this characteristic, enolase has been proposed as a candidate vaccine against *Mycobacterium tuberculosis* (Rahi et al., 2017; Wang and Jeffery, 2016).

Over 300 intracellular/surface moonlighting proteins have been identified in bacteria (Wang and Jeffery, 2016). Many are important for bacterial virulence; being enzymes and chaperones the major groups (Henderson and Martin, 2011).

Most moonlighting proteins do not follow classical secretion pathways, and therefore the study of signal peptides cannot always predict their external location. Thus, proteomic approaches are very important for the characterization of these proteins as they are capable of determining the actual location and expression of the proteins, and provide hints on the diverse protein functions.

1.2.1.3 Immunoproteome

The subset of the proteome that contains all the proteins involved in a particular immune response is known as the immunoproteome. In this work we use the term immunoproteome to refer to the pathogen counterpart of the interaction (i.e. the bacterial proteins playing a role in the host-immune response and, more specifically, the bacterial immunogenic proteins). A more adequate term would have been the *antigenome* (antigen-ome). Unfortunately this is a term already used with the meaning of anti-genome. Another term, *antigome*, with a sibling construction to that of *prote(in)-ome*, would be more appropriate but we have not used it before in our works and its potential acceptance is not yet clear to us.

With the aim of proposing suitable vaccine candidates against a pathogen, it is very important to determine this pathogen immunoproteome, i.e. the proteins that are recognized by the immune system and elicit the production of antibodies. One of the most popular approaches for this uses a combination of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) with Western blotting, commonly known as serological proteome analysis (SERPA).

SERPA has been used in the characterization of the immunoproteome of several bacterial pathogens including *Borrelia burgdorferi*, *Bifidobacterium longum*, *Actinobacillus pleuropneumoniae* (Górska et al., 2016; McGill et al., 2010; Zhang et al., 2011). A recent study compared the immunoproteomes of multidrug-resistant (MDR) strains and non-MDR strains of *E. coli* and *Klesbiella pneumoniae* and provided data on the immunoreactive profile, intensity of immunoreactivity and identity of proteins involved (Magalhães et al., 2016).

The limitations of the 2D-gel technique used in SERPA, such as its small detection dynamic range and the difficulty for separation of some groups of

proteins like membrane (hydrophobic) proteins, have been overcome by non-gel approaches such as protein arrays (Barbour et al., 2008; Fulton and Twine, 2013). Still SERPA approaches have the advantage over protein arrays that they do not require the characterization of potential ORFs and their expression and that SERPA analyzes the actual bacterial protein (with its particular folding and PTMs), not a recombinant form (Davies et al., 2005). Thus, SERPA can be more convenient in many cases. The major technical problem with SERPA is performing western blots on 2D gels. In this Thesis we propose an alternative approach (Paper #3) where 2D-electrophoresis is performed offline, coupling Offgel IEF fractionation (separation based on protein isoelectric point) and 1D-PAGE (separation based on protein molecular mass) (Figure 1.7).Western blotting is then performed on small size gels making the process more convenient and effective than conventional SERPA.

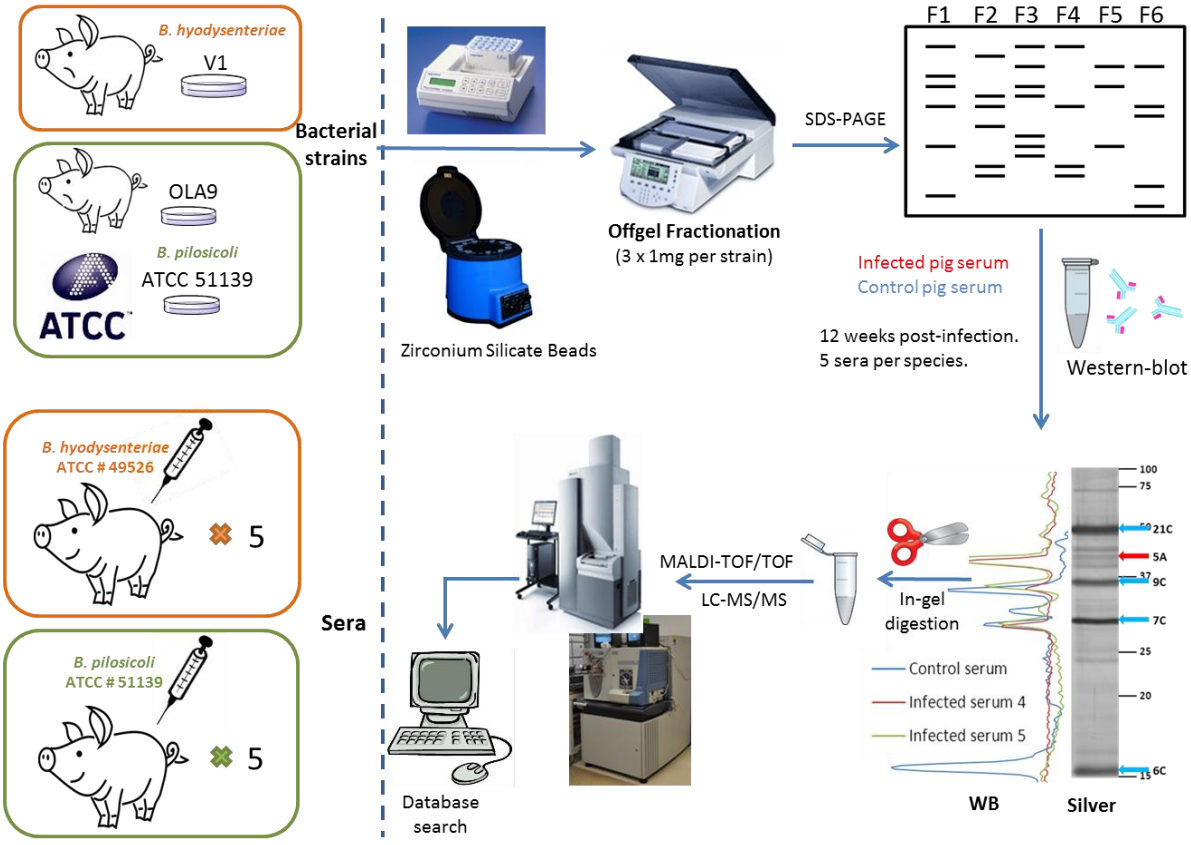

Figure 1.7. Immunoproteome analysis workflow developed in our studies.

1.2.2 Bacterial PTMs

In the 1940´s Beadle and Tatum proposed a hypothesis in which each gene produces a single enzyme that affects a single metabolic pathway. This hypothesis termed one gene-one enzyme was based on the study of the mold *Neurospore* and confirmed in *E. coli* by Horowitz (Horowitz, 1995). Although this concept was overcome in the 1950´s, the consideration of prokaryotes being simple organisms drove researchers to neglect the potential importance of PTM in bacterial cells for many decades.

Post-translational modifications (PTMs) of bacterial proteins were almost unknown until the advent of mass spectrometry. Mass spectrometry-based proteomics has revealed that prokaryotes are able to modify their proteins with a high number of PTMs which have influence on bacterial physiology and virulence (Cain et al., 2014).

The number of classes of PTMs described in bacteria is increasing continuously, and in some cases, the ratio of modified sites in prokaryotic organisms is even higher than the described in eukaryotic cells (Weinert et al., 2013).

These modifications introduce further molecular diversity and modulate protein functions. By means of PTMs, pathogens are able to manipulate pathways in host organisms, increasing their probabilities of survival (Broberg and Orth, 2010; Ribet and Cossart, 2010) and adapting themselves to different environments (Bernal et al., 2014; Cain et al., 2014). Something similar happens with mutations, as microbes replicate at a fast pace and mutations enable evolution and adaptation to new conditions, including exposure to antimicrobial treatments (National Institute of Allergy and Infectious Diseases). Most mutators (strains with a high mutation rate) are isolated from pathogenic bacteria and have been related to pathogenicity and antibiotic resistance (Denamur and Matic, 2006).

Although new modifications are continuously added to the list of bacterial PTMs, the most important—according to their known roles in microorganisms—are acetylation, phosphorylation, and methylation (Cao et al., 2010). The main drawback in the detection of PTMs is related with its low stoichiometry compared

to the non-modified peptide. Thus, for an efficient detection of these modified sequences, a specific enrichment step if often necessary before analysis.

1.2.2.1 Acetylation

Acetylation is the most studied PTM in prokaryotic organisms. This process consists of the addition of an acetyl moiety at the protein N-terminus or at the sidechains of Lys, Ser and Thr residues (Soufi et al., 2012). Due to the development of efficient, highly specific anti-acetyl Lysine antibodies, most acetylation studies are focussed on Lys acetylation.

It has been observed that Lysine acetylation may occur through metabolic regulation. Lysine acetylation is a reversible process driven by acetyltransferases and deacetylases. However, acetylation can also occur via a non-metabolic process (Choudhary et al., 2014).

Microbial species such as *E. coli* (Yu et al., 2008), *Leptospira interrogans* (Cao et al., 2010), *Salmonella enterica* (Wang et al., 2010b), *Mycoplasma pneumoniae* (van Noort et al., 2012), *Bacillus subtilis* (Kim et al., 2013a), *Mycobacterium tuberculosis* (Liu et al., 2014), *Spiroplasma eriocheiris* (Meng et al., 2016) and *Mycobacterium abscessus* (Guo et al., 2016) have their acetylomes mined.

Acetylation appears to be more frequent in prokaryotic than eukaryotic cells (Weinert et al., 2013). In *Spiroplasma eriocheiris,* 44.7% of the total proteome was detected acetylated (Meng et al., 2016). For other bacteria, the published acetylomes range from 1% to 34% of the total proteome (Ouidir et al., 2016).

Acetylation has been detected in proteins involved in gene transcription, motility and chemotaxis, protein stability, and virulence (Bernal et al., 2014; Ouidir et al., 2016). However, the higher group of acetylation targets (Lysine-acetylated proteins) correspond to proteins involved in metabolism (Bernal et al., 2014; Guo et al., 2016; Kim et al., 2013a; Meng et al., 2016; Xie et al., 2015) and, for example, in the case of *Salmonella enterica,* 90% of central metabolism enzymes are acetylated (Wang et al., 2010b).

Curiously, in a study where the acetylomes of 21 different bacterial species (including different species of cyanobacteria, spirochetes, actinobacteria, proteobacteria, tenericutes and firmicutes) were compared, 4% of the acetylated proteins were shared by more than 11 species. However, acetylated sites were different among the conserved acetylated proteins and different strains of the same species showed different acetylation sites. In particular, strains with different virulence from *E. amylovora* showed different acetylation sites (Ouidir et al., 2016).

A study of acidic stress in *Salmonella enterica* showed that the bacteria uses protein acetylation to adapt to acidic environments (Ren et al., 2015). In this species, mutants of genes related to acetylation/deacetylation processes were shown to be less virulent. It has been reported that acetylation in *S. enterica* is involved in virulence by means of invasion, survival and systemic infection (Sang et al., 2016). Protein acetylation has also been related to bacterial persistence in *M. tuberculosis* (Xie et al., 2015).

1.2.2.2 Phosphorylation

Phosphorylation in eukaryotic organisms mainly occurs in Ser, Thr and, to a lesser extent, Tyr. In bacteria, in addition to these amino acids, phosphorylation has been detected in Lys, Arg, His, Asp, Glu and Cys, with His and Asp the most common. Phosphorylation of His is thought to be exclusive to bacteria (Cain et al., 2014).

This modification has been studied in several bacteria species, such as the cyanobacterium *Synechococcus* (Yang et al., 2013), *Leptospira interrogans* (Cao et al., 2010) and *Leptospira biflexa* (Stewart et al., 2015).

Many phosphorylated proteins in *E. coli* have also been detected acetylated This group of *E. coli* proteins contains highly conserved sequences among different species and may be related to the regulation of cellular processes in bacteria (Soufi et al., 2012). In a species with a smaller genome, such as *Mycoplasma pneumoniae*, 72% of phosphorylated proteins have also been detected Lysine-acetylated (van Noort et al., 2012). In recent years, the interaction between

phosphorylation and acetylation has been reported in several bacterial species. For example, the effect of the modulation of S/T/Y phosphorylations in the K acetylome and that of the acetylation on the phosphoproteome has been described in *Mycoplasma pneumoniae* using kinase/phosphatase knockouts and acetyltransferase mutants (van Noort et al., 2012).

1.2.2.3 Methylation

Methylation is the attachment of methyl groups to Lys/Arg/Gln (N- methylation) or Glu (O-methylation).

Methylated proteins have been identified in species such as *Leptospira interrogans* (Cao et al., 2010; Witchell et al., 2014), *Leptospira biflexa* (Stewart et al., 2015) and *Shewanella oneidensis* (Bubendorfer et al., 2013).

In *Leptospira biflexa*, methylated proteins have been detected mainly in membrane-associated fractions (Stewart et al., 2015). In *Leptospira interrogans*, (Cao et al., 2010), Glx-methylation (methylation in Gln/Glu) was more abundant than Lys/Arg-methylation (135 vs 64 sites) .The Glx-methylated proteins were enriched in proteins related to cell motility, such as chemotaxis proteins and flagella, the Lys/Arg methylated proteins were enriched in membrane proteins. It has been suggested that Lys-methylation of surface proteins could participate in bacterial adherence to the environment (Lanouette et al., 2014). Lys-methylation was also reported in flagellar proteins from *Salmonella typhimurium* (Yoshioka et al., 1995).

The main antigen of the typhus agent *Rickettsia typhi*, OmpB, was found heavily methylated and this methylation was related with its antigenicity. Thus, the serological reactivity (antibody titres) of a recombinant methylated OmpB was found higher than the non-methylated form although lower than that observed for the native protein (Chao et al., 2008). In the lipoprotein LipL32 from *Leptospira interrogans*, methylation occurs in the epitope recognized by the host´s immune system, which decreases recognition by the host during infection (Witchell et al., 2014). This could be a mechanism of evasion from the immune system.

In *Pseudomonas aeruginosa*, trimethylation of a Lys residue in Elongation Factor-Tu is involved in bacterial invasion. The results observed suggest that the modification is specific and critical to the initial stages of the disease (Barbier et al., 2013).

1.2.2.4 Other PTMs

As mentioned above, the most studied modifications for prokaryotic cells are acetylation, methylation and phosphorylation. However, other PTMs have been described for bacteria, such as pupylation (i.e. attachment of prokaryotic ubiquitin-like protein molecules), which is analogous to eukaryotic ubiquitination (Cain et al., 2014), or glycosylation (addition of carbohydrate moieties), which is common in the surface and flagellar structures of bacteria (Chaban et al., 2015).

Using an algorithm that allows searching for all possible PTMs, up to 23 different PTMs could be identified in *Synechococcus* sp. PCC 7002, including ADP Ribose addition, succinylation, palmitoylation, crotonylation, biotinylation and geranylation (Yang et al., 2014).

1.2.3 Antimicrobial resistance and proteomics

A quick response to stress is essential for bacterial survival. The development of defence mechanisms is necessary to survive stressful situations, like those promoted by all major classes of antibiotics through the generation of hydroxyl radicals, which induce the stress oxidative response (Kohanski et al., 2010).

Proteomics can be applied to the study of bacterial resistance through the global characterization of protein expression, post-translational modifications and mutations

Bacteria respond to environmental changes by modulating protein expression and thus proteome composition. Up and downregulation of specific proteins in strains resistant to antibiotics has been described in *E. coli*. In particular, outer membrane proteins were proposed as new targets for drug development as they were found altered in resistant strains (Radhouani et al., 2012).

A fast response to surrounding changes may also occur via post-translational modifications (Ribet and Cossart, 2010). In *Mycobacterium* species, protein modifications have been related to antibiotic resistance. For example, in *Mycobacterium tuberculosis*, many proteins related to persistence, virulence and antibiotic resistance were acetylated (Xie et al., 2015) and in *Mycobacterium abscessus,* Lysine-acetylated proteins were also related to drug resistance (Guo et al., 2016).

It is known that when pathogens are exposed to stressful environments such as antibiotic treatments, mutations are favoured (Denamur and Matic, 2006). One of the broadest classes of antibiotics is composed of drugs that inhibit protein synthesis. Their primary targets are the ribosomal subunits 30S or 50S (Kohanski et al., 2010). Notably, mutations in ribosomal protein L3, a component of the 50S subunit, are involved in *Brachyspira* resistance to antimicrobials (Klitgaard et al., 2015; Pringle et al., 2004). In this protein, the mutated amino acids are near the peptidyl transferase centre, which is the binding site for ribosomal antibiotics to block protein translation.

1.3 *Brachyspira* and proteomics

Although *Brachyspira* were discovered and classified in the 1980s and the genomes of *B. hyodysenteriae* and *B. pilosicoli* were sequenced in 2009-2010, knowledge of these species at the proteome level is scarce.

Available studies on *Brachyspira* proteins often focus on specific targets inferred from the information available for other species. A global characterization of the *Brachyspira* proteomes has not yet been performed.

On the other hand, most of the available studies were performed during the 1990s and rely primarily on techniques such as SDS-PAGE and immunoblotting (Western-blotting). Using these approaches, the observed bands were assigned to proteins based on published properties for proteins of other species. In most cases, no further protein identification was performed.

Proteins more commonly targeted in these studies were those located on the exterior of the bacteria, due to their probable relationship to antigenic response, and thus vaccine development. This set of proteins primarily includes flagellar proteins (Kent et al., 1989; Koopman et al., 1992) and outer membrane components (Trott et al., 2001).

1.3.1 Flagellar proteins

In 1989, the extracts of axial filaments of *Brachyspira hyodysenteriae* and other non-pathogenic species strains were compared (Kent et al., 1989). Using an approach based on SDS-PAGE and Western blotting, the molecular mass and the immunoreactivity of the proteins in these structures were determined. The bands detected ranged from 46 to 29 kDa, some of these signals were doublets or triplets. Sera from convalescent pigs reacted with the proteins observed in all strains, even the non-pathogenic ones. The profile observed in Western blots was similar for all strains and those observed for other *Treponema* species.

In the same year, it was found that the administration of endoflagellar proteins elicited a protective response in mice against *B. hyodysenteriae* (Boyden et al., 1989). The approach used SDS-PAGE and Western blotting. Characterization of the proteins involved in the process was based on the similarity of the gel bands with previously published data for treponemal endoflagellar proteins.

In 1992, in addition to SDS-PAGE and Western blotting, Koopman et al. performed an immunogold labelling study to establish the composition of axial filaments for *B. hyodysenteriae* (Koopman et al., 1992). They detected 5 major bands with molecular masses at approximately 44, 37, 35, 34 and 32 kDa. In some flagellar preparations, doublets were observed for some bands. The antisera used were raised in rabbit against periplasmic flagella from *B. hyodysenteriae* and other *Treponema* species. All the bands reacted with anti-flagella sera, confirming their flagellar nature. In addition, the protein location was confirmed through immunogold labelling. The 44- and 35-kDa proteins were observed all along the flagella. The 37, 34 and 32 kDa proteins were observed only in some damaged points or the ends of periplasmic flagella. Thus, the 44-

and 35-kDa proteins were proposed to be sheath proteins and the other three core proteins.

The N-terminal sequence obtained for the core proteins was very similar, among species and with other spirochetes. However, the 44- and 35-kDa proteins were different from other species. When comparing different isolates from diseased pigs, all isolates contained the 37, 35, 34 and 32 kDa proteins, but the 44-kDa protein varied in molecular mass between the different isolates. This difference appeared to be related to haemolytic activity; the 44 kDa protein was observed in strong haemolytic isolates and a band between 44-47 kDa in weak haemolytic isolates was also seen.

In 1993, Li et al. detected a 46-kDa protein that was initially thought to be an outer membrane protein, but in fact it was a periplasmic flagellar protein (Li et al., 1993). This protein was observed in *B. hyodysenteriae* strains but not in *B. innocens* strains. It was identified as the sheath protein FlaA1, and unlike those observed in the core, exhibited no cross-reactivity with periplasmic flagella from *B. innocens*. FlaA1 was different in the species of *Brachyspira* included in the study, showing different mass and antigenic properties. Furthermore, it was glycosylated in *B. hyodysenteriae*.

In 1995, in addition to the typical approach of SDS-PAGE and Western blotting, an HPLC separation of the components of the extracytoplasmic fraction obtained from *B. hyodysenteriae* was performed (Gabe et al., 1995). The N-terminal and/or an internal region of each separated component was sequenced and subjected to a homology search. The 38 kDa band was characterized as the FlaB1 protein. The difference between the theoretical mass (32 kDa) and the observed mass (38 kDa) was not explained, but post-translational modifications were posited to be the cause. The FlaB1 protein was also tested as a vaccine, but no reduction in the incidence or severity of disease was observed in experimentally infected swine.

On a subsequent work, specific antibodies to the 44-kDa protein (sheath protein FlaA1) and to the core proteins FlaB were used for the study of flagella in several strains of *B. hyodysenteriae*, *B. innocens* and *B. pilosicoli* (Fisher et al., 1997).

The N-terminal sequences were obtained (10 amino acid residues) for FlaA (*B. hyodysenteriae* and *B. pilosicoli*) and FlaB (*B. hyodysenteriae*) proteins. This enabled comparison of sequences and established a degree of homology between species. The FlaA protein was conserved among different species of *Brachyspira*, and FlaB proteins had a high homology with proteins from other species of bacteria. In contrast with previous studies, FlaA was not exclusive to *B. hyodysenteriae*. However, it had more variability than other periplasmic flagellar proteins and showed varying solubility among strains.

The importance of flagellar proteins was proposed in 1997 through the study of deletion of the correspondent coding genes (Kennedy et al., 1997). In this work, they observed the effect of *flaA1* and *flaB1* genes deletion on the motility and colonization capacity for *B. hyodysenteriae* and noticed that the deletion of *flaB1* had more impact on bacteria, *flaB1* mutants were less motile, and their capacity for infection was much lower than that of the wild-type. Another interesting finding from this work was that the *flaB1* mutant obtained from a laboratory cell culture was “weaker” than the mutant obtained from the same strain passed through an animal (fed and re-isolated). The cells isolated from the animal appeared more apt for colonization.

1.3.2 Outer membrane proteins

In addition to flagellar proteins, some outer membrane proteins have been studied in *Brachyspira* species (Trott et al., 2001). The presence of proteins such as the 16-kDa lipoprotein SmpA, a *B. pilosicoli* 72-kDa outer membrane protein, variable surface proteins, and the 36-kDa lipoprotein MglB were determined in different species and strains, primarily by SDS-PAGE and Western blotting.

The serpulina membrane protein A (SmpA) gene was cloned in *E.coli* and sequenced in 1993 (Thomas and Sellwood, 1993). In 1995, using an ELISA and a Western blotting approach with an antibody against the *Brachyspira hyodysenteriae* SmpA protein (Sellwood et al., 1995), it was observed that this protein was immunoreactive against pig sera from pre-challenge and post-challenge animals. However, this protein was detected only in *in vitro* cultures and not in samples from infected animals concluding that the *smpA* gene was not

expressed in diseased pigs and mice. This was explained as the result of a process of antigenic variation during infection (microbial pathogens create variation, structural and antigenic changes to persist inside the host and escape phenotypes (Palmer et al., 2016)) or the switching-off of the gene expression.

In further studies of the outer envelope for these species, a 72-kDa outer membrane protein specific of *B. pilosicoli* was identified (Tenaya et al., 1998). The study included different strains of *B. hyodysenteriae*, *B. pilosicoli, B. innocens, B. intermedia, B. murdochii and B. aalborgi*. Using SDS-PAGE and Western blotting it was shown that most outer envelope proteins were immunoreactive towards anti-sera raised against *B. pilosicoli*, *B. hyodysenteriae* and *B. innocens*. However, using pig antisera raised against *B. pilosicoli and* cross-adsorbed ('filtered' antisera obtained by incubation with outer envelope preparations from *B. hyodysenteriae* and *B. innocens*) they detected only one protein at 72 kDa in *B. pilosicoli* strains. This effect was not observed in the other species. Immunogold labelling confirmed that this protein was located on the surface of *B. pilosicoli* but not in *B. hyodysenteriae* (other species were not tested).

The Vsp proteins are the most abundant outer membrane proteins of *B. hyodysenteriae* (Gabe et al., 1998) , in which they have been postulated to have an antigenic role either as protein complexes or as individual forms (McCaman et al., 1999; Witchell et al., 2011). Two members of the Vsp family, VspH and VspD, have been included as potential vaccine components against *B. hyodysenteriae* (Bellgard et al., 2015). However, little is known about the expression of these proteins in *Brachyspira species*. The expression of VspH was reported in a B204 strain of *B. hyodysenteriae* (Witchell et al., 2006); however, in further studies the absence of the gene in other strains (ATCC WA1 and X576) is described (Witchell et al., 2011). In the latter study, the expression of the VspD protein (together with VspF, VspE and VspI) in a virulent Australian isolate of *B. hyodysenteriae* (Witchell et al., 2011) was reported. These authors suggested that Vsp proteins form complexes and are immunoreactive only in that form.

Some studies also used genomic tools to identify/characterize *Brachyspira* proteins. For instance, based on previous knowledge of antigens recognized by sera obtained from pigs recovered from colonic spirochetosis, Zhang et al. selected the product of the gene *mglB* (Zhang et al., 2000). In a study of nucleotide homology for the *mglB* gene, primers from a highly conserved domain with other bacterial species were selected and amplified by PCR. The recombinant protein expressed in *E. coli*, was a 36-kDa lipoprotein with high amino acid-sequence identity with other bacterial glucose-galactose transport proteins (MglB proteins).

Using a similar genetic approach, Movahedi et al. expressed different *Brachyspira* proteins. In 2007, the gene coding the ClpX protein, a clip protease, was found in all strains from the seven *Brachyspira* species included in the study (Movahedi and Hampson, 2007). In Western blots, the recombinant protein was immunoreactive towards mice sera raised against whole cell preparations from *B. pilosicoli*. In another attempt to develop a vaccine (Movahedi and Hampson, 2010), genes encoding the oligopeptide-binding proteins (OppA) were selected and the recombinant proteins were expressed. The immunoreactivity of these recombinant proteins was confirmed by Western blotting with mouse antisera raised against *B. pilosicoli* preparations, and with sera from experimentally and naturally infected pigs.

2. Justification

The main objective of this research is the identification of the potential antigenic proteins of *B. hyodysenteriae* and *B. pilosicoli*.

This issue was addressed through the following studies:

1. Bacterial proteome: Casas, V., Abian, J., Carrascal, M., 2017. "**An insight into the *Brachyspira*'s proteome and PTMs"** *(in preparation).*

The *Brachyspira hyodysenteriae* and *B. pilosicoli* genomes were sequenced in 2009-2010. These genomes are predicted to contain approximately 2200-2500 protein-coding genes, respectively, but information on the actual expressed gene products is scarce.

In Uniprot (The UniProt Consortium, 2014), only 12 proteins are described with evidence at the protein level for *B. hyodysenteriae*; no protein was found in this category for *B. pilosicoli*. Thus, most protein sequences available for these species are theoretical and were directly translated from the genome; there is no factual expression for these proteins. In addition, the available databases are highly redundant, particularly for *B. pilosicoli*. Curation of these databases is necessary to develop accurate proteomic research.

Numerous studies of the genomics, epidemiology and pathogenicity of *Brachyspira hyodysenteriae* and *B. pilosicoli* are available, but few investigate at the protein level, and none give a "full picture" of the proteome.

In this work, we used a methodology based on maximal sample preparation efficiency and sought to develop a combined search engine workflow to reach maximal coverage of the proteome for these *Brachyspira* species. These data allow us to have a more extensive view of the proteome, including post-translational modifications (PTMs) and mutations, which are important issues for pathogenic bacteria due to their potential relation to pathogenicity.

2. Exposed proteome: Casas, V., Vadillo, S., San Juan, C., Carrascal, M., Abian, J., 2016. "**The Exposed Proteomes of *Brachyspira hyodysenteriae* and *B. pilosicoli*.**" Front. Microbiol. 7, 1103. doi:10.3389/fmicb.2016.01103

A proteomic study of these species is necessary not only because inaccurate and scarce information are available at proteomic level but also because proteomic characterization of bacterial pathogens allows elucidation of host-pathogen interactions. These interactions are very important to find new vaccine targets and sequences related to antimicrobial resistance, which is an increasing concern (Cohen et al., 2015). To characterize them, the study of secreted and surface-exposed proteins is very important because these proteins are more likely prompted to interact with the host. The study of these proteome subsets is very important for vaccine development and identification of infection factors. A proteomic approach to these pathogens provides data for the validation of the actual protein expression and localization.

In the exposed proteome, analysis by means of a cell-shaving strategy and a shotgun proteomic approach yielded a large-scale characterization of proteins located on the bacterial surface and in the extracellular medium. In this work, five isolates (three of *B. hyodysenteriae* and two of *B. pilosicoli*) obtained from an Iberian pig breed in farms in central Spain were used.

3. Immunoproteome: Casas, V., Rodriguez-Asiain, A., Pinto-Llorente, R., Vadillo, S., Carrascal, M., Abian, J., 2017. "***Brachyspira hyodysenteriae* and *B. pilosicoli* proteins recognized by sera of challenged pigs".** Front. Microbiol. 8, 723. doi:10.3389/FMICB.2017.00723

A proteomic approach can be used to determine immunogenic proteins, develop deeper knowledge of the pathogenic process and propose new vaccine candidates against these *Brachyspira* species.

In this study, we extended our proteomic approach to identify potential immunogenic proteins from *B. hyodysenteriae* and *B. pilosicoli*. For this purpose, we studied the immunoproteome of two *B. pilosicoli* strains (the isolate OLA9 and the commercial ATCC strain P43/6/78) and one *B. hyodysenteriae* isolate (isolate V1). Cell lysates were fractionated using preparative off-gel isoelectrofocusing, and the fractions were separated by SDS-PAGE. The gels were immunoblotted using pig immune-sera, and the reactive bands were identified by mass spectrometry.

3. Paper#1
Bacterial proteome

An insight into the *Brachyspira's* proteome and PTMs

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ABSTRACT

Spirochaetes of genus *Brachyspira* are known gut pathogens. Due to their relevance in disease, *Brachyspira hyodysenteriae* and *B. pilosicoli* have been the most studied species. *B. hyodysenteriae* is the causative agent of swine dysentery, a severe mucohaemorrhagic colitis which affects the large intestine. There is an increased concern for this species because of the appearance of strains resistant to antibiotics and the lack of a commercial vaccine. *Brachyspira pilosicoli* causes colonic spirochetosis in pigs and is responsible for a human form of the disease.

In the last decade these species have had their genomes sequenced, but the information at the proteomic level is still scarce and not full proteome study is available yet. The characterization at proteomic level of bacterial pathogens is a way to the elucidation of interaction host-pathogen and to find new vaccine targets.

We applied a proteomics approach using Silicate Zr beads for cell lysis and FASP digestion combined with long acquisition gradients in 60-cm long nanocolumns to obtain the highest coverage possible of the proteome for these species. We describe 1531 and 1539 proteins for *B. hyodysenteriae* and *B. pilosicoli*, respectively, covering around 70% of each species proteome. Information on PTMs, including acetylation, methylation and phosphorylation was also obtained. These analyses showed the prevalence of acetylation as the major PTM in these bacteria (5579 and 3221 acetylation sites for *B. pilosicoli* and *B. hyodysenteriae*, respectively) as well as important differences on its level between the two species.

INTRODUCTION

Anaerobic spirochaetes of the genus *Brachyspira* are gram negative bacteria that have long been known as important gut pathogens of pigs and have appeared as disease-causative agents in birds, dogs and other species including human. The most notorious, because of the magnitude of their effects are *Brachyspira hyodysenteriae* and *Brachyspira pilosicoli*.

Brachyspira hyodysenteriae is frequently related to swine dysentery, a severe mucohaemorrhagic colitis which affects the large intestine. This disease causes economic losses, due to animals' treatment, reduction in the exploitations production and death (Burrough, 2016; Stanton, 2006). Therapeutic treatments are mainly dependent on antibiotics, and resistance to these compounds has been detected among strains of this species. In fact, resurgence of the disease, which seemed to have disappeared from the U.S. during the nineties, could be related with a higher resistance to antimicrobials of the bacteria (Mirajkar and Gebhart, 2014). In a recent study including 108 Italian isolates of *B. hyodysenteriae*, more than 50% were found resistant to antibiotics (Rugna et al., 2015). This resistance to antibiotics is related to the fact that many intestinal diseases characterised by diarrhoea may have a non-infectious cause. However, due to the lack of diagnostic tests, it is common that veterinarians resolve to treat diarrhoea cases with antibiotics without demonstration of bacterial infection (Pedersen et al., 2015).

Brachyspira pilosicoli is the causative agent of intestinal spirochetosis (IS), a disease milder than swine dysentery. This species has been isolated from a wider number of hosts than *B. hyodysenteriae* including pigs, humans, birds and dogs (Neo et al., 2013b).The corresponding human disease (HIS) seems to be more widespread in developing countries and rural areas; it was detected in 32.6 % of stool cultures in North West Australia against 1.5% in United Kingdom (Walker et al., 2015). In Sweden, HIS was detected with a prevalence of 2% and it was associated with irritable bowel syndrome (Walker et al., 2015). Similar results have been published in Japan from 4254 patients, of which 1.7% were diagnosed with HIS. This percentage was increased to 5.5% when the population

studied were patients with Human Immunodeficiency Virus infection (Tateishi et al., 2015).

When pathogens are exposed to stressful environments, such as antibiotic treatments, mutations are favoured (Denamur and Matic, 2006). Another fast way of response to surrounding changes is by post-translational modifications (PTMs) (Ribet and Cossart, 2010) (Cain et al., 2014). Mutations and post-translational modifications are behind antimicrobial resistance and bacterial virulence. It is fundamental to know the protein sequences expressed by pathogenic species and perform a deep study of polymorphisms and modifications that could explain differences in virulence among species and strains.

The genomes of several *Brachyspira* species have been characterized (Bellgard et al., 2009; Black et al., 2015; Håfström et al., 2011; Lin et al., 2013; Mappley et al., 2012; Neo et al., 2013; Osorio et al., 2013; Wanchanthuek et al., 2010) and a number of studies addressing genomics, epidemiology and pathogenicity of *Brachyspira hyodysenteriae* and *B. pilosicoli* are available. However few of these studies are focussed at protein level. Information of proteins is mainly restricted to groups for which their immunogenic properties have been tested (Holden et al., 2006; La et al., 2004; Lobova et al., 2011; McCaman et al., 2003; Song et al., 2015) and no large-scale study of the proteome is available. Several large-scale studies of bacterial proteomes have been performed, some of them with proteome coverages higher than 60%. These are the cases of *E.coli* with a 63% of proteome coverage (Iwasaki et al., 2010) and *Leptospira interrogans* with 64% (Cao et al., 2010). In the latter work, an exhaustive study of protein PTMs was also reported. In the same line, the acetylomes of *Salmonella enterica* (Wang et al., 2010) and *Mycobacterium tuberculosis* (Liu et al., 2014) have been reported.

The proteome of *B. hyodysenteriae* WA1 strain ,which is considered the reference proteome for this specie*s* in the Uniprot database (The UniProt Consortium, 2014), contains 2642 proteins. Only 111 of them have been reviewed, and for 5 proteins there is evidence at protein level (2017_06 version). There is no reference proteome for *Brachyspira pilosicoli*, although the genomes of several strains have been completely sequenced (Mappley et al., 2012; Wanchanthuek et al., 2010). *B. pilosicoli* database in Uniprot (2017_06 version)

includes four strains, P43/6/78 (102 entries), 95/1000 (2302 entries), B2904 (2638 entries) and WesB (2638 entries). In total it contains more than 7600 proteins, only 3 of them reviewed, none found at evidence level.

In the present work the proteomes of two commercial strains of *Brachyspira hyodysenteriae* (strain WA1) and *B. pilosicoli* (strain P43/6/78) were characterized. This large-scale analysis included the characterization of PTMs such as methylation, oxidation, phosphorylation and acetylation as well as of unexpected sequences (polymorphisms, mutations) by De Novo sequencing. This is the first collection of protein data for these species with a high coverage of proteome, around 70%. We described 79 / 91 phosphorylation sites and 3221/ 5579 acetylation sites, for *B. hyodysenteriae* and *B. pilosicoli*, respectively.

MATERIAL AND METHODS

Brachyspira culture

Brachyspira hyodysenteriae ATCC 49526 (strain WA1) and *Brachyspira pilosicoli* ATCC 51139 (strain P43/6/78) reference strains were obtained from the American Type Culture Collection (Manassas, VA, USA). These two strains had been previously sequenced and described at genomic level (Bellgard et al., 2009; Lin et al., 2013). The collection strain B-78 (ATCC 27164) and several environmental isolates (INFE1, LL1, OLA9, Vi13, Vi32) were included in the acetylation validation study.

Isolation and culture of the strains were done as described before (Casas et al., 2016). The grown cultures were centrifuged at 12900 xg for 10 min, and the pellet washed twice with TE buffer (10 mM Tris pH 8.0, 1 mM EDTA, both from Sigma-Aldrich, St. Louis, MO, USA) before store at -80ºC.

Sample preparation and digestion

The lysis method used is based on the combination of a buffer with a high content in detergent and mechanical lysis with beads as follows. Wet pellets (around 40-50 mg) were suspended in lysis buffer (4% (w/v) SDS, 100mM Tris/HCl pH 7.6, 0.1M DTT) and incubated at 95ºC for 1 hour with agitation, followed by homogenization (Bullet Blender, Next Advance) with Silicate Zr beads (100µm, BioSpec,11079101z). Then, samples were centrifuged for 3 min at 14100xg and the supernatant was recovered. Protein concentration determined by using the Bio-Rad RC/DC protein assay kit (Bio Rad laboratories, California) according to the manufacturer´s instructions.

Protein was digested with Sequencing Grade Modified Trypsin (Promega, Madison, WI, USA) using the FASP (Filter Aided Sample Preparation) digestion protocol (Wiśniewski et al., 2009). Two hundred and 80 µg of protein from ATCC 51139 (strain P43/6/78) and ATCC 49526 (strain WA1), respectively, were loaded to a 10-kDa Amicon Ultra-0.5 centrifugal filter (Millipore, Watford, UK), washed three times by adding 200 µL of UA buffer (8 M Urea, 0.1 M Tris/HCl pH 8.5) to the filter and centrifuging at 14000xg for 10 min at 13ºC. Previously to

enzyme addition proteins were alkylated with 100 µL of alkylation buffer (0.05M IAA, 8M Urea, 0.1 M Tris/HCl pH 8.5) in the dark for 20 min at 25 ºC and the protein extracts were washed three times with 100 µL of UA buffer and three times with 100 µL of ammonium bicarbonate 20 mM. Trypsin digestion was performed at 37 °C for 18 h using an enzyme-to-protein ratio of 5:100. Tryptic peptides were eluted by the addition of 3×100 µL of ammonium bicarbonate 20 mM followed by a centrifugation at 14000xg for 15 min at 13 ºC.

LC-MS/MS

1. Shotgun mass spectrometry

All samples were analyzed by LC-MSn using LTQ Orbitrap XL instrument (ThermoFisher, CA) equipped with a nanoESI ion source (Proxeon, Odense, Denmark). A volume of 20 µL, corresponding to 6 µg of total protein or 45% of the PTM-enriched extracts, was loaded into the chromatographic system consisting in a C18 preconcentration cartridge (Agilent Technologies) connected to a 60-cm x 100 µm i.d. C18 column (NanoSeparations) for total proteome or to a 15-cm x 100 µm i.d (Nikkyo Technos Co) for PTM-enriched samples. The separation was done at 0.25-0.40 µL/min in a 480-min (360-min for PTM) acetonitrile gradient from 3 to 40% (solvent A: 0.1% formic acid, solvent B: acetonitrile 0.1% formic acid). The HPLC system was composed of an Agilent 1200 capillary nano pump, a binary pump, a thermostated micro injector and a micro switch valve. The LTQ Orbitrap XL was operated in the positive ion mode with a spray voltage of 1.8 kV. The spectrometric analysis was performed in a data dependent mode, acquiring a full scan followed by MS/MS scans of the 10 most intense signals detected in the MS scan. The full MS (range 300-1800) was acquired in the Orbitrap with a resolution of 60.000. The MS/MS spectra were done in the linear ion-trap. For phosphorylated-enriched samples, a subsequent MS^3 scan was performed when a neutral loss of -98, -49, or -32.7 m/z (loss of H_3PO_4 for the +1, +2, and +3 charged ions, respectively) was detected in the CID MS/MS among the 5 most intense ions. Dynamic exclusion was set to 1 with a time window of 45 s to minimize the redundant selection of precursor ions. Before each total proteome sample analysis 2 short blanks (70 minutes) and a long one (540 minutes) were run in order to minimize cross-contamination. For the same

purpose, each sample was injected six times but the first one was discarded for data analysis.

Additionally, acetylated-enriched samples were analysed in an Orbitrap Fusion Lumos™ Tribrid (ThermoFisher, CA) equipped with the Advion Triversa Nanomate (Advion BioSciences, Ithaca, NY, USA) as the nanoESI source. Samples were loaded into the Thermo Scientific Dionex Ultimate 3000 chromatographic system using a Acclaim PepMap100 trap (100 µm × 2 cm Thermo Scientific) connected to a Acclaim PepMap® RSLC (75 µm × 50 cm, nanoViper, C18, 2 µm, 100Å) analytical column. The separation was done at 0.2 µL/min in a 120-min acetonitrile gradient from 1 to 35% (solvent A: 0.1% formic acid, solvent B: acetonitrile 0.1% formic acid). The analyses were performed in a Top Speed (most intense) data dependent mode using CID. The full MS (range 400-1600) was acquired in the Orbitrap with a resolution on 120.000 and the MS/MS spectra were done in the linear ion-trap. Target ions already selected for MS/MS were dynamically excluded for 15s.

2. Target mass spectrometry

Analysis by LC-MS/MS in target mode was carried out in two different systems, a LTQ Velos and a LTQ Orbitrap XL. The LTQ Orbitrap XL was configured in the same way as for the shotgun analysis. A volume of 20 µL, corresponding to 2 µg of protein, was loaded and the separation was done at 0.40 µL/min in a 360-min acetonitrile gradient from 3 to 40%. The LTQ Velos instrument was equipped with a microESI ion source (ThermoFisher, CA). The volume correspondent to 3 µg was taken from each sample and diluted to 5 µL with 5% methanol, 1% formic acid. Samples were loaded into a chromatographic system consisting in a C18 preconcentration cartridge (Agilent Technologies) connected to a 15-cm long, 150 µm i.d. C18 column (Nikkyo Technos Co.). The separation was done at 1 µL/min in a 120-min acetonitrile gradient from 0 to 40% (solvent A: 0.1% formic acid, solvent B: acetonitrile 0.1% formic acid). The HPLC system was composed of an Agilent 1200 capillary micro pump, a loading pump, a thermostated micro injector and a switch valve. The Velos LTQ was operated in the positive ion mode with a spray voltage of 2 kV.

The spectrometric analysis was performed in target mode, acquiring the MS/MS scans of the signals included in the corresponding mass list (Tables S1 and S2).

Database search and peptide identification

1. Total proteome

PeptideShaker (Vaudel et al., 2015) and PEAKS (Zhang et al., 2012) were used in a search workflow as it is shown in Figure 1. PeptideShaker allows using several search engines in one process, here X! Tandem (Bjornson et al., 2008), MyriMatch (Tabb et al., 2007), MS-GF+ (Kim and Pevzner, 2014), Amanda (Dorfer et al., 2014), Comet (Eng et al., 2013) and OMSSA (Geer et al., 2004) were run for each raw file against the corresponding species database, either *Brachyspira hyodysenteriae* (reference proteome, Uniprot organism ID 565034) or *Brachyspira pilosicoli* (organism database ,Uniprot). For *B pilosicoli* search we resolved in using the database filtered at the species taxonomical level because there is no reference proteome for this species and the database for the P43/6/78 strain has few entries annotated. The homology between *B. pilosicoli* strains has been reported between 54.9% and 68.4% (Mappley et al., 2012).Thus, it is important to take into account other strains databases for the analysis of poorly characterized P43/6/78 strain. *B. pilosicoli* database contains mostly non-curated sequences from 4 different strains, P43/6/78 (102 entries), 95/1000 (2302 entries), B2904 (2638 entries) and WesB (2638 entries). In total it contains more than 7600 proteins. PeptideShaker default validation parameters were applied to search results (1% FDR (false discovery rate) at PSM (Peptide Spectrum Match), peptide and protein level).

Spectra not assigned by PeptideShaker in any of the different levels of analysis (PSM, peptide and protein) were exported as mgfs and combined for further analysis by PEAKS software. PEAKS analysis comprises several steps, as follows: De Novo, Database search, PTM and Spider (Han et al., 2011; Yuen, 2011; Zhang et al., 2012). In this step, the full *Brachyspira* database from Uniprot (2016_06) was used for searches to increase the chances of identification of sequences. This was based on previous findings (Casas et al., 2016) for these

species showing the existence of trans-species-only matches. I.e., spectra from some species only have a match in a species database different than the corresponding to the analysed sample. The main contributors to *Brachyspira* database are *B. hampsonii*, *B. hyodysenteriae*, *B. innocens*, *B. intermedia*, *B. murdochii* and *B. pilosicoli*. Results obtained from the different appliances contained in PEAKS were validated by 0.1% FDR at PSM level, and ±5 ppm at peptide level.

Those peptides detected only by De Novo were filtered by a 70% of ALC (average local confidence) assignation. The inspection of De Novo only results showed a high percentage of sequences with PTMs. This effect was due to the fact that when two sequences were possible for the same spectrum (one with PTM and other without it) only one result was exported and most times it was the modified sequence, even when both of them had the same probability. I.e., if the spectra could be explained by two equivalent sequences, the first containing a methylated Ser and the second a Thr, only the first one would be exported in results. In order to correct this effect, all De Novo results obtained from PEAKS were revised and when a modified aminoacid with equivalent mass to “normal” aminoacids was detected, the option of non-modified peptide was added to the original PEAKS result list. Tags of at least 5 consecutive aminoacids with local amino acid confidence higher than 90% were BLASTed against the *Brachyspira* database (Figure 1 and Figure S1).

Finally, the remaining non-matching sequences were BLASTed against a mammal database (containing *Homo sapiens* and *equus*, *bovidae* and *sus*) (Figure 1) to detect possible contaminants.

All search engines were used with the following parameters: peptide mass tolerance 20 ppm, fragment tolerance 0.6 Da, enzyme set as trypsin and allowance up to two missed cleavages, dynamic modification of methionine oxidation (+15.99 Da) and acetylation N-term (+42.01 Da), fixed modification of cysteine carbamidomethylation (+57.02 Da) .For PEAKS workflow, phosphorylation (HCDR, +79.97 Da), Lysine acetylation (+42.01 Da) and methylation (+14.02 Da) were added as dynamic modifications.

Protein reports generated PeptideShaker and PEAKS express the protein number of identifications in terms of protein groups. For convenience purposes, in this paper the protein groups are referred as proteins and the information given is about the head protein of the group, unless otherwise is explained in the text. Full information about the protein group composition is available in Supplementary Information tables.

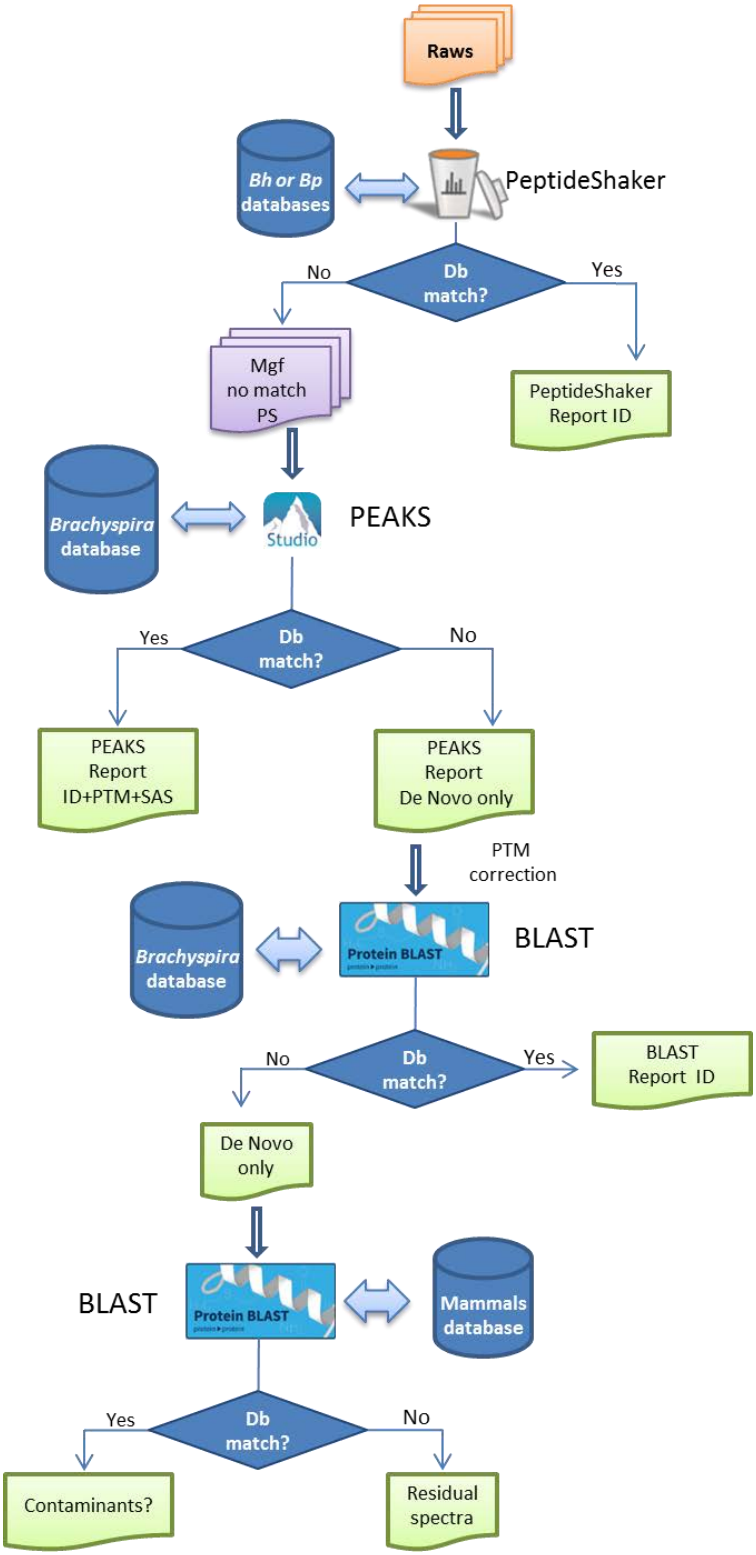


Figure 1: Workflow for peptide and protein identification using combined software, PeptideShaker (PS) and PEAKS. ID (identifications), Db (database), Bh (*B. hyodysenteriae*), Bp (*B. pilosicoli*), PTM (post-translational modification), SAS (single aminoacid substitution).

2. PTM enriched samples

The raw data were processed using Proteome Discoverer (version 1.4.0.288 and 2.1, Thermo Scientific). The fragmentation spectra were searched with the SEQUEST engine against the corresponding species database using the following parameters: trypsin, maximum 3 missed cleavages, 20 ppm precursor mass tolerance, 0.6 Da fragment mass tolerance, cysteine carbamidomethylation as a fixed modification, oxidation of methionine and Lysine acetylation, or STY-phosphorylation and ST-dehydration, as dynamic modifications for acetylated and phosphorylated peptides, respectively. Peptide spectral matches were filtered at 0.1 % FDR using Percolator. The localization probability for each site was calculated using ptmRS (Taus et al., 2011).

Immunoblotting

Forty µg of protein were diluted with loading buffer (NuPAGE® LDS Sample Buffer (4X), Life Technologies), boiled for 5 min and resolved by polyacrylamide gel electrophoresis (SDS-PAGE), (NuPAGE® 4-12% Bis-Tris Gel 1.5 mm, 10 well, Life Technologies).

After SDS-PAGE, the gel proteins were transferred to a nitrocellulose membrane using an iBlotTM system (Life Technologies, CA, USA). Following Ponceau staining of the protein (Sigma-Aldrich, St. Louis, MO, USA), the membranes were blocked for 1 h in blocking buffer (TBS 0.2 % Tween-20, 5 % (w/v) skimmed milk) at 20 ºC and then incubated for 16 h at 4ºC with 1/5000 dilution of anti-acetyl Lysine antibody (ImmuneChem Pharmaceuticals, Canada) under gentle agitation. The membrane was washed 3×5 min at 20 ºC in TBS/Tween-20 buffer before incubating with the horseradish peroxidase (HRP)-conjugated IgG secondary antibody (#ab99697, Abcam) for 1 h at 20 ºC. Afterwards, the membrane was washed 3×10 min with TBS/Tween-20 and visualized by using the ECL method.

Enrichment of acetylated peptides

Acetylated peptides were enriched as previously described by Svinkina et al. with minor modifications (Svinkina et al., 2015). Briefly, 500 µg of protein from P43/6/78 and WA1 strains were digested using LFASP method (Casanovas et al., 2017) and

the tryptic peptides were evaporated, reconstituted in immunoprecipitation buffer (50 mM MOPS, 10 mM Na_2HPO_4, 50 mM NaCl) and centrifuged. For immunoprecipitation, the supernatants were incubated (2 h at 4ºC with rotation) with an amount of antibody beads equivalent to 1/10 of the commercial vial (PTMScan Pilot Acetyl-Lys Motif, CST14541T). The beads were washed twice with immunoprecipitation buffer, and three times with NH_4HCO_3 20 mM. Peptides were eluted from beads with 2 x 50 µL of 0.15% TFA by incubating for 10 minutes at room temperature. The eluted fractions were desalted with Mediterranean C18 (Teknokroma).

Phosphopeptide enrichment

Enrichment of phosphopeptides by Titanium oxide (TiO_2) was carried out using the non-retained fractions from the immunoprecipitation of acetylated peptides. Thus, these fractions were evaporated to dryness and resuspended in 600 µL of 1M glycolic acid, 80% ACN, 1% TFA. Three mg of titanium beads (Titansphere, GL Sciences, Japan) were added to each sample and they were incubated for 30 min at 20ºC with end-over-end rotation. Subsequently, samples were transferred to a Mobicol with 10 µm filter (Sigma) and the beads were washed 2 x 150 µl of 60% acetonitrile, 1% TFA. The elution of the phosphopeptides was done in a tube containing 10 µL of 20% HCOOH with 2 x 15 µl 5% NH_4OH followed by 2 x 15 µl of 25% acetonitrile , 14% NH_4OH.

RESULTS AND DISCUSSION

Combined shotgun analyses

A common strategy used to increase the coverage of samples analysed by LC-MS/MS is the use of multiple analysis of the same sample. Long chromatographic columns and extensive gradients can be used to reduce the peptide co-elution in the MS/MS selection window, avoiding pre-fractionation or multidimensional separation. Several works have been published using this strategy in order to reach the highest proteome coverage expending the minimum amount of protein and time (Iwasaki et al., 2010, 2012; Pirmoradian et al., 2013; Thakur et al., 2011; Yamana et al., 2013).

Using 60-cm long columns with gradients of up to 8 h, a single analysis provides of 4000 peptide identifications pointing to 1000 proteins (Figure 2). Additional injections increase these figures for a maximum of 10%, this increase diminishing with the number of injections accumulated. Third and following injections give less than 5% of new identifications in average, 27 new proteins for *B. hyodysenteriae* and 16 for *B. pilosicoli* were identified per injection. The same tendency was observed in peptides. This low number of new identifications from the third injection showed that 5 analyses were enough to characterize these samples.

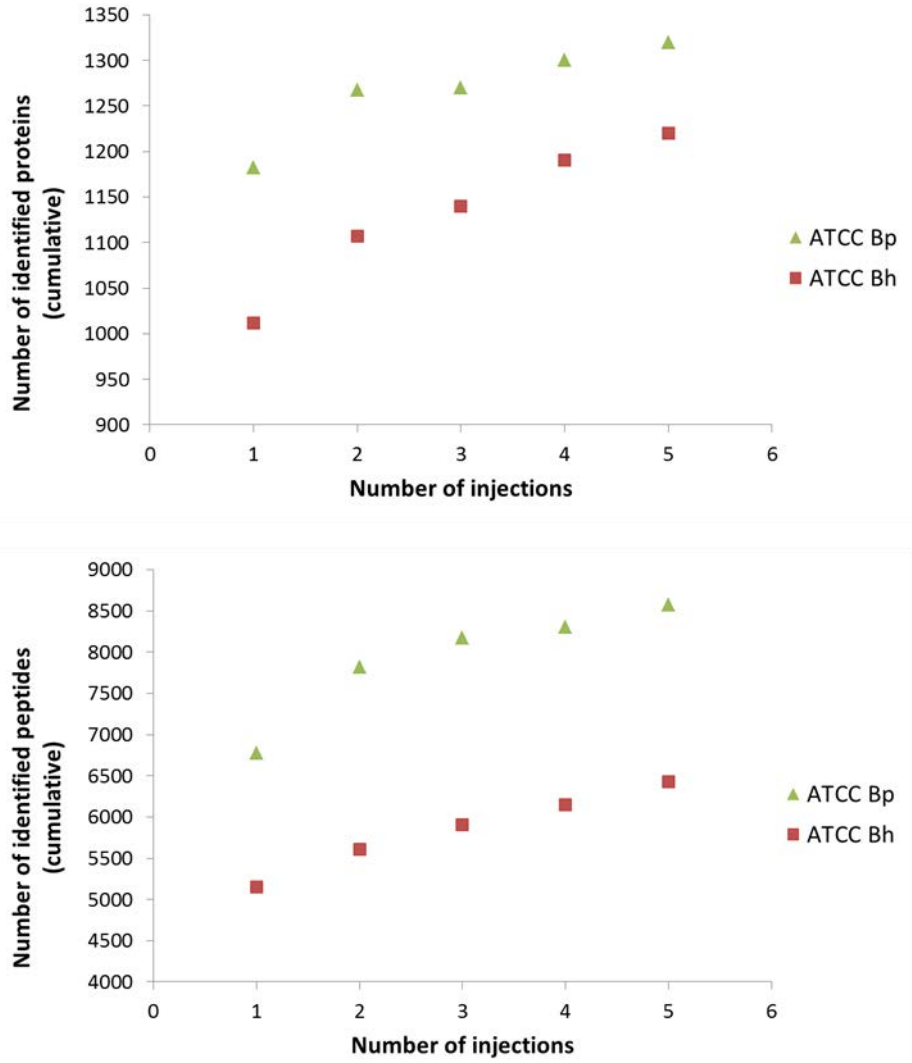


Figure 2: Increase in the total number of identified peptides and proteins with the number of analyses. Values were obtained by searching with PeptideShaker.

With this system we obtained a proteome coverage of 60-70% for *B. hyodysenteriae* and *B. pilosicoli*, respectively. This is in the same order that the coverage reported for the *E. coli* strain BW25113 (63% coverage) using only triplicate injections but much longer monolithic silica column (350 cm) and gradients (41 h) with an LTQ Orbitrap XL (Iwasaki et al., 2010). Considering only the 3 first replicates our system could provide a proteome coverage of 50-60% for *B. hyodysenteriae* and *B. pilosicoli*, respectively.

Database search

In a first step of our data analysis (Figure 1), *B. hyodysenteriae* spectra were confronted to the reference database for the WA1 strain. In the case of *B. pilosicoli*, the complete organism database was used. This database contains four different strains and is highly redundant (see Material and Methods). In fact, searches against this database produced less than 13% of peptide identifications pointing to individual proteins, making of protein reporting a major issue. PeptideShaker was selected for protein inference and data reporting over other available applications due to the flexibility and completeness of the tools provided for data analysis, including FDR filtering at different levels and its management of protein groups (Vaudel et al., 2015). For the analysis, PeptideShaker was used with 6 different search engines and quite restrictive criteria of validation to ensure high confidence results. From a total of 137,121 and 180,693 MS/MS spectra from *B. hyodysenteriae* and *B. pilosicoli*, respectively, a total number of 1220/1323 proteins and 6428/8573 peptides for *B. hyodysenteriae* and *B. pilosicoli* remained (Table 1 and Tables S3-S4). The FDR at the protein level for the set of protein identifications was 0.82% for *B. hyodysenteriae* and 0.98% for *B. pilosicoli.*

Table 1. Summary of the spectra analysed and the identifications performed using Peptide Shaker (PS) and PEAKS.

		B. hyodysenteriae	*B. pilosicoli*
PS	Total number spectra	137,121	180,693
	Protein groups	1220	1323
	Peptides	6428	8573
	Non-assigned spectra	61,081	66,975
PEAKS	Peptides no PTM/SAS	552	771
	Peptides with PTM/SAS	231	349

Those scans that did not give a positive identification in PeptideShaker were exported as mgfs and submitted to PEAKS. PEAKS workflow included steps for De Novo sequencing analysis, PTM identification and homology search, all combined with database search (Zhang et al., 2012).

A total of 61,081 spectra from *B. hyodysenteriae* and 66,975 from *B. pilosicoli*, 44% and 37% of the initial number respectively, were loaded into PEAKS workflow (Figure 1).PEAKS analyses recovered 2744 PSMs of the *B. hyodysenteriae* previously unmatched spectra, identifying a total number of 783 non redundant peptides, of which 231 had some type of PTM/SAS (single aminoacid substitution) in their sequence (Table 1, Table S5). Some of these peptides have a match in species different than *B. hyodysenteria*e. In order to check if they have homologue sequences in *B. hyodysenteriae* species, the sequences were searched in BLAST. The results showed that 61 of them only have a 100% homology with species different than *B. hyodysenteriae,* mainly with *B. pilosicoli* and 9SPIR (a nomenclature from the database that includes several *Brachyspira* species, such as *Brachyspira hampsonii*, *Brachyspira murdochii*, *Brachyspira alvinipulli* and others).Thus, from these 61 trans-species-only matches, 33% were peptides exclusive of 9SPIR, 15% of *B. pilosicoli*, 3% of *B. intermedia* and 2% of *B. murdochii*. Forty-seven percent of peptides pointed to various species.

In the same way, in *B. pilosicoli* dataset, 3399 PSMs previously unmatched spectra could be assigned to 1120 non-redundant peptide, 349 of them with some PTM or single-amino acid change in their sequence (Table 1, Table S6). Homology searches confirmed that 64 of them had 100% match with other *Brachyspira sp.* different than *B. pilosicoli*, 26% were peptides exclusive of 9SPIR,14% of *B. murdochii* and 60% were contained in proteins from various species .

Overall, PEAKS analyses added 311 and 216 new protein groups to the initial *B. hyodysenteriae* and *B. pilosicoli*, collections. Among these 216 new *B. pilosicoli* protein groups there are 116 that contain *B. pilosicoli* among their entries. In the other 100 protein groups, *B. hyodysenteriae* and *B. hampsonii* proteins are the most frequent species. In *B. hyodysenteriae* samples, we have identified proteins from *B. hyodysenteriae* species in 249 of 311 new protein groups from PEAKS. From these 249 new protein groups, 235 do not contain accessions from WA1, but from

Brachyspira hyodysenteriae BRAHO (accession identifier in the database from Uniprot). This denomination includes the strains B204, C5 and ATCC 27164.

Strain and species cross-matches could evidence the low curation level of these databases. Even for the reference proteome of *B. hyodysenteriae* (WA1) strain, a high number of assignations to other *Brachyspira* strains was found. In many cases, differences correspond to single amino acid changes. In other cases, sections of the protein are not annotated in the database. For instance, the peptide TQDVLFQYEGMPK was identified in the WA1 sample. However, this peptide belongs to the N-terminal sequence of the BHAHO protein A7LAV0. A7LAV0 is 100% homologue to the WA1 protein C0QVY4, but the sequence of the later is shorter and does not contain this peptide (Table S7). Overall 53 peptide sequences exclusive of BRAHO species were identified in the analysis of WA1 sample but not in the WA1 reference database (Table 2). Remarkably, 30% of these sequences are annotated in WA1 as the protein N-terminal sequence and with a Methionine as the first amino acid while the peptide identified correspond to the same sequence (keeping the M or substituting L(subM)) extended on that terminal. As these protein sequences are translated from genomic data, these results could point to DNA sequence errors or errors in the characterization of the actual codifying gene sequence.

Table 2. Some WA1 peptides identified exclusively in BRAHO database

PEPTIDE	STRAIN	%HITS	BLAST MATCH	MASS	START STOP	PROTEIN NAME
DMAALSSGMR	BRAHO	100	.....**DMAALSSGMR**.....	1037.46	26-35	Flagellar filament core protein flaB2
	WA1	90	----------**MAALSSGMR**.....	922.44	1-9	Flagellin
HMISLSEEEKEK	BRAHO	100	----------.....**HMISLSEEEKEK**.....	1458.7	7-18	Uncharacterized protein
	WA1	91	----------**MISLSEEEKEK**.....	1321.64	1-11	Uncharacterized protein
KDSAMISSGMR	BRAHO	100	.....**KDSAMISSGMR**.....	1181.55	25-35	Flagellin
	WA1	63	----------**MISSGMR**.....	780.36	1-7	Flagellin
LANKPISIPQGVEVK	BRAHO	100	----------...**LANKPISIPQGVEVK**.....	1591.93	4-18	50S ribosomal protein L6
	WA1	93	----------**MANKPISIPQGVEVK**.....	1609.89	1-15	50S ribosomal protein L6
NVDLSKDMAALSSGMR	BRAHO	100	-.....**NVDLSKDMAALSSGMR**.....	1693.81	20-35	Flagellar filament core protein flaB2
	WA1	56	----------**MAALSSGMR**.....	922.44	1-9	Flagellin
SMYSLLIEPILTEK	BRAHO	100	----------M**SMYSLLIEPILTEK**.....	1766.92	2-15	50S ribosomal protein L23
	WA1	92	----------**MYSLLIEPILTEK**.....	1548.85	1-13	50S ribosomal protein L23
TYLIALNQIDK	BRAHO	100	----------.....**TYLIALNQIDK**.....	1290.72	9-19	DNA processing protein DprA
	WA1	72	----------**MIALNQIDK**.....	1044.56	1-9	Putative DNA protecting protein DprA
VTKEELIQAVR	BRAHO	100	-----.....**VTKEELIQAVR**.....	1284.74	16-26	Ubiquinone biosynthesis protein
	WA1	90	----------**MTKEELIQAVR**.....	1316.71	1-11	Uncharacterized protein
YIMNYYYK	BRAHO	100	.....**YIMNYYYK**.....	1156.53	42-49	30S ribosomal protein S3
	WA1	75	----------**MNYYYK**.....	880.38	1-6	30S ribosomal protein S3

%HITS: percentage of peptide homology (BLAST) in BRAHO and WA1 databases.
BLAT MACH: sequence with higher homology identified by BLAST in each strain database.
MASS: protein molecular mass.
START, STOP: start and end positions, aminoacids, of the peptide inside the protein sequence.

Peptides De Novo only

In addition of proteins identified in the database, PEAKS provides a set of non-database-matching peptide sequences obtained De Novo (Figure 1). This set includes SAS sequences as well as sequences with PTMs. This dataset was strictly filtered (70% average local confidence (ALC), 90% tag confidence and 5 ppm) to increase the confidence on the sequences and manually corrected for a software bug (described in Material and Methods).

Thus, from the selected 1637 tags from *Brachyspira hyodysenteriae*, 151 matched with proteins in the *Brachyspira* database, 63 of them only with species different than *B. hyodysenteriae* (Table S8). Similarly, from the 1504 tags of *B. pilosicoli*, 170 tags matched against *Brachyspira* proteins, 70 only with species different than *B. pilosicoli* (Table S9).

The remaining De Novo only sequences were BLASTed against a mammal database (Figure 1).This search gave us 423 peptides from mammals (279 and 144 peptides identified in *B. hyodysenteriae* and *B. pilosicoli* samples, respectively). Most of these peptides correspond to plasma proteins indicating that many of the initially unmatched spectra correspond to contaminations coming from the blood agar *Brachyspira* cultures (Table S10).

Post-translational modifications and SAS

Microbes replicate at a fast pace and mutations are a way of evolution and adaptation to new conditions, including exposure to antimicrobials (National Institute of Allergy and Infectious Diseases). Most mutators (strains with a high mutation rate) are isolated from pathogenic bacteria, and have been related with pathogenicity and antibiotic resistance (Denamur and Matic, 2006). Although much less studied in bacteria, something similar occurs with PTMs. Through PTMs, pathogens are able to manipulate pathways in the host organism, increasing their probabilities of survival (Broberg and Orth, 2010; Ribet and Cossart, 2010) and adapting themselves to different environments (Bernal et al., 2014; Cain et al., 2014).

PEAKS search gives 231 peptides in *B. hyodysenteriae* and 349 in *B. pilosicoli* with PTMs or SAS in their sequence. A summary of all confident SAS identified

(alterations with respect to available sequence on database) are listed in Table S11. A total number of 50 classes of SAS have been detected, the most frequent changes common to both species correspond to SNP (single nucleotide polymorphism), the D (subN) (22 peptides), which is a conservative change, and S (subA) (total 4 peptides). In this dataset, other 4 SNPs have been identified; H (subQ), D (subE), N (subK) and M (subI) (see Table S5 and S6, column "SNP_score"). Further information on PEAKS SAS for these samples can be found in Document S1.

Methylation was the more frequent PTM observed in these bacteria (Table 3). Other frequent modifications included phosphorylation, oxidations and acetylation. Interestingly, when comparing the two species, acetylation seems to be exclusive of *B. pilosicoli*.

Table 3: Summary of identification features for the more frequently detected PTM in the direct analysis and after the enrichment procedures.

		B. hyodysenteriae		*B. pilosicoli*	
	PTM	**Peptides**	**PSMs**	**Peptides**	**PSMs**
Direct analysis	Methylation	26	111	46	132
	Hydroxylation	5	31	6	19
	Dihydroxylation	19	82	3	4
	Oxidation	15	53	5	9
	Phosphorylation	1	5	1	4
	Acetylation K	0	0	13	51
Enrichment	Acetylation K	3142	5393	5496	12471
	Phosphorylation	74	499	86	305

- Methylation, oxidation and phosphorylation

PTMs were almost unknown in prokaryotic microorganisms until the development of mass spectrometry. In the recent years with the huge advance in some technologies, it has been possible to prove that PTMs have a big influence in bacterial physiology

and virulence (Cain et al., 2014). Although new PTMs are continuously added to the list of bacterial PTMs, the most important, taking into account the role they play in microorganisms, are phosphorylation, acetylation and methylation (Cao et al., 2010).

Methylation was the most frequent modification observed. In *Brachyspira hyodysenteriae,* 26 methylated peptides pointing to a total of 72 proteins were identified. Proteins of identical sequence or description were grouped to produce 14 protein groups. This dataset was enriched in proteins related to movement, flagella or chemotaxis in agreement with that reported for the methylome of *Leptospira* (Cao et al., 2010). For *Brachyspira pilosicoli,* 46 methylated peptides pointed to 180 protein accessions that could be classified in 50 protein groups. Differently to *B. hyodysenteriae* the most important group of methylated proteins corresponded to enzymes.

Other abundant PTMs detected were modifications by oxidation, hydroxylation and dihydroxylation (Tables 3, S5, and S6). This group of oxidative modifications is enriched in external proteins, especially in *B. hyodysenteriae*, where from 39 peptides (pointing to 233 non-redundant protein accessions) 62% of proteins with known cellular location were annotated as outer membrane, periplasmic space or plasma membrane. In *B. hyodysenteriae*, the same site was detected with different oxidative PTMs. For instance, the sequence VGFAGESDYQIWDPIVAK (from lipoprotein C0R281) has been identified oxidized (+15.99), dihydroxylated (+31.99) and oxidized to kynurenin (+3.99) at W_{44}. Something similar has been observed in LILATGSWPVTPPIEGLK and SISSWVYGR, which W residues (C0QXS8-115 and C0R0T5-149) have been both detected dihydroxylated and oxidized to kynurenin. It has been reported the relationship between protein oxidation to kynurenin-like forms and the decrease of neutrophil killing activity in *S. aureus*, due to the necessity of oxidised species for phagocytes to carry out their microbiocidal activity and the interference that other molecules susceptible of being oxidised can suppose (Silva et al., 2006).

The number of phosphorylated and acetylated residues was relatively low in the direct analysis of samples. Only two phosphorylated peptides could be identified (one for each species). After the TiO_2 enrichment, 74 and 86 phosphorylated peptides could be identified in *B. hyodysenteriae* and *B. pilosicoli* (Table S12). In *B.*

hyodysenteriae, 315 of the total 499 PSMs obtained corresponded to a single peptide GIKPEEAGDAQIAAISGATISSTSVVNAVNSAK of the Electron transport complex subunit G protein (C0R204).This protein, which was detected in low abundance in the total proteome (35 PSMs), is a membrane protein involved in the binding with Flavin mono nucleotide and in the electron transport chain. The potential role of the basal phosphorylation of Thr-167 in this protein is unknown.

In *B. pilosicoli*, the top three most phosphorylated proteins are GAPDH, PEPCK and enolase (30, 28 and 25 phosphorylated PSMs, respectively). When a functional enrichment analysis was performed (STRING vs 10.0, confidence level highest 0.9), no enrichment was observed in *B. hyodysenteriae* phosphorylated proteins but in *B. pilosicoli* enrichment in 10 different KEGG pathways was detected, being "metabolic pathways" the one with more gene counts.

- *Brachyspira's* acetylome

Interestingly, K-acetylation seemed to be exclusive of *B. pilosicoli* in the direct analysis of samples. A targeted analysis of the homologous counterparts in *B hyodysenteriae* of the acetylated sites detected in *B. pilosicoli* was performed to confirm this trait (see Table S1). Two of the acetylated sequences in *Brachyspira pilosicoli* TSTSNVGK(+42.01)AEVGK and VIISAPGK(+42.01)GDLR, had identical counterparts in *Brachyspira hyodysenteriae*. These sequences were searched both acetylated and de-acetylated. For target selection, it was taken into account the different cleavage points between the forms (the Trypsin K cleavage site is protected by acetylation) (Table S1). For both peptides, the targeted analysis confirmed the presence of the acetylated form only in *B. pilosicoli* (Figure S2). The existence of the unmodified form of VIISAPGK could be confirmed in both species (Figure S2_A), suggesting that differences observed for this peptide were due to differences in protein modification rather than protein expression. Expression of the corresponding form of the TSTSNVGK(+42.01)AEVGK peptide in *B hyodysenteriae* could not be confirmed (Figure S2_B).

The same results were obtained for sites in homologous sequences of B*. hyodysenteriae* and *B pilosicoli* proteins (Table S13). For example, *B. pilosicoli* sequence AVGEVLPVTKGK was detected acetylated (AVGEVLPVTKGK) and de-

acetylated (AVGEVLPVTK) while the corresponding moiety in *B. hyodysenteriae* (AVGEVLPATKGK) was only found non-acetylated (AVGEVLPATK).

Ratio of acetylated to non-acetylated forms was very low in all cases, ranging from 124:1 for sequences LLITCPLNWK/LLITCPLNWKTPDDMGK to 752:1 for sequences AVGEVLPVTK/AVGEVLPVTKGK in agreement with the low stoichiometry commonly expected for PTMs and underlying the importance of PTM enrichment for a more complete characterization of the modified entities in the proteome.

The predominance of acetylation in *B. pilosicoli* was further confirmed in several different *Brachyspira* isolates by Western-blot using antibody anti-acetyl Lysine (Figure 3). These analyses showed strong acetylated bands in all *B. pilosicoli* strains, while only in the case of the *B. hyodysenteriae* LL1 strain a weak band could be detected around 75kDa.

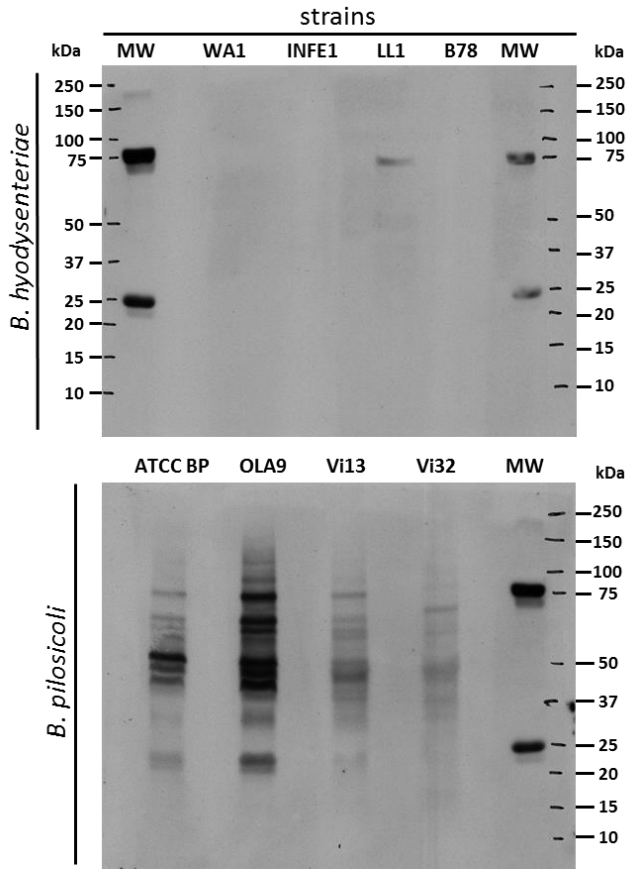


Figure 3. Western blots prepared with anti-acetyl Lys antibody of different strains for the two *Brachyspira* species. 30 seconds of exposition

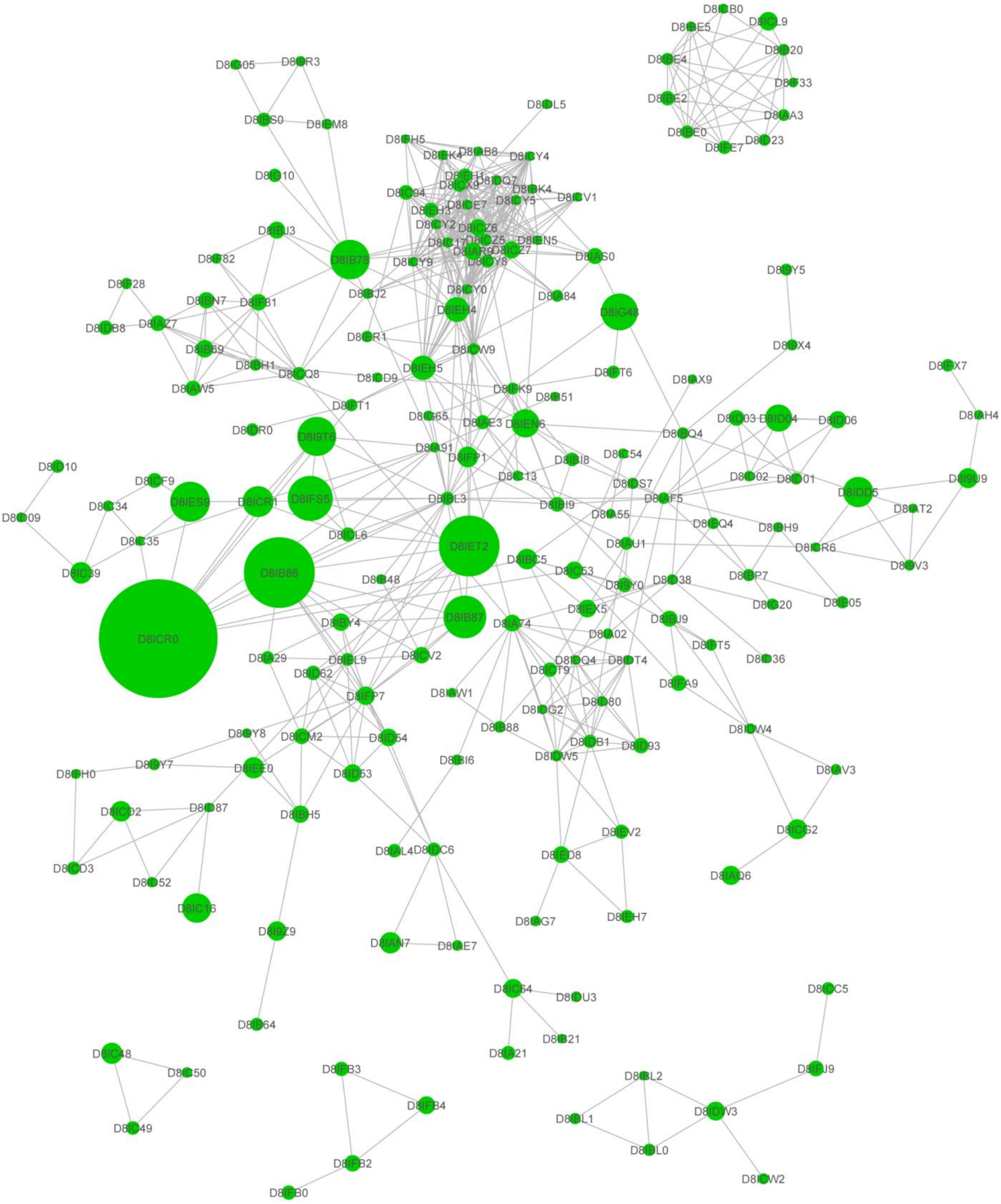
To determine the relative acetylation levels of proteins in these species, acetylated peptides were immunopurified with an anti-acetyl Lysine antibody and enriched and non-enriched samples were analyzed by shotgun proteomics on a LTQ Orbitrap XL system. Information for the full proteome, obtained from non-enriched samples, was used to normalize the acetylation data relative to the expression levels observed for the corresponding proteins (estimated from the total number of PSMs). The analysis of enriched samples provided of 730 acetylated peptides in *B. hyodysenteriae* and 2848 in *B. pilosicoli* .These results confirmed the higher acetylation level observed in *B. pilosicoli*. The semiquantitative analysis showed that proteins with a high acetylation ratio (normalized value higher than 200) were more frequent in *B. pilosicoli.* Only the protein Beta-galactosidase was found acetylated in both species with similar values of normalized acetylation (Table S14).

For a more exhaustive characterization of the acetylome in these species, enriched samples were analyzed with higher sensitivity and resolution in an Orbitrap Fusion

Lumos mass spectrometry system. The total number of acetylated peptides was 3142 (of 4645 peptides) for *B. hyodysenteriae* and 5496 (of 6548 peptides) for *B. pilosicoli* (Table S15). These values suppose an enrichment efficiency of 68-84% for *B. hyodysenteriae* and *B. pilosicoli*, respectively, while in the LTQ Orbitrap XL was 53-83%. The increase in the enrichment for *B. hyodysenteriae* with the more sensitive Orbitrap Fusion Lumos could be explained because of the low acetylation levels in this species that requires higher sensitivity to detect its minor components. This is supported by the fact that the number of *B. hyodysenteriae* acetylated peptides which were detected with only 1 PSM, at the highest sensitivity, was increased by 9-fold while for *B. pilosicoli* were increased by only 3-fold (Table S15).

The ratio of acetylated peptides between *B. pilosicoli* and *B. hyodysenteriae* is 1.7, but this figure increases to 2.3 when compare the number of acetylated PSMs (12471 in *B. pilosicoli* versus 5393 in *B. hyodysenteriae*).This corroborates the fact observed through the normalized data of acetylation, where not only a higher number of acetylated proteins was observed for *B. pilosicoli*, but also acetylation levels were higher. A total number of 3221/5579 high confidence acetylation sites were identified (ptmRS), for *B. hyodysenteriae* and *B. pilosicoli*, respectively. High abundant acetylation has been observed in these species, this agrees with several works where acetylation is one of the most prominent PTM in bacteria (Bernal et al., 2014; Cain et al., 2014)

The two sets of acetylated proteins were found enriched in proteins involved in metabolic pathways and biosynthesis of secondary metabolites (KEGG pathway descriptions, String v10.0, score 0.9). This was in agreement with that observed for *Salmonella enterica*, where the 90% of enzymes of central metabolism are acetylated (Wang et al., 2010)(Figures 4 and S3).


D8ICB0
D8ICL9
D8IBE5
D8IB20
D8IBE4
D8IF33
D8IBE2
D8IAA3
D8IBE0
D8IFE7
D8ID23
D8IG05
D8I9R3
D8IBS0
D8IEM8
D8IDL5
D8IFH5
D8IAB8
D8IEK4
D8ICY4
D8IEH1
D8IDQ7
D8ICX9
D8IC94
D8IBK4
D8ICV1
D8IEH3
D8ICE7
D8ICY5
D8IG10
D8IBU3
D8ICY2
D8ICZ6
D8IF82
D8IC17
D8ICZ5
D8IEN5
D8IAR9
D8ICZ7
D8IB78
D8ICY9
D8ICY8
D8IAS0
D8I9Y5
D8IF28
D8IBJ2
D8ICY0
D8IA84
D8IBN7
D8IF81
D8IAZ7
D8IDB8
D8IEH4
D8IG48
D8IER1
D8IB69
D8ICW9
D8I9X4
D8IBH1
D8IEH5
D8IFX7
D8ICQ8
D8ICD9
D8IFT6
D8IAW5
D8IAX9
D8IFK9
D8IB51
D8IFT1
D8IG65
D8IAE3
D8IEN6
D8ID03
D8ID04
D8ID06
D8IAH4
D8IDR0
D8I9T6
D8IBQ4
D8IA91
D8IFP1
D8IBI8
D8IC54
D8IC13
D8ID02
D8ID01
D8ID10
D8ICF9
D8IDS7
D8I9U9
D8IES9
D8ICR1
D8IFS5
D8IBL3
D8IAF5
D8IDD5
D8IC34
D8IBI9
D8IAT2
D8ID09
D8IA55
D8IEQ4
D8IBH9
D8ICL6
D8IC35
D8IAU1
D8ICR6
D8IET2
D8IC39
D8IBC5
D8IB86
D8IC53
D8I9V3
D8IB48
D8I9Y0
D8ID38
D8IBP7
D8IG20
D8IE05
D8IEX5
D8IB87
D8IBY4
D8IA74
D8IBU9
D8ICR0
D8IA02
D8IFT5
D8IA29
D8IEL9
D8ICV2
D8ID36
D8IDQ4
D8IDT4
D8ID62
D8ICT9
D8IFA9
D8IFP7
D8IAW1
D8ID80
D8IDG2
D8IB88
D8IDW4
D8I9Y8
D8ICM2
D8ID54
D8IDB1
D8ID93
D8IDW5
D8IBI6
D8IFH0
D8I9Y7
D8IEE0
D8ID53
D8IAV3
D8ICD2
D8ID87
D8IBH5
D8IEV2
D8ICG2
D8IAL4
D8IDC6
D8IED8
D8ICD3
D8ID52
D8IAQ6
D8IC16
D8IAG7
D8IEH7
D8I9Z9
D8IAN7
D8IAE7
D8IC64
D8IDU3
D8ICC5
D8IB64
D8IB21
D8IA21
D8IC48
D8IC50
D8IFB3
D8IFU9
D8IBL2
D8IFB4
D8IDW3
D8IBL1
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D8IFB2
D8ICW2
D8IFB0

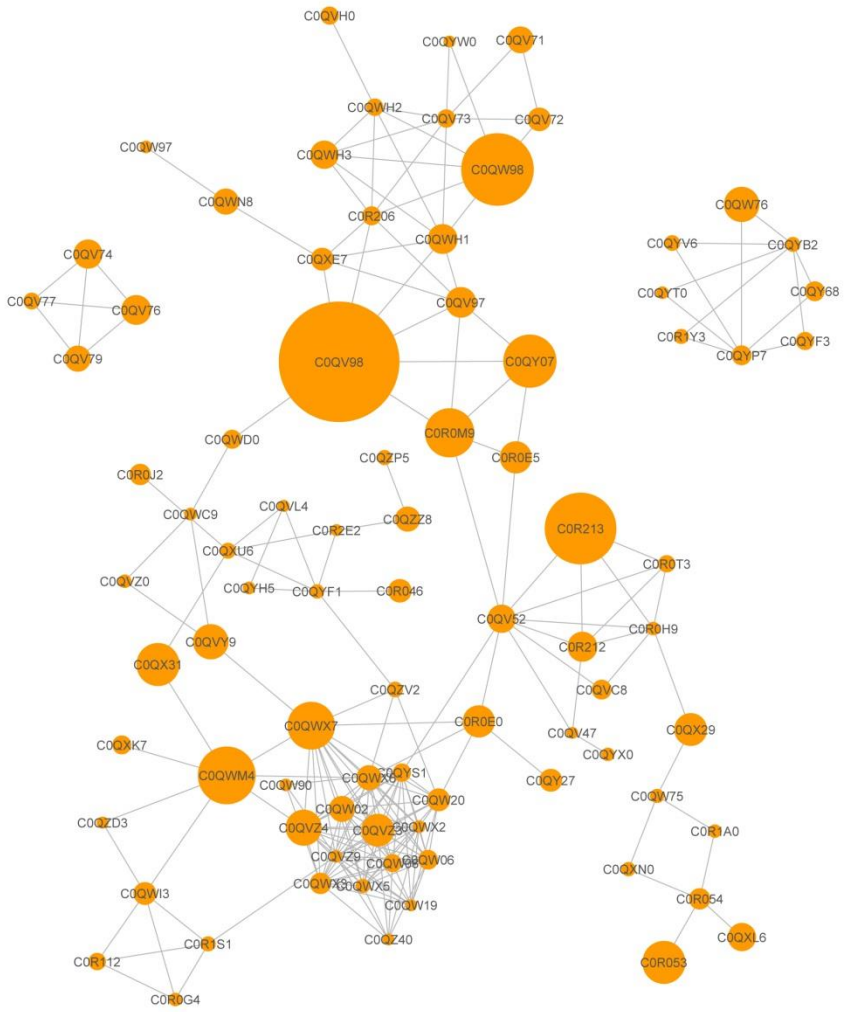


Figure 4. Network of acetylated proteins detected for *B. pilosicoli* (green) and *B. hyodysenteriae* (orange) in Orbitrap Fusion Lumos. The node size is proportional to the number of acetylated PSMs detected for the protein, proteins with less than 10 PSMs acetylated were filtered out (those filtered at 5 PSMs are available in Figure S3). Networks generated with Cytoscape v3.0.

GAPDH was one of the proteins detected with a higher number of PSMs acetylated for both species; the normalized acetylation values (acetylated PSMs/total PSMs) for this protein were 0.22 and 0.58 for *B. pilosicoli* and *B. hyodysenteriae*, respectively. In *B. pilosicoli* it has 31 different acetylation sites; the same protein in *B.*

hyodysenteriae has 20 acetylation sites. This protein was also detected acetylated in *Salmonella* and *R. palustris* (Bernal et al., 2014). It was classified as virulence factor for several microbial pathogens, for instance the gram negative *Pseudomonas syringae* (Elkhalfi et al., 2014).

Another highly acetylated protein was enolase. This protein has also been detected acetylated in other bacteria (Ouidir et al., 2016). Despite being a typical cytoplasmic enzyme, it has been detected exposed on the surface of different pathogenic species. It has been included in a list of vaccine candidates against *Mycobacterium tuberculosis* (Rahi et al., 2017; Salazar et al., 2017; Wang and Jeffery, 2016). *B. hyodysenteriae* and *B. pilosicoli e*nolases were found immunoreactive against sera obtained from infected pigs and thus proposed as vaccine candidate for these species (Casas et al., 2017).

Enolase in the bacterial surface is involved in the host-plasminogen binding. The sequence which binds to plasminogen is well characterized for several bacteria, including the spirochete *Leptospira interrogans* (Salazar et al., 2017). The homologous binding site of the *B. pilosicoli* enolase contains two Lysines, K_{251} and K_{253}, which were found acetylated in our study (ptmRS confidence higher than 99%) (Figure 5).

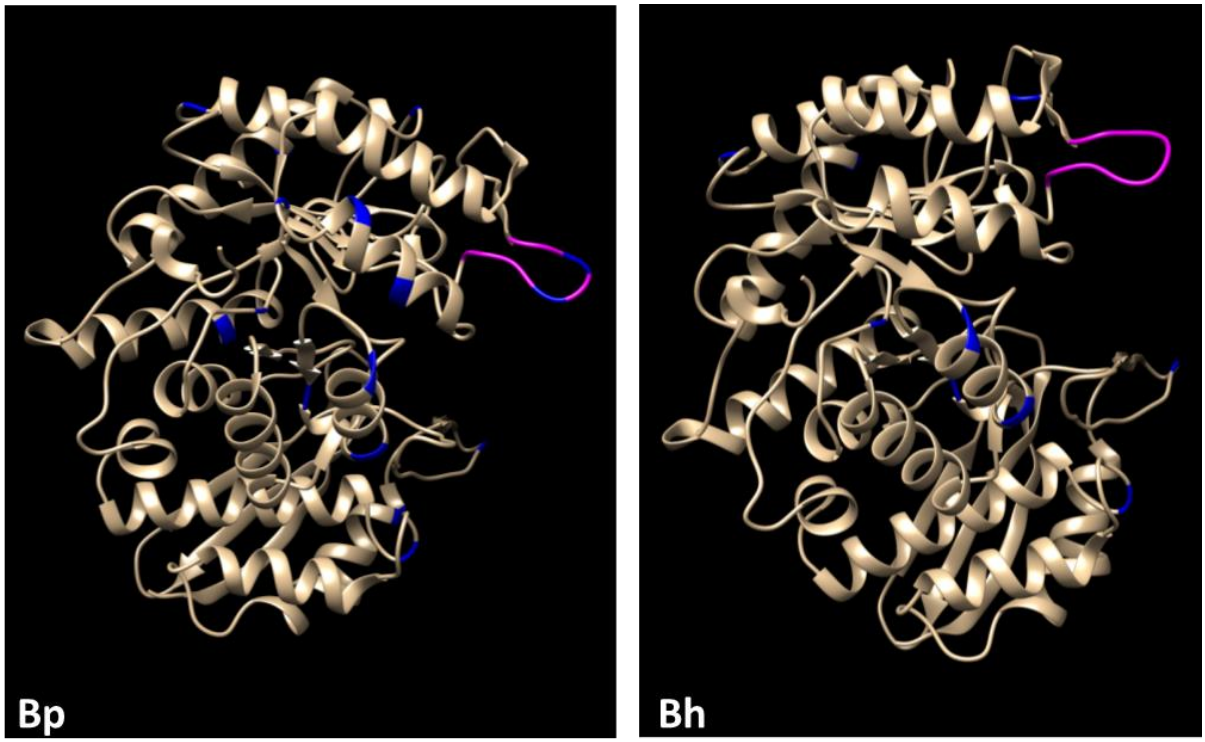


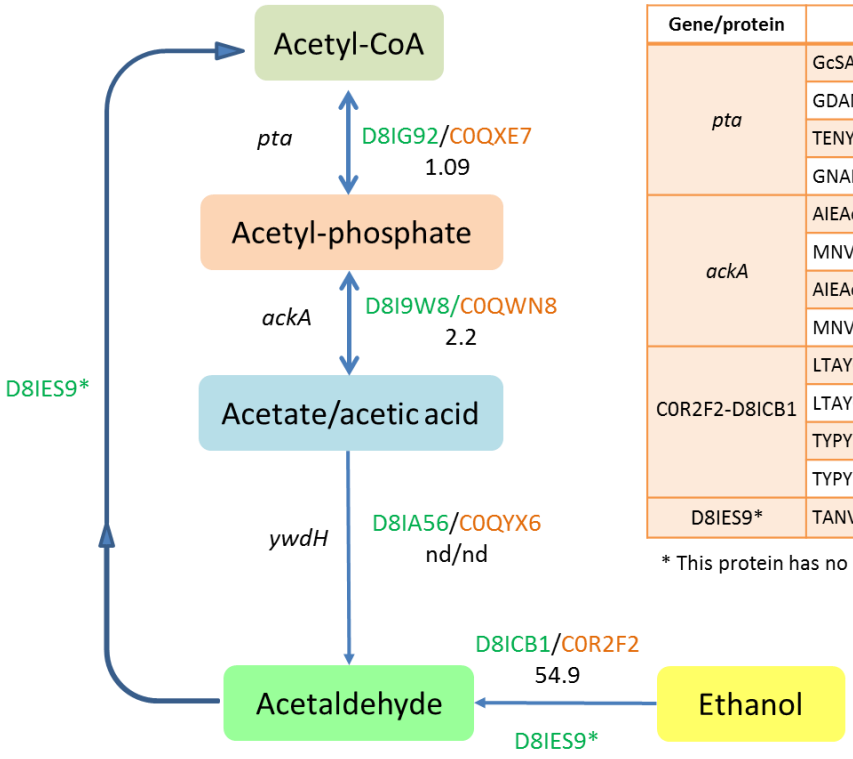
Figure 5: High confident acetylation sites (blue) detected in enolase from *B. pilosicoli* (Bp) and *B. hyodysenteriae* (Bh). The binding site to plasminogen is labeled in pink.

These sites are analogous to those of the enolase from *E.coli* (K_{254} and K_{257}) that were described as acetylation sites glucose/time dependent, no Acetyl-phosphate (ACP) dependent (Schilling et al., 2015). Interestingly, these sites are not present in *B. hyodysenteriae*. Enolase acetylation could affect the binding to plasminogen which is known to occur mainly through the Lysine residues (Nogueira et al., 2013). This could be related to a more effective binding of *B. hyodysenteriae* molecules to plasminogen and its subsequent conversion to plasmin and the start of the cascade which ends with the spread of bacteria within the host's tissues. This finding would agree with the different mechanisms of adherence and virulence observed for both species.

Acetylation and metabolism

Soufi et al. reported an increase in the acetylation level of *E. coli* due to ethanol stress (Soufi et al., 2015). It is known that *B. pilosicoli* produces ethanol to the media during bacterial growth. Also it has a specific enzyme (D8IES9) capable of converting ethanol to acetaldehyde and Acetyl-CoA (Figure 6). With the aim of going

deeper into the differential acetylation observed between both species, the proteins involved in the acetylation/de-acetylation process were monitored by target mass spectrometry analysis (Table S2 and Figure 6).



Gene/protein	peptide	area	sp
pta	GcSAQDIADLAVITSLQAK	919633	hyo
	GDANVFIFPELNSGNIGHK	1938388	hyo
	TENYIELLFK	792187	pilo
	GNANVLIFPNLNAGNIGYK	2332185	pilo
ackA	AIEAccDIAPLHNPAGLQGISAcK	3936019	hyo
	MNVLVINSGSSSIK	573747	hyo
	AIEAccDIAPLHNPAGLQGIR	8842965	pilo
	MNVLVINSGSSSIK	1071903	pilo
C0R2F2-D8ICB1	LTAYQGFDALFHSTEGYINK	8756	hyo
	LTAYQGFDALFHSTEGYINK	739971	pilo
	TYPYLSIVDPELMK	16932	hyo
	TYPYLSIVDPELMK	671581	pilo
D8IES9*	TANVFEPDVIIALGGGSPIDAGK	477014	pilo

* This protein has no homologue in hyo.

Figure 6: Acetyl-CoA/Acetate metabolic pathway (left) and MS identification results for the proteins involved (right). *B. hyodysenteriae* and *B. pilosicoli* accessions in orange and green, respectively. When the protein was described for both species the ratio of areas detected is indicated. From KEGG pathways (Kanehisa et al., 2017). sp. species.

The *pta* gene (proteins D8IG92 and C0QXE7 for *B. pilosicoli* and *B. hyodysenteriae*, respectively) was found expressed at the same level in both species. However, *ackA*, the gene that interconverts Acetyl-phosphate (ACP) and acetate was found 2-fold more expressed in *B. pilosicoli.* During the glycolysis, in the conversion of glucose to acetate, the lack of this enzyme increases the proteome acetylation level, because acetylation is related with the production of ACP (Schilling et al., 2015; Weinert et al., 2013). The Iron-containing alcohol dehydrogenase D8ICB1/C0R2F2 (accessions for *B. pilosicoli* and *B. hyodysenteriae*, respectively), was 50-fold more expressed in *B. pilosicoli*. This protein is responsible of oxidizing alcohol to aldehyde. This action is also carried out by the protein Aldehyde-alcohol dehydrogenase,

D8IES9, which is involved in a metabolic route exclusive of *B. pilosicoli*, the production of Acetyl-Coa from ethanol and acetaldehyde. This protein had been detected previously, in the study of the exposed proteome for these *Brachyspira* species (Casas et al., 2016). In that work, D8IES9 was more abundant in the exoproteome compartment and although it was detected in the two *B. pilosicoli* strains included in the study (OLA9 and Vi13), its expression was higher in OLA9 (569:165 PSMs). The higher expression of D8IES9 in this strain correlates with its higher acetylation levels, suggesting that this protein and/or the differentially expressed D8ICB1 could be related with the higher acetylation observed in *B. pilosicoli* species (Figure 3).

Ethanol is produced in small amounts only by *B. pilosicoli* and although both species produce large amounts of acetate, *B. pilosicoli* generates almost double than *B. hyodysenteriae* (Stanton and Lebo, 1988; Trott et al., 1996). A higher production of Acetyl-CoA and ACP by part of *B. pilosicoli* due to the conversion of ethanol and acetate present in the media through the action of differentially expressed proteins (D8ICB1, D8I9W8, D8IES9) could explain the acetylation ratio detected.

Proteome coverage

The initial report on the genome of the reference strain WA1 identified 2122 protein coding genes (Bellgard et al., 2009). This number increased up to 2613 in subsequent analyses (Mappley et al., 2012; Wanchanthuek et al., 2010). Here we use an average (2260 protein coding genes) in order to estimate the proteome coverage. Our search workflow combining PeptideShaker and PEAKS gives 1531 protein groups for the *B. hyodysenteriae* strain WA1; i.e. 67% of proteome coverage.

In the same way an estimation of the proteome coverage for *Brachyspira pilosicoli* strain P43/6/78 was obtained. For this strain only one reference reporting the number of protein coding genes is available (Lin et al., 2013), so the number proposed in this work (2208 protein coding genes) was used for calculation. The workflow developed in the present work gives 1539 protein groups for *Brachyspira pilosicoli* P43/6/78 strain; this is 70% of proteome coverage for this specie.

This range of proteome coverage is similar to those reported for other bacteria such as *E. coli* (63%) (Iwasaki et al., 2010),*S. pneumoniae* (63%) (Bittaye and Cash, 2015), or *L. interrogans* (51% (Malmström et al., 2009), 64% (Cao et al., 2010)) .

By searching with PeptideShaker (using each species database) 1220 and 1323 protein groups of *B. hyodysenteriae* and *B. pilosicoli* were identified, this is 53% and 60% of proteome coverage. Adding PEAKS software to search workflow, we incorporate 311 and 216 new protein groups for *B. hyodysenteriae* and *B. pilosicoli*, respectively, which is a 20-14% of the total number of identifications. These new proteins groups only identified by PEAKS come mainly from peptides with PTM in their sequence (101 and 64 for *B. hyodysenteriae* and *B. pilosicoli*) or from species different than the one analysed (62 and 100 for *B. hyodysenteriae* and *B. pilosicoli*).These trans-species-only matches had been reported previously for these species (Casas et al., 2016). It is important to notice that 235 of 311 new protein groups identified by PEAKS for WA1 reference strain are proteins of the database identifier BRAHO.

Thus, the combination of search software enables to go into detail in these species proteome. In a first step with PeptideShaker and each species database, we identified the main part of proteins/peptides in a fast and straightforward way. Applying PEAKS software with total *Brachyspira* database to those spectra non-identified by PeptideShaker, we can identify a wide range of PTMs and SAS. This is quite useful when working with species whose protein databases are mainly theoretical and predicted. As our dataset shows, including PTMs and searching homology with other species proteins we increase the proteome coverage of each species between 14-10% (*B. hyodysenteriae* and *B. pilosicoli*, respectively).

CONCLUSIONS

The alliance of PeptideShaker and PEAKS software proved to be highly efficient, providing a collection of high confidence identifications, including other sequence traits such as post-translational modifications (PTMs) and single aminoacid substitutions (SASs). Moreover, this workflow contains De Novo peptides, which is very important information for this kind of species with a high rate of genomic

variability among strains. By this methodology, proteome coverage of 67% for *Brachyspira hyodysenteriae* and 70% for *Brachyspira pilosicoli* was reached.

Although in the past PTMs were almost neglected in prokaryotic organisms, during the last years have become relevant for bacteria. Pathogenicity and adaptation to media are concepts that may be related with sequence modifications. Among the most abundant PTMs in the shotgun approach for these species are methylation and acetylation.

One significant finding regarding to acetylation in *Brachyspira* species was the higher level detected in *B. pilosicoli*. This fact was initially observed in direct analysis and after that confirmed by target mass spectrometry. Moreover, the immunoblotting with anti-acetyl Lysine antibody of several *Brachyspira* strains (including commercial ATCCs and isolates) showed that the *Brachyspira pilosicoli* ones were highly acetylated while in *Brachyspira hyodysenteriae* the signal was almost insignificant. All these observations were reinforced by the analysis of enriched samples by immunopurification which showed that acetylated PSMs were 2.5 times higher in *B. pilosicoli* than in *B. hyodysenteriae*.

A target mass spectrometry study focus on the Acetyl-CoA/Acetate metabolism involved in acetylation showed that Acetate kinase, Iron-containing alcohol dehydrogenase and Aldehyde-alcohol dehydrogenase were expressed in higher amounts in *B. pilosicoli* than in *B. hyodysenteriae*. These proteins are related to the conversion of acetate and ethanol to ACP and Acetyl-CoA This fact could correlate the different composition of products generated by these species to the media with the acetylation ratio detected between both species.

In summary, this is the first work addressed to characterize the total proteome of *Brachyspira* species, giving experimental evidence of proteins expressed, in addition to full profile of PTMs, SAS, acetylome and phosphoproteome

Supporting Information Available

Tables_S1_S2.xlsx, Mass list for target analysis of selected acetylated proteins**.**

Table_S3.xlsx, Results obtained from PeptideShaker for *B. hyodysenteriae* sample.

Table_S4.xlsx, Results obtained from PeptideShaker for *B. pilosicoli* sample.

Table_S5.xlsx, Results obtained from PEAKS for *B. hyodysenteriae* sample.

Table_S6.xlsx, Results obtained from PEAKS for *B. pilosicoli* sample.

Table_S7.xlsx, BLAST results obtained for peptides TQDVLFQYEGMPK and LDSLEFGK.

Table_S8.xlsx, DeNovo only results obtained from PEAKS and corrected for *B. hyodysenteriae* sample.

Table_S9.xlsx, DeNovo only results obtained from PEAKS and corrected for *B. pilosicoli* sample.

Table_S10.xlsx, BLAST matches of De Novo only peptides (PEAKS software) searched against mammals database. Results for *B. hyodysenteriae* and *B. pilosicoli* samples.

Table_S11.xlsx, SASs results (PEAKS).

Table_S12.xlsx, Phosphorylated peptides detected in TiO_2 enriched samples (Proteome Discoverer v1.4).

Table_S13.xlsx, Results of acetylation target analysis (Proteome Discoverer v1.4).

Table_S14.xlsx, Acetylated proteins normalized ratios for *B. hyodysenteriae* and *B. pilosicoli*.

Table_S15.xlsx, Results for acetylated enriched samples analysed in LTQ Orbitrap XL and Orbitrap Fusion Lumos (Proteome Discoverer v2.1).

Table_S16.xlsx, Blast result for SAS peptides (assigned by PEAKS) classified as non-confident.

Document_S1.docx, PEAKS SASs study.

Figure S1: A case of PEAKS De Novo only peptide annotated with PTMs.

Figure S2: Chromatograms (left) and selected retention time spectra (right) obtained in acetylation target analysis for the homologue peptides VIISAPGK (A) and TSTSNVGKAEVGK (B) in *B. pilosicoli* and *B. hyodysenteriae***.**

Figure S3: Network of acetylated proteins detected for *B. pilosicoli* (A) and *B. hyodysenteriae* (B) in Orbitrap Fusion Lumos. The node size is proportional to the number of acetylated PSMs detected for the protein, protein with less than 5 PSMs acetylated were filtered out. Networks generated with Cytoscape v3.0

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4. Paper#2
Exposed proteome

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The Exposed Proteomes of *Brachyspira hyodysenteriae* and *B. pilosicoli*

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Brachyspira hyodysenteriae and *Brachyspira pilosicoli* are well-known intestinal pathogens in pigs. *B. hyodysenteriae* is the causative agent of swine dysentery, a disease with an important impact on pig production while *B. pilosicoli* is responsible of a milder diarrheal disease in these animals, porcine intestinal spirochetosis. Recent sequencing projects have provided information for the genome of these species facilitating the search of vaccine candidates using reverse vaccinology approaches. However, practically no experimental evidence exists of the actual gene products being expressed and of those proteins exposed on the cell surface or released to the cell media. Using a cell-shaving strategy and a shotgun proteomic approach we carried out a large-scale characterization of the exposed proteins on the bacterial surface in these species as well as of peptides and proteins in the extracellular medium. The study included three strains of *B. hyodysenteriae* and two strains of *B. pilosicoli* and involved 148 LC-MS/MS runs on a high resolution Orbitrap instrument. Overall, we provided evidence for more than 29,000 different peptides pointing to 1625 and 1338 different proteins in *B. hyodysenteriae* and *B. pilosicoli*, respectively. Many of the most abundant proteins detected corresponded to described virulence factors and vaccine candidates. The level of expression of these proteins, however, was different among species and strains, stressing the value of determining actual gene product levels as a complement of genomic-based approaches for vaccine design.

Keywords: shotgun proteomics, *Brachyspira*, surface proteins, virulence factors, membrane shaving

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INTRODUCTION

The genus *Brachyspira* (previously *Treponema*, *Serpula*, and *Serpulina*) includes several pathogenic species affecting humans and other animals such as pigs, dogs, and birds. In pigs, *Brachyspira hyodysenteriae* and *Brachyspira pilosicoli* are well-known intestinal pathogens. These species are flagellated, anaerobic, aerotolerant Gram-negative spirochetes that inhabit the large intestine, where they are intimately associated with the colonic mucosa. *B. hyodysenteriae*, an obligate anaerobe with strong β-hemolysis on blood agar, is the causative agent of swine dysentery (Taylor and Alexander, 1971; Harris et al., 1972). *B. hyodysenteriae* colonizes the large intestine and can be found on the luminal surface and within the crypts of the caecum, colon, and rectum. The first evidence of disease is usually soft, yellow to gray feces that usually progress to mucohemorrhagic

diarrhea. On the other hand, *B. pilosicoli* (weakly β-hemolytic) produces porcine intestinal/colonic spirochetosis, with gray-wet diarrhea, sometimes with mucus, and occasionally mucohemorrhagic (Mappley et al., 2012).

Swine dysentery, with a mortality rate of 50–90% (Alvarez-Ordóñez et al., 2013), is a disease with an important impact on pig production due to the costs associated with mortality, morbidity, inefficient production, and continual in-feed medication of the animals. Although the disease can affect animals of all ages, it is rarely detected in piglets younger than 3 weeks of age; it occurs more frequently during growing/finishing periods, thereby aggravating economic losses.

Strategies to treat these diseases include the use of antibiotics such as tiamulin, valnemulin, tylosin, tylvalosin, and lincomycin. Unfortunately, the emergence of *B. hyodysenteriae* strains that are resistant to one or several of these antibiotics has been reported in several countries in Europe and Asia and in the US (Alvarez-Ordóñez et al., 2013; Rugna et al., 2015). Although it has long been known that pigs generate resistance to *B. hyodysenteriae* (Joens et al., 1979) after recovering from an infection, no vaccine is currently available. Administration of killed or attenuated bacteria has been of limited success (Alvarez-Ordóñez et al., 2013). Several bacterial recombinant proteins, including membrane and flagellar proteins, have been tested as candidates for this purpose. Experimental infection with the outer-membrane lipoprotein Bhlp29.7 of *B. hyodysenteriae* (also known as BmpB or Blpa) resulted in a 50% reduction in the incidence of disease (La et al., 2004). The search for possible vaccine candidates has been facilitated by the publication of the genome sequences of *B. hyodysenteriae* (WA1 strain; Bellgard et al., 2009) and *B. pilosicoli* (Wanchanthuek et al., 2010). This allows *in silico* analysis of the full genome sequence in the search of possible vaccine candidates that can be expressed and screened. Song et al. demonstrated the potential of this reverse vaccinology approach in a study in which partial genomic data from *B. hyodysenteriae* were used to identify 19 ORF-encoding candidate proteins, including lipoproteins, proteases, toxins, flagella-associated proteins, and membrane proteins. Although the results were not conclusive, a prototype vaccine prepared from four of the recombinant proteins produced antibodies in pigs, and conferred some protection against infection (Song et al., 2009). More recently, a US patent was registered for the development of a vaccine that is proposed to include up to 33 bacterial gene candidates selected from outer-surface and secreted proteins and from virulence factors described in public databases (Bellgard et al., 2015).

The characterization of the secreted and surface-exposed proteins of *B. hyodysenteriae* and *B. pilosicoli* is thus of special interest both for the development of vaccines and for the identification of factors involved in *Brachyspira* infection. Due to their localization, these protein groups are key for the induction of the host immune response (Zagursky and Russell, 2001; Grandi, 2010). As for other pathogens, proteins exposed on the surface of *B. hyodysenteriae* and *B. pilosicoli* play an important role in colonization and disease expression (Trott et al., 2001; Gömmel et al., 2013). On the other hand, secreted proteins such as β-hemolysin, which is considered a major virulence factor in *B. hyodysenteriae*, can act as cytotoxins against the host (Barth et al., 2012).

In reverse vaccinology approaches, vaccine candidates are searched in the microbial genome, mainly among predicted secreted and outer-membrane proteins and lipoproteins. Protein location predictions based on homology comparisons as well as on predictions of the actual levels of the protein molecules in that location have, however, an inherent degree of uncertainty. For example, most secreted proteins are synthesized as precursors with N-terminal signal sequences, but a significant fraction of secreted proteins are secreted by non-classical pathways that do not involve signal peptides (Armengaud et al., 2012). Although signal peptides are necessary for the targeting of many proteins to the membrane-embedded export machinery, the presence of an N-terminal signal peptide does not necessarily mean that the protein will be secreted; it could be released in the periplasmic space, or anchored to the outer membrane. Another important point to be considered is that some secreted proteins are not free in the extracellular milieu; instead, they remain attached to outer membrane components or to macromolecular structures such as flagella. Moreover, application of bacterial prediction algorithms to spirochetes can give inaccurate results due to the high plasticity of the lipobox in these bacteria compared to that of other Gram-negative species (Setubal, 2006). In this context and in comparison with *in silico* approaches, direct analysis of the bacterial proteome using proteomics approaches can provide a more accurate description of the protein profile in a given subcellular location.

Proteomics analysis of the cell surface can give a high-resolution view of the molecular components exposed by the cell, the surfaceome. The surfaceome includes membrane integral proteins as well as other proteins, such as secreted or exported proteins, that are bound to the outer membrane. One efficient method of characterizing the protein sequences exposed to the cellular milieu is cell "shaving." This strategy uses proteases to partially digest intact cells, resulting in preferential cleavage of the exposed portions of proteins. The resulting peptides are released to the supernatant and can then be identified by mass spectrometry (Solis et al., 2010). This method has been previously used with Gram-negative (Gesslbauer et al., 2012) and Gram-positive bacteria (Tjalsma et al., 2008). One surface-associated protein identified using this strategy has been validated in mice as a potential vaccine candidate (Doro et al., 2009).

On the other hand, direct analysis of the cell milieu provides a view of the extracellular proteome or exoproteome. As defined by Desvaux et al. (2009), the exoproteome includes actively secreted proteins as well as other extracellular, non-secreted proteins resulting from cell lysis, cell friction, and protein degradation, which can be also relevant to immune recognition and pathogenesis. Relevant exoproteome components can range from full-size, high-mass proteins to smaller protein fragments, and oligopeptides. Due to the potential relevance of extracellular oligopeptides, e.g., as protease inhibitors or to cell communication, specific characterization of these components, grouped under the term "exopeptidome," would be desirable. Bottom-up shotgun proteomics involves, however, technical limits to the characterization of protein

fragments and to differentiation between such fragments and full-length proteins. Conventional shotgun proteomics approaches to protein identification based on sequence analysis of proteome digests identify a protein on the basis of the characterization of several of its peptides. Although analysis of protein coverage can provide hints as to whether a given set of peptides reflects the presence of a complete protein, a protein fragment, or a polypeptide, a confident assignation is generally not possible.

Despite the fact that many genome sequences are already available, thus allowing protein characterization in databases, proteomic information for *Brachyspira* species is still scarce. Only a few outer membrane proteins of the genus have been characterized to date. Only 14 proteins are described in Uniprot with existence evidenced at the protein level, and only four of these are annotated as membrane proteins. Moreover, most of these proteins are from *B. hyodysenteriae*; no proteins from *B. pilosicoli* have been described.

In the present work, we used a proteomic shotgun approach to characterize the proteins bound to the cell surface (proteomic surfaceome) and the subset of proteins present in the extracellular milieu (exoproteome; Desvaux et al., 2009; Armengaud et al., 2012) of *B. hyodysenteriae* and *B. pilosicoli*. To specifically describe the components of the exopeptidome, we also analyzed cell media extracts that were not treated with protease. Because our analytical conditions do not preserve cell viability and because the outer membrane of this species is labile, it can be expected that many of these exogenous peptides and proteins potentially derive from cell leakage or from the periplasmic space rather than from active cellular mechanisms. Still, their characterization can be important to determine possible factors involved in the pathogenicity and recognition of these species. In these experiments, we sought to gather the greatest possible coverage of the detectable proteome by performing multiple biological replicates and including several strains of *Brachyspira*.

MATERIALS AND METHODS

Brachyspira Strains

Three isolates of *B. hyodysenteriae* (INFE1, V1, LL1) and two isolates of *B. pilosicoli* (OLA9 and Vi13) obtained from the Iberian pig breed in farms in central Spain (Extremadura and Castilla-León) were used in this study (**Table 1**). Strain identification was carried out by PCR using species-specific primers for nox (*B. hyodysenteriae*) and 16S rRNA (*B. pilosicoli*) (La et al., 2006).

Brachyspira Cultures

Culture media were based on those described by Calderaro (Calderaro et al., 2001, 2005). In addition, various antimicrobial agents to which *Brachyspira* bacteria are resistant were added to the culture medium to remove most of the fecal micropopulation (Feberwee et al., 2008). The medium was composed of blood agar base n°2 (40 g/L) supplemented with 5% defibrinated horse blood (50 mL; Oxoid, Thermo Scientific, Waltham, MA, USA), beef extract (3 g/L), Bacto-peptone (5 g/L; Difco, BD, Franklin Lakes, NJ, USA), and spectinomycin (0.2 g/L), spiramycin (0.025 g/l), rifampicin (0.012 g/l), vancomycin (0.0062 g/L), and colistin (0.00625 g/L; all from Sigma-Aldrich, St. Louis, MO, USA), and 810 mL distilled water. The plates were incubated for 4–7 days at 42°C in an anaerobic jar with H_2 and CO_2 produced by an AnaeroGen TM 3.5 L (Oxoid, Thermo Scientific, Waltham, MA, USA). The colonies were examined by contrast microscopy (40x).

To obtain sufficient mass for analysis, solid subcultures in blood agar were used as the starting material. Samples were seeded in BHI medium (Laboratorios Conda, Pronadisa Torrejón de Ardoz, Spain) enriched with horse serum (15%) and incubated with stirring in anaerobiosis jars at 42°C for 4–7 days.

The cells were recovered by several centrifugations in 50 mL tubes at 12,900 × g for 10 min. The cell pellets were washed three times with TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA, both from Sigma-Aldrich, St. Louis, MO, USA).

Sample Preparation

Bacterial pellets were resuspended in PBS (Sigma-Aldrich, St. Louis, MO, USA), and aliquots of 1 mL were transferred to 1.5 mL Eppendorf tubes (Eppendorf AG, Hamburg, Germany). The samples were pelleted again by centrifugation at 13,800 × g for 1 min. The amount of material in each aliquot was determined by weight (23 mg per aliquot on average), and the samples were stored at −80°C until further analysis.

Two aliquots of each strain were used per experiment, one for the analysis of the surfaceome and one for the analysis of the exoproteome and exopeptidome fractions. Three independent experiments, each one comprising the preparation and proteomic analysis of these three cell fractions from the five isolates, as described below, were performed during this study (Figure S1).

For surfaceome analysis, the cell pellets were resuspended in 150 μL of 25 mM ammonium bicarbonate (Sigma-Aldrich, St. Louis, MO, USA), and 100 μL of 0.1 μg/μL trypsin (sequencing grade, Promega, Fitchburg, WI, USA) was added. The samples were incubated at room temperature for 1 h and subsequently harvested by centrifugation at 2000 × g for 20 min at 13°C. The supernatants were transferred to fresh tubes and incubated for 7 h at 37°C.

For exoproteome/exopeptidome extracts, the cell pellets were resuspended in 250 μL of 25 mM ammonium bicarbonate, incubated at room temperature for 1 h, and harvested at 2000 × g for 20 min at 13°C. The supernatants were transferred to fresh tubes. Two aliquots (100 and 90 μL) were obtained from each supernatant for the exoproteome and exopeptidome analyses, respectively. For exoproteome analysis, the samples were incubated for 7 h at 37°C after addition of 40 μL of 0.1 μg/μL trypsin (sequencing grade). Samples for exopeptidome analysis were incubated at 37°C for 7 h without addition of trypsin. Digestion was terminated by the addition of TFA (final concentration 1%), and the samples were stored at −40°C until LC-MS/MS analysis.

Nano-LC-MS/MS

Aliquots of each extract corresponding to 2 mg of the original bacterial pellet were concentrated to approximately 5 μL and brought to 20 μL volume with 1% formic acid, 5% methanol. Depending on sample availability, each extract was injected at

TABLE 1 | PCR characterization of the *Brachyspira* strains studied*.

Strain	Pig breed	Region	Date	PCR	*clpX*	*ftnA*	*ACP*	*bitC*	*tlyA*	*hlyA*	nox_{hyo}	*smpA*	*smpB*	*vspF*
INFE1	Iberian Duroc	Badajoz	3/10/2009	*BRAHW*		X	X	X	X	X	X		X	X
V1	Iberian	Badajoz	10/16/2008	*BRAHW*		X	X	X	X	X	X	X		X
LL1	Iberian Duroc	Llerena	11/6/2009	*BRAHW*		X	X	X	X	X	X	X		X
Vi13	Iberian	Salamanca	11/17/2011	*BRAPL*	X		X			X				
OLA9	Iberian	Badajoz	10/26/2011	*BRAPL*	X	X	X			X				

**The isolates were classified on the basis of the nox and 16S rRNA genes (La et al., 2006). Data from San Juan (2015). Primers used for PCR amplification were those described in Barth et al. (2012).*

least in duplicate; in most cases, triplicate or quadruplicate samples were injected.

LC-MS/MS peptide analysis was performed using an Agilent 1200 nanoflow system (Agilent Technologies, Santa Clara, CA, USA) coupled to an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a nanoelectrospray ion source (Proxeon Biosystems, Odense, DK). The HPLC system consisted of an Agilent 1200 capillary pump, a binary pump, a thermostatted microinjector and a microswitch valve. A 12-cm long, 100-μm-I.D., 5 μm, C18 column (Nikkyo Technos Co., Bunkyo-ku, Tokyo, Japan) preceded by a C18 preconcentration cartridge (Agilent Technologies) was used.

Chromatography was performed at 0.4 μL/min (Solvent A, 0.1% formic acid in water; Solvent B, 0.1% formic acid in ACN) using a multisegment linear gradient of buffer B as follows: 3–10% in 9.5 min; to 40% in 170.5 min, to 90% in 1 min, and to 100% B in 5 min. Mass spectra (400–1800 m/z) were acquired in data-dependent acquisition (DDA) mode at a resolution of 60,000. The 10 most abundant ions in the linear ion trap were sequentially selected for sequencing by collision-induced dissociation, using collision energy of 35%. Ions already selected for fragmentation were dynamically excluded for 45 s. The spray voltage was 1.8 kV, and the heated capillary temperature was 200°C.

To control analytical performance along the study, each file was processed using Proteome Discoverer 1.4v (Thermo Fisher Scientific, Waltham, MA, USA) and the Uniprot Database *Brachyspiraceae* (August 2014, 28,436 entries). The resulting data were used to monitor the number of new peptides and proteins identified per run. The searches used the following parameters: Enzyme, trypsin allowing 1 missed cleavage (no enzyme for peptidome fractions); dynamic modifications, acetyl N-terminus, methionine oxidation, and asparagine and glutamine deamidation. Precursor mass tolerance was set to 20 ppm and fragment mass tolerance to 0.8 Da. For database search and False Discovery Rate (FDR) calculation, Proteome Discoverer uses a decoy database automatically generated from the target database. Filtering of the search results is carried out by the Proteome Discoverer tool Percolator. For this purpose FDR limits were set at 0.001 and 0.01 for the Percolator FDR strict and FDR relaxed parameters, respectively. Final results were filtered by protein filters (peptide rank 1 and 2 peptides per protein) and peptide filters (set to medium peptide confidence which corresponds to FDR < 0.005).

Database Search

PeptideShaker (version 1.6.0; Barsnes et al., 2011) was used for peptide and protein identification from the full MS data collection. Through its SearchGUI user interface (version 2.1.4), this application combines six different search engines: OMSSA (version 2.1.9; Geer et al., 2004), Amanda (version 1.0.0.5242; Dorfer et al., 2014), X-tandem! (version 2013.09.01.1; Bjornson et al., 2008), MS-GF+ (version Beta v10282; Kim and Pevzner, 2014), Comet version 2015.02 rev.1 (Eng et al., 2013), and MyriMatch version 2.2.140 (Tabb et al., 2007). Searches were carried out against concatenated target/decoy versions of the Uniprot Databases for *Brachyspiraceae* and for *B. hyodysenteriae* and *B. pilosicoli* (all from November 2015, 40,573, 14,301, and 7670 entries, respectively). The decoy sequences were created by reversing the target sequences with SearchGUI.

Search parameters included acetyl N-terminus, methionine oxidation, and pyrrolidone from glutamic, glutamine, and carbamidomethylated cysteine as dynamic modifications. Precursor mass tolerance was set to 20 ppm and fragment mass tolerance to 0.6 Da. Surfaceome and exoproteome samples were searched setting trypsin as the enzyme and allowing two missed cleavages. Peptide analyses were carried out with no enzyme set.

After database search, PeptideShaker uses protein inference algorithms for protein characterization. Peptide Spectrum Matches (PSM), peptides and proteins were validated at 1% FDR estimated using the decoy hit distribution. Post-translational modification localizations were scored using the D-score (Vaudel et al., 2013) as implemented in the compomics-utilities package (Barsnes et al., 2011).

The mass spectrometry data, along with the PeptideShaker identification results, have been deposited to the ProteomeXchange Consortium (Vizcaíno et al., 2014) via the PRIDE partner repository (Martens et al., 2005) with the dataset identifier PXD003900.

Data Analysis

MS sequencing data were fed to PeptideShaker grouped by strain and cell fraction, originating a total of 15 PeptideShaker output files. PeptideShaker reports for peptide and protein identifications were exported to MS Excel format; from there, the data were read, combined, and further processed using Python scripts. Total PSM in the samples were normalized taking into account the corresponding number of replicates for each sample. For searches in the full *Brachyspiraceae* database,

when different accessions of identical probability were assigned for an entry, the first accession corresponding to the species studied was selected as the group head. To obtain the list of surfaceome-specific proteins, the peptides, or proteins identified in the cell supernatants (exoproteome) were subtracted from those identified in the surfaceome sample. For this, a peptide or protein was considered to be present in a compartment when it represented more than 5% of the total counts for the three compartments and the two species. Otherwise, its presence was suspected to result from analytical or non-specific compartment cross-contamination. To compare the protein collections between species, proteins were indexed by their Uniprot names. For this purpose, Uniprot names were standardized by applying a group of simple rules (elimination of commas, hyphenations, etc.) to facilitate the comparison of identical protein names with small differences in their database annotations.

The properties of the protein collections were described using Gene Ontology Annotation (GOA), obtained through STRAP v1.5 annotation software, and freely available prediction software (LipoP v1.0, SignalP v4.0, PSORTb v3.0.2). LipoP v1.0 detects putative lipoproteins in Gram-negative bacteria and predicts the cleavage site of the signal peptide. This software has been trained on SPaseI-cleaved proteins, lipoproteins (SPase II-cleaved), and cytoplasmic and transmembrane proteins and is able to assign proteins to one of these classes on the basis of the protein's N-terminal sequence (Juncker and Willenbrock, 2003). SignalP v4.0, which detects potential signal peptides using neural networks, was designed to discriminate between signal peptides and transmembrane regions (Petersen et al., 2011). PSORTb predicts protein subcellular location in Gram-negative bacteria, classifying proteins according to five major locations (cytoplasmic, inner membrane, periplasmic space, outer membrane, and extracellular; Yu et al., 2010).

Hierarchical clustering of the peptides detected in the different strains was performed using GENE-E software version 3.0.204 (http://www.broadinstitute.org/cancer/software/GENE-E/). The column and row distance metric was one minus Pearson's correlation, and an average linkage method was used.

Ethical Statement

No animals were housed, infected, or pharmacologically treated for the study. Data presented was obtained from laboratory-grown bacterial strains. Original bacterial strains conserved at the cell bank were isolated from pig feces provided by field veterinarians carrying out their routine activity.

RESULTS AND DISCUSSION

Shotgun Proteomics Characterization

We applied a shotgun proteome approach to the study of the surfaceome, exoproteome, and exopeptidome of two *B. pilosicoli* and three *B. hyodysenteriae* strains, aiming to obtain the highest possible coverage of the host-exposed proteome of these *Brachyspira* species.

Despite recent advances in the field of discovery proteomics, comprehensive characterization of proteomes is still a major challenge, and requires extensive resources in terms of time, sample amount, and instrumentation (Gstaiger and Aebersold, 2009). Thus, no study of proteome mapping has reached 100% coverage of the proteins predicted from a genome (Ahrens et al., 2010). In addition to biological factors (i.e., lack of expression of some proteins under some conditions), limitations of the current MS instrumentation for the shotgun approach, in terms of scan speed and sensitivity, determine the degree of coverage obtainable from one analysis. As a consequence and due to the mechanics of data-dependent MS analysis, repeated analysis of the same sample provides different, partially overlapping protein collections. When sample amount is not limiting, combination of data from replicate experiments can increase the number of detected peptides, and proteins to a point determined by the detection limit of the technology. Following this strategy, in our study each sample was processed thrice and in each of these experiments the different fractions were analyzed by LC-MS/MS at least in triplicate. A total of 148 injections were carried out to reach the maximum coverage for each species and compartment (**Figure 1**).

More than 29,000 different peptides were identified in the PeptideShaker search of the MS/MS data against the complete *Brachyspira* database (Tables S1–S3). This database contains mainly annotations from the proteomes of *B. hyodysenteriae*, *B. pilosicoli*, *"B. hampsonii," B. intermedia, B. suanatina,* and *B. murdochii* (88% of all annotations) and a few sequences from other species such as *B. innocens*, *B. alvinipulli*, "*B. canis,*" and "*B. corvi*". Overall, 16,970 and 15,493 peptide sequences were identified in *B. hyodysenteriae* and *B. pilosicoli*, respectively. Most of these peptides corresponded to the sequences expected from the annotated proteome of the respective species. Approximately 1.9% (*B. hyodysenteriae*) and 3.5% (*B. pilosicoli*) of the peptide matches in each of these collections, however, corresponded to sequences from proteomes other than those of the species analyzed (**Figure 2**). These figures are higher than that of the FDR for these collections (<1% at the peptide level). The origin of these trans-species matches is likely diverse. Matches of spectra from *B. pilosicoli* samples with *B. hyodysenteriae* sequences and vice versa can derive from residual contamination of the analytical system. Other trans-species-only matches may result from errors in assignation by the search engines, errors in database annotations, or even from a lack of the corresponding protein sequence in the database of the analyzed species. In fact, a previous study of the *B. pilosicoli* data using the smaller database version of 2014 produced approximately 8% trans-species-only matches, of which nearly 30% fully matched *B. pilosicoli* proteins in the current, more complete database (not shown). Trans-species-only matches could also reflect genetic or transcriptional differences between our strains and those used as the source of the Uniprot annotations, which include 14 different strains of *B. hyodysenteriae*, with strain ATCC 49526 as a reference proteome, and the *B. pilosicoli strains* ATCC BAA-1826, B2904, P43/6/78, and WesB. To test these hypotheses, we performed a BLAST analysis on a random sample of 100 peptides detected in *B. pilosicoli* samples that matched sequences from other species. More than half of these sequences (54%) showed more than 80% amino acid identity with the sequences of the studied

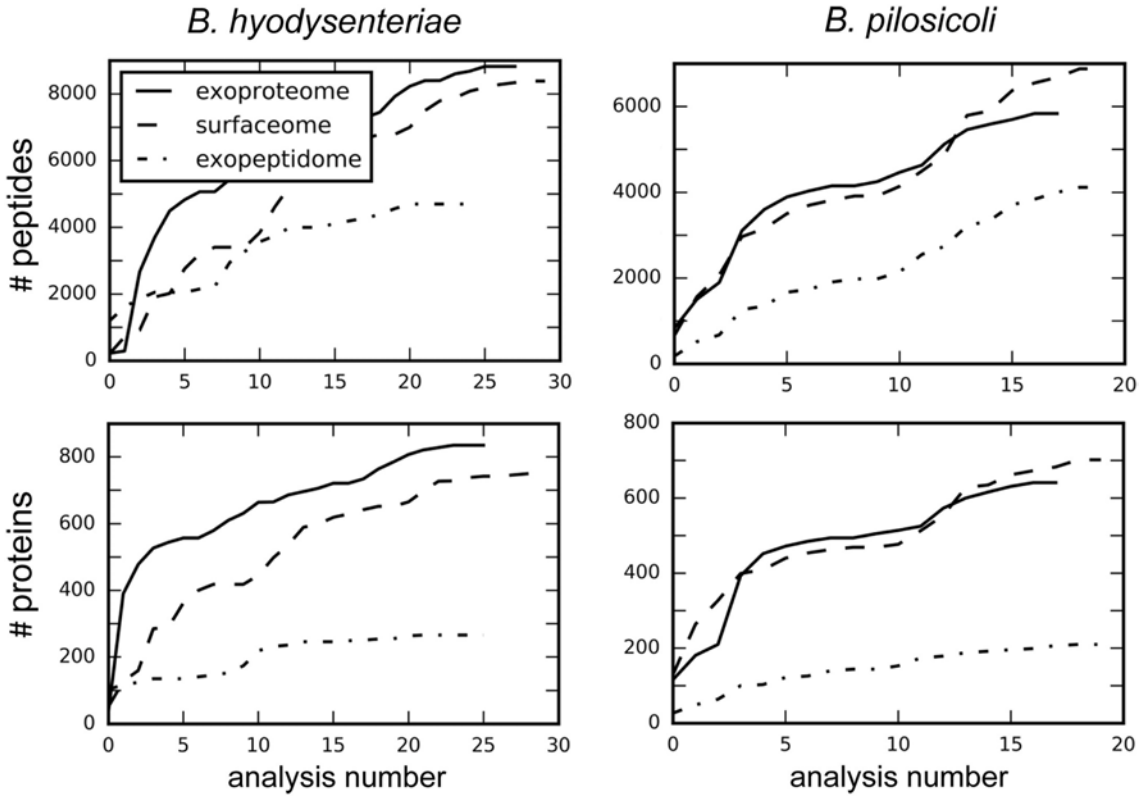

FIGURE 1 | Increase in the total number of identified peptides and proteins with the number of combined analyses. Values were obtained by searching each independent file with Proteome Discoverer 1.4.

species (differences in 1–3 amino acids, depending on the peptide size). In a significant proportion of cases in this group, the observed differences between the alternative sequences could be explained by a single nucleotide change. The four most common substitutions, D/N, I/V, K/R, and Q/K, represented more than 40% of the total observed differences. Assignment to other species due to Q/K differences most probably results from search engine limitations associated with assignment to the isobaric counterpart in the case of L and I or Q and K. D/N substitutions imply a difference of one mass unit between the alternative sequences that can also produce search engine mis-assignments. This can also be the case in combinations of two or more amino acid changes, which may produce isobaric sequences, or sequences with small mass differences. Leaving aside these possible assignment errors, the other cases observed support the idea that some of these trans-species-only matches are a reflection of both strain variability, and the still incomplete annotation/curation of the available *Brachyspira* databases.

The sequences of many of the peptides identified in *B. hyodysenteriae* were conserved in the proteome of *B. pilosicoli* and other species (80% of common peptides). In the case of *B. pilosicoli*, only 44% of the identified peptides were common to other species, whereas 54% of the sequences were *B. pilosicoli*-specific. This characteristic of the *B. pilosicoli* collection of sequences is consistent with differences observed in the comparison of several species of *Brachyspira* by MALDI-TOF protein profiling (Calderaro et al., 2013). Hierarchical clustering of the MALDI profiles produced a dendrogram in which the different species were located in two major branches. *B. hyodysenteriae*, *B. murdochii*, and *B. intermedia* clustered in different sub-branches of one of the major branches, whereas *B. pilosicoli* and *B. alborgii* were located in the other. Similar differences are observed in phylogenetic trees constructed from the 16S rRNA genes of several species of *Brachyspira* (Wanchanthuek et al., 2010).

A similar number of peptides was identified in the surfaceome and exoproteome fractions. In comparison, the number of exopeptidome peptides in the exopeptidome was lower (**Figure 1**, Table S2). When considering the number of assigned proteins the difference between the exopeptidome and the other two fractions increases. This could be due in part to the different composition of the digested and non-digested fractions (peptides vs. proteins) and the bias induced by our filtering protocols. Thus, for the Proteome Discoverer searches used to monitor individual analyses, the results of which are depicted in **Figure 1**, the identification of at least two different peptides per protein was required for a protein to be considered identified. This is a reasonable filter for the surfaceome and exoproteome collections but produces an underestimate of the actual confident identifications in the exopeptidome analysis, in which only

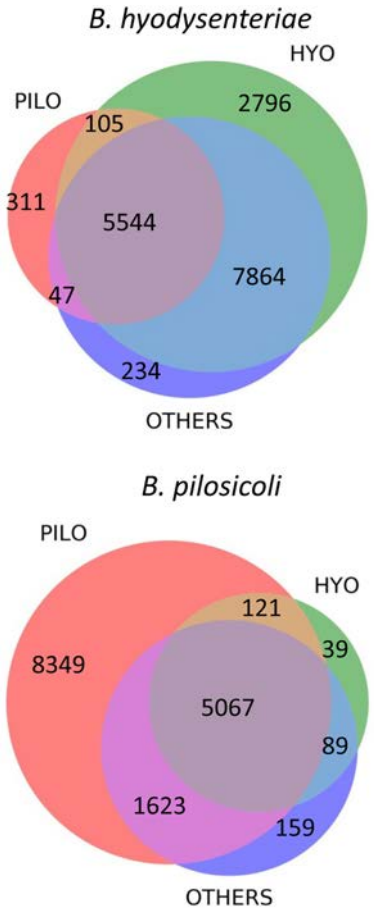

FIGURE 2 | Distribution of sequence matches in the different *Brachyspira* annotated proteomes for the collection of peptides identified in the *B. hyodysenteriae* and *B. pilosicoli* samples. PILO, *B. pilosicoli*; HYO, *B. hyodysenteriae*; OTHERS, other *Brachyspira* species.

small individual peptides are susceptible to being identified. PeptideShaker, which was used for the analysis of the full data, does not include the two-peptide requirement for protein inference; therefore, differences between the yield of peptides and proteins in the different compartments are smaller.

On average, ca. 300 proteins were identified per run in the surfaceome and exoproteome, and ca. 80 proteins were identified in the exopeptidome fractions. Due to the low degree of overlap of the identifications obtained between analyses, the total number of identifications of peptides and proteins increased rapidly for the first replicates (Tabb et al., 2010). This speed steadily decreased with the number of analyses performed, although a plateau was not clearly reached even after more than 40 analyses (**Figure 1**).

Considering all fractions, the PeptideShaker protein inference analysis using 1% FDR at all levels (PSM, peptide, and protein) pointed to 1625 and 1338 different protein accessions in *B. hyodysenteriae* and *B. pilosicoli* samples, respectively (Tables S4–S6). To facilitate large-scale analysis of the functional information derived from these data, for some studies we indexed the collections by gene, and protein name instead of by accession. Grouping by protein name has the disadvantage of including in the same group proteins with completely different sequences but with a common protein name. In return, grouping by gene or name tends to eliminate the redundancy derived from the existence of protein isoforms and homologous or identical protein sequences identified in different strains, sequencing projects that are present in the current *Brachyspira* databases. Overall, 2963 protein accessions were identified among the three fractions and the two species analyzed (surfaceome and exoproteome/exopeptidome analysis, non-redundant data). When indexed by gene and name, this figure was reduced to 1793 entries (1243 and 1060 for *B. hyodysenteriae* and *B. pilosicoli*, respectively). The genomes of these species have been described to contain 2153 (*B. hyodysenteriae*, WA1) and 1987 (*B. pilosicoli* 95/1000) protein-coding genes (Wanchanthuek et al., 2010). Current UniProt databases include 14 *B. hyodysenteriae* strains with an average of ca. 2640 protein sequences. Data for *B. pilosicoli* are fewer, restricted to four strains and still unreviewed. In this case, the largest database contains 2638 entries. Considering these figures, the number of accessions in our collection would roughly correspond to 62% (*B. hyodysenteriae*) and 50% (*B. pilosicoli*) of the proteins annotated for these species.

Distribution by Compartment

Peptidome and exoproteome samples were prepared from aliquots of the same cell supernatants that were analyzed directly (peptidome) or after tryptic digestion (exoproteome). Although the exoproteome samples contain all the peptides detectable in the peptidome samples, many of these peptides will be further hydrolyzed by trypsin, yielding sequences that are too small to be detected, or from which the original state of the peptide cannot be inferred. Thus, the exopeptidome analysis provides information on the low-molecular-weight components of the sample, including oligopeptides and protein hydrolysis products present in the supernatants. The surfaceome collection was prepared from the same cell aliquots using a cell shaving strategy. Cell shaving, which is based on limited proteolytic digestion of the whole bacterial cell, has been described as an efficient method for characterization of the cell surfaceome (Tjalsma et al., 2008; Doro et al., 2009; Solis et al., 2010; Gesslbauer et al., 2012). This method allows characterization of exposed outer membrane proteins and exposed sections of internal membrane proteins as well as extracellular proteins that could be bound to the membrane.

The 10 most abundant peptides detected in each compartment in terms of validated PSM (**Table 2**; complete data are provided in Table S2) point to a few proteins, including glyceraldehyde-3-phosphate dehydrogenase, flagellar filament proteins FlaB3, and FlaA1, elongation factor Tu, 60-kDa chaperonin, acyl carrier protein, rubrerythrin, thiol peroxidase, and NADH oxidase. These proteins are also among the 10 more abundant proteins in the collection of proteins inferred by PeptideShaker (**Table 3**).

Characterization of compartment-specific components from these collections is not straightforward, especially for the exoproteome and surfaceome components. The procedure for

TABLE 2 | The 10 most abundant peptides identified in each compartment for *B. pilosicoli* and *B. hyodysenteriae; Values correspond to the PeptideShaker validated PSM for each sequence.**

Peptide	*Hyodysenteriae*			*Pilosicoli*			Protein	Accessions	Species
	pep	prt	surf	pep	prt	surf			
EIDVVGVVDVSTDAK	5	0	3	0	1323	1140	G3PDH	D8IB86; J9UE83; K0JJQ2	BRAPL
DLGVEYVIESTGLFTDKEK	4	0	0	0	558	643			
DLGVEYVIESTGLFTDK	0	0	0	0	776	53			
ADITTEGEDVLVVNGNK	4	85	57	2	782	332		A0A0H0UCR6; C0R213; D8IB86; J9UE83; K0JJQ2; Q8VNZ1	C
AEGHIAAGAK	0	45	219	0	326	568			
EKAEGHIAAGAK	0	69	112	0	25	63			
EIEVVGVVDVSTDAK	0	262	94	0	0	0			
ALGVEYVIESTGLFTEK	0	132	96	0	0	0		A0A0H0UCR6; C0R213; Q8VNZ1	BRAHW
ALIQVEVNQLVAEVDR	0	308	94	0	841	80	Flagellar protein FlaB3	A0A0H0USH8; C0R1D6; D8IDG1; J9UXQ6; K0JLS4; Q9F0F6	C
SLMIATENTIASESVIR	4	306	76	3	152	39			
INTAGDDASGLAVSEK	2	114	80	0	98	68			
ELAIQSANGIYSDSDR	0	142	42	0	68	25			
IDEGIQMVVSQR	0	119	81	0	1	0		A0A0H0USH8; C0R1D6; Q9F0F6	BRAHW
NMITGAAQMDGAILVVSAEDGVMPQTK	2	85	78	0	534	364	Elongation Factor Tu	A0A0H0TPC4; C0QVZ4; D8ICZ6; J9UB96; K0JL99; P52854	C
TTLTSAITAVSSAMFPATVQK	0	79	99	0	7	14			
SLETSLSLVEGMQFDR	7	197	200	2	276	259	60 kDa chaperonin	A0A0H0TIS1; A0A0H0V6M8; C0QWM4; D8IB78; J9USS2; K0JLL7; Q3YLA1; Q3YLA3	C
AMLEDIAILTGGQVISEDLGMK	0	155	104	0	192	145		A0A0H0TIS1; A0A0H0V6M8; C0QWM4; D8IB78; J9USS2; K0JLL7	C
EVIITDIPEPEKPMPPMPGGGMGGMY	45	3	0	102	26	11			
ITDIPEPEKPMPPMPGGGMGGMY	36	7	3	92	30	14			
TAEVIITDIPEPEKPMPPMPGGGMGGMY	38	5	0	87	15	7			
VIITDIPEPEKPMPPMPGGGMGGMY	10	0	0	106	26	9			
ITDIPEPEKPMPPMPGGG	44	0	0	75	0	0			
FGPPTIINDGVTIAKE	2	0	0	77	22	4			
AKEIELEDPFENMGAQIVKEV	56	0	0	7	0	0			
DAIKLENPDEQVGVNIVKR	0	0	0	100	2	0	60 kDa chaperonin	D8IB78; J9USS2; K0JLL7	BRAPL
ISNMKELLPILEK	44	11	17	0	0	0		A0A0H0TIS1; A0A0H0V6M8; C0QWM4; Q3YLA1	BRAHW
TAELEDALLLIYDKK	43	9	6	0	0	0			
TVENPDEQVGVNIVK	40	9	5	0	0	0		A0A0H0TIS1; A0A0H0V6M8; C0QWM4	
LTVENPDEQVGVNIVKRAIEEPIRM	50	0	0	0	0	0			
ALIDEIKDVVANQLNISDK	0	113	63	2	548	188	Acyl carrier protein	A0A097BU34; C0QYL1; D8IBL1; J9USC6; K0JK36;O34163; Q6VAN3	C

(Continued)

TABLE 2 | Continued

Peptide	*Hyodysenteriae*			*Pilosicoli*			Protein	Accessions	Species
	pep	prt	surf	pep	prt	surf			
FFEVRVESY	8	2	0	629	202	36	Rubrerythrin	D8ICG1; J9UBZ5; K0JKT4	BRAPL
KFFEVRVESY	0	1	0	79	68	49			
FEVRVESY	0	0	0	146	40	0			
AIIAEVFEEFASLSGR	0	0	0	0	427	267	Putative pyruvate oxidoreductase	D8ICR0; J9URY6; K0JMR2; O87445	BRAPL
AIILDVFEEFASLSGR	0	79	111	0	0	0		A0A0H0UY93; A0A0H0VE59; C0QV98	BRAHW
ITFQGGEVHLEGVSLVEGAK	1	0	0	0	483	194	Thiol peroxidase	D8IFX8; J9UV33; K0JFG9	BRAPL
AAGGGAQITAK	0	37	159	0	18	108	50S ribosomal L2	A0A0H0W8S2; C0QVZ9; D8ICZ1; J9UBA7; K0JL93	C
IKDAGIELHLGETVK	5	0	0	0	65	207	NADH oxidase	B1NIM3; D8IAM5; F1B291; G8DZP2; J9UGQ4; K0JKC2; Q7BTH4; Q9R903; Q9R904; Q9ZHJ2; S4UT40; T1W0C5; W0FBQ9	BRAPL
IREQAELR	0	72	146	0	0	0	Flagellar filament outer layer protein flaA1	A0A0H0TRE1; A0A0H0V489; A0A0H0WR23; C0R0T5; P32520	BRAHW
DADLIIEAAFENLEVK	0	207	0	0	0	0	3-hydroxybutyryl-CoA dehydratase	A0A0H0TSQ1; C0QWH3	BRAHW
DGVIQNVGLELIGEAK	0	51	97	0	0	0	Electron transfer flavoprotein beta subunit	A0A0H0UWP8; A0A0H0VH59; C0QV71	BRAHW
TLEYDIIISGR	0	20	97	0	0	5		A0A0H0U588; C0QV72; D8ID09; J9TR46; K0JMS4	C
IPGGEATPAPPLGPALGQKQ	40	2	0	39	3	0	50S ribosomal protein L11	A0A0H0XFL7; C0QWX2; D8IEH0; J9UXV3; K0JHR3	C
TINQKQLEEIAQEKMA	40	0	0	23	0	0			
RIPGGEATPAPPLGPALGQKQ	39	0	0	8	0	0			

**The accessions column lists the accession number of all proteins containing the corresponding peptides. Exclusive indicates whether the corresponding protein was found in the samples from the two Brachyspira species (C) or was specific (>98% of total PSM) to one of them (BRAPL, BRAHW for B. pilosicoli, and B. hyodysenteriae, respectively). Full tryptic peptides were not considered for the peptidome list. Complete data is presented in Table S2.*

the preparation of the surfaceome sample is identical to that used for the exoproteome except that, in the former case, trypsin is already present during the first hour of incubation prior to cell elimination. Thus, the surfaceome sample contains both surface and exogenous proteins, and characterization of the surface-specific proteins requires subtraction of the exoproteome collection from that of the surfaceome. Determination of these differences is also complicated by the fact that the studied compartments are not hermetic. Proteins strictly considered membrane proteins can also be found in the culture medium due to cell lysis or contamination of the sample with cell debris, and elimination of these proteins would constitute a false negative for the surfaceome. Thus, as expected, most of the peptides and proteins identified in our experiments were detected in several compartments, although in some cases this was supported by a high number of validated peptide sequence matches. Because peptide sequence matches can be considered a rough estimate of protein abundance, we used this parameter to distinguish tentative compartment-specific proteins from proteins derived from compartment cross-contamination. For this, we filtered out from each compartment all peptides and proteins that were supported by fewer than 5% of the total peptide sequence matches pointing to them in the three fractions and considered only peptides and proteins with a minimum number of validated peptide spectra (5 and 15 for peptides and proteins, respectively).

TABLE 3 | The 10 most abundant proteins identified in each compartment for *B. hyodysenteriae* and *B. pilosicoli*.

Protein	*Hyodysenteriae*			*Pilosicoli*		
	Pep	Prt	Surf	Pep	Prt	Surf
60 kDa chaperonin	5636	1817	1342	10564	2070	2168
Glyceraldehyde-3-phosphate dehydrogenase	146	1041	1164	2004	5077	4098
Flagellin	1708	2663	968	414	4531	1616
Rubrerythrin	650	457	325	1941	1133	578
Elongation factor Tu	155	881	762	120	1705	1210
NADH oxidase	25	892	958	42	1348	1464
Acyl CoA dehydrogenase	776	939	1527	183	294	967
50S ribosomal protein L7/L12	159	706	460	762	1329	1159
Alcohol dehydrogenase	40	476	174	148	2742	990
Pyruvate phosphate dikinase	38	600	1184	0	171	2476
Enolase	367	1155	722	67	1689	285
Pyruvate ferredoxin oxireductase	122	825	778	31	1772	1944
Flavoprotein	9	697	564	0	484	1391
Chaperone protein DnaK	11	979	379	70	802	422
Acyl carrier protein	25	278	168	182	1208	502
Pyruvate oxireductase	72	98	123	830	388	702
50S ribosomal protein L2	41	546	902	81	71	505
Methyl-accepting chemotaxis protein B	72	188	799	12	2	882
10 kDa chaperonin	21	285	144	1177	95	142
50S ribosomal protein L11	898	87	82	528	52	131
Flagellar filament outer layer protein flaA1	23	903	540	0	0	0
ATP-dependent 6 phosphofructokinase	691	151	102	41	226	60
Lipoprotein	1	97	860	15	140	145
2-isopropylmalate synthase	293	51	40	711	56	42
50S ribosomal protein L10	169	119	107	539	70	141
50S ribosomal protein L18	597	92	79	182	20	51
Rubrerythrin fusion protein	388	125	75	0	0	0

Values correspond to the total validated PSM for all proteins in the described group. The complete data are presented in Table S5.

Using the above criteria, 192 accessions from *Brachyspira* proteins (53 and 139 for *B. hyodysenteriae* and *B. pilosicoli*, respectively) were classified as surfaceome-specific, whereas 119 were classified as specific to the exoproteome sample (55 and 64 for *B. hyodysenteriae* and *B. pilosicoli*, respectively; **Figure 3**).

Whereas the total number of *B. pilosicoli* proteins is slightly lower than that of *B. hyodysenteriae*, the number of proteins identified as exclusive to the surfaceome in *B. pilosicoli* is nearly triple that found in *B. hyodysenteriae*. This difference between species, which is observed independently of whether the collections are indexed by name or by accession and at any PSM cutoff, could reflect higher resistance of *B. hyodysenteriae* to trypsin hydrolysis. In fact, the different composition of the outer membrane LOS (Lipooligosaccharides) of these species makes the *B. pilosicoli* membrane more easily disruptable than the membrane of *B. hyodysenteriae* (Trott et al., 2001).

Surfaceome

The most abundant of the surfaceome-specific proteins, in terms of validated PSM, are indicated in **Table 4** (the full collection, indexed either by accession or name, is provided in Table S5). Among the proteins with GO annotation, more than 60% of the surfaceome-specific proteins are likely located on the membrane, and an additional small fraction is defined as extracellular or located in the cell periphery (**Figure 4**). This enrichment in membrane proteins is also observed using the PSORTb prediction tool for bacterial protein location (Figure S2). In this case, nearly 50% of the proteins with predicted location are classified as from the periplasmic space, inner membrane or outer membrane, whereas another 10% of all proteins could have multiple locations. In contrast, near 90% of proteins in the exoproteome fraction with predicted location by PSORTb are cytoplasmic. According to LipoP predictions for a total of 184 surfaceome-exclusive proteins from *Brachyspira* (>2 PSM), 91% were classified as lipoproteins (SPaseII-cleaved proteins), SPaseI-cleaved, or transmembrane proteins (Figure S3). Considering only high-confidence assignations (margin > 4, 114 proteins assigned), 57 corresponded to lipoproteins, 44 to transmembrane, and 13 to proteins with SpI signal (Table S7).

Approximately 5 and 20% of the surfaceome-specific proteins are classified as cytoplasmic according to GO annotation for *B. hyodysenteriae* and *B. pilosicoli*, respectively. As discussed

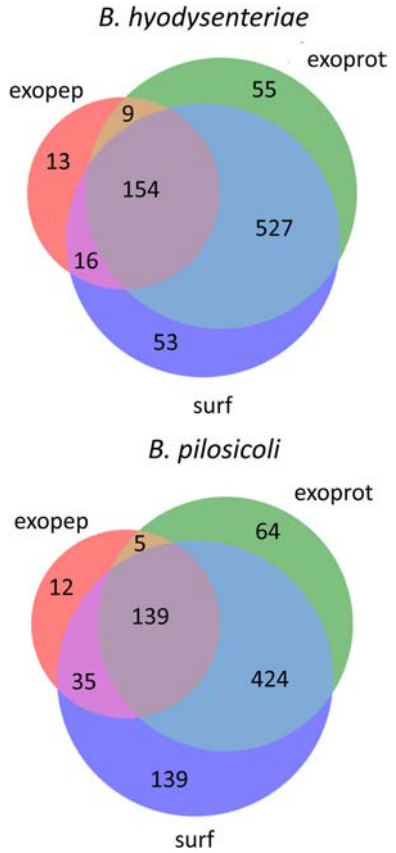

FIGURE 3 | Distribution of identified proteins (unique accessions) among compartments. Only proteins with more than 15 validated PSM are shown in the figure. Proteins considered exclusive must have more than 80% of total validated spectra in one compartment, and any other compartment, considering each species separately, must contain <5% of the total validated spectra.

above, the presence of cytoplasmic proteins in the extracellular compartments can be explained as the product of cell lysis (Christie-Oleza and Armengaud, 2010; Christie-Oleza et al., 2012). Gram-negative cells have been reported to be less resistant to the shaving process, with a higher level of cell death (Grandi, 2010), a characteristic that would be in agreement with an increased amount of cytoplasmic proteins. To reduce the contribution of possible leakage proteins, the surfaceome specific collection was obtained by subtracting the proteins detected in the exoproteome from the original surfaceome data. Thus, the presence of cytoplasmic proteins in our surfaceome-specific collection could also reflect the genuine presence of some of these proteins on the surface of the cells. In any case, only a small fraction of the proteins classified as cytoplasmic by GOA or the PSORTb prediction tool in the surfaceome-exclusive collection are predicted to bear a signal peptide-cleavage site by LipoP. This suggests that their presence on the cell surface, if genuine, would result from non-canonical transport mechanisms.

Surfaceome-specific proteins display a wide range of molecular weights (**Figure 5**) and predicted isoelectric points. According to GO annotation, these proteins are involved in many different cell functions (**Figure 6** and Figure S4). Many of them (ca. 48%) are classified as binding proteins, and a high proportion are transferases, hydrolases, or have some catalytic activity, with an overrepresentation of signal transducers and transporters relative to the peptidome or exoproteome.

Exoproteome and Exopeptidome

A total of 2053 proteins (non-redundant) were identified in the external proteome of *B. hyodysenteriae* and *B. pilosicoli* strains studied in this work. In contrast with the surfaceome collection, a high proportion of these proteins are annotated as cytoplasmic or ribosomal by GOA (**Figure 4**). PSORTb predictions assign a cytoplasmic location to approximately 81% of all proteins with known location (Figure S2). Specially, in the case of the *B. pilosicoli* exoproteome, cytoplasmic proteins account for more than 86% of these proteins.

According to LipoP predictions, 263 of a total of 301 exoproteome proteins from the two analyzed *Brachyspira* species (>2 PSM) were classified as lipoproteins (SPaseII-cleaved proteins), SPaseI-cleaved, or transmembrane proteins (see Table S8 for LipoP, PSORTb, and SignalP predictions for this collection). In contrast to the surfaceome-strict collection, in which transmembrane helix assignations were more frequent (42%, no margin cutoff) and only 17% showed an SpI signal, SpI proteins represented up to 34% of the proteins in the exoproteome fractions and only 17% of TMH assignations (Table S8).

Taking into account the protocols used in the preparation of the different samples, the protein collection from the exoproteome fraction would be expected to be included in that of the surfaceome compartment. Nevertheless, 119 protein accessions, representing nearly 9% of the total exoproteome assignations, were identified in *Brachyspira* samples as exoproteome-exclusive with high confidence (>15 PSM, **Table 5**). Due to the random character of the data-dependent MS scanning method, it is possible for some proteins with low numbers of validated peptide matches to be detected in one compartment and missed in another compartment in which they are also present. However, a number of exoproteome-exclusive proteins with high numbers of validated spectra (>100 PSM) were detected, making their presence in this category a result of limited spectral count statistics unlikely (**Table 5**). A tentative explanation for these identifications could be related to protein or peptide loss during the preparation of the surfaceome extract. In this process, trypsin is added to a cell suspension from which the cells are removed after 1 h. Some of the digested peptides produced from proteins in the medium could be lost in this step due to adsorption to the cellular pellet. In contrast, during the preparation of the exoproteome fraction, the cells are removed prior to digestion, and these losses may not occur, potentially resulting in the false positive identification of some peptides in the exoproteome fraction as exclusive.

Although exoproteome/exopeptidome proteins display a high degree of overlap (**Figure 3**), some proteins were only identified in the exopeptidome samples (**Table 5**). Because the

TABLE 4 | The 20 most abundant proteins specifically located in the surfaceome*.

Protein	PSM	Accessions	Genes	Species
Ribonuclease Y	582	A0A0H0V3X2, D8IB75, A0A0H0U8G9, C0R0C9	rny	C
DNA-directed RNA polymerase omega subunit family protein-like protein	322	D8IA88, K0JKN7	BP951000_2245, WESB_1288	BRAPL
Apolipoprotein A1/A4/E domain-containing protein	306	J9ULC7	B2904_orf1166	BRAPL
N-acetylglucosamine-1-phosphate uridyltransferase	203	C0QY93, D8IB64	glmU	C
Lon protease	169	D8ICT7, C0R248, J9URW0	lon	C
Preprotein translocase YajC subunit	135	D8IEE4	yajC	BRAPL
ATP synthase subunit b	128	C0QW62, D8IBP4	atpF	C
UPF0365 protein BP951000_0575	120	D8IBQ3	BP951000_0575	BRAPL
Unchar D8ICF3	114	D8ICF3	BP951000_0828	BRAPL
Phosphomannomutase phosphoglucomutase	112	D8I9Z9	manB	BRAPL
Transposase	106	K0JKA8	WESB_1932	BRAPL
Inositol-1-monophosphatase	98	C0QX44, D8IBH8	suhB	C
Unchar D8IEP8	90	D8IEP8	BP951000_1640	BRAPL
PTS system glucose subfamily IIA subunit	90	D8IBR5	ptsG	BRAPL
PTS system fructose-specific IIABC component	88	D8ID87	fruA	BRAPL
Transcriptional regulator XRE family	86	J9TSW8	B2904_orf609	BRAPL
PTS system fructose specific transporter subunit IIABC	83	K0JLH4	WESB_2470	BRAPL
Methyl-accepting protein	80	D8IBS9, C0R060	BP951000_0603, tar5	C
Unchar J9UB61	79	J9UB61	B2904_orf310	BRAPL
Transcriptional regulator CarD family	71	D8ID15	carD	BRAPL

**The members column lists protein accessions identified by PeptideShaker contributing to the total validated PSM. Note that not all accessions individually fulfil the filtering criteria. Species indicates whether the corresponding protein was found in the samples from the two Brachyspira species (C) or was specific (>98% of total PSM) to one of them (BRAPL, BRAHW for B. pilosicoli, and B. hyodysenteriae, respectively). When PSM are lower than 50, species specificity is not determined (X) except when all counts belong to a single species and there are at least 25 PSM. Complete data is presented in Table S5.*

exopeptidome samples consist of cell supernatants that were not treated with trypsin, only small polypeptides of less than ca. 3–4 KDa are expected to be detected. In addition to protein fragments resulting from protein degradation, this compartment potentially includes bioactive peptides that either leak from or are actively secreted by the cells and are important for the interaction of the bacterium with its environment. It is interesting to note that the size distribution of proteins pointed to by the peptidome peptides shows a much higher proportion of molecules <10–20 KDa in size than the proteins specific to the surfaceome fraction, which have an average size of ca. 50 KDa (**Figure 5**), or those specific to the exoproteome (not shown).

When considering the peptides identified with a higher number of PSM in our collection (**Table 2**), it can be observed that many of these highly abundant peptides constitute groups of nested sequences of varying lengths. This is the case for three peptides with the common core FEVRVESY from rubrerythrin and many of the sequences from the 60 KDa chaperonin. In trypsin-treated samples, the occurrence of nested sets is primarily due to incomplete digestion of the samples, which produces sequences with missed cleavages. In the case of the peptidome samples, which were not treated with trypsin, these sets may reflect the activity of cell proteases present in the media. The analysis of the N- and C-terminal amino acid sequences of these peptides seems to indicate a slight enrichment of some amino acids, such as alanine and glutamine, in the peptide terminal sides but, apart from this, no clearly recognizable specific peptidase motif could be detected (**Figure 7**). The frequency of methionine in the N-terminal region (positions 6–7 in **Figure 7**) and that of off-sequence positions (indicated by an asterisk in **Figure 7**) in the C-terminal region suggested that many of the observed peptidome sequences correspond to C- and N-terminal peptides that have been further processed by the action of exopeptidases.

Comparison between Species and Strains

Several differences between *B. pilosicoli* and *B. hyodysenteriae* have already been mentioned, including the significantly greater number of species-specific peptide matches in *B. pilosicoli* and the higher proportion of surfaceome-exclusive proteins in this species.

The different strains analyzed produced similar numbers of protein identifications, with a high degree of overlap between collections (**Figure 8**, Left). Among *B. hyodysenteriae* strains, however, more differences were found than among *B. pilosicoli* strains (an average of 16% of strain-exclusive accessions for any pair of *B. hyodysenteriae* strains compared with 8% for

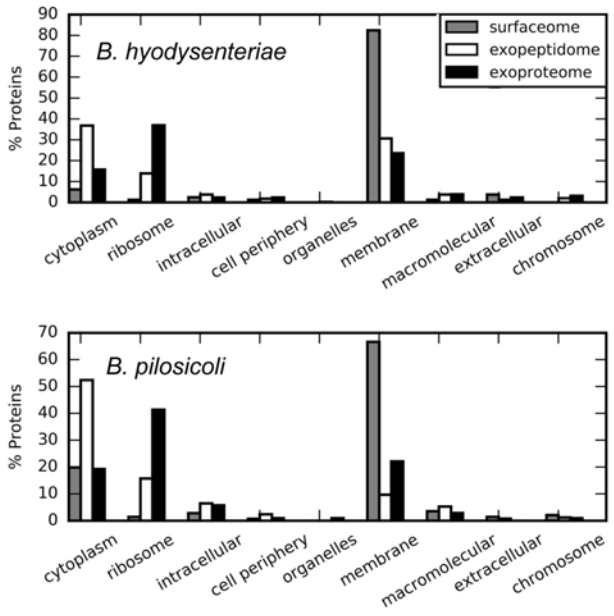

FIGURE 4 | Cellular location of the compartment-specific proteins obtained using STRAP. GO annotation, Cell component.

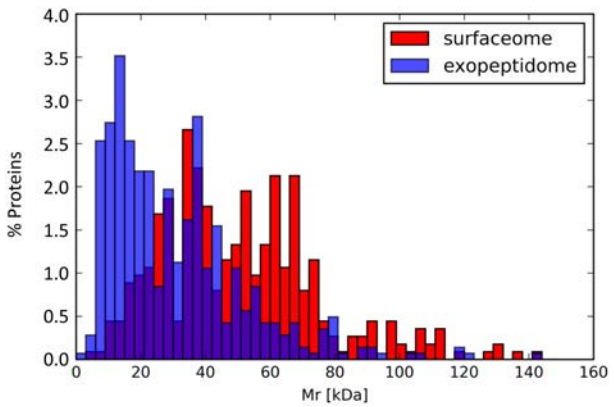

FIGURE 5 | Molecular mass distribution for the proteins annotated in the exopeptidome compared with those specific to the surfaceome.

B. pilosicoli). These differences increase when only surfaceome-exclusive proteins are considered. In this case, an average of only 42 of 307 surfaceome proteins were common to any pair of *B. hyodysenteriae* strains (Table S9), whereas on average 140 of 385 surfaceome proteins were common to the two *B. pilosicoli* strains (Table S10). This is in contrast to observations made at the genomic level in a study in which the genomes of 20 strains of *B. hyodysenteriae* were sequenced, aligned, and compared (Black et al., 2015). As a reference for *B. pilosicoli*, the study used the genome of four *B. pilosicoli* strains described by Mappley et al. (2012). The comparison was also extended to the gene protein prediction level; this indicated that protein homology between strains ranged from 75.6 to 88.5% for *B. hyodysenteriae* and from 54.9 to 68.4% for *B. pilosicoli*. Based on the high homology between strains of *B. hyodysenteriae* observed in that study, the authors concluded that the genome of this species is clonal whereas that of *B. pilosicoli* is recombinant. The disagreement with our findings could be due to the small number of strains considered in our study, to the fact that our collection of proteins represents a subset of the proteome (exposed proteome) or to the difference between predicted gene products and their actual expression levels. Proteomic analysis of a larger number of strains would be necessary to decide among these possibilities.

Cluster analysis of the abundance data (indirectly measured as the number of PSM) of the identified peptide sequences shows a clear distinction between the two species (**Figure 8**, Right). Using either the surfaceome or the peptidome data, *B. pilosicoli* and *B. hyodysenteriae* strains cluster in two different branches of the dendrograms. In the case of the *B. hyodysenteriae* strains, the *V1* strain presents differences with respect to the *LL1* and *INFE1* that place these strains in two different sub-branches.

Genes Related to Virulence

By analogy with secreted and surface proteins of other pathogenic organisms, secreted proteins and proteins expressed on the surface of *B. hyodysenteriae* and *B. pilosicoli* can play an important role in colonization and disease expression (Trott et al., 2001). Bellgard et al. screened 314 protein coding sequences potentially involved in pathogenesis and virulence in the *B. hyodysenteriae* WA1 genome (Bellgard et al., 2009). The potential virulence genes included genes coding for proteins involved in lipopolysaccharide biosynthesis, adhesion, chemotaxis, and motility, host cell membrane degradation, nutrition, immunoevasion, and immunosuppression. The same group also described 235 genes that are potentially involved in pathogenesis and virulence in *B. pilosicoli* 95/1000 (Wanchanthuek et al., 2010). Many of the products of these virulence-related genes were found in the fractions analyzed in our study (examples are given in Table S11).

The large number of genes involved in chemotaxis and motility in these *Brachyspira* species reflects the importance of these functions in relation to their enteric lifestyle and the colonization process (Bellgard et al., 2009). To induce disease, the highly motile spirochetes colonize colonic crypts and enter goblet cells, from which they induce a characteristic outpouring of mucus (Bellgard et al., 2009; Hampson and Ahmed, 2009). The capacity for movement in this dense environment is one determinant of the bacterium's virulence, and some non-chemotactic strains have been demonstrated to be avirulent (Milner and Sellwood, 1994). The chemotaxis-related protein assignations with higher numbers of validated peptide matches in our collection are methyl-accepting chemotaxis proteins B (McpB) that are present mainly in the surfaceomes of both species (**Table 3**). Other methyl-accepting proteins detected included several accessions from McpA and McpC proteins that were observed only in *B. hyodysenteriae*. The absence of the *mcpC* gene in *B. pilosicoli* strains was previously noted by other authors, whereas *mcpA* has been detected in both species (Wanchanthuek et al., 2010; Mappley et al., 2012). The most highly represented chemosensory transducer gene

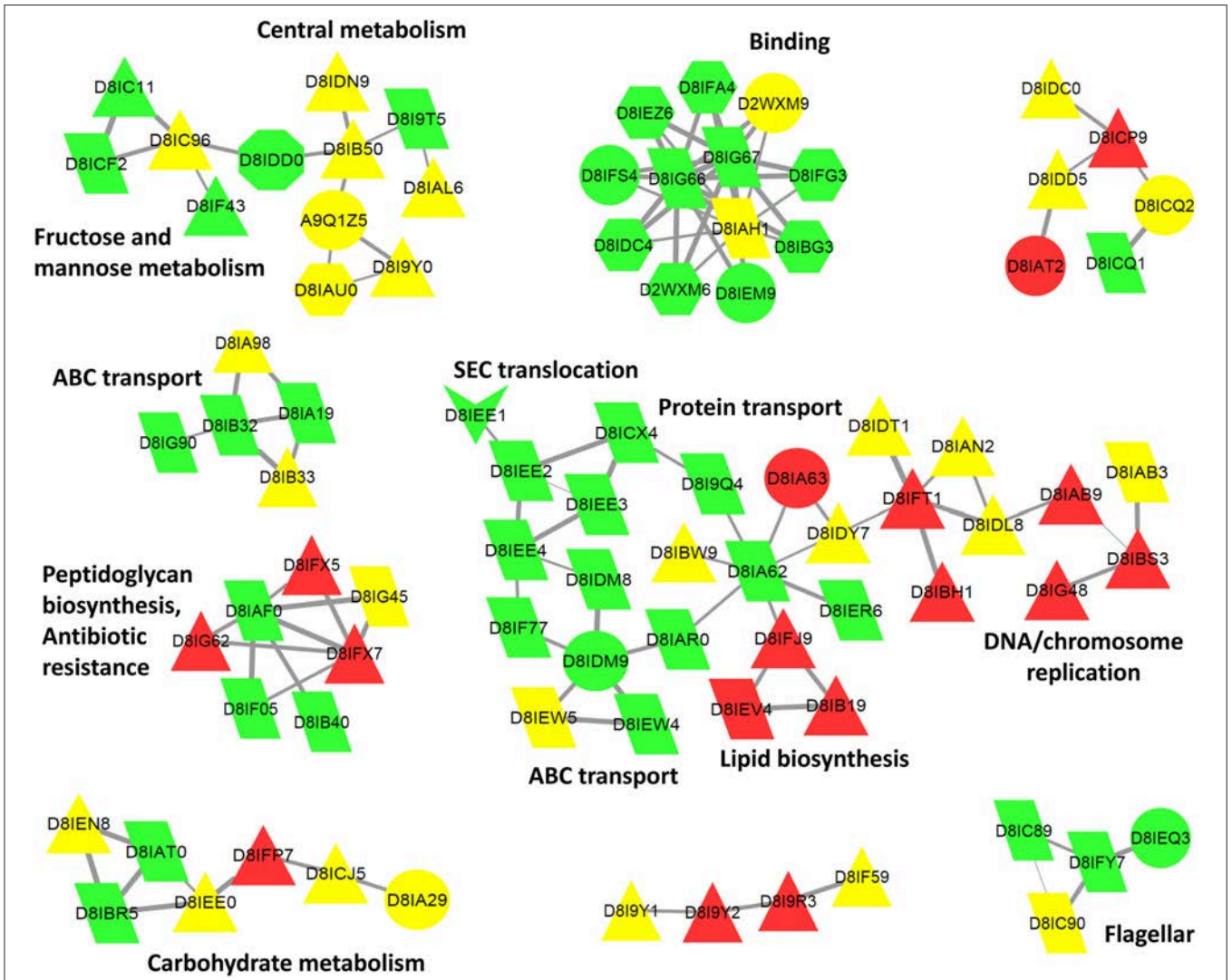

FIGURE 6 | Cytoscape biological network 47 of *B. pilosicoli* surface-exclusive proteins. GOA Cellular Component Annotation (node color): green, membrane, and cell periphery; red, cytoplasm, and intracellular; yellow, unknown. PSORTb prediction (node shape): triangle, cytoplasmic; trapezoid, cytoplasmic membrane; octagon, outer membrane; hexagon, periplasmic; arrow, extracellular; circle, unknown. STRING Database: *B. pilosicoli*, Interaction Score ≥0.8.

product was the chemotaxis protein CheY, which is found in the surfaceome, and exoproteome fractions of the two species studied. Other highly expressed proteins in this class were the chemotaxis histidine kinase CheA and the chemotaxis response regulator protein-glutamate methylesterase CheB. Less abundant chemotaxis proteins identified were, in decreasing order of PSM, CheW, CheX, CheD, CheR, and CheC. Unlike the other members of the family, CheW showed higher counts (ca. 3:1) in the exoproteome than in the surfaceome fractions.

Previous studies have described as many as 33 (Wanchanthuek et al., 2010) and 42 (Mappley et al., 2012) genes related to flagella in *B. hyodysenteriae* and *B. pilosicoli*, respectively (Hampson and Ahmed, 2009). In current *Brachyspira* databases, a total of 52 different flagella-related gene names are annotated (42 and 40 for *B. hyodysenteriae* and *B. pilosicoli*, respectively), and a few others are annotated as ORF names. Here, we present evidence for 49 protein accessions covering 29 different genes related to the production and function of these organelles (including the *fla*, *flh*, *flg*, *fli,* and *mot* families). The abundance of flagellar proteins was found to differ between the two species, with ca. 4905 validated PSM in *B. hyodysenteriae* vs. 2577 PSM in *B. pilosicoli*. This could be related to the different numbers of flagella in these cells (*B. hyodysenteriae* is reported to have approximately 7–14 flagella/cell and *B. pilosicoli* to have 4–7 endoflagella; Hampson, 2012).

Several membrane proteins of *B. hyodysenteriae* have been described as mediators of adherence to host cells (Gömmel et al., 2013). This group includes Vsps, lipoproteins such as BmpB and SmpA and SmpB, and other integral/inner membrane proteins (Wanchanthuek et al., 2010).

Vsp proteins are considered the major protein component of the outer membrane of *B. hyodysenteriae*, and it has been

TABLE 5 | Top 10 exoproteome-exclusive and exopeptidome-exclusive proteins (Table 4 for details).

Protein	PSM	Accessions	Gene	Species
EXOPROTEOME				
Superoxide dismutase	937	A0A0H0V406, C0QW83, D8ICG2	SR30_05435, sodA	C
Endoribonuclease	363	D8IFH7, C0R0L6	BP951000_1922, BHWA1_01174	C
Unchar D8IB10	248	D8IB10	BP951000_0328	BRAPL
Unchar D8I9Q8	195	D8I9Q8	BP951000_0016	BRAPL
CoxL protein	146	J9U0I3, K0JKH7	coxL	BRAPL
Variable surface protein VspD	144	D8ICU0, J9UQJ0	vspD, B2904_orf389	BRAPL
Unchar J9UGA5	122	J9UGA5	B2904_orf1414	BRAPL
Unchar J9U2W9	116	J9U2W9	B2904_orf2565	BRAPL
Flagellar filament protein FlaA	105	A0A0H0UUF0	SR30_02310	BRAHW
Nitroreductase	96	D8IFL9	BP951000_1964	BRAPL
Phosphate ABC transporter phosphate binding protein	94	D8IAV1, C0QY26	ptsS	C
Yqi (Fragment)	86	A9Q1Z1	yqi	BRAPL
Peptidase T	83	D8IFQ9, J9TSR4, C0QV99, K0JHK6	WESB_0904, pepT	C
Cytoplasmic protein	77	J9UX05	B2904_orf2396	BRAPL
EXOPEPTIDOME				
Rod shape-determining protein MreB	162	A0A0H0TUE9	SR30_05720	BRAHW
Electron transfer flavoprotein subunit alpha	98	A0A0H0VH59	SZ51_09315	BRAHW
Ferric uptake regulator	59	D8IDK7	fur	BRAPL
Ferredoxin 4Fe 4S	40	D8IA49	BP951000_2206	BRAPL
Phage terminase large subunit	26	D8IE54	xtmB	BRAPL
Pyruvate ferredoxin/flavodoxin oxidoreductase	23	D8IED5	porA	X
Unchar D8IDT5	20	D8IDT5	BP951000_1320	X
Unchar C0QVB6	19	C0QVB6	BHWA1_01956	X
Fer2/BFD BFD like 2Fe 2S binding domain-containing protein	19	J9UVB4	B2904_orf1553	X
Enzyme of poly gamma glutamate biosynthesis (Capsule formation)-like protein	18	C0QYZ6	BHWA1_00588	X

shown that sera from affected pigs are immunoreactive toward these proteins (Witchell et al., 2011). Vsp proteins form stable multimeric complexes that, in *B. hyodysenteriae,* have been described as consisting mainly of VpsF but that can contain also VpsE, VpsI, and VpsD. We have identified 6 products of the genes *vspD*, *vspE*, *vspF,* and *vspH*, all of them found either preferentially or exclusively in the exoproteome fraction. The most-represented members are Q9AEX5 (VpsH) and F1CJQ5 (VpsF) from *B. hyodysenteriae* and D8ICU0 (VpsD) from *B. pilosicoli*. VspD was among the most abundant exoproteome-exclusive proteins identified in our analyses (**Table 5**); it was detected only in *B. pilosicoli* samples (Uniprot accession numbers D8ICU0 and J9UQJ0). Interestingly, we also detected this protein in western blot analyses in which protein extracts from two *B. pilosicoli* strains were incubated with sera from infected pigs; however, it was not detected when the same experiment was performed with *B. hyodysenteriae* strain *V1* (Rodriguez-Asiain, in preparation). The *vspD* gene has been described as a virulence factor in *B. hyodysenteriae* and was recently included in a list of 33 potential targets for the development of a vaccine against *B. hyodysenteriae* (Bellgard et al., 2015). Up to four different VspD main accessions have been annotated for *B. hyodysenteriae* (C0R1D9, F1CJQ3, COR1D9, and C0R1E2). Sequences O68157 and F1CJQ3 include one or several identical sequences annotated with different accessions. Whereas C0R1E2 is a short polypeptide, the other three sequences are proteins >40 KDa in size, a size similar to that of *B. pilosicoli* VspD proteins, and show more than 90% identity among them and with other surface proteins, such as VspC (F1CJQ2, O68156, and other identical sequences) and VspB. No peptide from any of these sequences was identified in any *B. hyodysenteriae* fraction despite the fact that, except for C0R1E2, these proteins are expected to be as detectable as the analogous proteins of *B. pilosicoli*. These results suggest that VspD may be expressed in very low amounts in *B. hyodysenteriae*. A search of VspD peptides in extracts from the full proteomes of *B. pilosicoli* (ATCC #51139, strain P43/6/78) and *B. hyodysenteriae* (ATCC #49526, strain WA-1) confirmed these results (Casas et al., in preparation). Whereas up to 10 peptides from VspD could be found in the *B. pilosicoli* extract, only one was detected in the *B. hyodysenteriae* sample. The most abundant *B. pilosicoli* VspD peptide was found with more than 100 validated PSM, whereas the only *B. hyodysenteriae* VspD peptide was found with 4 PSM. These figures suggest a difference in expression of approximately 25-fold.

Several membrane lipoproteins and membrane proteins involved in lipoprotein biosynthesis were identified. The detected

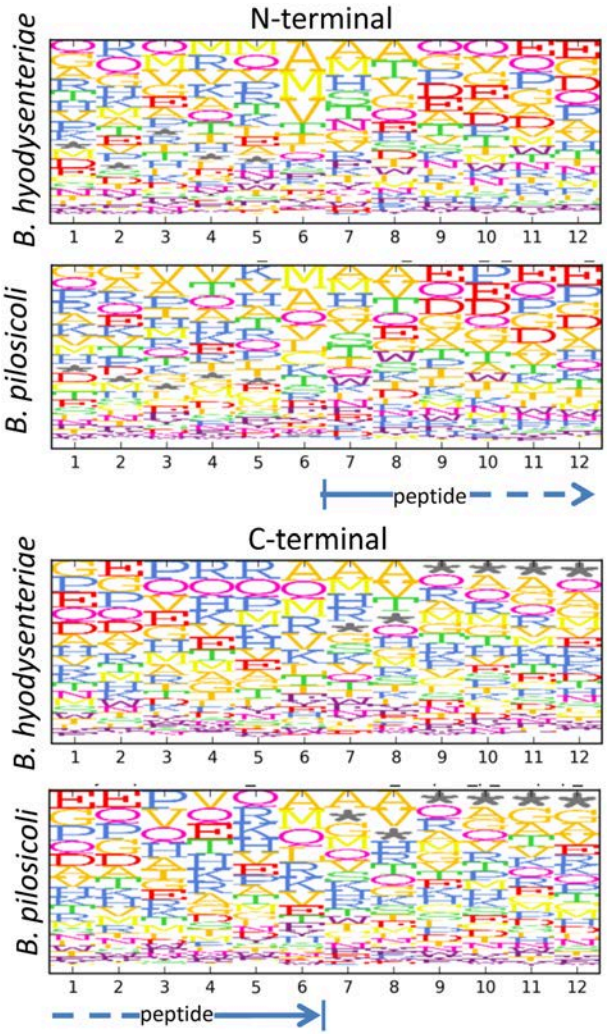

FIGURE 7 | Motifs in the N- and C-terminal regions of peptides in the exopeptidome fraction. Size and top to bottom position of the amino acid letters are related with the frequency at which the amino acid is observed on that position. Frequencies are corrected for the amino acid frequency in the full *Brachyspira* database. An asterisk denotes the absence of an amino acid (off-sequence positions in protein terminal peptides).

lipoproteins included five BmpB sequences that were present in the exoproteome and surfaceome fractions; the most abundant were C0R281 (*B. hyodysenteriae*) and J9UFV3 (*B. pilosicoli*). Two accessions for the outer membrane proteins SmpA and SmpB were also detected in the *B. hyodysenteriae* samples and in the surfaceome fraction. SmpA, a membrane-associated lipoprotein localized in the outer surface of the spirochete, has been proposed as a vaccine candidate (Holden et al., 2006; Hidalgo et al., 2010). The genes *smpA* and *smpB* are related, although different. Individual strains of *B. hyodysenteriae* contain either *smpA* or *smpB* but not both. In a study of ca. 40 isolates from Spanish pigs, all presented the *smpA* gene (Hidalgo et al., 2010), whereas the two genes were equally represented in a small group of Australian, UK, and North American strains (Holden et al., 2006). In our study, strain *INFE1* expressed the *smpB* product (seven validated peptides, 47% protein sequence coverage), whereas strain *LL1* expressed SmpA (four validated peptides, 30 % protein sequence coverage). These results agree with the PCR characterization of the strains (**Table 1**). However, for strain *V1,* whose PCR characterization indicated the presence of the *smpA* gene sequence, neither SmpA nor SmpB could be identified in the proteomics analysis. A search of the PeptideShaker unfiltered data allowed us to detect two non-validated assignations, each with just one spectral match, to SmpB peptides in *V1*. The matches corresponded to very low-quality spectra, so our results may indicate low, or no expression of any of these two proteins in *V1*.

Many other membrane proteins were identified in our collection, including members of the carbohydrate phosphotransferase system (PTS), the most abundant of which was C0QW75, a transmembrane protein of *B. hyodysenteriae*. These proteins were mainly identified in the surfaceome, and several members of this class (Ptsg and FruA) were among the more abundant surfaceome-specific proteins (**Table 4**). Phosphoenolpyruvate PTS components have recently been described as modulators of virulence in *Borrelia burgdorferi* (Khajanchi et al., 2015).

Hemolysins are considered major virulence factors that may be involved in the disruption and shedding of colonic enterocytes, a process that exposes the underlying lamina propria to polymicrobial invasion, and inflammation. Up to 8 and 12 genes have been predicted to encode hemolysin in *B. hyodysenteriae* and *B. pilosicoli*, respectively (Wanchanthuek et al., 2010; Mappley et al., 2012; Black et al., 2015). We identified seven hemolysin proteins from the *hly* and *tly* genes, the most abundant of which was C0R0R9 (*hlyB*, *B. hyodysenteriae*) and, with slightly fewer validated spectra (18 vs. 65), D8IFI0 (*B. pilosicoli*). Except for C0R0R9, which was also present in the exoproteome fraction, the hemolysins were preferentially located in the surfaceome.

Another important set of virulence factors are those related to aerotolerance. Although *Brachyspiras* are anaerobic bacteria, they support aerobic conditions inside their hosts (i.e., oxygen in mucosal tissues; Stanton, 2006). The *nox* product, NADH oxidase, plays an important role in the metabolism of oxygen and the response to oxidative stress in these *Brachyspira* species. Mutant strains lacking *nox* genes were described as being 100- to 10,000-fold more sensitive to oxygen exposure than normal cells and were also shown to be less virulent (Stanton et al., 1999). Several highly abundant *nox*-derived proteins were identified both in the surfaceome and exoproteome fractions; NADH oxidase was among the 10 most abundant proteins detected in these fractions (**Tables 2–3**). In addition, several aerotolerance proteins (BatA, BatB, BatC, and BatE) were detected in much lower amounts.

Proteins related to iron uptake and metabolism are crucial for these bacteria, which live in an environment in which this essential element is stored intracellularly by the host. In Gram-negative bacteria, transport of free iron from the periplasmic space into the cytoplasm is proposed to occur by a classic ABC transporter system (Higgins, 1992). In *B. hyodysenteriae*, a transport system composed of three periplasmic

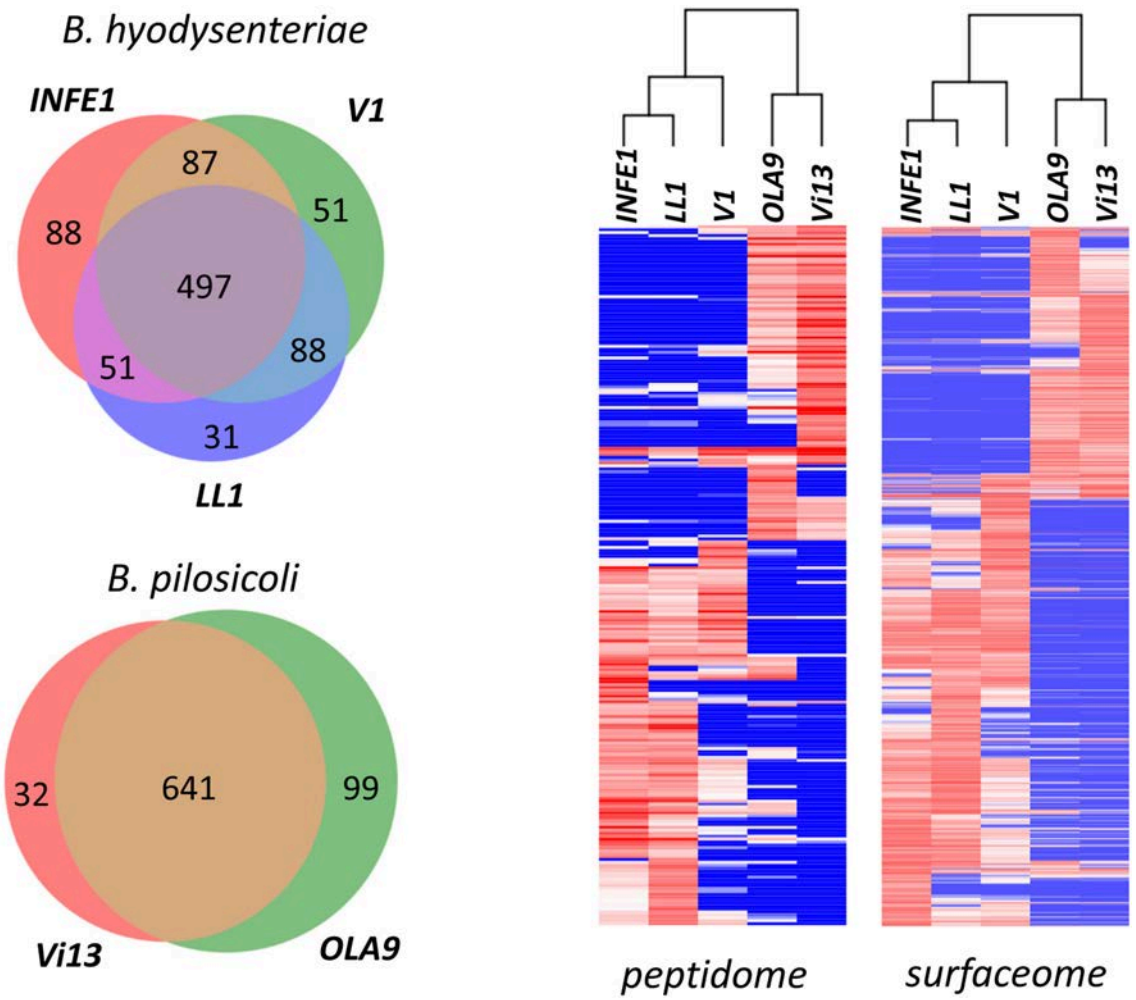

FIGURE 8 | Comparison of the *Brachyspira* strains. (Left) Number of proteins identified in the different *Brachyspira* strains analyzed. Only proteins with more than 15 PeptideShaker-validated spectra were considered. **(Right)** Hierarchical clustering of the peptide sequences identified in the different fractions from each *Brachyspira* strain. Only sequences showing a standard deviation >10 for the PSM observed in the different strains were considered for clustering. The cluster images are highly compressed because they include 240 and 853 peptides for the peptidome and surfaceome, respectively. Full-size cluster figures, including peptide sequence clusters, are shown in Figure S5.

iron-binding lipoproteins (BitA, BitB, and BitC), an ATP-binding protein (BitD) and two cytoplasmic membrane permeases (BitE and BitF) has been described (Dugourd et al., 1999). In our collection we detected 9 Bit proteins, the most frequent being C0QZS5 (BitD), C0QZS3 (BitB), C0QZS4 and O54369 (BitC), and C0QZS2 (BitA). These proteins were found in the exoproteome and surfaceome fractions, with a preference for the latter. We also detected one of the permeases, BitE (C0QZS6). This protein was found only in the surfaceome, although with a low number of validated matches.

Ankyrin-like proteins are delivered to the host cell by many bacterial pathogens. These proteins are known to bind to the host chromatin and to play a critical role in the interaction of the bacterial pathogen with the host cell (Alvarez-Ordóñez et al., 2013). Up to 55 ankyrin accessions from *B. hyodysenteriae* and *B. pilosicoli* were present in our collection. Those with the highest number of peptide matches (57% of total for ankyrins) were C0QW34 (BRAHW) and D8IDE2 (BRAPL). Ankyrins were primarily present in the exoproteome and surfaceome fractions (in ca. 2:1 proportion) and were practically absent in the peptidome data. Among the proteins with more than 10 validated PSM, five ankyrin accessions were characterized as exoproteome-exclusive, whereas five were exclusively found in the surfaceome. All the exoproteome-exclusive proteins with LipoP assignation (three proteins) had an SPI signal peptide, also predicted by SignalP, whereas all the surfaceome-exclusive proteins assigned by LipoP showed an SPIIase cleavage site or were assigned as transmembrane proteins.

Several genes with gene transfer agent (GTA) functionality have been detected in *Brachyspira* species (Motro et al., 2009). These elements permit the interchange of DNA fragments among cells and contribute to the genomic diversity of the species (Lang et al., 2012). The virus of *Serpulina hyodysenteriae* (VSH-1) genes are responsible for the first natural gene transfer mechanism described in spirochetes. This phage-like element consists of three modules encoding the capsid (head, seven genes), tail

(seven genes), and proteins involved in cell lysis (seven genes); these genes are flanked by the bacterial genes *mcpB*, *mcpC*, *glt,* and *oxd* (Matson et al., 2005; Motro et al., 2009). Production of VSH-1 in *B. hyodysenteriae* is related to cell death and occurs after treatment of the cells with mitomycin C, H_2O_2, or antibiotics such as carbadox or metronidazole (Stanton et al., 2008; Lang et al., 2012). The VSH-1 structure contains random packages of DNA that can be incorporated into other cells and transfer antibiotic resistance or other virulence characteristics. It has been shown that *B. hyodysenteriae* strains acquire antibiotic resistance when exposed to VSH-1 particles from antibiotic-treated cells (Stanton et al., 2008). Several proteins from the VSH-1 capsid and tail were identified, including products of *hvp19*, *hvp28*, *hvp38,* and *hvp53*. In addition, our collection included several accessions with no gene assignation corresponding to sequences with 100% homology with products of *hvp13* and *hvp45*. The most abundant members were B9US97, B9US99, and B9US98 (*B. hyodysenteriae*) and B9US83 (*B. pilosicoli*), all of which were present mainly in the surfaceome and exoproteome fractions of the corresponding species (Table S5).

To gain more insight into the possible virulence factors present in our collection, we compared our data with the data available in the VFDB virulence factor database (http://www.mgc.ac.cn/VFs/; Chen et al., 2015). The VFDB (January 2016) describes nearly 26,400 virulence factors derived from 75 bacterial genera. For the analysis, we BLASTed our protein collection against VFDB, selecting the matches with higher BLAST scores (*e*-value <0.01) and higher percentages of amino acid identities (>10%). The filtered collection (459 proteins) corresponded to virulence factors from 85 different bacterial species, with *Yersinia enterocolítica* the most frequent (Table S12). The most frequent virulence classes corresponded to flagellar proteins, followed by capsule-related proteins (mainly enzymes involved in carbohydrate biosynthesis), proteins of the Dot/lcm system (ankyrins), and oligopeptide-binding proteins. Many virulence factors corresponded to proteins related to sugar biosynthesis and metabolism. Among them were proteins related to galactose and mannose metabolism, the pentose phosphate pathway and peptidoglycan biosynthesis, all of which are involved in lipopolysaccharide (LPS) biosynthesis (Wanchanthuek et al., 2010). LPS are endotoxins situated in the outer membrane of Gram-negative bacteria. *Brachyspira* LPS differ from other Gram-negative species in that they contain the lipid A-sugar core of approximately 10–16 kDa but lack the repeating O-sugar ladder characteristic of these molecules. These lipooligosaccharides (LOS) are thought to be involved in the colonic damage produced by *B. hyodysenteriae*. *B. hyodysenteriae* LOS are known to be antigenic and are related to protective immunity against a specific serogroup (Wannemuehler et al., 1988). Among the genes involved in LOS biosynthesis, the more abundant products found were NagA (N-acetylglucosamine-6-phosphate deacetylase), NagB (glucosamine-6-phosphate deaminase), and the PTS N-acetylglucosamine-specific IIBC component, NagE. The *B. pilosicoli* deaminase D8IEE0 (NagB) was the most frequently detected (57% more validated PSM than the second most frequently detected protein); it was found exclusively in the surfaceome fraction, whereas the other *nag* products were detected both in the surfaceome and in the exoproteome. Other abundant proteins were products of the *murA, murC*, *glmU*, *lpxA, lpxM*, *kdsA*, and *rfbF* genes, but many other components of these biosynthetic pathways were also detected (see Table S11, LPS biosynthesis class).

CONCLUSIONS

Considerable effort is being directed to the genome characterization of *Brachyspira* species and isolates; this is reflected in the rapid growth of the corresponding databases and the current availability of a reference proteome for *B. hyodysenteriae*. However, the number of genome products for which there is experimental evidence in these databases is still scarce, and no large-scale project has been directed to the study of the *Brachyspira* proteome. In this work, we present evidence for nearly 30,000 peptide sequences pertaining to more than 1000 proteins from different isolates of *Brachyspira*. This information constitutes a rich source of sequence data for proteogenomic studies. The large-scale characterization of peptides and proteins in the extracellular media as well as of exposed proteins on the bacterial surface provides evidence of the expression of proteins related to virulence factors associated with chemotaxis and motility, iron intake, aerotolerance, and LPS/LOS biosynthesis, and these proteins could be considered as candidates for the production of antibacterial vaccines. The quantitative information on the expression levels and location of these gene products supplied by the mass spectrometry analyses performed in this work gives further valuable information in this respect, as shown here for the reduced expression of vspD on the *B. hyodysenteriae* surface.

AUTHOR CONTRIBUTIONS

JA, VC, and MC conceived the project and design the work. SV and CS supplied the biological samples and characterized the isolates by PCR. VC collected proteomics data. VC and JA analyzed and interpret the data. VC redacted manuscript draft and JA made the critical revision and produced the final manuscript. VC carried out this work in the framework of the Immunology Ph.D. program of the Autonomous University of Barcelona. All the authors approved the final version of the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01103

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5. Paper#3
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Brachyspira hyodysenteriae and *B. pilosicoli* Proteins Recognized by Sera of Challenged Pigs

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The spirochetes *Brachyspira hyodysenteriae* and *B. pilosicoli* are pig intestinal pathogens that are the causative agents of swine dysentery (SD) and porcine intestinal spirochaetosis (PIS), respectively. Although some inactivated bacterin and recombinant vaccines have been explored as prophylactic treatments against these species, no effective vaccine is yet available. Immunoproteomics approaches hold the potential for the identification of new, suitable candidates for subunit vaccines against SD and PIS. These strategies take into account the gene products actually expressed and present in the cells, and thus susceptible of being targets of immune recognition. In this context, we have analyzed the immunogenic pattern of two *B. pilosicoli* porcine isolates (the Spanish farm isolate OLA9 and the commercial P43/6/78 strain) and one *B. hyodysenteriae* isolate (the Spanish farm V1). The proteins from the *Brachyspira* lysates were fractionated by preparative isoelectric focusing, and the fractions were analyzed by Western blot with hyperimmune sera from challenged pigs. Of the 28 challenge-specific immunoreactive bands detected, 21 were identified as single proteins by MS, while the other 7 were shown to contain several major proteins. None of these proteins were detected in the control immunoreactive bands. The proteins identified included 11 from *B. hyodysenteriae* and 28 from the two *B. pilosicoli* strains. *Eight* proteins were common to the *B. pilosicoli* strains *(*i.e.*,* elongation factor G, aspartyl-tRNA synthase, biotin lipoyl, TmpB outer membrane protein, flagellar protein FlaA, enolase, PEPCK, and VspD), and enolase and PEPCK were common to both *species.* Many of the identified proteins were flagellar proteins or predicted to be located on the cell surface and some of them had been previously described as antigenic or as bacterial virulence factors. Here we report on the identification and semiquantitative data of these immunoreactive proteins which constitute a unique antigen collection from these bacteria.

Keywords: *Brachyspira*, vaccine, immunoblot, mass spectrometry, antigen, flagellar protein, swine dysentery, spirochaetosis

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INTRODUCTION

Bacteria in the *Brachyspiraceae* family are gram-negative and spiral-shaped *Spirochaetes*. Members of this phylum are characterized by their unique motility and a loosely coiled morphology caused by the existence of periplasmic flagella (Charon and Goldstein, 2002). Species of the *Brachyspiraceae* family are anaerobic, host-associated intestinal bacteria in pigs, humans and other species, and can cause gastrointestinal pathologies and mortality (Stanton, 2006). Currently, the *Brachyspiraceae* comprises 16 species. *B. hyodysenteriae* and *B. pilosicoli* are well-known *Brachyspira* intestinal pathogens in pigs, responsible for swine dysentery (SD, a severe mucohaemorrhagic colitis) (Alvarez-Ordóñez et al., 2013) and porcine intestinal spirochaetosis (PIS, porcine spirochaetal diarrhea, a mild, non-haemorrhagic colitis), respectively (Trott et al., 1996; Stanton, 2006).

SD is produced by *B. hyodysenteriae*, a restricted pig-associated species, and is a disease present worldwide that has an important economic impact on the farming business. SD mainly affects pigs in the growing-finishing periods. This contributes to the high cost of the disease, which is associated not only with mortality, which is relatively low when animals are treated (Hamdy, 1974), but also with high morbidity, growth retardation and the need for continual in-feed medication.

Unlike most *Brachyspira* species, *B. pilosicoli* has a wide host range. It is zoonotic in pigs, poultry, dogs and humans (Hampson et al., 2006), in which it can lead to intestinal spirochaetosis (IS). In humans, the prevalence of IS is very uneven, much higher in developing regions than in industrialized regions (Tsinganou and Gebbers, 2010).

The treatment for the control of a *Brachyspira* infection involves the use of multiple antimicrobial agents (Alvarez-Ordóñez et al., 2013). Nevertheless, several studies have highlighted the increasing occurrence of *B. hyodysenteriae* and *B. pilosicoli* isolates resistant to these antibiotics in many countries (Molnar, 1996; Karlsson et al., 2001; Rohde et al., 2004; Hidalgo et al., 2009; Ohya and Sueyoshi, 2010; Pringle et al., 2012), greatly compromising the efficacy of this global treatment.

Since Joens et al. described that pigs that recovered from SD acquired immunological protection, and therefore rarely relapse when re-exposed to the infective agent (Joens et al., 1979), several endeavors have been launched to design a vaccine. Unfortunately, these attempts have not been successful to date, and no effective vaccine against *B. hyodysenteriae* or *B. pilosicoli* is available. Early approaches in this area included immunization with an inactivated bacterin (Hampson, 2000) or attenuated strains (Hyatt et al., 1994). In addition to conferring only partial protection in the best cases, these strategies entailed a cumbersome anaerobic-culture of *Brachyspira* which was not appropriate for large-scale production.

In recent years, the development of subunit vaccines based on *Brachyspira* recombinant proteins has also been explored. Molecules studied included flagellar proteins such as FlaA and FlaB (Boyden et al., 1989; Kent et al., 1989; Gabe et al., 1995; Ge and Charon, 1997) and some structural and metabolic proteins, such as outer-membrane proteins BmpB (La et al., 2004), SmpB (Holden et al., 2008), or variable surface proteins (Vsp) (Witchell et al., 2006, 2011). Similar to an attempt to formulate a vaccine based on the ferritin protein FtnA (Davis et al., 2005), none of these antigens provided enough protection for SD. On the other hand, several surface proteins of *B. pilosicoli* (ClpX and two putative oligopeptide-binding proteins) have been evaluated as candidates for vaccination against IS (Movahedi and Hampson, 2007, 2010).

The recent publication of the genome sequences of *B. hyodysenteriae* and *B. pilosicoli* provided a useful tool for the exploration of new candidates for inclusion in vaccination processes. To date, the genome sequence of 20 strains of *B. hyodysenteriae* (including the reference strain WA1, ATCC 49526) (Black et al., 2015) and three strains of *B. pilosicoli* (porcine isolates P43/6/78 and 95/1,000, and an avian isolate B2904) (Wanchanthuek et al., 2010; Mappley et al., 2012; Lin et al., 2013) have been published. The availability of these data enables extensive *in silico* analysis to identify vaccine candidates, which can then be expressed and tested together in a subunit vaccine. The potential of this reverse vaccinology approach was demonstrated by Song et al. who explored the development of a vaccine against SD using a partial genome sequence of the *B. hyodysenteriae* WA1 strain (Song et al., 2009). More recently, a list of 33 ORF candidates to vaccine targets has been patented (Bellgard et al., 2015). These genes were selected on the basis of their homology with known amino acid sequences of surface proteins, secreted proteins and virulence factors from other species.

Despite these important advances on the genomic level, its translation to proteomic knowledge of *B. hyodysenteriae* and *B. pilosicoli* is still a challenge. Undoubtedly, future studies focused on the description of *Brachyspira* proteomes will be necessary to design an effective vaccination strategy. In this regard, we recently characterized a subset of proteins exposed on the cell surface (surfaceome) of *B. hyodysenteriae* and *B. pilosicoli* (Casas et al., 2016). This will not only contribute to select good candidates for a vaccine, but will also impart biological knowledge about invasive and pathogenic mechanisms of *Brachyspira*.

In this study, we extended our proteomic approach to identify potential immunogenic proteins from *B. hyodysenteriae* and *B. pilosicoli*. For this purpose we studied the immunoproteome of two *B. pilosicoli* strains (the isolate OLA9 and the commercial ATCC strain P43/6/78) and one *B. hyodysenteriae* isolate (isolate V1). Cell lysates were fractionated using preparative off-gel isoelectrofocusing and the fractions were separated by SDS-PAGE. The gels were immunoblotted using pig immune-sera, and the reactive bands were identified by mass spectrometry. *Brachyspira* isolates came from Spanish farms. It was reported that during 2001-2003, more than 30% of commercial pig farms in Spain had at least one positive for *B. pilosicoli* or *B. hyodysenteriae* (Carvajal et al., 2006). There is thus a major concern in relation to intestinal diseases caused by *Brachyspira* species in the country (Osorio et al., 2012, 2013), which is the world's fourth largest producer of pig meat and where the porcine industry has a huge socioeconomic impact. We propose the

reported proteins as suitable candidates to be included in vaccines for the treatment of SD and porcine IS.

MATERIALS AND METHODS

Brachyspira Cultures

Two isolates of *Brachyspira pilosicoli* and *B. hyodysenteriae* (strains OLA9 and V1, respectively) and a commercial *B. pilosicoli* (ATCC strain P43/6/78, ATCC 51139) were used for the immunoproteomics study. Three additional *B. hyodysenteriae* strains (the commercial ATCC strains WA1 and B-78, and the isolate INF1) were included in the study of Vsp profiles.

The isolates came from Iberian pigs that showed disease symptoms on two different farms in the Badajoz province (Spain). The medium for the isolation of *Brachyspira* was based on the blood agar modified medium described by Calderaro et al. (2001, 2005), supplemented with antibiotics to remove most of the fecal micropopulation (Feberwee et al., 2008). The medium was composed of blood agar base n° 2 (40 g/l) supplemented with 5% defibrinated horse blood (50 ml) (Oxoid, Thermo Scientific, Waltham, MA, USA), beef extract (3 g/l), Bacto-peptone (5 g/l), (Difco, BD, Franklin Lakes, NJ, USA) and spectinomycin (0.2 g/l), spiramycin (0.025 g/l), rifampicin (0.012 g/l) vancomycin (6.2 g/l), and colistin (6.25 mg/l) (all from Sigma-Aldrich, St. Louis, MO, USA) and 810 ml distilled water. The plates were incubated for 4–7 days at 42°C in an anaerobic jar with CO_2 produced by an AnaeroGen TM 3.5 L (Oxoid, Thermo Fisher Scientific, MA, USA). The colonies were examined by phase contrast microscopy (40x). The isolates were characterized by PCR using species-specific primers for *nox* (*B. hyodysenteriae*) and 16S rRNA (*B. pilosicoli*) as previously described (Casas et al., 2016) and stored at −80°C.

Blood agar solid subcultures of the isolates were seeded in Brain-heart infusion (BHI) broth (Laboratorios CONDA Pronadisa, Torrejón de Ardoz, Spain) enriched with horse serum (15%) and incubated with shaking in anaerobiosis jars at 4°C for 4–7 days. The grown cultures were centrifuged at 12,900 × g for 10 min, and the pellet washed twice with TE buffer (10 mM Tris pH 8.0, 1 mM EDTA, both from Sigma-Aldrich, St. Louis, MO, USA). All bacterial growth and handling procedures were carried out under biosafety level 2 conditions.

Brachyspira Cell Lysate and Protein Quantification

Bacterial pellets (∼150 mg) were resuspended in 500 μl denaturing lysis buffer containing 4% SDS, 0.1 M DTT and 100 mM Tris-HCl pH 7.5. After incubation in a Thermomixer (Eppendorf model Comfort, 600 rpm, 1 h, 95°C), the samples were homogenized in a Bullet Blender (Next Advance Storm, NY, USA) for 3 min at level 8, using 250 μl zirconium silicate beads (0.1 mm diameter, BioSpec, 11079101z). The beads were then pelleted by centrifugation at 14,000 × g for 3 min and the *Brachyspira* cell lysate was recovered from supernatant.

Prior to protein quantification, the excess SDS was removed from the sample using an SDS-Out ™ Precipitation Kit (Thermo Fisher Scientific). The sample was diluted with 100 mM Tris-HCl pH 7.5 (1/2 v/v) for this procedure and the SDS was precipitated with the precipitation reagent (1/20 v/v). After incubation in an ice bath for 20 min, the anionic detergent was pelleted by centrifugation (10,000 × g, 10 min) and the *Brachyspira* SDS-free lysate was recovered from supernatant. After clarification by an additional centrifugation (10,000 × g, 1 min) in a spin cup column, the cell lysate was aliquoted and kept at −40°C until use. A small aliquot was used to measure the protein concentration using the Bradford method (Bio-Rad Laboratories, CA, USA).

Off-Gel Protein Fractionation

Proteins in the *Brachyspira* lysates were fractionated by off-gel isoelectric focusing (Ros et al., 2002; Michel et al., 2003) using an OFFGEL Fractionator 3100 (Agilent Technologies CA, USA). For this procedure, 1 mg of protein (ca. 120–140 μl of cell lysate) was diluted to a final volume of 3.6 ml with a 1.25X protein off-gel stock solution (PROSS 1.25X: 7 M urea, 2 M thiourea, 1.5 M glycerol and 0.1 M DTT) supplemented with ampholytes (GE Healthcare Life Sciences, Barcelona, Spain). Large 24-cm dry strips with a wide immobilized pH gradient of 3–10 units (GE Healthcare Life Sciences) were used for the separation. After placing the 24 well frame, the strips were rehydrated with 50 μl/well of IPG strip rehydration solution (PROSS-ampholytes 1X) for 15 min, and the sample was loaded by pipetting 150 μl/well. To have enough material for an immuno-analysis, the experiments were carried out in triplicate so a total of 3 mg of each *Brachyspira* isolate was processed. The samples were focused for 36 h at 64 kVh with a maximum current of 50 μA and power of 200 mW (4,500 V final voltage). Twenty-four fractions were collected per sample. Three replicates were fractionated per sample. Triplicates of each fraction were pooled in a single tube, aliquoted and stored at −40°C.

Brachyspira-Challenge and Control Pig Sera

Pig sera were provided by the company Laboratorios Larrasa S.L. (Badajoz, Spain) in the frame of a project funded by the Spanish Ministry of Economy and Competitivity (MINECO, IPT-2011-0735-010000). Laboratorios Larrasa S.L. was in compliance with Spanish legislation (R.D. 1201/2005 and Law 32/2007) and EU Council Guidelines (2003/65/CE) for the use of experimental animals. These sera were obtained from pigs from a healthy herd in Extremadura (South-Western Spain) which were inoculated at the age of 14 weeks with active viable cells of *B. hyodysenteriae* strain WA1 (ATCC 49526) or the *B. pilosicoli* porcine strain P43/6/78 (ATCC 51139). The serum was extracted from challenged animals at 12 weeks post-infection. Five different sera from each *Brachyspira* species challenge (*B. hyodysenteriae*-challenge serum 1–5 and *B. pilosicoli*-challenge serum 6–10) were used in this study.

SDS-PAGE Separation of Off-Gel IEF Fractions

Five (for the WB analyses) or ten (for silver staining) microliters of the off-gel fractions were separated by SDS-PAGE. A sample of 20 μl of total lysate (input) was also prepared as a control. The samples were prepared in sample loading buffer (2% w/v SDS,

10% glycerol, 0.002% w/v bromophenol blue and 25 mM Tris-HCl pH 6.8), heated at 56°C for 30 min and resolved on 12 or 7.5%-polyacrylamide gels.

Six replicates were done for each separation. The protein bands in one replicate were visualized by mass spectrometry-compatible silver staining (Shevchenko et al., 1996; Casanovas et al., 2009). The other five replicates were used for immunoblotting with the appropriate antisera.

Immunoblotting

After SDS-PAGE, the proteins in the gel were transferred to a nitrocellulose membrane using an iBlot ™ system (Life Technologies, CA, USA). Following Ponceau staining of the proteins (Sigma-Aldrich, St. Louis, MO, USA), the membrane was blocked with TBS-T (20 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.2% Tween-20) containing 5% (w/v) skimmed milk (1 h, room temperature). The membrane was incubated with the appropriate pig serum diluted in blocking solution (1/3,000 for control serum and 1/5,000 for *Brachyspira* challenge serum) with gentle agitation (1 h, room temperature). The membranes were incubated (1 h) with a Rabbit Anti-pig IgG H&L horseradish peroxidase-conjugated secondary antibody (Abcam ab6777, 1/5,000 in blocking solution) and visualized with luminol.

Characterization of Immunoreactive Bands

Optical density (OD) profiles of the immunoblots and SDS-PAGE gels for all fractions were acquired using ImageJ Version 1.47 (NIH). The profiles were measured between 15 and 100 kDa, and the OD values were normalized relative to the total lane intensity. The Rf value was determined with the Quantity One 1D Analysis Software (Bio-Rad) and position of each band expressed as its molecular mass calculated from the Rf value using molecular mass markers. Calculations using bands for the same protein in a gel showed an average coefficient of variation of 0.85% ($n = 14$) for the calculated mass. As a consequence, bands in different lanes of the same gel with a mass difference less than 1.7% ($2 \times$ standard deviation) were considered the same band and numbered accordingly.

Bands observed in at least two OD profiles of challenge sera but absent in control sera were designated as challenge-specific, differential immunoreactive bands. Some bands were also considered differential when also potentially present in only one of the control sera but observed at a very low intensity. Other bands observed both in the control and challenge sera were considered as challenge-non-specific bands (Supplementary Tables S1, S2).

Mass Spectrometry Analysis and Protein Identification

Silver stained bands corresponding to the reactive bands detected in the immunoblots were excised and digested using an automatic device (DigestPro MS, Intavis, Cologne, Germany). The process involved reduction with dithiothreitol, derivatization with iodoacetamide, and enzymatic digestion with trypsin (37°C, 8 h) (Casanovas et al., 2009). The tryptic digests were evaporated and redissolved in 5 μl of methanol/water/trifluoroacetic acid (30/70/0.1 v/v).

Proteins in the tryptic digests (0.5 μl) were identified by MALDI-TOF peptide mass fingerprinting combined with MS/MS ion search in a 4800 TOF/TOF mass spectrometer (ABSciex, Barcelona, Spain) in the reflectron mode. The spectra were externally mass calibrated using a standard peptide mixture. Alpha-cyano-4-hydroxycinnamic acid (3 mg/ml) was used as the matrix. The five signals with the greatest intensity in each MALDI-TOF spectrum were automatically analyzed by TOF/TOF. The combined TOF and TOF/TOF spectra were interpreted by database search (Mascot, Matrix Science, MA, USA) using the following parameters: peptide mass tolerance, 50 ppm; fragment mass tolerance, 0.5 Da; fixed modification, carbamidomethyl cysteine; variable modification, oxidation of methionine; significance threshold of the MOWSE score, $p < 0.05$. All identifications were manually validated.

Samples which did not produce a positive identification by MALDI were reanalysed by LC-MS/MS in a Velos-LTQ or an Orbitrap-XL mass spectrometer (Thermo Fisher Scientific) equipped with a microESI ion source. Four microliters of each sample digest were diluted to 20 μl with 5% methanol and 1% formic acid, and loaded into a chromatographic system consisting of a C18 preconcentration cartridge (Agilent Technologies) connected to a 15-cm long, 150 μm i.d. (Velos-LTQ) or 100 μm i.d. (Orbitrap-XL) C18 column (Nikkyo Technos Co.). The separation was performed at 1 μl/min (Velos-LTQ) or 0.4 μl/min (Orbitrap XL) in a 30-min gradient from 3 to 40% acetonitrile (solvent A: 0.1% formic acid, solvent B: acetonitrile 0.1% formic acid). The instruments were operated in the positive ion mode with a spray voltage of 1.5 kV. The spectrometric analysis was performed in a data dependent mode. The scan range for full scans was m/z 400–1,800. The LC-MS/MS spectra were searched using SEQUEST (Proteome Discoverer v1.4, Thermo–Fisher Scientific) with the following parameters: peptide mass tolerance, 1 Da (Velos-LTQ) or 20 ppm (Orbitrap-XL); fragment tolerance, 0.6 Da; enzyme, trypsin; two missed cleavages allowed; dynamic modification, methionine oxidation (+16 Da); fixed modification, cysteine carbamidomethylation (+57 Da). The peptide identifications were filtered at 0.1% FDR and only proteins identified with two or more peptides and peptide rank 1 were considered. Relative abundance of the identified proteins in each sample was roughly estimated from the product of the total peptide sequence matches pointing to that protein and its sequence coverage. The group of more abundant proteins bearing more than 80% of the total abundance in the sample were considered for discussion (Full data is available in Supplementary Tables S3–S9).

Searches for the MALDI and LC-MS/MS methods described above were carried out against the Uniprot database (2015_11 version) restricted to *Brachyspira*. When results pointed to indistinguishable different accessions to the same protein in different strains, the accession for the reference ATCC strains was reported in **Table 1**, Supplementary Tables S1, S2.

Mass Spectrometry Target Analysis of Vsp Proteins

LC-MS/MS analyses of Vsps were carried out in a Velos-LTQ using the configuration described above for protein

TABLE 1 | Proteins identified in the immunoreactive bands from the two *Brachyspira* species.

Band id[a]	Sample	Specific[b]	Observed Mass (kDa)		Protein identified		
			12%	7.5%	Accession[c]	Name	Theor. Mass (kDa)
2	OLA9	X	86	76	D8ICZ7	Elongation factor G	75.8
3	OLA9		83		D8IDC2	Uncharacterized protein	62
4	ATCCBP	X	81	78	D8ICZ7	Elongation factor G	75.8
5*	OLA9	X	78	72	J9UT37	Putative polymerase	72.3
					D8IEM8/ A0A0G4K4U5	Chaperone protein HtpG	73.9
					D8IBS0	Chaperone protein DnaK	67.6
6	ATCCBP		77		D8IDC2	Uncharacterized protein	62
7	OLA9		75		D8IDC2	Uncharacterized protein	62.1
9	V1		73		G0EJY7	Uncharacterized protein	65.4
10*	ATCCBP	X	72	70	D8IEM8	Chaperone protein HtpG	73.9
					J9UT37	Putative polymerase	72.3
					D8ICG5	Uncharacterized protein	85
12	V1		72		C0R0R7	Putative treponemal membrane protein	63.9
13	OLA9	X	72	68	D8IE58	Aspartyl-tRNA synthase	67.5
15	ATCCBP		69		D8IDC2	Uncharacterized protein	62.1
16	ATCCBP	X	69		D8IFS5	Phosphoenol pyruvate carboxykinase	67.5
17	ATCCBP	X	69	70	D8IE58	Aspartyl-tRNA synthase	67.5
18	V1	X	68		C0R0E5	Phosphoenol pyruvate carboxykinase	67.5
19	OLA9	X	65		D8I9T6	Biotin lipoyl	65
20	OLA9		64	61	D8IB78	60 kDa chaperonin	58.1
22	ATCCBP	X	63		D8I9T6	Biotin lipoyl	65.0
24	ATCCBP		62	61	D8IB78	60 kDa chaperonin	58.1
25	V1		61		C0QWH4	60 kDa chaperonin	58.2
26*	OLA9	X	58	53	J9USS2	60 kDa chaperonin	58.1
					D8IAM3	Amidohydrolase 3	60.6
					J9UU81	2-isopropylmalate synthase	54.4
					D8IF78	Outer membrane efflux protein	54.7
					D8IDP7	Trigger factor, C-terminal domain protein	50.6
27	OLA9	X	57		D8IBK7	TmpB outer membrane protein	42.5
28	ATCCBP	X	56	55	K0JIZ9	Carboxyl terminal protease	54.9
29*	ATCCBP	X	54		D8IBK7	TmpB outer membrane protein	42.4
31	ATCCBP	X	51	50	D8IBH9	ATP synthase subunit beta	45.6
32	ATCCBP	X*	50		D8IET2	Enolase	47
36	V1	X	45		C0QW84	Uncharacterized protein	38.6
37	OLA9		44		Q9FA06	Putative elongation factor Tu	16.3
					D8ICZ6	Elongation factor Tu	44.8
38	ATCCBP		44		Q9FA06	Putative elongation factor Tu	16.3
					D8ICZ6	Elongation factor Tu	44.8
39	ATCCBP	X	44		D8ICG3	Uncharacterized protein	38.9
40	OLA9	X	43	42	D8IBY6	FlaA	35.7
41	V1		43		C0QVZ4	Elongation factor Tu	44.7
					Q9FA06	Putative elongation factor Tu	16.3
42	V1		42		C0QXS8	NADH oxidase	50.5
					C0QVZ4	Elongation factor Tu	44.7
43	V1		42		C0R0T5	FlaA	24.5
					P32520	FlaA1	36
44	ATCCBP	X	41		D8IBY6	FlaA	35.7
45	OLA9	X	40	38	D8ICU0	VspD	42.7

(Continued)

TABLE 1 | Continued

Band id[a]	Sample	Specific[b]	Observed Mass (kDa)		Protein identified		
			12%	7.5%	Accession[c]	Name	Theor. Mass (kDa)
46*	ATCCBP	X	38	38	D8ICU0	VspD	42.7
					J9TU32	Ribonucleotide-diphosphate reductase subunit beta	41.1
					D8IEW7	Mannose-1-phosphate guanylyltransferase	40.5
					J9URY6	2-oxoacid:ferredoxin oxidoreductase subunit alpha-like protein	82.8
					J9UBH8	ATP-dependent 6-phosphofructokinase	34.8
					D8ICA2	Toxic anion resistance family protein	40.9
					D8I9T4	Uncharacterized protein	39.9
					D8ICR1	Pyruvate oxireductase	35.5
47	OLA9		36		D8IAP2	Flagellin FlaB2	32
48	V1	X	35		C0R1L9	UDP-glucose 4 epimerase	36
					C0QYC2	Galactose-glucose binding protein	38.2
49	ATCCBP		35		D8IAP2	Flagellin FlaB2	32
50	V1		35		Q26501	FlaB1	32
51*	V1	X	35		A0A0H0W3D6	Enolase	47
					Q26501	Flagellar protein FlaB1	32
					A0A0HOUMF4/ G0EJK5	Fructose-bisphosphate aldolase	35.4/35.4
					C0QYC2	Methyl-galactoside ABC transporter substrate-binding protein	38.2
					C0QZV6	Pseudouridine-5'-phosphate glycosidase	33.9
52	OLA9	X*	33		D8ICA8	Putative FlaA	26.7
53	OLA9		32		K0JHQ4	Flagellin FlaB2	31.3
54	V1		31		P80160	Flagellin FlaB2	31.1
55	ATCCBP		31		K0JHQ4	Flagellin FlaB2	31.3
56	OLA9		29		K0JLS4	Flagellin FlaB3	30
57	V1	X*	29		C0QV52	Enolase	47
					Q9F0F6	Flagellin FlaB3	30.4
58	ATCCBP		29		D8IDG1	Flagellin FlaB3	26.6
59	V1		29		Q9F0F6	Flagellin FlaB3	30.4
61	V1	X	26		C0QWY9	Putative FlaAL	24.7
62	ATCCBP	X	24		D8ICA7	FlaA-2	24

Complete identification data is available in Supplementary Table S1 (MALDI TOF/TOF) and Supplementary Tables S3–S9 (LC-MS/MS). Specific, indicates immunoreactivity is only detected in challenged animals. Observed mass, correspond to the experimental mass calculated from the gel bands. ATCCBP, ATCC 51139 (P43/6/78) B. pilosicoli strain.

[a] *All identifications by MALDI TOF/TOF except those indicated with an asterisk which were identified by LC-MS/MS.*

[b] *Specific bands with X* have been detected in a control serum with an intensity of one.*

[c] *Group head accession, full data in Supplementary Tables S1, S2.*

identification. The equivalent to 1 μg of protein in 20 μl of 5% methanol, 1% formic acid was loaded into a system and a 120-min acetonitrile gradient from 0 to 40% was used. The spectrometric analysis was performed in the target mode, acquiring the MS/MS scans of the signals included in the corresponding mass list (Supplementary Table S10). The mass list for each species included at least two unique peptides for each Vsp protein. Potential peptide targets detected in previous shotgun analyses were preferentially selected to build the mass lists. Other targets were selected among the best proteotypic peptides predicted by the PeptideRank software (Qeli et al., 2014).

RESULTS

Protein Fractionation of *B. pilosicoli* and *B. hyodysenteriae* Strains and Selection of Immunogenic Fractions

Proteins from the cell lysates were fractionated by preparative isoelectric focusing (IEF) prior to SDS-PAGE. Via this technique, we could fractionate up to 1 mg of each *Brachyspira* lysate using 24-cm pH 3-10 Immobiline Drystrips. The Off-gel system provided adequate resolution and reproducibility for protein fractionation as verified in a parallel study in which replicate SDS-PAGE analyses were carried out on an arbitrary selection

of 7 out of the 24 fractions collected (Supplementary Figure S1). This reproducibility allowed the pooling of fractions from the three independent IEF fractionations, thereby providing of the equivalent of a total of 3 mg of fractionated proteins for each *Brachyspira* strain.

To select the fractions that contained the immunogenic proteins, the 24 recovered fractions were immunoblotted with sera from pigs challenged with *B. pilosicoli* (serum 6–10, for OLA9 and ATCC 51139 fractionations) or *B. hyodysenteriae* (serum 1–5 for V1 fractionation). Most of the immunogenic bands from the three *Brachyspira* strains appeared in the early-middle fractions (numbers 3–13) that had isoelectric points between 3.5 and 6.5 (**Figures 1**, **2** and Supplementary Figure S2). Note that for all strains, an immunoblot analysis with control serum from non-challenged pigs also revealed some reactive bands (Supplementary Figure S3). This cross-reactivity was consistent with previous reports that showed that healthy pig serum detects *B. hyodysenteriae* surface antigens (Wannemuehler et al., 1988) or some of the recombinant proteins tested for a vaccine against SD (Song et al., 2009).

Protein Identification

IEF fractions from the different *Brachyspira* strains showing the highest immunogenic response (fractions 4–9, 11, 13 and 3–9, 11–12, for *B pilosicoli* and *hyodysenteriae* respectively) (**Figures 1**, **2**) were selected for a more detailed image analysis and band characterization. For this purpose, these fractions were reanalysed by SDS-PAGE and immunoblotted. Immunoreactive bands were identified by densitometry and the immunoblot images were matched with those obtained by silver staining on replicate SDS-PAGE separations (**Figures 3**, **4**). The bands were then excised, trypsin digested and analyzed by mass spectrometry. Overall the components in 51 gel bands (36 from *B. pilosicoli* and 15 from *B. hyodysenteriae* strains) were identified (**Table 1**, Supplementary Tables S1–S9).

Most of the bands (45) could be identified by MALDI, except for six of them that required an LC-MS/MS analysis. The failure of MALDI in the analysis of these bands was probably due to the presence of several major proteins in the band as confirmed from the LC-MS/MS identification data. Thus, when LC-MS/MS identifications were filtered to select the most abundant components, only one of these bands produced a single protein while the others showed the presence of 3–8 major components.

B. pilosicoli Immunoreactive Proteins

Sixteen and 20 immunoreactive bands were detected for the *B. pilosicoli* OLA9 and ATCC 51139 strains, respectively (Supplementary Figures S4, S5A–F and **Table 1**, Supplementary Tables S1, S2). Several of these bands were common to both strains. Some of the fractions (#8 and #9) showed a complex profile at the high mass range of the silver-stained gels. To increase the resolution of the more complex fractions, parallel SDS-PAGE and Western blot analyses were carried out using 7.5% acrylamide gels (**Figure 5**, Supplementary Figures S5B,E). The mass spectrometric analysis of the corresponding silver-stained SDS-PAGE bands (**Figures 3**, **4**) produced the identification of 28 different proteins in challenge-specific immunoreactive bands (**Table 1**). Eighteen of the immunoreactive specific bands yield a single protein (7 for OLA9 and 11 for ATCC 51139), while 4 other bands were shown to contain more than one protein.

The two *B. pilosicoli* strains shared eight proteins in common in the bands with specific reactivity toward the challenge sera (the outer membrane protein of the TmpB family, a flagellar filament outer layer protein FlaA, the variable surface protein VspD, the chaperone protein htpG, a putative polymerase, the aspartyl-tRNA synthase, the biotin lipoyl and the elongation factor G) (**Table 1**).

Seven other proteins common to both strains were found in challenge-non-specific bands including a 60 kDa chaperonin, flagellins B2 and B3 and the elongation factor Tu.

B. hyodysenteriae Immunoreactive Proteins

Fifteen immunoreactive bands were detected in the selected IEF fractions from the *B. hyodysenteriae* farm isolate V1 using sera from *B. hyodysenteriae*-challenged animals (**Figures 3**, **4**, Supplementary Figures S4, S5G,H). Six of these bands showed challenge specific immunoreactivity (#18, #36, #48, #51, #57, #61) while nine others also cross-reacted with control sera from healthy pigs (#9, #12, #25, #41, #42, #43, #50, #54, and #59). Three of the challenge-specific bands, produced a single protein identification, while the other bands were shown to contain several proteins up to a total of 11 (**Table 1**, Supplementary Tables S1–S9). Among the challenge-specific proteins PEPCK (phosphoenolpyruvate carboxykinase) and enolase had also been identified as antigenic in the *B. pilosicoli* isolates.

Cross-Reactivity with Control Sera

For all strains, the immunoblots with sera from control, non-challenged pigs also revealed several immunoreactive bands (Supplementary Figure S3).

Cross-reactivity was observed for all flagellar proteins except *B. pilosicoli* FlaA. The FlaB proteins were the primary targets of the control sera for *B. pilosicoli* and *B. hyodysenteriae* (**Table 1**, Supplementary Tables S1, S2). The three isoforms of FlaB that constitute the inner core of the spirochaetal flagella (FlaB1, FlaB2, and FlaB3) were identified in the immunoreactive bands (**Table 1**, **Figure 4**).

Other proteins immunoreactive to control sera were Elongation factor Tu, which was detected in both species and the uncharacterized protein D8IDC2, which was identified in the two B. *pilosicoli* strains.

Distribution of Vsp Among *Brachyspira* Species and Strains

Although the variable surface protein VspD has been suggested as a potential vaccine candidate against *B. hyodysenteriae* we could only identify VspD in the immunoreactive bands from *B. pilosicoli* samples (**Table 2**, Supplementary Tables S11–S17). To confirm these findings, a more detailed LC-MS/MS analysis

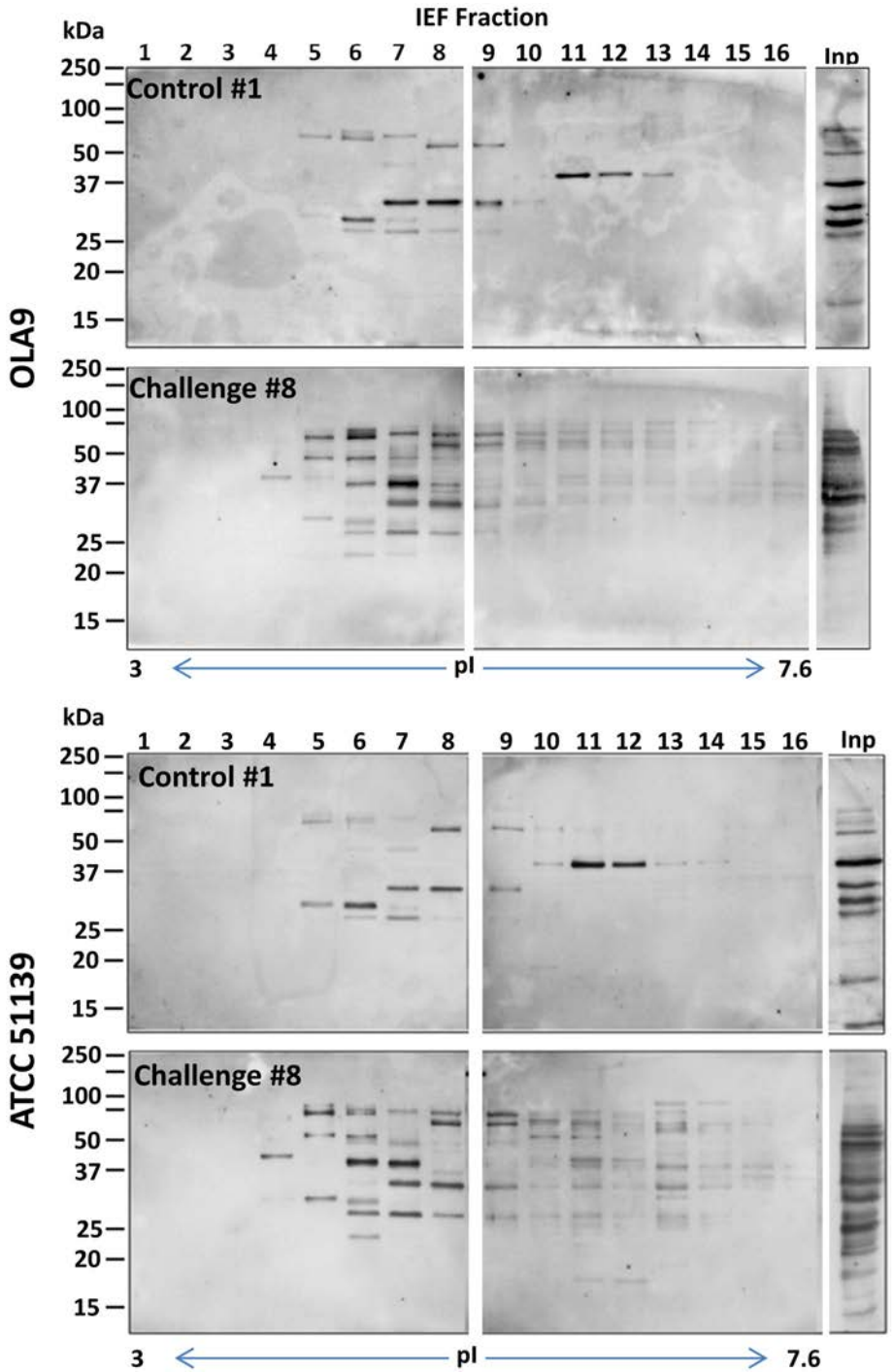

FIGURE 1 | General view of the immunoreactive proteins in each of the IEF fractions of the protein extracts of OLA9 (top) and ATCC 51139 (bottom) *B. pilosicoli* strains. The images are examples of the Western blots prepared with sera #1 and #8 (from a control and a challenged animal, respectively) (upper and lower gels for each strain). Twenty four consecutive IEF protein fractions, covering a pI range from 3 to 10, were analyzed in the corresponding lanes of three SDS-PAGE gels. Fractions presenting intense immunoreactive bands in these preliminary experiments were submitted to a detailed immunoproteomics analysis using all the individual sera available. The Figure shows only the images for the two first gels (lanes for IEF fractions 1–16), containing the more acidic fractions, and the lane corresponding to the crude proteome extract (lane Inp). IEF fractions 17–24 did not show relevant immunoreactive bands (complete images for all the fractions and individual sera tested can be found in Supplementary Figures S2A–C).

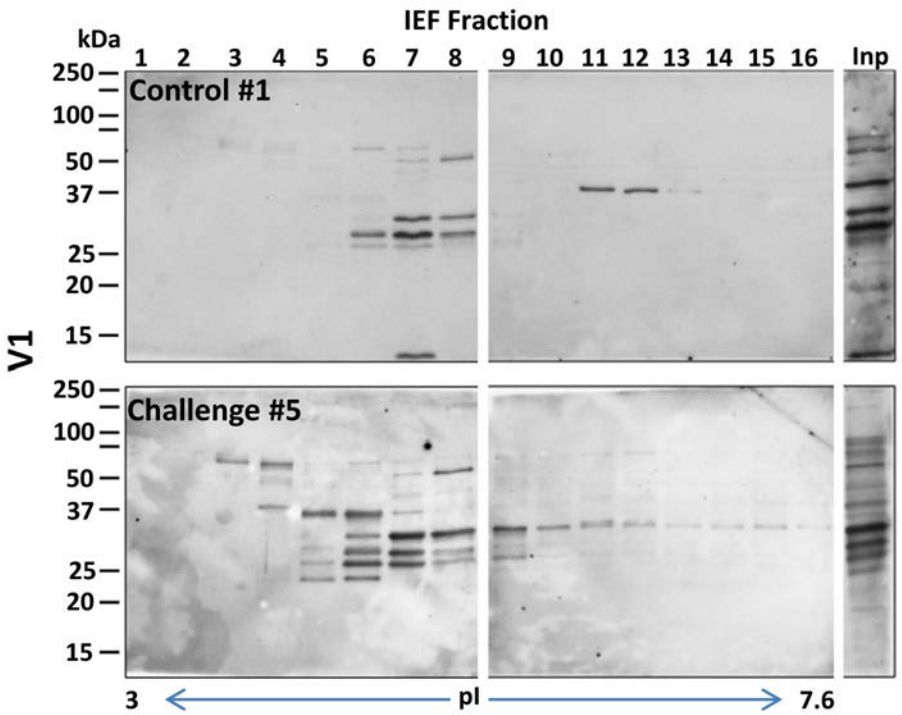

FIGURE 2 | General view of the immunoreactive proteins in each of the IEF fractions of the protein extracts of the *B. hyodysenteriae* strain V1. The images correspond to examples of the Western blots prepared with sera #1 and #5 (a control and a challenged animal, respectively) (upper and lower gels for each strain). Fractions presenting intense immunoreactive bands in these preliminary experiments were submitted to a detailed immunoproteomics analysis, see **Figure 1** for details. Inp, lane corresponding to the crude extract before IEF separation.

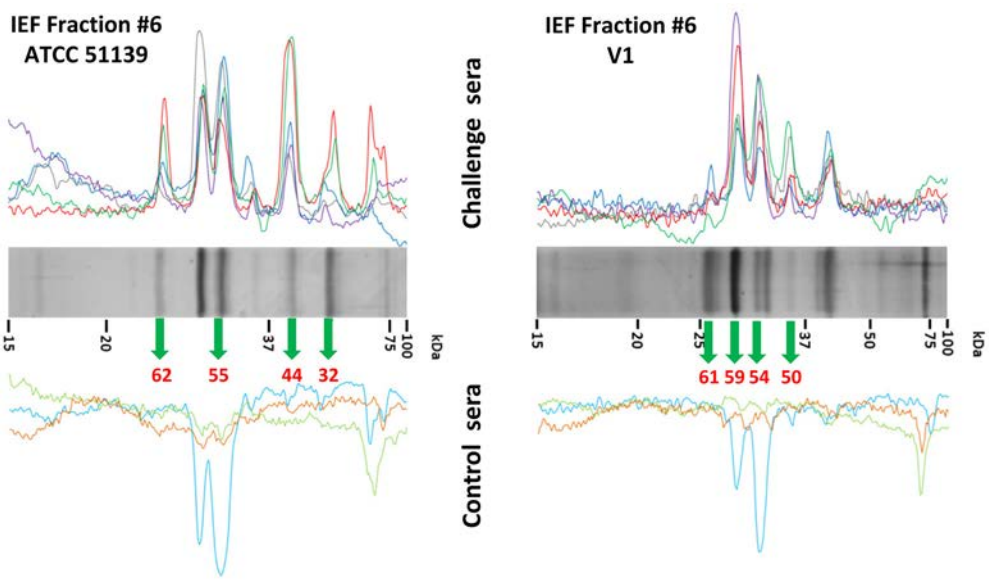

FIGURE 3 | Identification of immunoreactive proteins in the IEF fractions of the *Brachyspira* proteomes. The image shows the Western blot densitometry profiles **(top and bottom)** and the protein band profile of the corresponding silver-stained gel lane (center). Immunoreactivity traces for the 5 sera from challenged pigs **(top)** and the 3 sera for control pigs **(bottom)** are shown with different colors (see SI for color codes). Bands identified as immunoreactive were sliced from the SDS gel lane and submitted to MS analyses for identification. Code numbers for the bands analyzed from these specific lanes are indicated in red. The example given corresponds to the IEF fractions #6 from ATCC 51139 (B. *pilosicoli*) (left) and V1 (*B. hyodysenteriae*) (right) strains. The full set of images for all the fractions is given in Supplementary Figures S5A–H.

was performed targeting the family of Vsp proteins. For this, each Vsp protein was monitored by targeting two or more tryptic peptides with sequences unique to that specific protein (Supplementary Table S10). Three strains of *B. hyodysenteriae* (two commercial ATCC strains and one isolate) and two strains of *B. pilosicoli* (one commercial ATCC strain and one isolate) were analyzed.

The analysis showed that the profile of Vsp proteins was very variable between species and strains (**Table 2**). In agreement with our previous results, VspD, was highly abundant in both B. *pilosicoli* strains (more than 200 PSMs per strain). Contrarily, it was detected with a very low abundance (<25 PSMs) (**Table 2**, Supplementary Tables S13, S17) in only one of the three *B. hyodysenteriae* strains (WA1). Another proposed vaccine candidate in this family, VspH, was found in both species but not in all strains.

The vspC and vspF genes have not been described in B. *pilosicoli*. Both proteins were detected in *B. hyodysenteriae* although the expression was strain-dependent. VspC was detected in the commercial strains (range 25–100 PSMs), but not in the isolate. To the best of our knowledge, this result constitutes the first experimental evidence at the protein level of the expression of this protein. On the other hand, VspF was found to be greatly abundant in the *B. hyodysenteriae* WA1 strain but was not detected in the others. This is similar to reports by Black et al. (2015), who described the absence of the vspF gen in some strains from *B. hyodysenteriae*. According to Witchell et al. (2011), VspF and VspE are found in the cell in a protein complex which can include also other Vsps. Differently to the other members discussed above, VspE was detected in both species and in all strains.

We could not identify VspA, VspG, or VspJ in any of the strains analyzed. Another member, VspB, described from the genome of *B. hyodysenteriae* was detected in all strains but with a very low abundance.

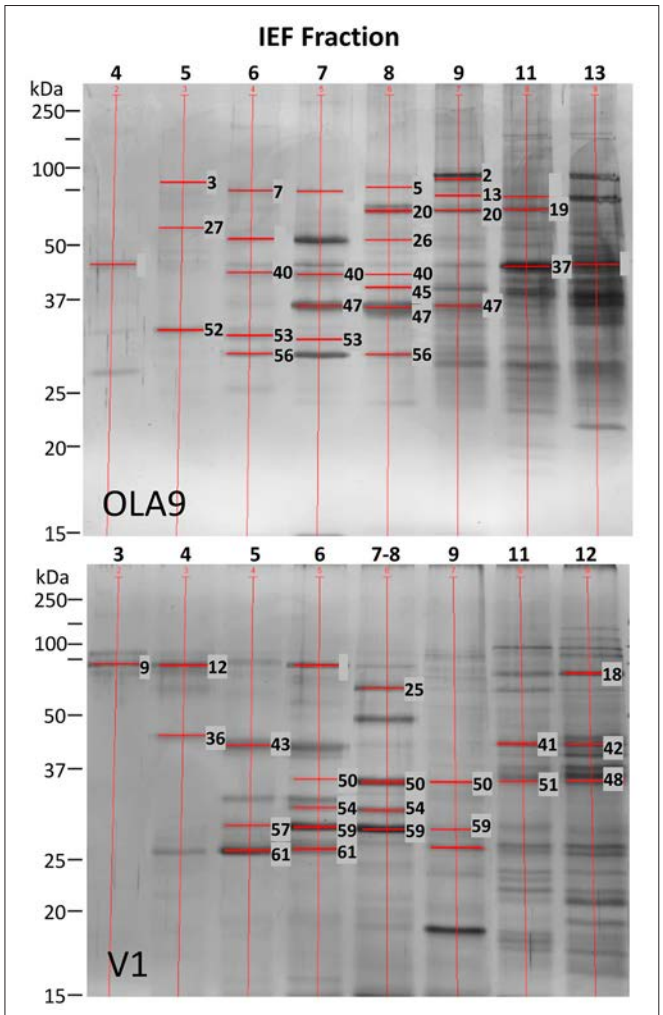

FIGURE 4 | Summary of the analyzed bands from SDS-PAGE silver stained gels for the selected IEF fractions of OLA9 strain (*B. pilosicoli*) and V1 strain (*B. hyodysenteriae*).

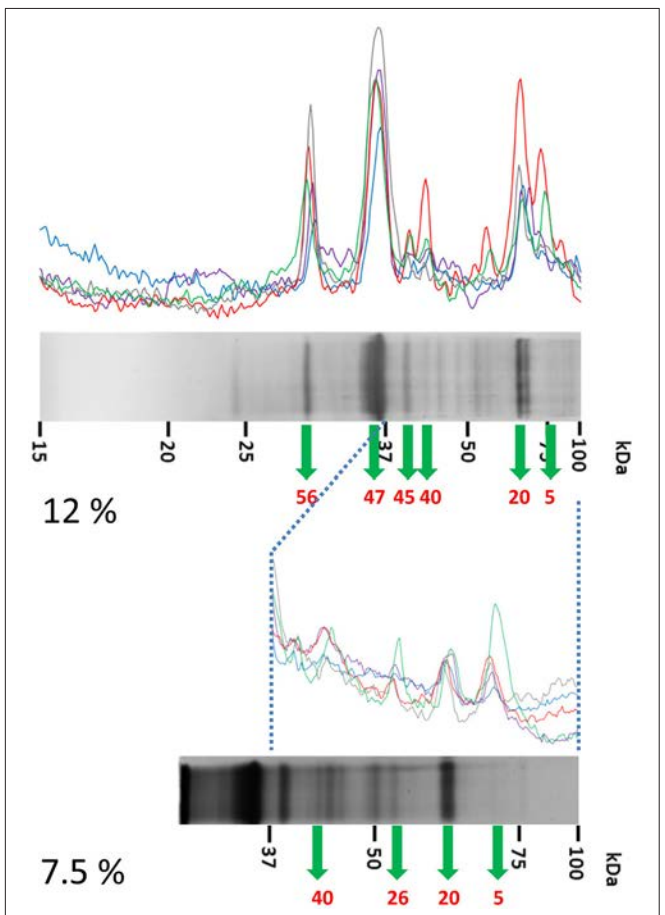

FIGURE 5 | Detailed analysis of the high-mass proteins in IEF fractions #8 and #9 from ATCC 51139. IEF fractions #8 and #9 from B. *pilosicoli* showed unresolved, complex profiles of bands in 12% acrylamide gels (up). These profiles were resolved by a parallel analysis of these fractions in 7.5% acrylamide gels (down). Data from the 12% and 7.5% separations were later combined. The example shown corresponds to IEF fraction #8 (see **Figure 3** for details).

TABLE 2 | Presence of Vsp proteins in *Brachyspira* strains.

	B. hyodysenteriae			*B. pilosicoli*	
	WA1	**B-78**	**INF1**	**ATCCBP**	**OLA9**
Protein	**Collection**	**Collection**	**Isolate**	**Collection**	**Isolate**
VspA	nd	nd	nd	na	na
VspB	*[a]	*[a]	*[a]	na	na
VspC	**	**	nd	na	na
VspD	*	nd	nd	***	****
VspE	*	**	**	**	*
VspF	****	nd	nd	na	na
VspG	nd	nd	nd	na	na
VspH	nd	***[a]	nd	**	nd
VspI	?	?	**	na	na
VspJ	nd	nd	nd	na	na

Asterisks indicate total number of peptide sequence matches (PSM) for the protein (, 0–25 PSM; **, 25–100 PSM; ***, 100–300 PSM; ****, > 300 PSM). PSM is correlated with protein abundance. ATCCBP, ATCC 51139 (P43/6/78) B. pilosicoli strain.*

[a]Identified from only one protein-exclusive peptide.

nd, not detected; na, not monitored (sequence not described for the species).

?, Identified from only one, non-protein-exclusive peptide (common with VspA).

DISCUSSION

Challenge-Specific Immunoreactive Proteins Common to Both *Brachyspira* Species

Two proteins (PEPCK and enolase) were revealed by challenged sera from both *Brachyspira* species. These proteins showed a high degree of identity between species (96% identity for *B. hyodysenteriae* and *B. pilosicoli* enolases and 95% of identity for the corresponding PEPCK).

In a vaccine search, candidates are often selected from membrane-exposed proteins, because surface exposure facilitates recognition by an antibody (Boyle et al., 1997). Still, many cytosolic proteins have been described as major antigens and some of them have also been explored as vaccine components (Davis et al., 2005). In our study, many of the proteins detected were proteins annotated as cytoplasmic. This is the case for PEPCK, whose antigenic capacity had been previously reported in other species. PEPCK was identified as the antigen triggering the cellular response responsible for the hepatic granulomatous inflammation in schistosomiasis (Asahi et al., 2000). Additionally, PEPCK from *Mycobacterium tuberculosis* has been demonstrated to induce a strong immune response in mice and, for this reason, was proposed as a component of a subunit vaccine for tuberculosis (Liu et al., 2006).

Enolase has been reported to be immunoreactive in several pathogenic species, such as *Mycobacterium tuberculosis* (Rahi et al., 2017), and *Borrelia burgdorferi* (Barbour et al., 2008). This protein has also been described as a differential, immunogenic protein in strains of *Bifidobacterium longum* ssp. longum (Górska et al., 2016) and showed protective activity against colitis in mice (Srutkova et al., 2015). Enolase is a moonlighting enzyme found on the surface of some pathogens and involved in the activation of plasminogen (Rahi et al., 2017). In a previous work (Casas et al., 2016), we reported enolase to be among the ten most abundant proteins detected in the surfaceome and exoproteome compartments for *B. pilosicoli* and *B. hyodysenteriae*.

These two proteins are thus tentative candidates for vaccines against *Brachyspira* infections that have not been included in earlier reported vaccines.

Two other putative uncharacterized proteins (C0QW84 and D8ICG3) were identified in challenge-specific bands (bands #36 and #39, respectively) (**Figure 4**) from *B. hyodysenteriae* and *B. pilosicoli*, respectively. Both are predicted (PSORTb v3.0 (Yu et al., 2010), SignalP v4.1 (Petersen et al., 2011) to be extracellular or located on the outer membrane, although C0QW84 lacks the signal peptide on the N-terminal side. These proteins have the same molecular weight and isoelectric point and 62% of identity and 72% similarity between them, suggesting they are different forms of the same functional molecule in these species. In this case, a potential vaccine candidate common to both species would require identifying possible common epitopes capable of inducing an immune response.

B. pilosicoli Challenge-Specific Immunoreactive Proteins

Twenty-eight *B. pilosicoli* proteins were identified in the challenge-specific bands, with 8 of them common to the ATCC 51139 and environmental OLA9 strains. More than half of these proteins could be identified as proteins potentially exposed on the surface of the bacteria or secreted into the media. Thus, 9 corresponded to known or predicted (Gene Ontology Annotation (GOA), PSORTb) membrane or membrane-exposed proteins (TmpB outer membrane protein, outer membrane efflux protein, VspD, enolase, D8ICG5, D8ICG3) or flagellar proteins (FlaA, FlaA2, D8ICA8) (Supplementary Table S2). Six of these proteins (ATP-dependent 6-phosphofructokinase, 2-isopropyl malate synthase, chaperone protein DnaK, pyruvate oxidoreductase, enolase and VspD) were identified in a previous work among the most abundant proteins in the exposed proteome of these bacteria (Casas et al., 2016). Especially VspD, similar to enolase, was among the 10 most abundant proteins exclusively found on the exoproteome (i.e., proteins present in the bacterial culture media). Variable surface proteins constitute a well-known family of antigenic bacterial components. However, no evidence at the protein level had been previously reported for the expression of any Vsp protein in *B. pilosicoli*.

The treponemal outer membrane protein B (TmpB, D8IBK7) was identified as challenge-specific for both *B. pilosicoli* strains. Two putative treponemal membrane proteins (C0R0R7 and C4MGG7) with 47 and 55% homology with *B. pilosicoli* TmpB were included in a reverse vaccinology study against *B. hyodysenteriae,* but they were not immunoreactive when immunoblotted with porcine sera from challenged animals (Song et al., 2009). Contrarily, we detected C0R0R7 in immunoreactive bands of *B. hyodysenteriae.* However, this reactivity was observed toward both the challenge and control sera, so this protein was discarded as a specific vaccine candidate.

The flagellar protein FlaA was also found in the immunoreactive bands of both *B. pilosicoli* strains. FlaA constitutes the outer sheath of periplasmic flagella in spirochetes, impacting the unusual morphology and motility of this bacterial phylum (Li et al., 2000, 2008; Rosa et al., 2005; Jiang et al., 2014; Zhao et al., 2014). Flagellar proteins are major immunoreactive proteins in *B. hyodysenteriae* (Kent et al., 1989). It has been reported that FlaA from *S. hyodysenteriae*, *S. innocens*, and *S. pilosicoli* was recognized by rabbit polyclonal and murine monoclonal antibodies produced against *S. hyodysenteriae* lysates (Fisher et al., 1997). However, no data were previously available on the antigenicity of *B. pilosicoli* FlaA. In our study, both *B. pilosicoli* and *B. hyodysenteriae* FlaA were found in immunoreactive bands although only *B. pilosicoli* evidenced a challenge-specific response.

Two other cytoplasmic proteins, the chaperone protein HtpG and aspartyl tRNA synthase, were found to be immunogenic in both *B. pilosicoli* strains. HtpG has been reported to be responsible for a strong humoural response in human periodontitis caused by *Porphyromonas gingivalis* (Shelburne et al., 2008). On the other hand, aminoacyl tRNA synthases are the targets of many antibacterial compounds (Chopra and Reader, 2014). They play an important role in bacterial resistance as described for *Mycobacterium tuberculosis* strains where mutations in this protein are involved in their resistance to whole-cell inhibitors (Ioerger et al., 2013). No data were available on the antigenicity of these molecules.

B. hyodysenteriae Challenge-Specific Immunoreactive Proteins

Flagellar proteins were the most frequent class of immunoreactive proteins identified in *B. hyodysenteriae* (**Table 1**, Supplementary Tables S1, S2). However, except for a putative flagellar filament outer layer-like protein (C0QWY9), other flagellar proteins identified such as FlaA1, FlaB1, and FlaB3 were also found in bands immunoreactive toward control sera. FlaA1 had been previously described as one of the molecules that produced a highly specific immune reaction in *B. hyodysenteriae* (Li et al., 1993).

Other proteins detected in the challenge-specific immunoreactive bands included the galactose-glucose binding protein, a periplasmic protein, and two cytoplasmic proteins, pyridine nucleotide-disulfide oxidoreductase and fructose-bisphosphate aldolase. These proteins have been detected in the extracellular space in other species involved in processes related to the interaction/ adhesion to the host cell (Tunio et al., 2010; Roier et al., 2015; Zhe et al., 2016). The galactose-glucose binding protein was identified as the main component of the outer membrane vesicles released from 5 different strains of *Haemophilus influenza* (Roier et al., 2015). Extracellular nanovesicles are released by all pathogenic and non-pathogenic gram-negative bacteria (Lusta, 2015). They are composed of outer membrane components such as LPS, glycerophospholipids as well as proteins from the outer membrane and the periplasm (Kuehn and Kesty, 2005; Bai et al., 2014; Lusta, 2015). Outer membrane vesicles are considered as potent virulence factors because they provide a means for the extracellular secretion of proteins and lipids that can interact with the host tissues. The pyridine nucleotide-disulfide oxidoreductase (Zhe et al., 2016) has been identified as one of the proteins that interacts with brain microvascular endothelial cells, which may contribute to invasion by *Streptococcus equi* ssp. zooepidemicus through the blood-brain barrier. Finally, fructose-bisphosphate aldolase has been reported to be immunogenic in *Candida albicans* (Calcedo et al., 2012) and *Madurella mycetomatis,* in which it has been proposed as a vaccine candidate (de Klerk et al., 2012).

Vsp proteins are the most abundant outer membrane proteins of *B. hyodysenteriae* (Gabe et al., 1998), in which they have been postulated to have an antigenic role either as protein complexes or as individual molecules (McCaman et al., 1999; Witchell et al., 2011). Two members of the Vsp family, VspH, and VspD, have been included as components of a potential vaccine against *B. hyodysenteriae* (Bellgard et al., 2015). However, little is known about the expression of these proteins in *Brachyspira* species. The expression of VspH was reported in a B204 strain of *B. hyodysenteriae* (Witchell et al., 2006), although in further studies they described the absence of the gene in other strains (ATCC WA1 and X576) (Witchell et al., 2011). In the latter study, the expression of the VspD protein (together with vspF, vspE, and vspI) in a virulent Australian isolate of *B. hyodysenteriae* (Witchell et al., 2011) was reported. These authors suggested that Vsp proteins form complexes and that they are immunoreactive only in that form.

Interestingly, the analysis of our immunoreactive bands identified VspD only in the *B. pilosicoli* samples. This was in agreement with our previous work (Casas et al., 2016) on the exposed proteomes of *B. pilosicoli* and *B. hyodysenteriae*. In that study, the VspD protein was classified as exclusive from the exoproteome of *B. pilosicoli* because it was not found in any compartment of *B. hyodysenteriae*. To confirm our findings about the differential presence of VspD and to depict the distribution of the Vsp proteins in different *Brachyspira* strains, a more detailed targeted LC-MS/MS analysis was performed. The study confirmed the high expression of VspD in both *B. pilosicoli* strains and the low or no expression in *B. hyodysenteriae* strains.

Thus, Vsp proteins have a broad expression profile in different strains and species, a trait that could determine the efficiency of proteins such as VspH or VspD as vaccine components. Vsp proteins are components of a mechanism used by pathogenic bacteria to adapt to host conditions and optimize colonization. These proteins can show reversible on/off switching of their expression (phase variation) or antigenic changes by the expression of alternative protein phenotypes (Lysnyansky et al., 2001). Variability in the expression of variable surface proteins has been reported for several species of *Mycoplasma*. In *in vitro* experiments with *Mycoplasma bovis*, it was observed that exposure of the bacteria to anti-Vsp antibodies induced a change in the Vsp expression pattern (Caswell et al., 2010). Vsp proteins are the major antigenic targets in *Mycoplasma bovis*, however the immune response observed was not protective. In *Mycoplasma mycoides*, the different Vsp expression pattern observed in several outbreaks was suggested to be related to different Vsp proteins triggering the immune response in each case (Hamsten et al.,

2008). These facts stress the value of determining the actual gene product levels present under different conditions as a complement of genomic-based approaches for vaccine design.

Cross-Reactivity with Control Sera

We observed immunoreactive bands using control sera from non-challenged pigs with all strains tested (Supplementary Figure S3). This was consistent with previous reports showing that healthy pig serum can detect *B. hyodysenteriae* surface antigens (Wannemuehler et al., 1988). Song et al. also observed that non-vaccinated pigs from a herd with no reported history of SD developed increasing systemic lgG and lgM levels to all antigens of a vaccine being tested as the body weight of the animals increased (Song et al., 2009).

The FlaB family of proteins was the primary target of control sera in both species. This finding could be related with reports indicating that endoflagellar FlaBs, but not FlaA, from *B. hyodysenteriae* cross-react with the corresponding proteins from the non-pathogenic *B. innocens* (Li et al., 1993). In fact, it has been shown that the immunoreactivity patterns of purified flagellar proteins from different strains of *B. hyodysenteriae* and several non-pathogenic spirochetes have similar distributions, suggesting the existence of shared epitopes in these species (Kent et al., 1989). Thus, the observed cross-reactivity of flagellar proteins could be due to exposure to other non-pathogenic bacteria. Vaccination of pigs with the endoflagellar protein FlaB1 (recombinant or purified from *B. hyodysenteriae*) generated antibodies against the protein (Gabe et al., 1995). However, that response was not sufficient to protect the animals against the disease. It was suggested that the efficiency of the anti-FlaB antibodies would be reduced by the reduced accessibility to the inner part of the *B. hyodysenteriae* flagella (Gabe et al., 1995).

Other identified targets of the control sera were the Elongation factor Tu and the uncharacterized protein D8IDC2. Elongation factor Tu, is a protein which is conserved in different bacterial species so its detection with sera from pigs that had not been infected with SD or IS is not surprising. This protein has been detected on the surface of *Leptospira interrogans* (the spirochete which is the aetiological agent of leptospirosis) and it is related to the binding with the host plasminogen (Wolff et al., 2013). It is interesting to note that D8IDC2, which was identified in the two *B. pilosicoli* strains, has a 97% of homology with Bpmp-72, a membrane protein whose sequence was patented by Hampson and La (2009) for its use as a vaccine and a therapeutic treatment against intestinal spirochaetosis.

CONCLUSIONS

The immunoproteomics approach applied in this study has been demonstrated to be very effective for the characterization of new *Brachyspira* antigens. Data reported here was restricted to IgG's immunoreactivity toward these molecules. Further studies focussing on serum and secretory IgA's could potentially increase this collection of potential candidates. Most previously reported vaccine candidates were selected on the basis of previous knowledge from other species and through *in silico* reverse vaccinology approaches. The advantage of an immunoproteomics approach is that it intrinsically takes into account the actual expression levels, molecular characteristics and exposure to the host of the specific antigens that elicit an immune response. Consequently, the immunoreactive proteins described are unrivaled candidates to be components of vaccines for the treatment of SD and porcine IS. As discussed, we identified two abundant antigenic proteins shared by the two *Brachyspira* species (enolase and PEPCK) that could be considered as candidates for common vaccines for these species. In addition, 8 and 11 challenge-specific immunoreactive proteins were described for *Brachyspira pilosicoli* and *B. hyodysenteriae*, respectively. Although some of the immunoreactive bands were shown to contain more than one component and the actual antigen should be confirmed, the collection of proteins described constitutes a unique antigen collection from these bacteria.

AUTHOR CONTRIBUTIONS

JA, VC, AR, and MC conceived the project and designed the work. SV prepared the bacterial cultures and characterized the isolates by PCR. AR performed the immunoblot assays and RP the image analysis. VC collected proteomics data. VC and JA analyzed and interpreted the data. AR and VC redacted the manuscript draft and JA made the critical revision and produced the final manuscript. All the authors approved the final version of the article. VC carried out this work in the framework of the Immunology Ph.D. program of the Autonomous University of Barcelona.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00723/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6. General Discussion

The large-scale characterization of pathogens at the proteomic level provides of an actual picture of the cell molecular machinery expressed under certain conditions. This knowledge is vital for the understanding of the molecular processes involved in the host-pathogen interaction, the immunogenic process, and antimicrobial resistance. In this context, we undertook the characterization of *B. hyodysenteriae* and *B. pilosicoli* proteomes, two pathogenic species for which protein data was almost inexistent when our work was started. The studies performed were carried out in the framework of an INNPACTO project directed to the development of vaccines against these species. In consequence, our large-scale approach was strategically directed not only to the analysis of the cell proteome as a whole but also to the subset of components of the exposed proteome (i.e. proteins located on the external part of bacteria or present in the media), a classical target location for the search of potential antigens. In addition we directly searched for antigens through an immunoproteomic approach that made use of sera from infected pigs to recognize bacterial immunoreactive proteins.

6.1 Providing Experimental evidence for *Brachyspira* proteins

The present work is the first approach to a total proteome description for the pathogenic bacteria *Brachyspira pilosicoli* and *B. hyodysenteriae*. This exhaustive analysis incorporated SASs, PTMs and *de novo* sequences. This information is very useful when working with non-curated databases and species with genetic variability. In particular, published databases for *Brachyspira* are poorly curated and had suffered important changes and reorganizations during the development of this work. Our data showed how, despite the ATCC 49526 strain WA1 being a reference proteome in Uniprot, we could identify many WA1 sequences not included in its database but present in other strains (B204, C5, ATCC 27164) databases. This finding also supports the need for curation of the available databases with real data for the expressed protein sequences.

The total proteome of these bacteria was studied using a strategy based on the combination of two different search engines to process the raw data obtained from the shotgun analysis (Figure 1,Paper·#1). In the first stage, using the PeptideShaker search engine platform (Vaudel et al., 2015), we identified 1220 and 1323 proteins in *B. hyodysenteriae* and *B. pilosicoli*, respectively. The spectra that did not match at this first stage were launched into PEAKS software (Zhang et al., 2012). This search revealed 311 new protein groups for *B. hyodysenteriae* and 256 for *B. pilosicoli*.

The exposed proteome was studied applying a cell-shaving protocol (controlled enzymatic treatment of intact cells) (Figure 6.1). Among the most abundant proteins identified in these compartments were proteins related to movement/chemotaxis, ribosomal proteins, enolase, NADH oxidase and Heat Shock Proteins. In total, 53 and 139 proteins exclusive of the surfaceome compartment and 55 and 64 proteins exclusive of the exoproteome compartment were identified for *B. hyodysenteriae* and *B. pilosicoli*, respectively.

In the immunoproteome study, potential *Brachyspira* immunogenic proteins were revealed by immunoblotting with pig-immune sera. Thus, 11 immunoreactive proteins for *B. hyodysenteriae* and 8 for *B. pilosicoli* (common to the two strains included in the study) were described.

Overall, 67-70% proteome coverage for *B. hyodysenteriae* and *B. pilosicoli* species was reached in the total proteome analysis (Paper #1). In the exposed proteome study (Paper #2); despite focusing on a cell sub-proteome, similar results for proteome coverage were obtained (72-60% for *B. hyodysenteriae* and *B. pilosicoli*, respectively). This can be explained because the most exhaustive analysis carried out in the exposed proteome study, where 148 LC-MS/MS analysis were performed, in comparison with the 5 analyses included in the total proteome study. Also, the sample fractionation step introduced in the analysis of the exposed proteome (to obtain the exopeptidome, exoproteome and surfaceome sets) could have contributed to the relative high proteome coverage obtained from this subset of the proteome.

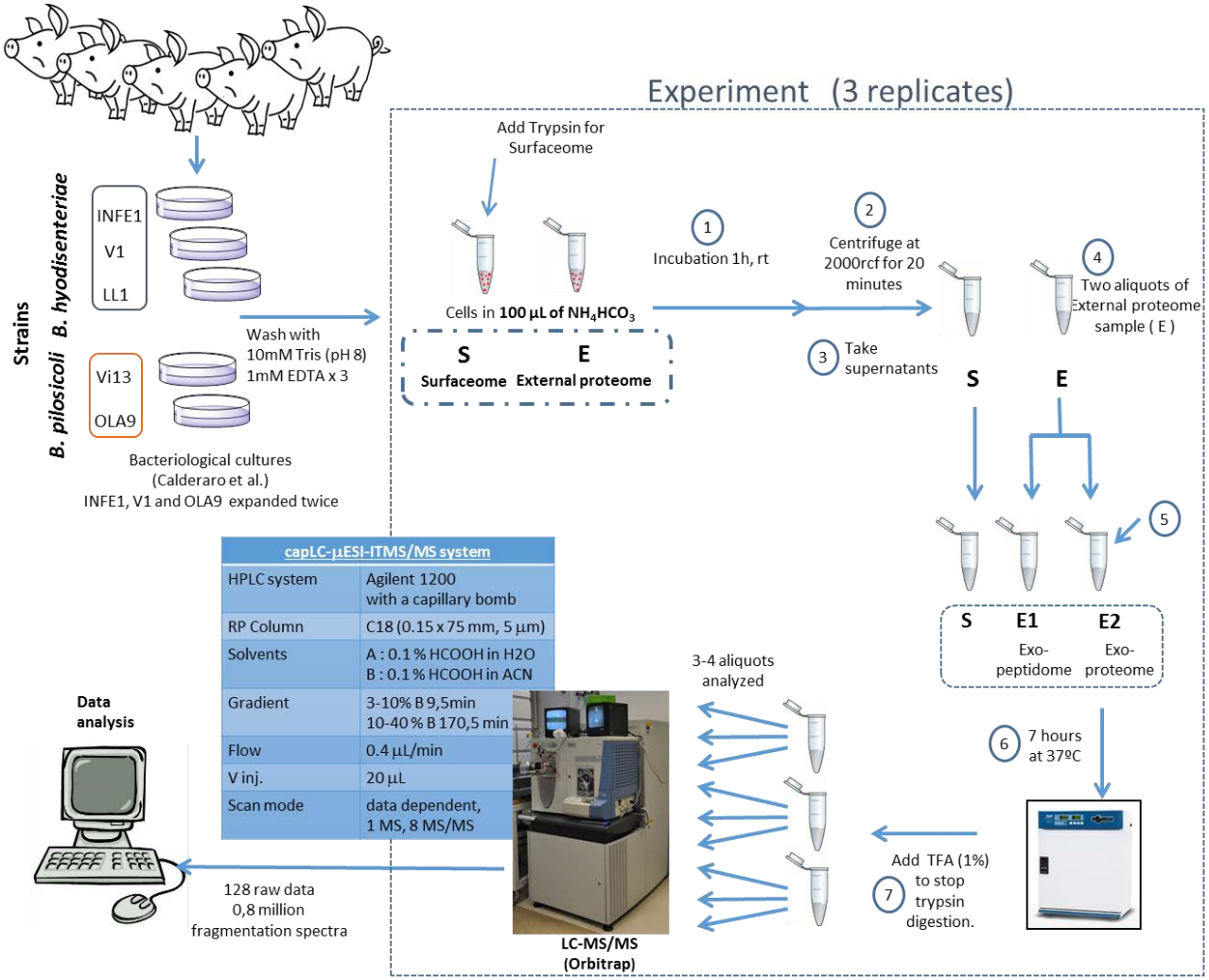


Figure 6.1. *Cell-shaving* workflow used in this Thesis.

6.2 Post-translational Modifications

Information on the PTM profile in these proteomes was obtained through the direct analysis and also using affinity-based enrichment procedures.

It has been reported that methylation, phosphorylation and acetylation are the most important PTMs in microorganisms (Cao et al., 2010) and these preferences were also observed in the *Brachyspira* species included in this study (Table 6.1).

Table 6.1. Summary of most frequently detected PTM in *Brachyspira* species.

		B. hyodysenteriae		*B. pilosicoli*	
	PTM	**Peptides**	**PSMs**	**Peptides**	**PSMs**
Direct analysis	Methylation	26	111	46	132
	Hydroxylation	5	31	6	19
	Dihydroxylation	19	82	3	4
	Oxidation	15	53	5	9
	Phosphorylation	1	5	1	4
	Acetylation K	0	0	13	51
Enrichment	Phosphorylation	74	499	86	305
	Acetylation K	3142	5393	5496	12471

In total, 74 and 86 phosphopeptides (79 and 91 sites) and 3142 and 5496 acetylated peptides (3221 and 5579 sites) were identified in *B. hyodysenteriae* and *B. pilosicoli*, respectively. In *B. hyodysenteriae*, 26 methylated peptides (72 proteins) were identified. The methylated dataset was enriched in proteins related to movement, flagella or chemotaxis, a distribution that was also reported for *Leptospira* (Cao et al., 2010). For *B. pilosicoli*, 46 methylated peptides (pointing to 180 different protein accessions) were identified, most being enzymes.

Other PTMs were detected in the total proteome study in significant numbers, such as oxidation, hydroxylation and dihydroxylation (Table 6.1). This group of oxidative modifications is enriched in external proteins, especially in *B. hyodysenteriae*, where 62% of proteins with known cellular location from the 72 proteins identified (39 oxidised peptides), were annotated as outer membrane, periplasmic space or plasma membrane proteins.

The major fraction of identifications obtained in these experiments corresponded to phosphorylated and acetylated peptides and were obtained from samples

previously affinity-enriched in the corresponding modified peptides (Table 6.1). Without affinity enrichment, only two phosphorylated peptides could be identified (one for each species). After TiO_2 enrichment, the number increased to 74 and 86 phosphorylated peptides for *B. hyodysenteriae* and *B. pilosicoli*, respectively. Taking into account the relative number of phosphopeptides (number of identifications/amount of sample purified) for other bacterial species (Table 6.2), the number of phosphopeptides characterized for *Brachyspira* species in this work is higher (between 19 and 39-fold), although much lower than the ratio identified in eukaryotic cells using the same enrichment method (between 170 and 200-fold).

Table 6.2. Phosphopeptides identified in samples enriched with TiO_2 for *Brachyspira* and other species.

Species	#Phosphopeptides	#Phosphopeptides/mg	Publication
Synechococcus sp.	280	14	(Yang et al., 2013)
Leptospira interrogans	34	8	(Cao et al., 2010)
B. pilosicoli	86	312	Paper #1 this Thesis
B. hyodysenteriae	74	269	Paper #1 this Thesis
Homo sapiens **(Jurkat cells)**	2512	55800	V. Casas (unpublished results)

In *B. hyodysenteriae*, 315 of 499 PSMs were derived from a phosphopeptide of the electron transport complex subunit G protein. This is a membrane protein involved in binding to flavin mononucleotide and the electron transport chain. In *B. pilosicoli*, the top three most phosphorylated proteins were GAPDH, PEPCK and enolase (30, 28 and 25 PSMs, respectively). PEPCK and enolase were also identified in the immunoproteome study (Paper#3) as immunoreactive-specific for both *Brachyspira* species.

The increase in the number of phosphorylated peptides detected after enrichment with respect to the initial number in the direct analysis highlights the importance of using selective enrichment techniques to study PTMs. The effect of the

enrichment was even more significant in the case of acetylation (Table 6.1). Acetylation shown to be highly frequent in these species; 3142 and 5496 acetylated peptides (892 and 1127 proteins) were detected for *B. hyodysenteriae* and *B. pilosicoli*, respectively. This is in agreement with several reports indicating that acetylation is one of the most prominent PTM in bacteria (Bernal et al., 2014; Cain et al., 2014). Thus, the percentage of proteome acetylated was 39% for *B. hyodysenteriae* and 51% for *B. pilosicoli*, which are higher values than the reported for other bacterial species, ranging from 2% to 34% (Ouidir et al., 2016).

The PTM abundance in these species and the fact that most proteins identified as immunoreactive in the immunoproteome study contain PTM in their sequence indicates the importance of characterizing bacterial pathogens at the proteomic level. Thus, describing the actual gene products expressed and how they are expressed (cellular levels and mechanism involved on protein modifications) is very important, not only for vaccine development but also for the expression of recombinant therapeutic proteins in bacteria such as *E. coli* (Wolfe, 2015).

6.2.1 The case of acetylation

In our dataset, we observed a higher acetylation level in *B. pilosicoli* than in *B. hyodysenteriae*. This was initially observed in non-enriched samples submitted to shotgun analyses and confirmed using targeted mass spectrometry. Moreover, immunoblotting with an anti-acetyl Lysine antibody of several *Brachyspira* strains (including commercial ATCCs and environmental isolates) showed that *Brachyspira pilosicoli* strains were highly acetylated, while in *Brachyspira hyodysenteriae*, the signal was insignificant, except for one band in one environmental strain. This fact was reinforced by the immunopurification of ATCC strains, which showed that PSMs for acetylated peptides were 2.3-fold more abundant in *B. pilosicoli* than in *B. hyodysenteriae*.

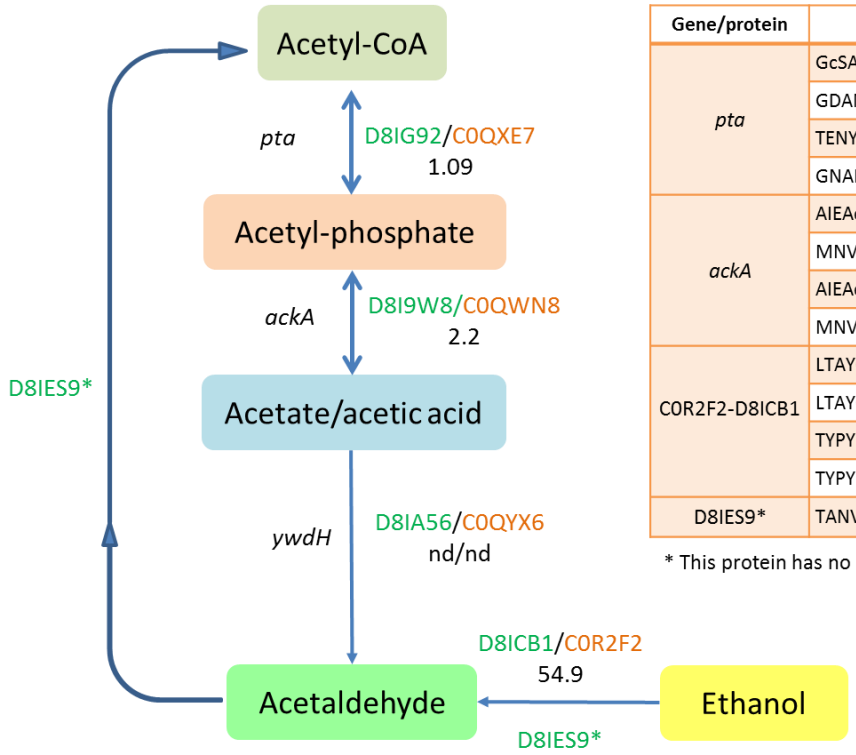
The statistical analysis of acetylated proteins dataset (String v10.0) showed that at the highest confidence interaction score (0.9), datasets from both species were significantly enriched in KEGG pathways such as metabolic pathways or the biosynthesis of secondary metabolites. The high abundance of metabolic proteins in the acetylomes of *B. hyodysenteriae* and *B. pilosicoli* is in accordance with the

observed for other bacterial species (Bernal et al., 2014; Guo et al., 2016; Kim et al., 2013b; Meng et al., 2016; Wang et al., 2010a; Xie et al., 2015).

These species show differences on the end products secreted to the media during bacterial growth (Stanton and Lebo, 1988; Trott et al., 1996). For instance, ethanol is produced in small amounts only by *B. pilosicoli* and although both species produce large amounts of acetate, the amount detected in *B. pilosicoli* is almost double that in *B. hyodysenteriae*. Ethanol is produced by many microorganisms under anaerobic conditions by the alcoholic fermentation process. Ethanol production has been observed in patients with overgrowth of *Candida albicans* and gastrointestinal abnormalities symptomatic of alcohol intoxication. Other pathogens, such as the gastric pathogen *Helicobacter pylori*, have significant alcohol dehydrogenase activity and are able to use ethanol to produce acetaldehyde (Salaspuro, 1997).

The presence of ethanol in the media produces cell stress. The effects of ethanol stress in the bacterial proteome has been studied in *E. coli* (Soufi et al., 2015). This stress produces several important changes at different levels, affecting PTMs and the proteome composition. For instance, it was observed that the acetylation level increased in just 10 minutes. The authors suggested that this fact was likely due to the conversion of ethanol to acetate and the acetate binding to susceptible amino acids via a non-enzymatic system.

Figure 6.2 shows proteins from Acetyl-CoA/Acetate metabolic pathway involved in the acetylation process for *B. hyodysenteriae* and *B. pilosicoli* (Kanehisa et al., 2017). Most of the reactions and components in this path are shared by both species, except for the conversion from ethanol to acetaldehyde and further to Acetyl-CoA, which is exclusive to *B. pilosicoli*.



Gene/protein	peptide	area	sp
pta	GcSAQDIADLAVITSLQAK	919633	hyo
	GDANVFIFPELNSGNIGHK	1938388	hyo
	TENYIELLFK	792187	pilo
	GNANVLIFPNLNAGNIGYK	2332185	pilo
ackA	AIEAccDIAPLHNPAGLQGISAcK	3936019	hyo
	MNVLVINSGSSSIK	573747	hyo
	AIEAccDIAPLHNPAGLQGIR	8842965	pilo
	MNVLVINSGSSSIK	1071903	pilo
C0R2F2-D8ICB1	LTAYQGFDALFHSTEGYINK	8756	hyo
	LTAYQGFDALFHSTEGYINK	739971	pilo
	TYPYLSIVDPELMK	16932	hyo
	TYPYLSIVDPELMK	671581	pilo
D8IES9*	TANVFEPDVIIALGGGSPIDAGK	477014	pilo

* This protein has no homologue in hyo.

Figure 6.2. Acetyl-CoA/Acetate metabolic pathway (left) and MS identification results for the proteins involved (right). *B. hyodysenteriae* and *B. pilosicoli* accessions in orange and green, respectively. When the protein was described for both species the ratio of areas detected is indicated. From KEGG pathways (Kanehisa et al., 2017). sp. species.

Our targeted analysis for the components of this pathway showed that the *pta* gene (proteins C0QXE7 and D8IG92 for *B. hyodysenteriae* and *B. pilosicoli*, respectively) was expressed at the same level in both species. However, *ackA*, the gene that interconverts acetyl-phosphate (ACP) and acetate, was expressed in a ratio of 2.2 to 1 in *B. pilosicoli* compared to *B. hyodysenteriae.* The effect of these enzymes (*pta/ackA*) in protein acetylation has been widely investigated before. It is known that, during glycolysis, in the conversion of glucose to acetate, the lack of *ackA* increases the acetylation level, because acetylation is related to the production of ACP (Schilling et al., 2015; Weinert et al., 2013).

ACP levels play an important role in proteome acetylation in *E. coli*, where acetylation at Lysine depends mainly on non-enzymatic acetyl phosphate-dependent processes (Schilling et al., 2015). This ACP-regulated acetylation is a response to carbon overflow to regulate central metabolism. Acetylation with ACP is specific, and requires an environment that is able to bind to the phosphoryl

group, locate the acetyl group and deprotonate the Lysine. The proteins with acetylated Lysine residues in response to this carbon overflow were mainly involved in central metabolism. Enzymes in central metabolism become acetylated due to glucose consumption.

The effects of ethanol on the *pta* and *ackA* genes has been estudied in *A. baumannii* (Camarena et al., 2010) where these genes showed 4.4- and 5.3-fold induction in the presence of the alcohol. The study of Camarena et al. postulates that *A. baumannii* converts ethanol to acetate and then to Acetyl-CoA. Thus*, A. baumannii* would assimilate ethanol as a C source. For this, genes related to central metabolism and ethanol/acetate assimilation are induced by the presence of ethanol. This increase in metabolic capacity and the expression of proteins related to the stress response has been proposed to be related to an increase in virulence.

Other protein involved in the acetylation process is the Iron-containing alcohol dehydrogenase C0R2F2/D8ICB1 (accessions for *B. hyodysenteriae* and *B. pilosicoli*, respectively). We found this protein expressed in a 50:1 ratio when compared *B. pilosicoli* to *B. hyodysenteriae*. This protein is responsible for oxidizing alcohol to aldehyde. This action is also performed by the Aldehyde-alcohol dehydrogenase (D8IES9), which is involved in the production of Acetyl-CoA from ethanol and acetaldehyde, a metabolic route exclusive to *B. pilosicoli*. We could measure D8IES9 in the study of the exposed proteome of these *Brachyspira* species where it was found preferentially in the exoproteome compartment. Although D8IES9 was detected in the two *B. pilosicoli* strains studied (OLA9 and Vi13), its expression was much higher in OLA9 (569:165 PSMs). This fact agrees with the higher acetylation level detected in OLA9 by Western blot with antibody specific anti-acetyl Lysine (Figure 6.3).

Central metabolism pathways of *B. pilosicoli* and *B. hyodysenteriae* have been described as being quite similar*,* suggesting that they share many metabolic capabilities (Bellgard et al., 2009; Wanchanthuek et al., 2010). In any case, it is known that *B. hyodysenteriae* and *B. pilosicoli* liberate different end products to the media. *B. pilosicoli* produces larger amounts of acetate than *B.*

hyodysenteriae; and ethanol, which is produced by *B. pilosicoli*, is not detected in *B. hyodysenteriae* cultures (Stanton and Lebo, 1988; Trott et al., 1996). The differences we observed in the analysis of the acetylome and the proteins involved in the Acetyl-CoA/Acetate pathway are the molecular reflection of these metabolic differences. Thus, the higher production of Acetyl-CoA and ACP by *B. pilosicoli* due to the increased *ackA*, D8ICB1, and D8IES9 expression could be involved in the higher protein acetylation level observed in *B. pilosicoli*.

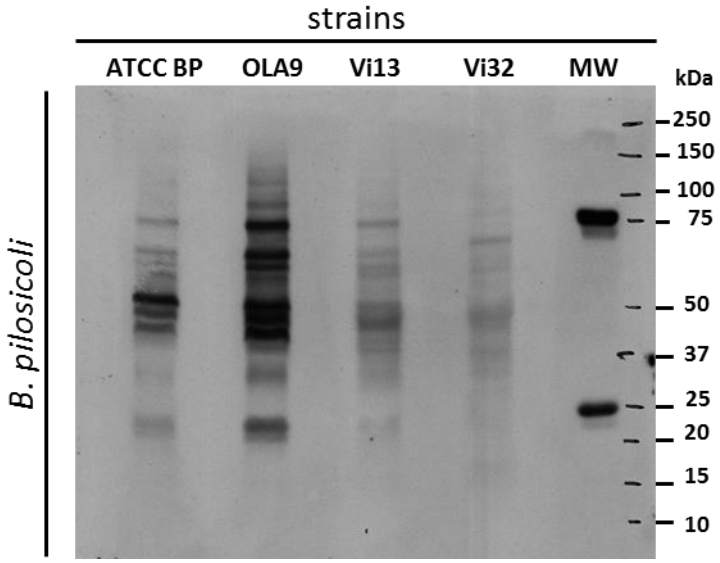


Figure 6.3. Western-blot prepared with anti-acetyl Lys antibody of different strains for *B. pilosicol*

6.3 Vsp proteins

Vsp proteins are the most abundant outer membrane proteins of *B. hyodysenteriae* (Gabe et al., 1998) in which they have been postulated to have an antigenic role either as protein complexes or as individual molecules (McCaman et al., 1999; Witchell et al., 2011). Two members of the Vsp family, VspH, and VspD, have been included as components of a potential vaccine against *B. hyodysenteriae* (Bellgard et al., 2015).

Interestingly, in our analysis of the immunoproteome, VspD was only detected in *B. pilosicoli* samples. This was in agreement with the results obtained in the study of the exposed proteome. In this study, VspD was classified as exclusive of the

exoproteome of *B. pilosicoli*, because it was not found in any compartment of *B. hyodysenteriae*. To confirm these findings and to clearly depict the distribution of the Vsp proteins in different *Brachyspira* strains, a more detailed, targeted LC-MS/MS analysis was performed. This study confirmed the high expression of VspD in both *B. pilosicoli* strains (P43/6/78 and OLA9) and the low or no expression in *B. hyodysenteriae* strains (WA1, B78, and INF1). The other vaccine candidate, VspH, was found in both species but not in all strains (Table 6.3).

Table 6.3. Presence of Vsp proteins in *Brachyspira* strains.

	B. hyodysenteriae			***B. pilosicoli***	
Protein	**WA1** *ATCC 49526*	**B78** *ATCC 27164*	**INF1** *isolate*	**P43/6/78** *ATCC 51139*	**OLA9** *isolate*
VspA	nd	nd	nd	na	na
VspB	*[1]	*[1]	*[1]	na	na
VspC	**	**	nd	n.a.	na
VspD	*	nd	nd	***	****
VspE	*	**	**	**	*
VspF	****	nd	nd	na	na
VspG	nd	nd	nd	na	na
VspH	nd	***[1]	nd	**	nd
VspI	?	?	**	n.a.	n.a.
VspJ	nd	nd	nd	n.a.	n.a.

(Note) Asterisks indicate total number of peptide sequence matches (PSM) for the protein (*, 0-25 PSM; **, 25-100 PSM; ***, 100-300 PSM; ****, > 300 PSM). PSM is correlated with protein abundance

[1] Identified from only one protein-exclusive peptide

nd, not detected. na, not monitored (sequence not described for the species)

?, Identified from only one non-protein-exclusive peptide (common with VspA)

The *vspC* and *vspF* genes have not been described in *B. pilosicoli*. Both proteins were detected in *B. hyodysenteriae*, although the expression was strain-dependent. VspC was detected in the commercial strains (range 25–100 PSMs)

but not in the isolate. To the best of our knowledge, this result constitutes the first experimental evidence at the protein level for the expression of this protein. In contrast, VspF was found to be highly abundant in the *B. hyodysenteriae* ATCC 49526 strain but was not detected in the others. This is similar to reports by Black et al., who described the absence of the *vspF* gen in some strains from *B. hyodysenteriae* (Black et al., 2015). According to Witchell et al., VspF and VspE are found in the cell in a protein complex that can also include other Vsps (Witchell et al., 2011). Unlike the other members discussed above, VspE was detected in both species and in all strains.

We could not identify VspA, VspG, or VspJ in any of the strains analysed. Another member, VspB, described from the genome of *B. hyodysenteriae*, was detected in all strains but with very low abundance. These data reflect the high variability of expression in this group of proteins. Another example of this variability is the VspD protein from *B. pilosicoli* (D8ICU0), which in the study of total proteome was identified with more than 50 SASs (single aminoacid substitutions) in its sequence. One of these altered sequences was detected with 57 PSMs.

Vsp proteins are components of a mechanism used by pathogenic bacteria to adapt to host conditions and optimize colonization. These proteins can show reversible on/off switching of expression (phase variation) or antigenic changes by the expression of alternative protein phenotypes (Lysnyansky et al., 2001). Variability in the expression of variable surface proteins has been reported for several species of *Mycoplasma*. In *in vitro* experiments with *Mycoplasma bovis*, it was observed that exposure of the bacteria to anti-Vsp antibodies induced a change in the Vsp expression pattern (Caswell et al., 2010). Vsp proteins are the major antigenic targets in *Mycoplasma bovis;* however, the immune response observed was not protective. In *Mycoplasma mycoides*, the different Vsp expression pattern observed in several outbreaks was suggested to be related to different Vsp proteins triggering the immune response in each case only in that form (Hamsten et al., 2008).

Thus, Vsp proteins have a broad expression profile in different strains and species. These results, especially those for VspD, pose important questions on the efficiency of the currently proposed vaccines and reinforce the need for the quantification of bacterial proteins for their design. This is important not only in the characterization of vaccines directed to several related species but also to different strains of the same species. This is the case of the other proposed vaccine candidate in this family, VspH, which was found in both species but was not present in all strains.

6.4 Immunoreactive proteins

Proteins showing specific immunoreactivity against sera from challenged pigs can be considered as potential candidates to vaccine components against *B. pilosicoli* and *B. hyodysenteriae* species (Table 6.4).
Notably, 62% of these proteins were detected modified in the analysis of the full proteome and of the acetylation/phosphorylation-enriched samples. This fact should be taken into account for vaccine development because the epitope recognized by the immune system could contain a PTM in its sequence.

6.4.1 Immunoreactive proteins common to both species

The proteins PEPCK and enolase from the two *Brachyspira* species were found immunoreactive against sera from challenge pigs. Immunoreactivity for these proteins was reported previously in other species. PEPCK was identified as the antigen triggering the cellular response responsible for hepatic granulomatous inflammation in schistosomiasis (Asahi et al., 2000). Additionally, PEPCK from *Mycobacterium tuberculosis* has been demonstrated to induce a strong immune response in mice and, for this reason, was proposed as a component of a subunit vaccine for tuberculosis (Liu et al., 2006). Enolase immunoreactivity has been described in several pathogenic species, such as *Mycobacterium tuberculosis* (Rahi et al., 2017) and *Borrelia burgdorferi* (Barbour et al., 2008). This protein has also been described as a differential, immunogenic protein in strains of

Bifidobacterium longum ssp. longum (Górska et al., 2016) and has shown protective activity against colitis in mice (Srutkova et al., 2015).

Enolase is a moonlighting enzyme found on the surface of some pathogens and is involved in the activation of plasminogen (Rahi et al., 2017). In our studies (Paper #2), enolase was among the ten most abundant proteins detected in the surfaceome and exoproteome compartments for *B. hyodysenteriae* and *B. pilosicoli*. Enolase has been found phosphorylated in *E. coli* (Dannelly et al., 1989) and has been detected acetylated in various bacterial studies (Ouidir et al., 2016). We detected enolase with a high level of acetylation in the acetylomes of *B. hyodysenteriae* and *Brachyspira pilosicoli*. When the abundance of enolase acetylated peptides was normalized with respect to enolase abundance in the total proteome (PSMs), the *B. hyodysenteriae* enolase (C0QV52) had an acetylation ratio of 29 and the *B. pilosicoli* enolase (D8IET2) an acetylation ratio of 500. These data indicate that enolase in *B. pilosicoli* is more acetylated.

As mentioned above, one role described for enolase pathogenesis is binding to plasminogen. The sequences that bind to plasminogen are well characterized for several bacteria, such as the spirochete *Leptospira interrogans* (Salazar et al., 2017). In this species, the binding motif is FYDKS___KKK. The homologue binding sequences are FY*NKD*KKK (*B. hyodysenteriae*) and FY*DKN*KKK (*B. pilosicoli*). We detected this binding site acetylated, and this acetylation was exclusive of *B. pilosicoli*. Specific acetylation residues were characterized as K_{251} and K_{253} (ptmRS, confidence > 99%) (Figure 5, Paper #1).The homologue binding sequence in enolase from *E. coli* has two acetylation sites (K_{254} and K_{257}). *E.coli* acetylation sites K_{254} and K_{257} were found glucose/time dependent and independent of acetyl-phosphate (Schilling et al., 2015).

Acetylation of these sites in enolase could probably be involved in its binding to plasminogen, as binding occurs mainly through Lysine residues (Nogueira et al., 2013). Thus, in *B. pilosicoli*, the acetylation of these sites could difficult the binding to plasminogen which initiates the conversion to plasmin and the cascade that results in tissue degradation and the spread of bacteria within the host.

These processes would be more favoured in *B. hyodysenteriae* and be behind of the different mechanisms of adherence and virulence between these two species.

6.4.2 *B. pilosicoli* immunoreactive proteins dataset is enriched in HSPC

Many proteins in the immunoproteome of *B. pilosicoli* have been described as Heat Shock Proteins (HSP) or components of the Heat Shock Protein Complex (HSPC) (Han et al., 2016), e.g., the Elongation factor G, ATP synthase subunit Beta, Trigger factor, DnaK (Hsp70), HtpG (Hsp90 family), Elongation factor Tu, and 60 kDa chaperonin. All these proteins except for Elongation factor Tu and the 60 kDa chaperonin are immunoreactive specific against sera from the *B. pilosicoli* challenge (exclusively detected in bands revealed with sera from challenged pigs, undetected with control sera).

Bacterial HSPs may modulate immunity by rapidly and directly increasing cytokine production in macrophages and are related to pathogen survival inside the host (Bajaj and Batra, 2012). The HSPs are immunodominant in infected subjects (Colaco et al., 2013). These proteins have been used as vaccines against different pathogenic bacteria, e.g., *Neisseria* species (Han et al., 2016), *Mycoplasma tuberculosis* (Bajaj and Batra, 2012), and *Mycoplasma hyopneumoniae* (de Oliveira et al., 2017). The HSPs are implicated in the stimulation of the innate and adaptive immune system. Their capacity to bind to antigenic peptides and deliver them to antigen-presenting cells (APCs) is the justification of the peptide-specific T-lymphocyte response (Javid et al., 2007).

The HSPs increase their expression under different stress conditions, such as hypoxia, nutrient starvation or ethanol in the media. Ethanol stress produces changes in proteome composition; the most altered proteins are related to general stress response and heat shock response. The chaperones Hsp70, HtpG and DnaJ are among the proteins that increased their expression during this stress situation (Soufi et al., 2015). In *A. baumannii*, the increase in this type of proteins (UspA, GroEL, Hsp90) was also observed when grown in ethanol (Camarena et al., 2010).

Approximately 20% of the immunoreactive specific proteins of *B. pilosicoli* are HSPs, which are acetylated (Table 6.4). The immunoproteome of *B. hyodysenteriae* does not contain HSPs. The recognition of these proteins by part of the immune system of infected pigs would point to HSPs, a stress response, involved in the pathogenic process for *B. pilosicoli*, but not for *B. hyodysenteriae*. In addition, the proteins DnaK, Elongation factor Tu and Elongation factor G have been related to the high adhesion capacity of *Bifidobacterium* (Vazquez-Gutierrez et al., 2017). Thus, HSPs could be related to the different interaction with the host and/or different pathogenic features that show both species.

6.4.3 *B. hyodysenteriae* immunoreactive proteins

The major group of *B. hyodysenteriae* immunoreactive proteins corresponded to flagellar proteins. However, except for C0QWY9, all flagellar proteins were also identified in bands revealed with control sera. Overall, eleven proteins were detected as immunoreactive specific for *B. hyodysenteriae* (Table 6.4).

The group of immunoreactive specific proteins of *B. hyodysenteriae* includes the periplasmic galactose-glucose binding protein and two cytoplasmic proteins, pyridine nucleotide-disulfide oxidoreductase and fructose-bisphosphate aldolase. These proteins have been detected in the extracellular space in other species involved in processes related to interaction/adhesion to host cells (Roier et al., 2015; Tunio et al., 2010; Zhe et al., 2016). The galactose-glucose binding protein was identified as the main component of the outer membrane vesicles released from five strains of *Haemophilus influenza* (Roier et al., 2015). These extracellular nanovesicles are released by all pathogenic and non-pathogenic gram-negative bacteria (Lusta, 2015). They are composed of outer membrane components such as LPS, glycerophospholipids and proteins from the outer membrane and the periplasm (Bai et al., 2014; Kuehn and Kesty, 2005; Lusta, 2015). Outer membrane vesicles are considered potent virulence factors because they provide a means for the extracellular secretion of proteins and lipids that can interact with host tissues. The pyridine nucleotide-disulfide oxidoreductase (Zhe et al., 2016) has been identified as one of the proteins that interacts with brain microvascular endothelial cells, which may contribute to invasion by

Streptococcus equi ssp. zooepidemicus through the blood-brain barrier. Finally, fructose-bisphosphate aldolase has been reported to be immunogenic in *Candida albicans* (Calcedo et al., 2012) and *Madurella mycetomatis,* in which it has been proposed as a vaccine candidate (de Klerk et al., 2012).

Table 6.4. Proteins detected in immunoreactive specific bands for the two *Brachyspira* species.

Species	Pilo strains	Protein accession	Protein description	GO cell component	psortB prediction	Exposed proteome ranking	PTM SAS
hyo		C0QV52	Enolase	cell surface, extracellular region	C	12	A
hyo		Q26501	Flagellar protein FlaB1	flagellum	P	32	A
hyo		Q9F0F6	Flagellin FlaB3	flagellum	P	34	M,A
hyo		C0QZD7	Fructose-bisphosphate aldolase		C	110	
hyo		C0QYC2	Galactose-glucose binding protein		P	297	O,D,A
hyo		C0R0E5	Phosphoenol pyruvate carboxykinase	cytoplasm	C	33	A,P
hyo		C0QZV6	Pseudouridine-5'-phosphate glycosidase		C	1296	
hyo		C0QWY9	Putative flagellar filament outer layer	outer membrane, periplasmic space	U	28	
hyo		C0QZ10	Pyridine nucleotide-disulfide oxidoreductase		C	232	A
hyo		C0R1L9	UDP-glucose 4 epimerase		C	357	
hyo		C0QW84	Uncharacterized protein		O	76	
pilo	1	J9UU81	2-isopropylmalate synthase		C	41	
pilo	1	J9URY6	2-oxoacid:ferredoxin oxidoreductase subunit alpha-like protein		C	nd	M,A
pilo	1	D8IAM3	Amidohydrolase 3		C	178	A
pilo	2	D8IE58	Aspartyl-tRNA synthase	cytoplasm	C	72	A
pilo	1	D8IBH9	ATP synthase subunit beta	plasma membrane	C	32	A
pilo	1	J9UBH8	ATP-dependent 6-phosphofructokinase	cytoplasm	C	132	A
pilo	2	D8I9T6	Biotin lipoyl		C	55	A
pilo	1	K0JIZ9	Carboxyl terminal protease		C	187	
pilo	1	D8IBS0	Chaperone protein DnaK		C	29	A
pilo	2	D8IEM8	Chaperone protein HtpG	cytoplasm	C	161	A

Species	Pilo strains	Protein accession	Protein description	GO cell component	psortB prediction	Exposed proteome ranking	PTM SAS
pilo	2	D8ICZ7	Elongation factor G	cytoplasm	C	22	M,A
pilo	1	D8IET2	Enolase	cell surface, extracellular region	C	15	M,A,P
pilo	2	D8IBY6	FlaA	outer membrane, periplasmic space	P	24	
pilo	1	D8ICA7	FlaA 2	outer membrane, periplasmic space	U	95	
pilo	1	D8IEW7	Mannose-1-phosphate guanylyltransferase		C	276	A
pilo	1	D8IF78	Outer membrane efflux protein		U	nd	
pilo	1	D8IFS5	Phosphoenol pyruvate carboxykinase	cytoplasm	C	20	A,P
pilo	1	D8ICA8	Putative FlaA	outer membrane, periplasmic space	U	nd	
pilo	2	J9UT37	Putative polymerase		C	nd	A
pilo	1	D8ICR1	Pyruvate oxireductase		C	16	M
pilo	1	J9TU32	Ribonucleotide-diphosphate reductase subunit beta		C	228	A
pilo	2	D8IBK7	TmpB outer membrane protein		O	159	A
pilo	1	D8ICA2	Toxic anion resistance family protein		C	147	
pilo	1	D8IDP7	Trigger factor, C-terminal domain protein		C	751*	A
pilo	1	D8I9T4	Uncharacterized protein		C	241	
pilo	1	D8ICG3	Uncharacterized protein		E/O	93	
pilo	1	D8ICG5	Uncharacterized protein	integral membrane	C	nd	A
pilo	2	D8ICU0	VspD		U	250**	SAS

Note:
Species; hyo (*B. hyodysenteriae*), pilo (*B. pilosicoli*).
Pilo strains: number of *B. pilosicoli* strains in which the protein has been detected; 1(only in one strain), 2 (in both strains P43/6/78 and OLA9) (Paper #3).
GO cell component: cell component annotation for the protein in Gene Ontology.
psortB prediction: result for cellular location prediction with psortB**;** P (periplasmic), U (unknown), C (cytoplasmic), O (outer membrane), E (extracellular).
Exposed proteome ranking: protein position in the ranking of protein abundance (PSMs) among the proteins identified in Paper #2; nd, non-detected, (*) exclusive of exopeptidome, (**) exclusive of exoproteome.
PTM SAS: post-translational modifications and single aminoacid substitution identified in the protein by direct analysis (Paper #1). A (acetylation), D (dihydroxylation), M (methylation), O (oxidation), P (phosphorylation), SAS (single aminoacid substitution).

6.4.4 Immunoreactive proteins proposed as vaccine components

Based on the information obtained for the immunoreactive specific proteins (Table 6.4) in the other studies included in this Thesis (Papers #1 and #2), those that had been detected among the 50 most abundant proteins in the exposed proteome (in terms of total PSMs) were selected as potential vaccine components (Table 6.5). This selection criterion was grounded in the fact that due to their location, directly exposed to the host, these proteins are crucial for the induction of the immune response.

Table 6.5. Selected proteins on the basis of immunoreactivity, location and expression level on exposed proteome (extracted from Table 6.4).

Species	Protein accession	Protein description	GO cell component	psortB prediction	Ranking exposed proteome	PTM SAS
hyo	C0QV52	Enolase	cell surface, extracellular region	C	12	A
hyo	C0QWY9*	Putative flagellar filament outer layer	outer membrane, periplasmic space	U	28	
hyo	Q26501*	Flagellar protein FlaB1	flagellum	P	32	A
hyo	C0R0E5	PEPCK	cytoplasm	C	33	A,P
hyo	Q9F0F6*	Flagellin FlaB3	flagellum	P	34	M,A
pilo	D8IET2	Enolase	cell surface, extracellular region	C	15	M,A,P
pilo	D8ICR1	Pyruvate oxireductase		C	16	M
pilo	D8IFS5	PEPCK	cytoplasm	C	20	A,P
pilo	D8ICZ7	Elongation factor G	cytoplasm	C	22	M,A
pilo	D8IBY6*	FlaA	outer membrane, periplasmic space	P	24	
pilo	D8IBS0	Chaperone protein DnaK		C	29	A
pilo	D8IBH9	ATP synthase subunit beta	plasma membrane	C	32	A
pilo	J9UU81	2-isopropylmalate synthase		C	41	

Note: Protein accessions with (*) have been previously included in vaccine tests with unsuccessful results, see text.

It is important to notice that 4 flagellar proteins, two FlaA (C0QWY9 and D8IBY6) and two FlaB (Q26501, Q9F0F6), fulfill the criterion used to select the proteins included in Table 6.5. Unlike FlaA, both FlaB proteins have been detected with PTMs in their sequence. Both classes of flagellar proteins were previously tested as vaccine components. FlaB1 was tested as vaccine against *B. hyodysenteriae* but it was not effective (Gabe et al., 1995). Likewise, a FlaA protein, was

proposed in a reverse vaccinology approach (Song et al., 2009), but the results were not conclusive.

Thus, a total number of 13 proteins are suggested as vaccine components, 5 proteins for *B. hyodysenteriae* and 8 for *B. pilosicoli*. Two proposed candidates, enolase and PEPCK, are common for both species.

In 10 of 13 from the proposed proteins, PTMs have been detected within their sequence (Table 6.5). This fact should be taken into account for vaccine development due to the potential role it could play in the recognition by the host´s immune system. The already mentioned vaccine tests with flagellar proteins were performed with recombinant proteins expressed in *E. coli*. In the case of FlaB proteins, which are modified, this could be related with the vaccine efficiency.

Aside from the flagellar proteins, some of the other proteins selected have been tested as vaccine candidates against different bacterial pathogens, such as DnaK against *Salmonella* Typhi (Paliwal et al., 2011) and enolase against *Streptococcus iniae* (Kayansamruaj et al., 2017). However, to the best of our knowledge, they have never been proposed as vaccine candidates against *Brachyspira* species.

7. Conclusions

1. The large-scale proteomics study presented here has provided the first experimental evidence for the expression of more than 1500 gene products from each of the targeted species, together with the characterization of hundreds of their modification sites. The estimated proteome coverage is near 70%.

2. We have reported a different degree of proteome acetylation in these species, being *B. pilosicoli* proteome the most acetylated one. We have shown that the main targets of acetylation were proteins involved in metabolism and that the differences observed are reflected in the different composition of the components involved in the Acetyl-CoA/Acetate metabolic pathway.

3. The large-scale characterization of peptides and proteins in the exposed proteome has provided quantitative evidence of the expression or location in these compartments of hundreds of bacterial proteins. Among the most abundant proteins in these compartments are proteins related to motility/chemotaxis and aerotolerance and many are classified as virulence factors in other species. This collection constitutes a unique database of potential vaccine candidates.

4. VspH and VspD proteins have been proposed as vaccine candidates against *B. hyodysenteriae*. VspD was found at high levels and generated strong immunoreactivity in *B. pilosicoli* strains. However we could only detect VspD (at very low levels) and VspH in one *B. hyodysenteriae* strain. These results support the need of quantitative gene product determination in the selection of vaccine components.

5. Immunoproteomics has proved to be an effective tool to characterize new *Brachyspira* antigens. Among the 11 (*B. hyodysenteriae*) and 35 (*B. pilosicoli*) immunoreactive proteins identified, a group of 13 proteins corresponds to bacterial exposed proteins that could constitute valuable vaccine candidates.

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