UNIVERSIDADE ESTADUAL DE MARINGÁ
CENTRO DE CIÊNCIAS AGRÁRIAS

VESÍCULAS DE MEMBRANA: UM COMPONENTE INEXPLORADO NO RÚMEM COM CAPACIDADE DE DEGRADAÇÃO DE PROTEÍNAS, CARBOXIMETILCELULOSE E AMIDO

Autor: Rodolpho Martin do Prado
Orientador: Prof. Dr. Geraldo Tadeu dos Santos
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TITULAÇÃO: Doutor em Zootecnia - Área de Concentração Produção Animal

APROVADA em 10 de julho de 2015.

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(Orientador)

“Everybody is a genius. But, if you judge a fish by its ability to climb a tree, it’ll spend its whole life believing that it is stupid”

Autor desconhecido

Às pessoas mais importantes da minha vida,
minha esposa, meus pais e meus irmãos

DEDICO

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BIOGRAFIA DO AUTOR

RODOLPHO MARTIN DO PRADO, filho de Ivanor Nunes do Prado e Marlene Martin Puertas, nasceu em Rennes, França, no dia 15 de janeiro de 1986.

Em dezembro de 2008 concluiu o Curso de Zootecnia pela Universidade Estadual de Maringá.

Em março de 2010 iniciou o mestrado em Produção Animal no Programa de Pós-Graduação em Zootecnia na Universidade Estadual de Maringá. Em março de 2010 foi contemplado com bolsa no Programme d'Adjoint de Recherche pelo governo do Canadá para estágio no Dairy and Swine Research and Development Centre – Agriculture and Agri-Food Canada, na cidade de Sherbrooke, província de Québec, Canadá, no qual participou de projetos na área de produção em bovinocultura leiteira. No dia 04 de abril de 2012 recebeu o título de Mestre em Produção Animal pelo Programa de Pós-Graduação em Zootecnia da Universidade Estadual de Maringá.

Em março de 2012 iniciou o doutorado em Produção Animal no Programa de Pós-Graduação em Zootecnia na Universidade Estadual de Maringá. Em agosto de 2013 foi contemplado com bolsa PDSE da CAPES para período sanduíche na Aberystwyth University até julho de 2014.

No dia 10 de julho de 2015 submeteu-se à banca de defesa de tese para obtenção do título de Doutor em Produção Animal pelo Programa de Pós-Graduação em Zootecnia da Universidade Estadual de Maringá.

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ABREVIAÇÕES

BAP - anti-bacterial alkaline phosphatase

BSA - bovine serum albumin

CMC - carboxymethylcellulose

CTM-T - casein–thiomersal buffer

EPS - extracellular polymeric substances

EV - extracellular vesicles

HOM - Hobson's M2 medium

IM - inner membrane

LAB - liquid-associated bacteria

LPS - lipopolysaccharide

MV - membrane vesicle

OD - optical density

OM - outer membrane

OMV - outer membrane vesicle

PP - periplasmic proteins

PQS - *Pseudomonas* quorum-sensing

QS - quorum sensing

RF-LAB - rumen fluid liquid-associated bacteria

RF-SAB - rumen fluid solid-attached bacteria

SAB - solid-attached bacteria

SDS-PAGE - sodium dodecyl sulphate polyacrylamide gel electrophoresis

SEM - scanning electron microscopy

TEM - transmission electron microscopy

TM-LAB - tris-magnesium liquid-associated bacteria

TM-SAB - tris-magnesium solid-attached bacteria

RESUMO

A formação de vesículas de membrana é um processo ubíquo em bactérias Gram-negativas e Gram-positivas. O número de pesquisas em relação as vesículas de membrana aumentou na última década, entretanto o foco são àquelas realacionadas a patógenos humanos. Além disso, na maioria dos estudos apenas culturas puras são utilizadas. Com o avanço da proteômica, várias funções foram atribuídas às vesículas de membrana. No entanto, até o momento nenhum estudo isolou vesículas de membrana do líquido ruminal, nem avaliou a possível capacidade de hidrolisar substratos. Assim, como primeiro estudo, vesículas de membrana externa foram isoladas de uma cultura axênica de *Prevotella ruminicola*, por meio de protocolo usualmente empregado para isolar vesículas de membrana externa em bactérias patogênicas, para avaliar a atividade hidrolítica das amostras. O protocolo foi empregado com sucesso e nenhuma contaminação bacteriana foi observada quando se utilizou microscopia de transmissão eletrônica de seção transversal. Foram distintos os perfis de proteínas da cultura pura, células lavadas, sobrenadante livre de vesículas e vesículas de membrana externa, como observado por SDS-PAGE. Atividade proteolítica foi avaliada em zimograma de gelatina e amostras de vesículas de membrana externa apresentaram bandas distintas e nítidas em relação à cultura pura, células lavadas e sobrenadante livre de vesículas. Inibidores de protease foram adicionados às amostras, mas nenhum efeito claro pôde ser observado. Nenhuma atividade pôde ser observada em zimogramas de carboximetilcelulose e amido. Embora fosse esperada maior atividade enzimática, os resultados fornecem evidências de que algumas enzimas de *P. ruminicola* são seletivamente transportadas nas vesículas de membrana externa. Como segundo estudo, as vesículas de membrana foram isoladas utilizando líquido ruminal e material sólido do rúmem. Foram separadas as frações de bactérias anexas à fibra e bactérias associadas ao

líquido para melhorar a caracterização das vesículas de membrana. Três vacas foram amostradas, mas apenas amostras de uma vaca foram utilizadas para gerar imagens de microscopia eletrônica de transmissão com coloração negativa. O perfil proteico foi semelhante entre as amostras quando avaliado em SDS-PAGE, mas a atividade proteolítica foi bastante diversa quando observado em zimogramas de gelatina e caseína. Ainda, a atividade em zimogramas com carboximetilcelulose e amido foi bastante diversa em todas as amostras. Estes resultados fornecem evidência de que vesículas de membrana no rúmen foram negligenciados pois em alguns casos houve mais atividade enzimática nas amostras de membranas de vesícula quando comparado com microrganismos somente. Compreender os fundamentos do microbioma é ponto chave para melhorar o uso de alimentos por ruminantes e pesquisas que utilizam vesículas de membrana isoladas do rúmen pode agregar conhecimento sobre as interações de microrganismos no rúmem.

Palavras-chave: amilase, celulase, *Prevotella ruminicola*, protease, vesículas de membrana, vesículas de membrana externa

ABSTRACT

Membrane vesicles production is a ubiquitous process in Gram-negative and Gram-positive bacteria. Research on membrane vesicles increased during the last 10 years, but it is mainly focused on human pathogens. Also, in most studies only bacterial pure cultures are investigated. With the advance of proteomics, several new functions are now associated with membrane vesicles. Yet, so far no study has isolated membrane vesicles from rumen fluid, or assessed their capacity to hydrolase substrate. Thus, as a first study, outer membrane vesicles were isolated from an axenic culture of *Prevotella ruminicola*, using a protocol used to isolate outer membrane vesicles from pathogenic bacteria, and hydrolytic activity from the samples was assessed. The protocol was successfully employed as no bacterial contamination was observed on cross-section transmission electron microscopy. The protein profile from the whole culture, washed culture, outer membrane vesicle-free supernatant, and outer membrane vesicles were distinct as observed on SDS-PAGE. Proteolytic activity was assessed on gelatin zymograms and outer membrane vesicle samples had distinct and clearer bands compared to whole culture, washed culture, and outer membrane vesicle-free supernatant. Protease inhibitors to assess protease classes were added to the samples but no clear effect could be observed. Furthermore, no activity could be observed on carboxymethylcellulose and starch zymograms. Although a greater enzymatic activity was expected, results provide evidence that some proteolytic enzymes from *P. ruminicola* are selectively loaded into outer membrane vesicles from this bacterium. As a second study, membrane vesicles were isolated using rumen liquor and particulate matter from the rumen. Fractions using solid-attached bacteria and liquid-associated bacteria were used to further improve membrane vesicle characterization. Three cows were sampled but only samples from one cow were imaged with negative staining

transmission electron microscopy. Protein profiles of samples were similar when assessed on SDS-PAGE, but proteolytic activity was diverse on gelatin and casein zymograms. Furthermore, activity on carboxymethylcellulose and starch zymograms was diverse across the samples. These results provide evidence that membrane vesicles in the rumen have been overlooked as in some cases more activity was observed in vesicle samples compared to within the microorganisms. Understanding fundamentals from microbiome is key to improve feed use in ruminants and research using membranes vesicles isolated from the rumen might add knowledge on rumen microbiome function.

Key-words: amylase, cellulase, membrane vesicle, outer membrane vesicle, *Prevotella ruminicola*, protease

I. LITERATURE REVIEW

I.1. Background

Ruminants are a group of animals with approximately 150 species distributed worldwide (Gould, 1986). The symbiotic relationship between the animal and the rumen microbiome allows the ruminant to digest plant material which would otherwise be unaccessible. Ruminants lack an enzyme to degrade cellulose, but microbes in the rumen are capable of breaking the β-1,4-glycosidic bond of plant cellulose (Hungate, 1966; Ferrer *et al.*, 2005; Hess *et al.*, 2011). Such capability is responsible for the success of ruminants and transformation of cellulose into meat, milk and wool. Despite the relatively low number of ruminant species, they provide products that are consumed and used every day by humans. Therefore, novel strategies to improve ruminant feed efficiency are needed and there is evidence that by 2050 the human consumption of milk and meat will have doubled compared to the beginning of the 21st century (FAOstat, 2009).

During the 20th century efforts to improve ruminant production was focused in the field of animal physiology and feedstuff evaluation. A great number of feedstuffs were fed to different species, mainly to evaluate animal performance. Information was scarce and needed to guide animal nutritionists to improve animal productivity. Furthermore, genetic selection and breeding of plants was and continues to be important to improve, and even breed optimal plants (e.g. canola), so that ruminant plant degradation could be improved (Kingston-Smith and Thomas, 2003). Also, genetic improvement of animals is now quickly developing due to increasing bioinformatics capacity. In general, much attention has being given to the animal and nutrient sources, and development in this field of research has been vast, but a fundamental understanding of the rumen

microbiome and its function is now required in order to further improve upon ruminant nutrient use efficiency.

Efforts have also been made to comprehend the rumen environment, where bacteria, protozoa, fungi, archaea and viruses interact (Hungate, 1966; Ogimoto and Imai, 1981; Hobson and Stewart, 1997; Krause *et al.*, 2003). Although the rumen is the site where most of the cellulose fermentation occurs inside the animal, culturable bacteria represent only 11% of the whole rumen bacteria (Edwards *et al.*, 2004; Kim *et al.*, 2011). Furthermore, nitrogen losses via faeces, and urine can account for over 70 % in some cases (Dewhurst *et al.*, 1996; Kingston-Smith *et al.*, 2010). This presents a challenge to explore and understand the rumen as a system, with the ultimate goal to maximize plant use, improve ruminant production and take advantage of compounds produced by rumen microbiome.

I.2. Prevotella ruminicola

Prevotella ruminicola is a Gram-negative, non-motile, rod-shaped, anaerobic bacterium, possessing numerous glycosides hydrolases (Purushe *et al.*, 2010). This bacterium is particularly known as a proteolytic species (McKain *et al.*, 1992; Wallace, 1996), although carboxymethylcellulose and starch activity was observed (Avguštin *et al.*, 1997). *Prevotella* spp. is the dominant bacteria in the rumen (Stevenson and Weimer, 2007; Huws *et al.*, 2010) and they are the predominant population of secondary colonizers of *Lolium perenne* after 4 h incubation in the rumen (Huws *et al.*, 2013). Therefore, *P. ruminicola* is an important bacterium regarding degradation of plant material.

I.3. Biofilms

The classical notion of an isolated bacteria is long gone and a much more complex bacterial interaction is now accepted (Hibbing *et al.*, 2010). Biofilms, which are defined as attached bacteria enveloped in extracellular polymeric substances (**EPS**), are the predominant phenotype of microbes in nature, and they offer microbes a source of protection, nutrition and stability (Absalon *et al.*, 2011). Attached microbes interact with their environment through the EPS – a mixture of protein, sugars, nucleic acids and lipids (Godefroid *et al.*, 2010). Extensive biofilms are found on ingested plant material within the rumen (Fig. I.1), and the EPS encompassing these plant-associated biofilms contains the majority of microbial digestive enzymes and a large amount of DNA

(Remis *et al.*, 2010). The EPS typically accounts for up to 90% of the biomass of a biofilm (Sutherland, 2001), and key components of the EPS are bacterial-derived vesicles (Schooling and Beveridge, 2006; Whitworth, 2011).

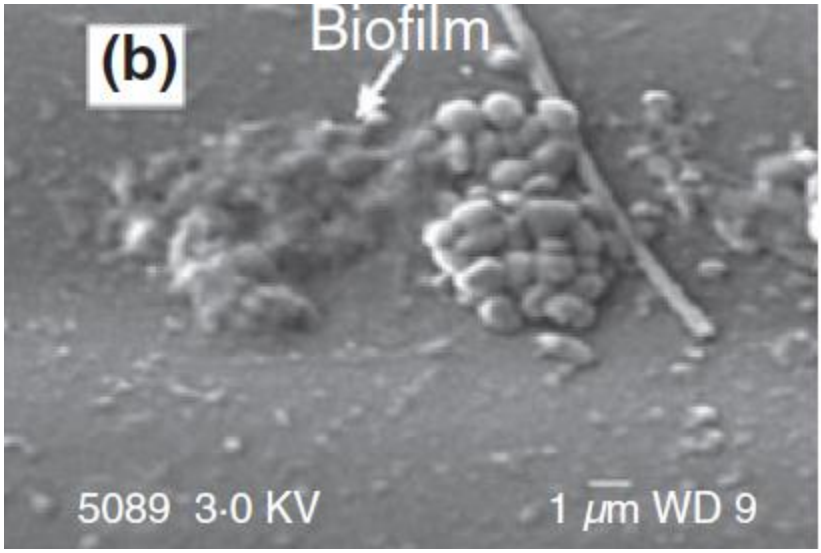

Fig. I.1. Biofilm imaged with scanning electron microscopy with cryo-stage of perennial ryegrass incubated in rumen for 1 h. (Huws *et al.*, 2013)

I.4. Vesicles

All organisms interact with the environment in order to feed, grow and reproduce. The outer membrane (**OM**) of a cell wall is the major site where interactions and several important functions, such as nutrient acquisition, adherence, secretion, signalling, and protection from the environment, take place (Kulp and Kuehn, 2010). Membrane disruption can result in cell death and this is possibly why organisms have evolved mechanisms to prevent damages on its envelope (Raivio, 2005). There are several ways in which bacteria, archaea and fungi interact with their surroundings, and one of them is through membrane vesicle (**MV**), a lipid bilayer sphere with lumen (Deatherage and Cookson, 2012).

Membrane vesicles are produced by Gram-negative (Kulp and Kuehn, 2010), Gram-positive (Gurung et al., 2011), and archaea (Soler et al., 2008), and 'extracellular vesicles' (**EVs**) are produced by eukaryote (Rodrigues et al., 2013). The production of vesicles is an ubiquitous physiological process (Schwechheimer *et al.*, 2013). The diameter of membrane vesicles ranges from 20 to 250 nm in Gram-negative (Kulp and Kuehn, 2010) (Fig. I.2); 15 to 100 nm in Gram-positive (Lee *et al.*, 2007; Olaya-Abril *et*

al., 2014); 50 to 230 nm in archaea (Soler *et al.*, 2008; Ellen *et al.*, 2009); and 40 to 200 nm in fungi (Wolf *et al.*, 2012).

Vesicles are found when bacteria are in planktonic state (Biller *et al.*, 2014), in biofilms (Schooling and Beveridge, 2006), in infected tissues (Shah *et al.*, 2012), in laboratory cultures (Kulp and Kuehn, 2010), riverbeds, domestic water drains, sewage and freshwater fish aquarium (Schooling and Beveridge, 2006).

The term 'outer membrane vesicle' (**OMV**) is often used for Gram-negative bacteria (Manning and Kuehn, 2013). In Gram-negative they are secreted when part of the OM and periplasm of bacteria bulges and pinches (Kulp and Kuehn, 2010), resulting in a structure composed of lipopolysaccharide (**LPS**), outer membrane proteins, phospholipids, periplasmic proteins (**PP**), and nucleic acids. *Prochlorococcus*, *Escherichia coli* and *Xylella fastidiosa* produce 2 to 5; 16; and 51 OMV per cell, respectively (Biller *et al.*, 2014; Ionescu *et al.*, 2014). The amount of different OMV-associated proteins are mostly species dependent, but they can range from 44 to 456 (Lee *et al.*, 2008; Choi *et al.*, 2014). Approximately 8-12 % of total protein and endotoxin of *Neisseria meningitidis* culture are packaged into vesicles, suggesting an efficient production mechanism, otherwise the cell death would be certain (Bonnington and Kuehn, 2014).

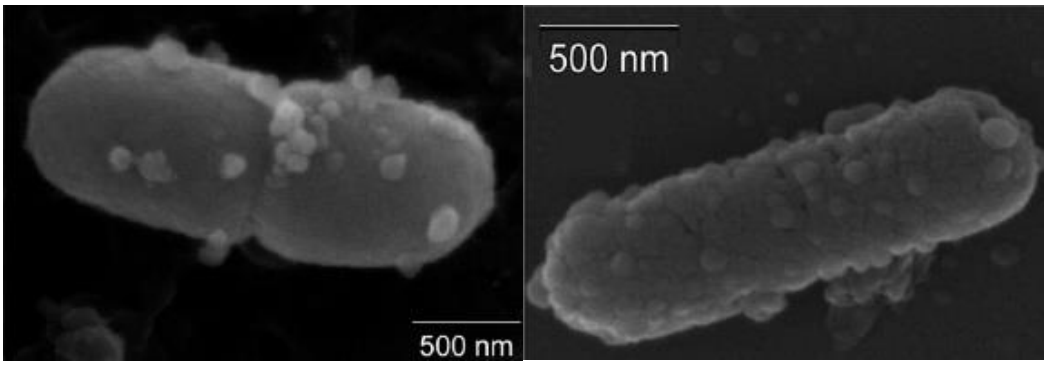

Fig. I.2. Scanning electron microscopy of *Escherichia coli* with OMVs on the surface. (Ellis and Kuehn, 2010)

Gram-positive bacteria have different cell wall architecture with a thicker peptidoglycan layer. The vesiculation process is different from Gram-negative bacteria as MVs originates from plasma membrane. The MV protein content is mostly cytoplasmic (~75 %), and MVs are enriched in lipoprotein and short fatty-acids (C12 to C16) compared to the plasma membrane (Olaya-Abril *et al.*, 2014). Gram-positive

bacteria transmission electron microscopy (**TEM**) micrographs showing MVs were thought to be artefacts from the cell division, but a recent study support that MV originates from the plasma membrane (Olaya-Abril *et al.*, 2014).

Eukaryotic extracellular vesicles can be termed as ‘exosomes’, ‘microvesicles’, ‘ectosomes’, or ‘shed vesicles/particles’, but the most used term is ‘extracellular vesicles’ (Witwer *et al.*, 2013). Fungal EVs were first shown by TEM micrographs in a *Cryptococcus neoformans* culture (Rodrigues *et al.*, 2007), and proteomics suggests that fungal extracellular proteins are not secreted by common pathways (Oliveira *et al.*, 2010; Vallejo *et al.*, 2012). It is now accepted that fungi produce EVs (Rodrigues *et al.*, 2013), but mechanisms by which EVs cross the cell wall is not known.

There is still some discussion as to whether the release of MVs, both in Gram-negative and Gram-positive, is a random event or if it is a well-conserved mechanism. One of the first studies to compare the protein profile of OMs and OMVs using SDS-PAGE suggest that the band patterns of purified OMV differ from the lysate of cells (Hoekstra *et al.*, 1976). On a global proteomic profile study with *E. coli*, Lee *et al.* (2007) observed a distinct protein profile from OMVs compared to envelope components. Recently, a proteomic study with *Bacteroides fragilis* has provided evidence that 46 proteins were OMV-exclusive and 33 proteins were OM-exclusive (Elhenawy *et al.*, 2014). *Pseudomonas putida*, a soil bacteria that is able to degrade aromatic hydrocarbons of organic solvents, was grown in three different media, one of which with benzoate. Proteome analysis from the culture grown in benzoate supplemented medium showed that out of 456 identified proteins, 186 were OMV-exclusive and 9 were exclusive related to benzoate metabolism (Choi *et al.*, 2014). This phenomenon is not protein specific, as LPS content from the OMs of a *Porphyromonas gingivalis* culture differs from the OMV (Kadurugamuwa and Beveridge, 1995; Haurat *et al.*, 2011). If OMV bulging was by pure chance, selective content would not be seen this commonly, and across different species. Also, if OMV are a result of cell lysis and encapsulation, cytosolic content would contribute more as an OMV component. Proteomics studies are more frequent these days and they provide much information on biogenesis and selective cargo of OMVs.

I.4.1. History of outer membrane vesicles

The first mention of OMVs was made during the 1960s (Bishop and Work, 1965; Knox *et al.*, 1966; Work *et al.*, 1966). The authors observed LPS in *E. coli* cell-free

supernatant, grown under lysine-limiting condition, and hypothesized that there was inhibition of the synthesis of peptidoglycan layer, but not OM production, therefore resulting in excess release of OM. Later, OMVs were also found to be produced under normal growth conditions. It was assumed that the energetic cost of sending great quantities of OM away could cost the stability of the bacteria, which suggested a significant function for OMVs existence. Hoekstra *et al.* (1976) compared OMVs to outer and inner membranes from *E. coli* with respect to protein content, phospholipid and fatty acid composition, and protein distribution on SDS-PAGE. It was suggested that the complexes (i.e. OMVs) originated from the OM, as the complexes were highly depleted in some cytoplasmic components.

I.4.2. Roles of vesicles

Bacteria have dynamic mechanisms to guarantee survivability and OMVs are continuously produced, even at high energetic costs, which leads to hypothesis that MV have several important roles for bacteria. Indeed, OMVs can act as secretor and delivery system, on defence and resistance (MacDonald and Kuehn, 2013), nutrient acquisition (Kadurugamuwa and Beveridge, 1996), message signalling (Mashburn and Whiteley, 2005), genetic material transport (Klieve *et al.*, 2005; Dubey and Ben-Yehuda, 2011), and can be even used to form network between *Myxococcus xanthus* bacteria (Remis *et al.*, 2014). Furthermore, OMVs are now known to have an important role in virulence, antibiotic resistance and competition with other bacteria (Kulp and Kuehn, 2010).

Studies from the 1990s focused on the OMV transport of toxic factors, as this feature has a direct impact in human health (Kadurugamuwa and Beveridge, 1995; Wai et al., 1995; Li et al., 1996; Garcia-del Portillo et al., 1997). Transportation of DNA, toxic factor to prokaryote and eukaryote, protection against antimicrobials agents have been observed on several bacterial species (Manning and Kuehn, 2013). Much has been elucidated, but recent research, mostly using proteomics, unveils several new functions, especially on cell-to-cell interaction (Table I.1).

I.4.2.1 Secretion

Outer membrane vesicles are one of the secretory pathways for Gram-negative bacteria. They have an important role for pathogenesis, as they carry complexes of inflammatory and virulence-associated factors (Ellis and Kuehn, 2010; MacDonald and Kuehn, 2012). With a hydrophobic layer of lipids, the OMV lumen is protected under several

conditions, which makes them perfect to be used as deployer of numerous components, increasing the effectiveness of such costly mechanism (MacDonald and Kuehn, 2012) (Fig. I.3). Indeed, protein complexes, periplasmic molecules and membrane proteins loaded in the OMV lumen are highly resistant to proteases (Kesty and Kuehn, 2004) and DNAses (Pérez-Cruz *et al.*, 2015). Moreover, OMVs can bind to specific ligands and receptors (Kulp and Kuehn, 2010).

Table I.1. Summary of OMV/MV/EV functions

Function	Species	Type	Reference
Transport DNA/transform	*P. aeruginosa*	-	(Renelli *et al.*, 2004; Schooling *et al.*, 2009)
	N. gonorrhoeae	-	(Dorward *et al.*, 1989)
	B. burgdorferi	Di	(Dorward and Garon, 1990)
	A. tumefaciens	-	(Dorward and Garon, 1990)
	E. coli	-	(Dorward and Garon, 1990; Yaron *et al.*, 2000)
	H. influenza	-	(Dorward and Garon, 1990)
	M. osloensis	-	(Dorward and Garon, 1990)
	S. typhimurium	-	(Dorward and Garon, 1990)
	S. marcescens	-	(Dorward and Garon, 1990)
	S. dysenteriae	-	(Dorward and Garon, 1990)
	S. flexneri	-	(Dorward and Garon, 1990)
	Y. pestis	-	(Dorward and Garon, 1990)
	Thermococcales	Archaea	(Soler *et al.*, 2008; Soler *et al.*, 2011)
	T. kodakaraensis	Archaea	(Gaudin *et al.*, 2013)
	Ruminococcus spp.	+	(Klieve *et al.*, 2005)
	M. catarrhalis	-	(Schaar *et al.*, 2011)
	A. baylyi	-	(Fulsundar *et al.*, 2014)
	S. vesiculosa	-	(Pérez-Cruz *et al.*, 2013)
Transport of toxic factor to eukaryote	*P. aeruginosa*	-	(Bomberger *et al.*, 2009)
	A. actinomycetemcomitans	-	(Rompikuntal *et al.*, 2012)
	V. cholera	-	(Chatterjee and Chaudhuri, 2011; Elluri *et al.*, 2014)
	N. meningitides	-	(Steeghs *et al.*, 1999)
	S. aureus	+	(Gurung *et al.*, 2011; Thay *et al.*, 2013)
	B. anthracis	+	(Rivera *et al.*, 2010)
	V. tasmaniensis	-	(Vanhove *et al.*, 2015)
	Mycobacterium spp.	+	(Prados-Rosales *et al.*, 2011)
	S. pneumonia	+	(Olaya-Abril *et al.*, 2014)
	P. gingivalis	-	(Mantri *et al.*, 2015)
	E. coli	-	(Wai *et al.*, 2003; Aldick *et al.*, 2009; Shah *et al.*, 2012)
	B. pertussis	-	(Donato *et al.*, 2012)
	H. pylori	-	(Olofsson *et al.*, 2010)
	C. neoformans	Fungi	(Huang *et al.*, 2012)
Respond to envelope stress	*E. coli*	-	(McBroom *et al.*, 2006; McBroom and Kuehn, 2007)
	S. typhimurium	-	(McBroom and Kuehn, 2007)
	P. aeruginosa	-	(Tashiro *et al.*, 2009; MacDonald and Kuehn, 2013)

Protect against antimicrobial agent	*E. coli*	-	(Manning and Kuehn, 2011)
	P. gingivalis	-	(Grenier *et al.*, 1995)
	P. aeruginosa	-	(Kadurugamuwa *et al.*, 1993)
	P. putida	-	(Baumgarten *et al.*, 2012)
	V. tasmaniensis	-	(Vanhove *et al.*, 2015)
	P. syringae	-	(Kulkarni *et al.*, 2014)
	M. catarrhalis	-	(Schaar *et al.*, 2011)
	S. aureus	+	(Cui *et al.*, 2003)
	N. meningitides	-	(Ferrari *et al.*, 2006)
Transport of toxic factor to prokaryote	*P. aeruginosa*	-	(Kadurugamuwa and Beveridge, 1996; Li *et al.*, 1996; Li *et al.*, 1998)
	S. acidocaldarius	Archaea	(Prangishvili *et al.*, 2000)
	E. coli	-	(Li *et al.*, 1998)
	S. pullorum	-	(Li *et al.*, 1998)
	S. arizonae	-	(Li *et al.*, 1998)
	S. choleraesuis	-	(Li *et al.*, 1998)
	E. agglomerans	-	(Li *et al.*, 1998)
	P. vulgaris	-	(Li *et al.*, 1998)
	S. marcescens	-	(Li *et al.*, 1998)
	K. pneumonia	-	(Li *et al.*, 1998)
	S. flexneri	-	(Li *et al.*, 1998)
	C. freundii	-	(Li *et al.*, 1998)
	M. morganii	-	(Li *et al.*, 1998)
	P. trifolii	-	(Li *et al.*, 1998)
	Lysobacter sp. XL1	-	(Vasilyeva *et al.*, 2008)
	P. fragi	-	(Thompson *et al.*, 1985)
	M. xanthus	-	(Evans *et al.*, 2012; Remis *et al.*, 2014)
Produce/maintain biofilm	*P. aeruginosa*	-	(Schooling and Beveridge, 2006; Schooling *et al.*, 2009)
	H. pylori	-	(Yonezawa *et al.*, 2009; Yonezawa *et al.*, 2011)
Biomineralization	Mixed culture		(Matlakowska *et al.*, 2012)
Adsorb UV	*V. cholera*	-	(Song and Wai, 2009)
Insecticidal activity	*X. nematophilus*	-	(Khandelwal and Banerjee-Bhatnagar, 2003)
Redox-reactivity	*S. putrefaciens*	-	(Gorby *et al.*, 2008)
	S. oneidensis	-	(Pirbadian *et al.*, 2014)
Carbon source in ocean-water	*Prochlorococcus*	Cyanobacteria	(Biller *et al.*, 2014)
Protect against phage	*K. intermedius*	Cyanobacteria	(Biller *et al.*, 2014; Kharina *et al.*, 2015)
Disturb cell adhesion	*X. fastidiosa*	-	(Ionescu *et al.*, 2014)
Hydrolyse substrate	*B. fragilis*	-	(Elhenawy *et al.*, 2014)
Degrade benzoate	*P. putida*	-	(Choi *et al.*, 2014)
Immune response decoy	*M. catarrhalis*	-	(Vidakovics *et al.*, 2010)

- Gram-negative; + Gram-positive; Di Diderm. Adapted from Manning and Kuehn (2013)

I.4.2.2 Delivery system

In competing niches, OMVs can be used to attack and prey upon other bacteria (Evans *et al.*, 2012; MacDonald and Kuehn, 2012). One of the soluble components observed in OMVs are peptidoglycan hydrolases (Li *et al.*, 1996), which could be used to disrupt bacteria, resulting in cell death and nutrient release in the environment.

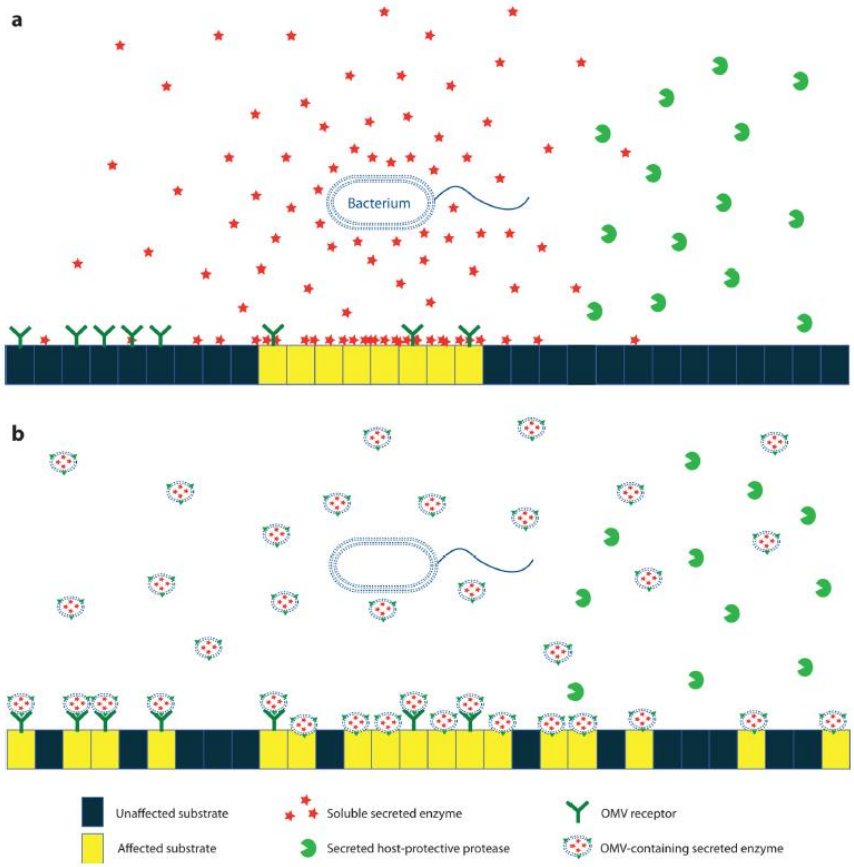

Fig. I.3. Benefits from packaging enzymes in OMVs. Soluble enzymes (a) secreted in the environment would be susceptible for host protease action and would lose efficiency. When packaged in OMVs (b) the bacteria can increase concentration of a single and/or multiple components, increase protection from environmental proteases and benefit from a long distance mechanism. (Kulp and Kuehn, 2010)

Two mechanisms are proposed for OMV delivery; fusion with membrane or autolysis close to a bacterial OM. In the first scenario, OMV membrane fuses with another membrane, either by chemical attraction or by a random contact, and releases its content in the periplasmic space, affecting the bacteria (Kulp and Kuehn, 2010). In the second proposed mechanism, OMVs spontaneously lyse near the targeted site and release their content, which may cause direct interactions (Kulp and Kuehn, 2010).

P. aeruginosa OMVs can fuse to Gram-negative bacteria (Kadurugamuwa and Beveridge, 1996), which suggest that interaction mechanisms used are similar to eukaryotic cells (Kadurugamuwa and Beveridge, 1999). Outer membrane vesicles can also be internalized in eukaryotic cells via endocytic pathway (Furuta *et al.*, 2009). This is supported by labelled gentamicin from *Shigella flexneri* (Kadurugamuwa and Beveridge, 1998) and labelled O-antigen from *Salmonella enterica* (Garcia-del Portillo

et al., 1997) observed in host cells. Fusion could also be explored for prey and horizontal gene transfer, although different quantities of OMVs might be needed for a successful membrane disruption, compared to a single OMV fusion needed for transformation. Horizontal gene transfer is an important feature for bacterial survivability, and OMV are implicated as a delivery system, which will be further discussed (Yaron *et al.*, 2000; Klieve *et al.*, 2005; Schaar *et al.*, 2011).

A positively charged vesicle-associated membrane protein might be implicated in electrostatic interaction with the plasma membrane (Williams *et al.*, 2009). Kadurugamuwa and Beveridge (1996) observed that OMVs can adhere to the OM of Gram-positive bacteria, possibly by Ca^{2+} or Mg^{2+} salt bridges, and that they have peptidoglycan hydrolases, suggesting a possible mechanism for disruption of peptidoglycan layer in bacteria.

I.4.2.3 Virulence

Outer membrane vesicles have an important role for bacteria as a front line trooper in bacterial infection and several bacterial species have been found to carry OMV-virulence factors (Kuehn and Kesty, 2005). The OM of Gram-negative bacteria are composed of adhesins, toxins, LPS and immunomudolatory compounds, which helps on the bacterial infection and are related with the host immune response (Ellis and Kuehn, 2010). The OM protein composition of pathogenic bacteria is genetically well defined and will vary according to the environment. *P. aeruginosa* OMVs from patients with cystic fibrosis were enriched with surface aminopeptidase and exhibited 3- to 4- fold greater lung adhesion when compared to a laboratory strain (Bauman and Kuehn, 2009), which also might influence coaggregation.

Outer membrane vesicles associated virulent cargo has greater virulence potential than purified components (Wai *et al.*, 2003; Bomberger *et al.*, 2009; Elluri *et al.*, 2014). The use of cell-free supernatant has been shown to result in infection responses in the host, which could be related to the presence of OMVs (Ellis and Kuehn, 2010). Outer membrane vesicle from a nonpathogenic strain of *E. coli* were shown to provoke inflammatory response in endothelial cells (Soult *et al.*, 2013) and this mechanism could have implications in general sepsis. Indeed, OMV-purified from an *E. coli* culture infused in rats were responsible for physiological, biochemical and histological changes consistent with sepsis (Shah *et al.*, 2012).

Like *E. coli* OMVs, *S. enterica* OMVs can trigger inflammatory responses (Alaniz *et al.*, 2007). Furthermore, *Salmonella* spp. isolated OMVs can be used as antigens and induce host immune system to respond to infection (Deatherage *et al.*, 2009). Within this scope, OMVs are now under study to be used as vaccines. The challenges to be faced are the low yield of purified OMV, the purity and reproducibility, as several minor stressors can influence the OMV cargo (Ellis and Kuehn, 2010).

Meningococcal OMV-vaccine was developed for human use in Cuba at the end of the 1980s (Sierra *et al.*, 1991). Since then, another 3 meningococcal OMV-vaccines were developed; one of them was recently approved by the European Medicines Agency (Gorringe and Pajón, 2012). Still, OMV-vaccine is more efficient against the homologous strain of the parental bacteria (Avila-Calderón *et al.*, 2015). Some work has been carried with OMVs from *Pasteurella multocida* and *Mannheimia haemolytica* as vaccines against bovine respiratory disease (Roier *et al.*, 2013). Outer membrane vesicles are safe as their antigen is less toxic compared to whole cell and has surface markers (i.e. proteins, lipids and LPS). Still, the major concern is reducing the LPS content, but tailor-made vaccines could be achieved by mutating specific genes, and enriching some antigens using overexpression of some genes (Baker *et al.*, 2014). In a recent study, the synergism between gold nanoparticles and OMV was achieved as bacterial membrane-coated with gold nanoparticles resulted in strong and durable antibody response (Gao *et al.*, 2015). Thus, such technique provides evidence of physicochemical alterations on OMVs, which means an enhanced OMV-vaccine.

I.4.2.4 Bacterial survival

Despite several functions and substances that OMV employs to act offensively, defensive functions were uncovered in the last years. Among them, OMV can inactivate β-lactam antibiotics (Kulkarni and Jagannadham, 2014), titrate harmful components from the bacteria (Kadurugamuwa and Beveridge, 1995), and act as a decoy in the milieu for chemical and bacteriophages (Manning and Kuehn, 2011; Biller *et al.*, 2014). Moreover, OMV is involved in biofilm stability (Schooling and Beveridge, 2006) which is linked to bacterial survival.

Strains of *P. aeruginosa* isolated from the lung of patients with cystic fibrosis were reported to have OMVs with β-lactamase activity in the lumen, and they were responsible for a inactivation of benzyl penicillin (Ciofu *et al.*, 2000). This feature is powerful inside a biofilm, especially as it might protect cells in the inner core of the

biofilm (Ciofu *et al.*, 2000). *Moraxella catarrhalis* OMVs were also reported to have a loaded lumen with β-lactamase, which inhibited amoxicillin, and supported growth of *M. catarrhalis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae* amoxicillin sensitive bacteria incubated with amoxicillin (Schaar *et al.*, 2011).

P. aeruginosa culture treated with gold immunolabeled gentamicin resulted in labelled OMVs on TEM micrographs, which suggests a potential for titration (Kadurugamuwa and Beveridge, 1995). Indeed, gold immunolabeled gentamicin incubated with OMVs resulted on labels to the OM of OMV and in the lumen (Schooling and Beveridge, 2006). Hypervesiculating mutant *E. coli* cultures treated with 1.5 µg/ml polymyxin B or 1 µg/ml colistin were found to have greater survivability when purified OMVs were added to the media (Manning and Kuehn, 2011). The addition of 250 µg/ml D-cycloserine and 2 µg/ml polymyxin B to the growth medium increased OMV production in 6.6-fold and 1.9-fold, respectively, in *P. putida* (Choi *et al.*, 2014). Also, the addition of 250 µg/ml of D-cycloserine or 4 µg/ml polymyxin B to cultures of *P. aeruginosa* PA14 increased OMV production by 9.2-fold and 6.3-fold, respectively (MacDonald and Kuehn, 2013). The protein profile from the treated and untreated cultures were similar, which support that OMV increase was not due to lysed material, but rather a process to protect the cell. The addition of different concentrations of OMVs (2 to 12 µg/ml) from *P. syringae* protected the culture against the membrane-active antimicrobials of 2 µg/ml colistin (19 % survivability) and 3 µg/ml mellitin (23% survivability), but not against 0.7 µg/ml streptomycin, which act on protein synthesis (Kulkarni *et al.*, 2014). It noteworthy that *P. syringae* is an antarctic bacterium which rarely encounters synthetic antimicrobials on the environment, which leads to speculation of a well-conserved mechanism for protection against such molecules.

In *E. coli*, high quantities of OMVs were found due to mutations in σ^E envelope stress response pathway genes (McBroom *et al.*, 2006). Outer membrane vesicles are implicated in unfolded proteins removal from the periplasmic space and mutants that undervesiculate were more sensitive to external stress (McBroom and Kuehn, 2007). On the other hand, hypervesiculating mutant strains were more likely to survive when ethanol and polymyxin B were used as external stressors. Indeed, OMVs from *Vibrio tasmaniensis* are able to titrate the antimicrobial peptide polymyxin B, but not degrade it (Vanhove *et al.*, 2015).

Outer membrane vesicles can act as a decoy to absorb cell surface-acting antimicrobial agents and the mutant *E. coli* OMV-hypervesiculating strain had a greater

survival rate compared to the wild strain (Manning and Kuehn, 2011). *P. gingivalis* use OMV to adsorb chlorhexidine and prevent interaction with the bacteria (Grenier *et al.*, 1995). Furthermore, the cyanobacteria *Prochlorococcus* OMV can act as a decoy against cyanophage (Biller *et al.*, 2014) (Fig. I.4). Although antimicrobial encounter is common for pathogenic bacteria, bacteriophages are an important threat to non-pathogenic bacteria. Manning and Kuehn (2011) observed that bacteriophage would bind to *E. coli* OMVs and strains with greater OMVs production have a higher chance of survival. Also, once the bacteriophage attaches to the OMV, there is evidence that it injects its viral DNA and therefore cannot infect again. When inside biofilms, OMV can act as decoy against bacteriophage, thus increasing bacteria survivability (Whitworth, 2011). This could be an important factor for biofilms stability and for resistance of a whole bacterial community.

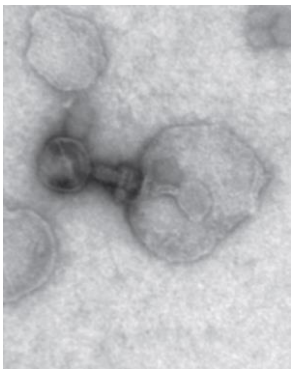

Fig. I.4. TEM micrograph of a cyanophage attached to a *Prochlorococcus* OMV. The shortened phage tail suggests OMV infection. Scale bar, 100 nm. (Biller *et al.*, 2014)

I.4.2.5 Message signalling

Three molecules are part of the quorum sensing (**QS**) network in *P. aeruginosa*, including the *Pseudomonas* quorum-sensing system (**PQS**), and they control 5% of all *P. aeruginosa* genes and biofilm formation (Mashburn and Whiteley, 2005). The hydrophobic nature of these molecules brought attention to them and researchers were interested on how these molecules were transported. This compound was found to be loaded in OMV and *P. aeruginosa* OMVs contain 86 % of the total PQS produced by the bacteria (Mashburn and Whiteley, 2005).

I.4.2.6 Nutrient acquisition

Outer membrane vesicles possess degradative enzymes and receptors that could support bacterial survival (Evans *et al.*, 2012; Berleman *et al.*, 2014; Vanhove *et al.*, 2015). *B.*

fragilis is an opportunistic pathogen that colonizes the human colon and OMVs from *B. fragilis* possess a wide range of hydrolytic compounds that can degrade proteins and carbohydrates (Elhenawy *et al.*, 2014). A *B. fragilis* culture was grown in media with glucose or fucose as carbon source, and OMV from the fucose media was enriched by 7-fold in α-L-fucosidase. *B. fragilis* α-L-fucosidase activity was detected exclusively in OMV. Therefore, when fucose macromolecules are decomposed, the cleaved molecules will be dispersed in the milieu and benefit the whole community. Vanhove *et al.* (2015) incubated a petri dish with a culture of *V. tasmaniensis* and covered it with cellophane for 20 h. The OMVs were recovered from the cellophane with EPS, isolated and 25 % of proteins identified by proteomics were proteases, sulfatases, phosphatases, lipases, haemolysins and murein hydrolases, with proteases corresponding to 45 %.

Myxococcus xanthus is an abundant soil delta-proteobacterium with a wolf-pack hunting behaviour (Whitworth, 2011). The OMV exclusive protein cargo with putative hydrolytic activity was found to be ~24% (Berleman *et al.*, 2014). Myxochelin, an iron chelating siderophore, was identified in OMV fraction and this compound could be responsible for aggregating iron from the environment (Berleman *et al.*, 2014).

Using global proteomic profile on OMVs from laboratory strain of *E. coli*, Lee *et al.* (2007) demonstrated that OMVs carry metal ion binding proteins. These binding proteins could serve to concentrate scarce ions of the environment and the ones that are released after cell lysis, which could have a similar function as the myxochelin.

I.4.2.7 Biofilm production and stability

Several strain of *Helicobater pylori* were evaluated for their biofilm production capacity. One strain had a thicker biofilm and this strain was reported as OMV producer (Yonezawa *et al.*, 2009). When *H. pylori* purified OMVs were added to *H. pylori* cultures in different concentrations, biofilms were thicker as OMV concentration increased (Yonezawa *et al.*, 2009). The ability of OMVs to support biofilm formation summed with OMVs intra and interspecies horizontal gene transfer ability (Yaron *et al.*, 2000) could be a powerful tool for community resistance in harsh environments.

When in planktonic phenotype, the LPS of the pathogenic bacteria *P. aeruginosa* is enriched in the B-band, which is more immunogenic than the A-band (Beveridge *et al.*, 1997). In the biofilm phenotype, *P. aeruginosa* LPS shifts to the less immunogenic form, which might benefit the bacterial community by tricking the host defence mechanisms (Beveridge *et al.*, 1997). Using mutant strains of *P. aeruginosa* with either

B-band or A-band expression gene mutated, biofilm formation decreased when A-band expression gene was mutated, and when B-band expression gene was mutated there was biofilm growth (Murphy *et al.*, 2014). When both genes were mutated, biofilm formation was greater than the wild type. Also, LPS charge can affect the adhesion of the biofilm by affecting surface hydrophobicity, which is an important feature for biofilm development. Proteome of A-band mutated expression gene had fewer proteins involved in surface and cell-cell contact (Murphy *et al.*, 2014), which could explain the fewer EPS secreted and decreased biofilm formation.

Outer membrane vesicles can mediate coaggregation of bacteria, assisting the production and stability of biofilms (Grenier and Mayrand, 1987). *X. fastidiosa* forms biofilm in the foregut of vector insects that feeds from sap, and use this mechanism to infect plants (Almeida and Purcell, 2006). When the gene regulating QS was mutated on *X. fastidiosa*, more OMVs and bacteria were observed, which means a more virulent behaviour, and bacteria were less adhesive, compared to the wild type (Ionescu *et al.*, 2014).

I.4.2.8 Sharing information

Acquisition of genetic information enables survival in stressful situations (Manning and Kuehn, 2013). To date there are three proposed mechanisms for DNA packaging into OMVs; a) free DNA resulting from cell lysis is encapsulate within OMVs; b) DNA passes through the IM, and peptidoglycan layer, and is encapsulated during the OMV formation process; c) breaches in IM and peptidoglycan layer facilitates cytoplasmic components to be present in the outer periplasmic space, which is entrapped during the bulging process (Pérez-Cruz *et al.*, 2013). One mechanism does not discard the other and all of them could happen at the same time.

DNA is present in OMV bound to the membrane and in the luminal content (Fulsundar *et al.*, 2014; Hagemann *et al.*, 2014), however the mechanism by which DNA leaves the cytoplasm to be packaged in the OMV is not yet understood (Kulp and Kuehn, 2010). Outer membranes can be responsible through DNA horizontal transfer between and across species (Dorward *et al.*, 1989; Yaron *et al.*, 2000). Indeed, *Ruminococcus* spp. OMVs transformed mutants (Klieve *et al.*, 2005). Such mechanism probably occurs in the rumen, possibly intra- and inter-species, although there is no study on this subject. However, how the DNA is used by the receiving cell is not yet

fully understood, but the most probable mechanism is via fusion reaction (Manning and Kuehn, 2013).

I.4.2.9 Outer membrane vesicle chains

M. xanthus have social self-organization behaviour and communication between bacteria is essential for the survival of the community. Recently, Remis *et al.* (2014) used combined cryo-electron microscopy, cryo-tomography and focused ion beam scanning electron microscopy (**SEM**) to demonstrate that *M. xanthus* creates OMV chains and use them as a link to communicate with other bacteria from the community. There is evidence of a continuous lumen formed by the OMV chains (Remis *et al.*, 2014). It is relevant to remark that authors found higher amounts of chains in biofilms compared to planktonic cells. These OMV chains could serve as a link between bacteria, which is important for communication, and could serve as a tunnel for signals and/or nutrients.

I.4.3. Factors affecting outer membrane vesicle production

Several environmental stressors were used to evaluate the ability of the bacterial cell to manage OMV production and so far no growth condition has resulted in the absolute absence of OMV production (Kulp and Kuehn, 2010). Some stressors were chosen to mimic a condition that the pathogenic bacteria would face in an infectious environment and when genes related to OMV production were mutated, the bacterial cell is more sensitive to external stressors (McBroom and Kuehn, 2007). Stressed bacteria in most of the cases will hipervesiculate and produce non-native OMVs.

I.4.3.1 Temperature

Proteins can be misfolded by temperature increase and OMVs can be used by the bacteria as a disposal mechanism for these proteins. *E. coli* mutants lacking DegP, a periplasmic chaperone at low temperatures and protease at high temperatures, hipervesiculate with temperature increase from 30°C to 37°C (McBroom and Kuehn, 2007). Hypervesiculation was observed with *P. putida* (Baumgarten *et al.*, 2012) when temperature peaked at 55°C. On the other hand, no effect was observed for temperatures of 25, 37 and 39°C with *P. aeruginosa* (MacDonald and Kuehn, 2013). Temperature increase affects cell division and metabolism, thus increasing the chance of misfolding

proteins, but a defined mechanism that affects OMV production is not totally understood.

I.4.3.2 Oxidative stress

When a pure culture of *P. aeruginosa* was treated with doses of hydrogen peroxide there was an increase in OMV vesiculation (MacDonald and Kuehn, 2013), but no effect was observed in culture of *P. putida* (Choi *et al.*, 2014).

I.4.3.3 Growth media

In an *A. baylyi* culture, OMV yield was 2.5-fold higher in a rich medium compared to a minimal medium, and the OMV protein concentration was 4-fold lower on the minimal medium (Fulsundar *et al.*, 2014). Outer membrane vesicle production of *P. putida* was decreased by 3-fold with a minimal media (Choi *et al.*, 2014). The OM proteins were halved in the minimal media and periplasmic protein doubled.

I.4.3.4 Genetic control

A single gene responsible for OMV production has not been found so far and OMV production is probably regulated by several mechanisms. Data on genetic control should be critically analysed, as species tend to respond differently to specific gene knockout. Kulp and Kuehn (2010) report that mutations on the Tol/Pal-envelope-spanning complex has been described either as hypervesisulator, or to cause considerable OM leakiness.

I.5. Outer membrane vesicle biogenesis

The biogenesis of OMV is not fully understood and several theories have been proposed in the last years (Kulp and Kuehn, 2010; Schertzer and Whiteley, 2012). When curvature of the OM occurs, a vesicle will be formed and released. Kulp and Kuehn (2010) suggest that negatively charges will gather and force the curvature of the membrane. During the curvature process, specific proteins that binds the OM to the peptidoglycan layer will be disrupted, periplasmic proteins will gather and this complex will be released as a vesicle. As a result, the vesicle will mostly be composed of OM, periplasm and some inner membrane (**IM**). DNA and ATP are constituents exclusive from the cytoplasm that have been found in OMVs but in less quantity compared to OM constituents (Schooling and Beveridge, 2006; Pérez-Cruz *et al.*, 2015).

A slight different type of OMV in Gram-negative, termed as ‘outer-inner membrane vesicle’ has been recently described (Pérez-Cruz *et al.*, 2013). During the bulging process part of the inner membrane, or plasma membrane, also bends. This results in double-bilayer membrane, with lumen enriched in cytosolic components, such as DNA, ATP, and cytoplasmic and inner membrane proteins. This type of vesicle has an electron dense lumen, as seen in TEM (Pérez-Cruz *et al.*, 2013). It is important to note that these structures account for about 0.1-1.2% of total OMVs from *Shewanella vesiculosa*, *Neisseria gonorrhoea*, *P. aeruginosa*, and *Acinetobacter baumannii* (Pérez-Cruz *et al.*, 2013; Pérez-Cruz *et al.*, 2015). Outer membrane vesicles were isolated after 120, 48 and 18 hours cultivation for *S. vesiculosa*, *N. gonorrhoea*, *P. aeruginosa*, and *A. baumannii*, respectively.

I.6 Implications

The rumen environment is a unique ecosystem and the dynamic of such a complex environment is far from being completely elucidated, but vesicles can possibly add further complexity. Vesicles from a single cell have a great number of different enzymes and the determinant factor for the cargo is dependent on several aspects. In the human pathogenic bacteria *P. aeruginosa*, OMV protein profile was slightly different when planktonic and biofilm states were compared (Schooling and Beveridge, 2006). Also, the occurrence of OMV is greater in biofilms than in planktonic environment (Remis *et al.*, 2014), which suggests that the ruminal biofilms could potentially host a large number of these structures. The rumen can be a 200 litres fermentation chamber with a myriad of bacteria, fungi, archaea, protozoa and viruses competing for survival, where different types of vesicles might be found. Yet, there is no study with rumen isolated vesicles and the possible role of it in the rumen environment. The EPS and membrane vesicles found within plant-associated biofilms in the rumen have been overlooked and their contribution to the function of the attached biofilm may be greater than the contribution of the attached microbes themselves. It appears that each species has a specific mechanism to regulate OMV/MV production. Therefore, the rumen has potential to help elucidate such mechanism. Understanding the relationship plant-cell is critical to improve plant degradation and utilization.

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II. GENERAL OBJECTIVES

To purify outer membrane vesicles from *Prevotella ruminicola* in Hobson's M2 medium or TM buffer;

To define physiological functions of outer membrane vesicles isolated from *Prevotella ruminicola* in Hobson's M2 medium or TM buffer, using zymograms with gelatine, carboxymethylcellulose or starch;

To purify membrane vesicles using rumen liquor;

To define physiological functions of membrane vesicles isolated from rumen liquor using zymograms with gelatine, casein, carboxymethylcellulose or starch.

III. Outer membrane vesicles from *Prevotella ruminicola* can degrade gelatine, but not carboxymethylcellulose or starch

(Normas: Environmental Microbiology)

SUMMARY

Outer membrane vesicles are linked to several functions in pathogenic bacteria, including transportation of toxic factors, protection against antimicrobials, maintenance of biofilm, and others. The aim of this study was to isolate and explore hydrolytic activity related to OMVs, which may aid plant degradation and bacterial survival. In this study we show that *Prevotella ruminicola*, a Gram-negative ruminal bacterium, produces and releases OMVs during stationary phase when incubated in Hobson's M2 medium and TM buffer. Outer membrane vesicles isolation protocol was successfully employed and a sterile OMV-preparation could be observed on cross-section TEM. Outer membrane vesicles imaged using cross-section TEM were immunolabelled for alkaline phosphatase, an enzyme which is preferably secreted. Protein profiles from samples of whole culture, washed culture, OMVs and OMV-free supernatant were distinct as observed on SDS-PAGE. Two clear and sharp bands for OMV samples from culture incubated in Hobson's M2 medium (**HOM**), and one band for OMV samples from culture incubated in TM buffer, both with ~150kDa can be observed on gelatine zymograms. Protease inhibitors were added to samples to evaluate proteases classes, but they had minor effect. No activity against carboxymethylcellulose (**CMC**) or starch was observed. These results provide evidence for the proteolytic activity related to OMVs from *P. ruminicola* and OMVs might have specific enzymatic activity. Thus, this secretory pathway remains to be explored on ruminal bacteria. These structures are used to degrade substrate, and understanding such process adds knowledge of ruminal microbiome interaction.

RUNNING TITLE: Outer membrane vesicles from *Prevotella ruminicola*.

INTRODUCTION

Outer membrane vesicles are bilayer lipid spheres with 20 to 250 nm of diameter produced by Gram-negative bacteria, which are bulged from the OM and sent away in order to perform several functions (Kulp and Kuehn, 2010). Outer membrane vesicles can: prey other bacteria (Evans *et al.*, 2012); be used as carbon source in sea-water

(Biller *et al.*, 2014); inactivate antibiotics (Kulkarni and Jagannadham, 2014); titrate harmful components away from the bacteria (Kadurugamuwa and Beveridge, 1995); act as a decoy in the milieu for chemicals and bacteriophages (Manning and Kuehn, 2011; Biller *et al.*, 2014); form chains between *Myxococcus xanthus* (Remis *et al.*, 2014); sustain biofilm formation (Schooling and Beveridge, 2006); share DNA between bacteria (Klieve *et al.*, 2005); among others.

Despite the diverse functions described in the literature, only a few studies exist with ruminal bacteria. Using TEM and SEM, researchers observed cellulosome-like structures in cultures of *Fibrobacter succinogenes* (Forsberg *et al.*, 1981), *Clostridium thermocellum* (Bayer and Lamed, 1986) and *Ruminococcus albus* F-40 (Kim *et al.*, 2001). In one study, most enzymatic activity of xylanase and endoglucanase of *F. succinogenes* culture was found to be related to a cell-free pellet obtained after ultracentrifugation (which was possible OMVs) (Forsberg *et al.*, 1981). More recently, OMVs containing DNA were isolated from *Ruminococcus* spp. and were able to transform two mutants that were unable to degrade crystalline cellulose (Klieve *et al.*, 2005).

It is likely that OMVs from different species have different enzymatic cargo which act on substrate degradation and improve bacterial survivability. *Prevotella* spp. is prevalent in the rumen (Stevenson and Weimer, 2007; Huws *et al.*, 2010) and *P. ruminicola* is an important species with peptidolytic (Wallace and McKain, 1991), carboxymethylcellulolytic and amylolytic activity (Avguštin *et al.*, 1997). *Prevotella* spp. are also prevalent colonizers of attached bacteria to perennial ryegrass after 4 h of incubation in the rumen (Huws *et al.*, 2013). Therefore, due to its diverse enzymatic capacity and its importance for the rumen microbiome, it makes sense to perform a study with *P. ruminicola*.

There is evidence that OMVs/MVs from ruminal bacteria have enzymatic activity (Forsberg *et al.*, 1981; Bayer and Lamed, 1986), but a more elaborate protocol to isolate OMVs and reduce contamination is needed. Thus, the aim of this study was evaluate the functions of isolated OMVs from *P. ruminicola* using gelatine, CMC and starch zymograms.

RESULTS AND DISCUSSION

Growth of P. ruminicola in Hobson's M2 medium magnification

P. ruminicola was cultured in a Hungate tube containing HOM. An aliquot with 2% formaldehyde was placed in a haemocytometer and observed with an optical microscope. Rod-shaped bacteria can be observed, although they are short rods (Fig. III.1).

Fig. III.1. Neat culture of *P. ruminicola* observed with an optical microscope at 40 x magnification.

Four Hungate tubes with HOM were used to cultivate *P. ruminicola* and prepare a growth curve (Fig. III.2). Readings had a maximum OD of 3.475 at 20 h, which was higher than those observed by Madeira and Morrison (1997) and Wang and Hsu (2005), probably due to the fact that these studies used simpler medium. Samples were diluted when readings were higher than 2 OD, to avoid saturation and incorrect readings. While the decline phase was not measured, it was concluded that a culture of *P. ruminicola* was in early stationary phase from 10 h and onwards, as OD was 3.285 at 10 h.

Growth phase affects both quantity and quality of OMVs (Tashiro *et al.*, 2010; McCaig *et al.*, 2013). The quantity of OMVs produced by *Francisella novicida* was increased by 4- to 8-fold from stationary to exponential phase (McCaig *et al.*, 2013). Furthermore, some physiochemical properties of OMVs from *P. aeruginosa* were affected from stationary compared to exponential phase (Tashiro *et al.*, 2010). Although there are not many studies with characterization of OMVs according to the growth phase, it seems reasonable to speculate that bacteria will produce OMVs according to their needs within each growth phase. Thus, it was important to choose a growth phase in which OMVs would be isolated for this experiment. It was decided to use OMVs

samples from the stationary phase, as diversity of bacterial population attached to the plant material is stable after 4-h inside a healthy rumen environment (Huws *et al.*, 2013).

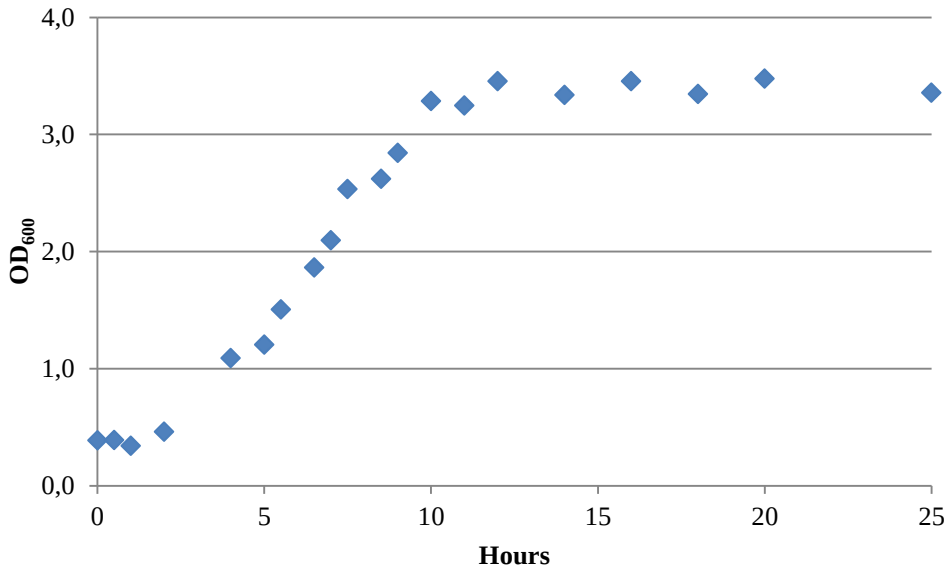


Fig. III.2. *P. ruminicola* growth curve, on optical density (OD), in 4 Hungate tubes containing Hobson's M2 medium and read at 600 nm using a Pharmacia Biotech Ultrospec 4000 UV:visible (Amersham Pharmacia Biotech, United States).

Outer membrane vesicle isolation from P. ruminicola incubated in Hobson's M2 medium or TM buffer

The bacterial pellet yield resulting from the first 13.000 *g* centrifugation was ~1.0%, and ~0.4% after the second 13.000 *g* centrifugation, for both cultures incubated in HOM or TM buffer. TM buffer was used to evaluate if OMVs produced in this maintenance medium would have different enzymatic cargo than OMVs produced in HOM. The OMV pellet after ultracentrifugation was ~1.33% of the total supernatant, for both cultures, which is consistent with OMV pellet yield observed with *P. aeruginosa* and *M. xanthus* (Bauman and Kuehn, 2006; Evans *et al.*, 2012). After all the steps to purify the OMVs, there was still pigmentation in the OMV pellet and supernatant (Fig. III.3). Colour intensity from supernatant was greater for HOM, compared to TM buffer (data now shown). In most cases, the ultracentrifuged pellet is bright-coloured (Schooling and Beveridge, 2006; Olaya-Abril *et al.*, 2014) and sometimes can even be colourless (Chutkan *et al.*, 2013).

Fig. III.3. OMV pellet after ultracentrifugation from *P. ruminicola* incubated in Hobson's M2 medium.

Cross-section TEM using OMV isolates from P. ruminicola incubated in Hobson's M2 medium

Cross-section TEM was used for 2 reasons: evaluate sterility and check OMVs morphology. No bacteria were observed on the micrographs, and a great number of OMVs can be observed (Fig. III.4-8). Variable OMVs diameters can be observed, from ~80 to 200 nm, which is consistent for OMV from Gram-negative bacteria (Kulp and Kuehn, 2010). Dark aggregates can be observed dispersed in the medium, possibly some humic compounds from the clarified rumen fluid present in the medium (Schneider *et al.*, 2012).

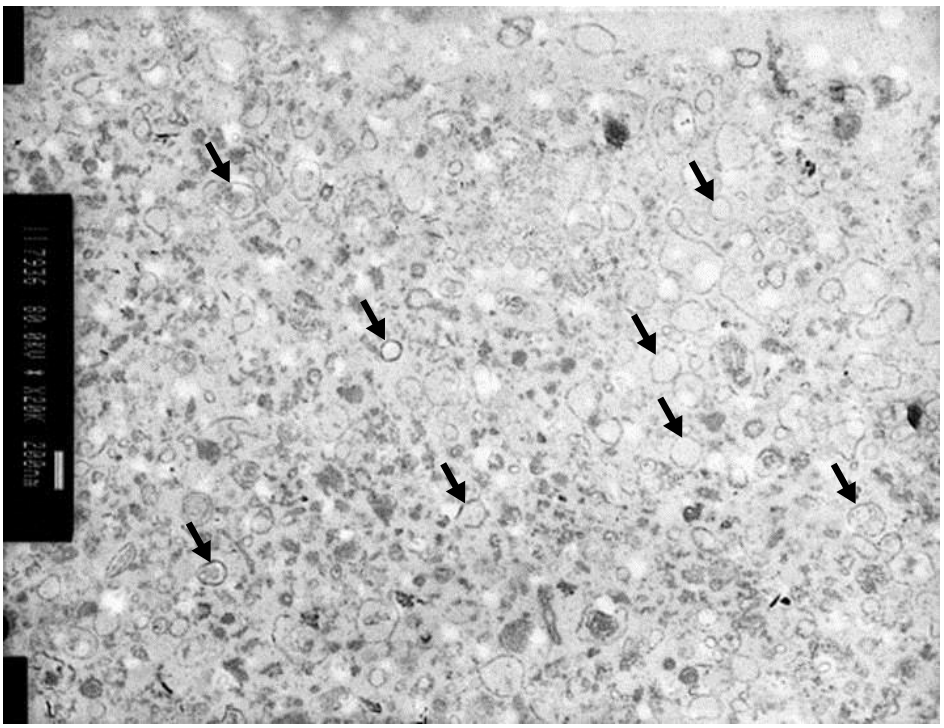


Fig. III.4. Electron micrograph of cross-section through *P. ruminicola* OMVs in Hobson's M2 medium. Black arrows indicate *P. ruminicola* OMVs. Vesicles with different diameters can be observed. Bar, 200 nm.

Some of the vesicles observed in Fig. III.5 have a bright-coloured centre. Some OMVs had a visible double bilayer, but they are less numbered than regular OMVs. Total double bilayer OMVs corresponds to 0.1% in *Shewanella. vesiculosa* (Pérez-Cruz *et al.*, 2013), 0.5% for *P. aeruginosa*, 0.2% for *Acinetobacter baumannii* and 1.2% for *Neisseria gonorrhoeae* (Pérez-Cruz *et al.*, 2015), all incubated in rich medium. Other media than Hobson' M2, without clarified ruminal fluid, might be employed to fully explore morphology of OMVs. Even after several low speed centrifugations to fully pellet *P. ruminicola*, and filtrations with 0.45 µm and 0.22 µm filters, artefacts can be observed on TEM micrographs (Fig III.4-5).

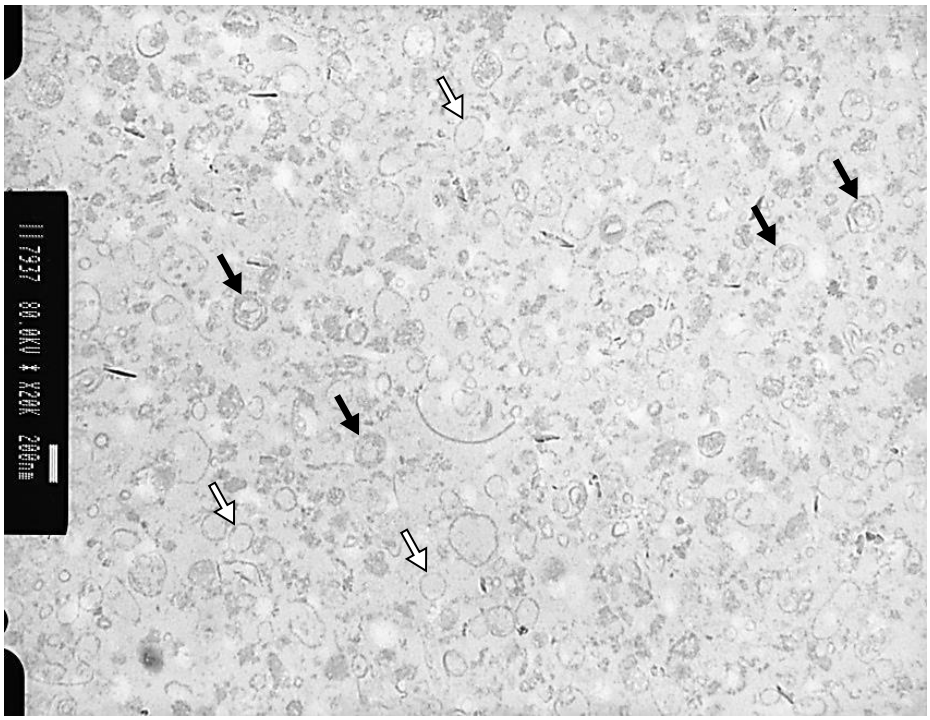


Fig. III.5. Electron micrograph of cross-section through *P. ruminicola* OMVs in Hobson's M2 medium. Black arrows indicate *P. ruminicola* double bilayer vesicle. White arrows with black edges indicate regular OMV from *P. ruminicola*. Vesicles with different diameters, shapes and cargo can be observed. Bar, 200 nm.

Cross-section TEM using OMV isolates from P. ruminicola incubated in TM buffer

Cross-section TEM micrographs of OMVs from *P. ruminicola* incubated in TM buffer was clearer (Fig. III.6) compared to Fig. III.4-5, where culture was incubated in HOM. This difference is probably due to clarified rumen fluid. Again, no bacterial cells were observed following TEM observations. Outer membrane vesicles with different

diameters ranging from ~80 to 200 nm were observed. This diameter range is similar to Fig. III.4-5, where *P. ruminicola* was incubated in a rich nutrient medium. It has been shown that OMVs from a culture of *Acinetobacter baylyi* from a nutrient deprived medium had smaller vesicle diameter compared to OMVs from a medium exposed to gentamicin (Fulsundar *et al.*, 2014), but this was not the case for this study. Furthermore, OMVs with a variation of the colour of the centre can be observed, which is implied with different luminal cargo. However, proteomics analysis would be needed to evaluate peptides.

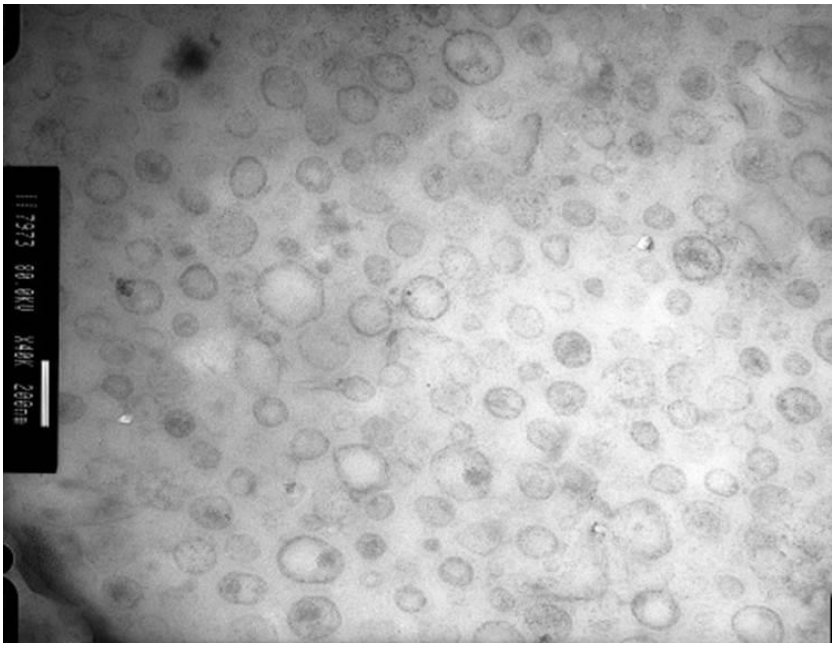


Fig. III.6. Electron micrograph of cross-section through *P. ruminicola* OMVs in TM buffer. Bar, 200 nm.

Cross-section TEM with OMVs immunolabeled for alkaline phosphatase isolated from P. ruminicola incubated in TM buffer

Gold immunolabelled alkaline phosphatase can be observed as black dots on micrographs (Fig. III.7). Several OMVs were labelled and some had more than 1 label. The chance of getting an OMV with multiple labels is unlikely, as sectioning has to expose more than one functional epitope in a very small area. *P. ruminicola* were suggested to be responsible for most of ruminal alkaline phosphatase activity (Forsberg and Cheng, 1980) and 96.5 % of alkaline phosphatase activity was detected in the supernatant (Madeira and Morrison, 1997), which is consistent with the OMV immunolabel observed in micrographs.

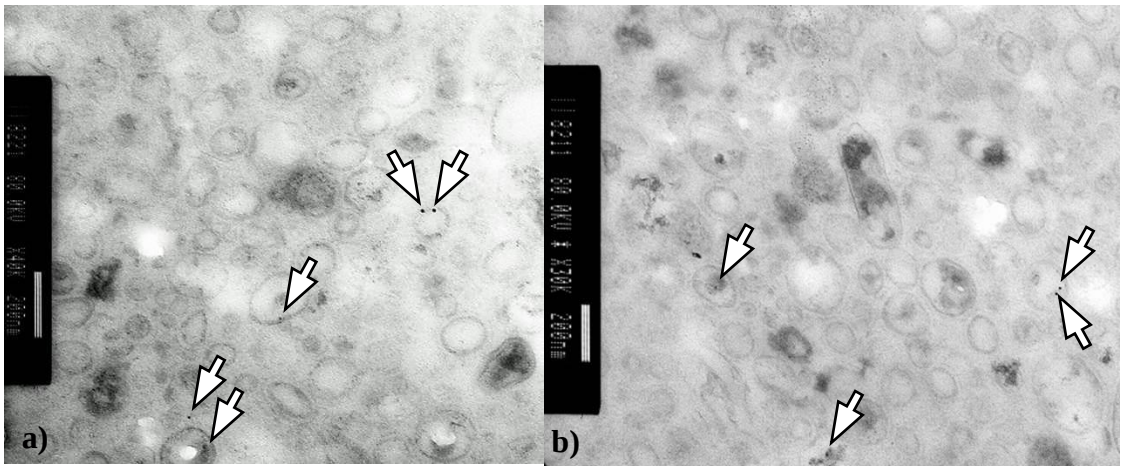


Fig. III.7. Electron micrograph of cross-section through *P. ruminicola* OMVs immunolabelled with bacterial alkaline phosphatase, in TM buffer. Black arrow with white edges indicates marked OMVs. Bar, 200 nm.

A second attempt to immunolabel samples was performed, this time to tyrosinase. Tyrosinase is an enzyme that catalyses the production of melanin. Although no marked OMV was observed, a lower magnification of the microscope shows the diversity of the OMVs (Fig. III.8). OMVs have several diameters, shapes and electron density cargo. Furthermore, sterility of the sample is further supported by the broader micrograph.

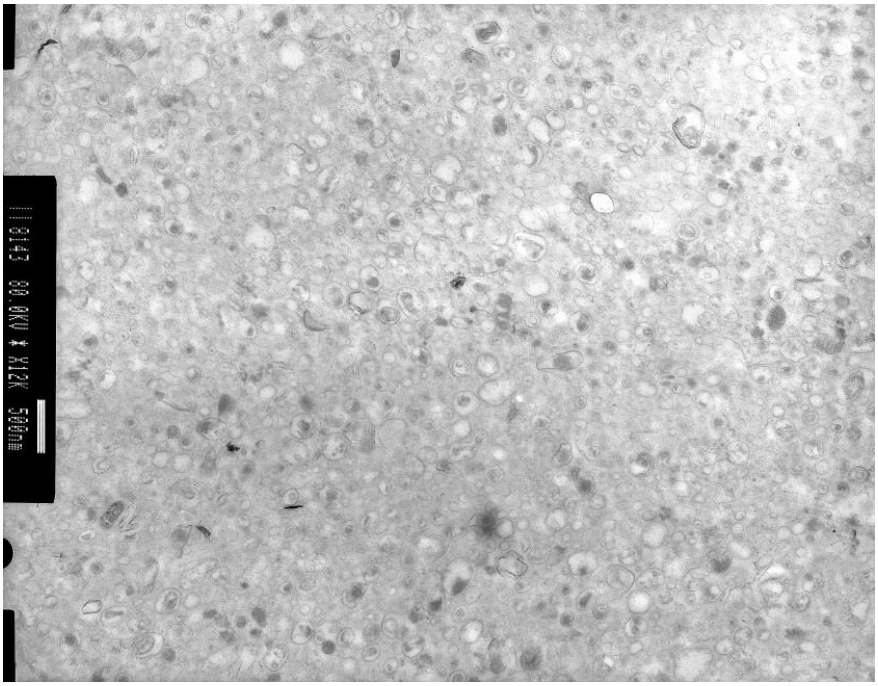


Fig. III.8. Electron micrograph of cross-section through *P. ruminicola* OMVs immunolabelled to tyrosinase, in TM buffer. Bar, 500 nm.

Low-temperature SEM from OMVs isolated from P. ruminicola incubated in TM buffer

In order to further evaluate sterility of the preparation, OMVs from TM buffer were imaged using SEM with cryo-stage. No bacteria were observed and sphere like structures were predominant (Fig. III.9). Although less resolute, a more homogenous pattern can be observed on micrograph c).

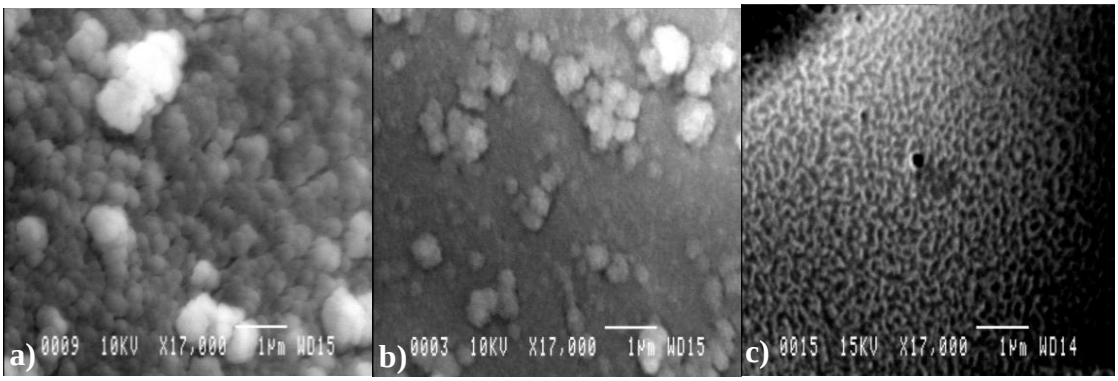


Fig. III.9. Low-temperature scanning electron microscopy micrographs showing morphology of OMVs from *P. ruminicola* in TM buffer. Bar, 1 µm.

Possible carry-over effect of Hobson's M2 medium

Using media with rumen content, like HOM, is challenging for vesicles purification due to the soluble and insoluble content which were seen in TEM micrographs (Fig. III.4-5). *P. ruminicola* was grown in HOM, a sterile culture medium which has 20% of clarified rumen fluid. To ensure that HOM was OMV-free and that protein profile would be different when compared to pure culture samples and OMVs, the same methodology used for OMV purification was employed in a pure autoclaved 500 ml of HOM. Thus, neat HOM was compared to whole culture of *P. ruminicola*; the supernatant-free HOM pellet was compared to washed culture; OMV-free ultracentrifuged supernatant from HOM was compared to supernatant from *P. ruminicola*; and OMV-free pellet obtained from ultracentrifugation at 115,000 *g* for 120 min was compared to *P. ruminicola* OMV isolates. This will further be explored on SDS-PAGE section.

Protein concentration within P. ruminicola incubated in Hobson's M2 medium

Protein concentration was determined using a colorimetric assay (Bradford, 1976) and a standard curve was built using bovine serum albumin (**BSA**) (Fig. III.10).

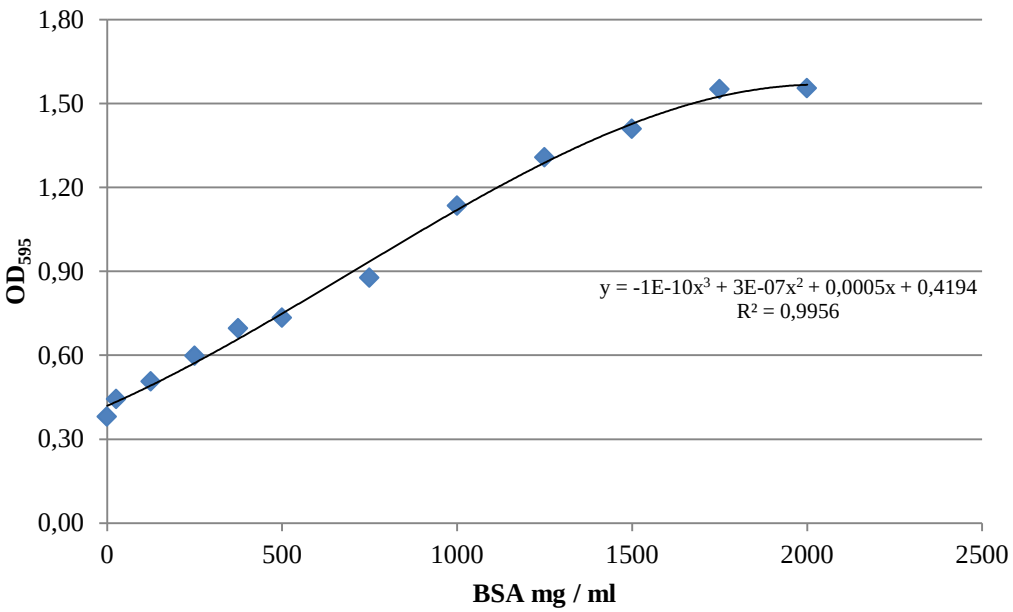


Fig. III.10. Protein concentration standard curve based on a BSA standard.

Outer membrane vesicles incubated in HOM had the highest estimated protein concentration (Table III.1), even higher than concentrated *P. ruminicola* washed cells (2.71 vs 0.79 µg/µl, respectively). This was expected as the OMV pellet was concentrated using the whole volume of culture. Protein content using incubation samples from HOM were always higher than samples from HOM alone, as expected. Protein values of samples from HOM have to be subtracted from *P. ruminicola* in HOM samples (e.g. value for the whole culture alone is 0.13 µg / µl) to correct protein carry-over content. Furthermore, most of the protein measured from *P. ruminicola* HOM incubation samples was in the medium itself. For the samples incubated in TM buffer, the rational is not the same as TM buffer has no protein on the medium composition.

SDS-PAGE using P. ruminicola incubated in Hobson's M2 medium samples

SDS-PAGE is an electrophoretic technique used to separate protein by its molecular weight (Silhavy *et al.*, 1984). A 12.5% SDS-PAGE gel was prepared to compare the protein profile of the samples from *P. ruminicola* incubated in HOM. Protease inhibitor cocktail was added to the samples to inhibit endogenous proteases. Although bands from *P. ruminicola* whole culture and washed cells were clear, supernatant and OMV bands were faint. The protein profile from whole culture was similar to washed cells, but different from OMV isolated from the TM buffer (Fig. III.11). Interestingly, protein bands were always sharper for the samples from TM buffer incubations.

Table III.1. Protein concentration estimation

Sample	µg / µl
P. ruminicola whole culture incubated in Hobson's M2 medium	0.79
P. ruminicola whole culture incubated in TM buffer	0.73
Neat Hobson's M2 medium	0.66
Washed *P. ruminicola* incubated in Hobson's M2 medium	0.79
Washed *P. ruminicola* incubated in TM buffer	0.93
Supernatant-free Hobson's M2 medium pellet	0.54
Supernatant from *P. ruminicola* incubated in Hobson's M2 medium	1.32
Supernatant from *P. ruminicola* incubated in TM buffer	0.54
Supernatant from neat Hobson's M2 medium	1.04
OMV from *P. ruminicola* incubated in Hobson's M2 medium	2.71
OMV from *P. ruminicola* incubated in TM buffer	0.81
Ultracentrifuged OMV-free pellet from neat Hobson's M2 medium	2.10

Silver staining is used to increase sensitivity of SDS-PAGE gels as silver ions interact with protein (Morrissey, 1981). Thus, Coomassie stain was removed and gels were silver stained to improve band visualization (Fig. III.11 b), but only really faint bands can be observed on OMV samples from HOM. It should be noted that humic compounds can and probably interfere with protein content estimation (Schneider *et al.*, 2012), thus affecting total protein content loaded on each lane. The dark background results from silver stain might be caused by humic compounds dispersed in the samples that spread throughout the lane during the electrophoresis process. Spaces between lanes were not stained with Coomassie blue or silver stain (Fig. III.11). Indeed, a pigmented line was seen running faster than the bromophenol blue line during the SDS-PAGE and zymograms electrophoresis (Fig. III.12), and there was yellow pigmentation on the top of the well, which means that some components could not enter the gel. Lane 12 (Fig. III.11) was loaded with ultracentrifuged OMV-free pellet from neat HOM (i.e. HOM was centrifuged at low speed, filtered and centrifuged at high speed) to verify presence of carryover of OMV/MV from the 20% clarified ruminal liquid that was used. The lane has no apparent bands, even after silver stain was used. Yet, the lane has the characteristic dark background. The HOM was autoclaved at 121°C in order to sterilize the media, probably denaturing proteins.

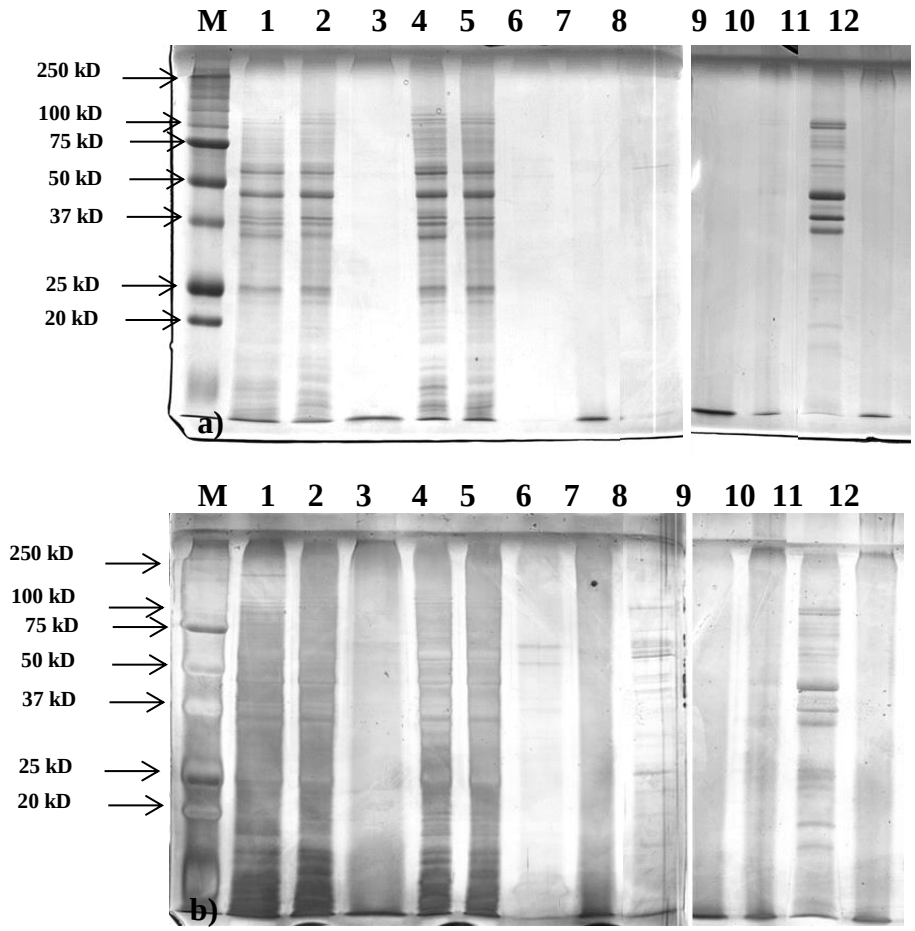


Fig. III.11. SDS-PAGE of pure culture samples in Hobson's M2 medium and TM buffer, and neat Hobson's M2 medium, stained with Coomassie blue (a) or silver stain (b).

M, protein marker; **1**, whole culture of *P. ruminicola* in Hobson's M2 medium; **2**, whole culture of *P. ruminicola* in TM buffer; **3**, neat Hobson's M2 medium; **4**, washed *P. ruminicola* in Hobson's M2 medium; **5**, washed *P. ruminicola* in TM buffer; **6**, supernatant-free Hobson's M2 medium pellet; **7**, ultracentrifuged supernatant from *P. ruminicola* in Hobson's M2 medium; **8**, ultracentrifuged supernatant from *P. ruminicola* in TM buffer; **9**, ultracentrifuged supernatant from neat Hobson's M2 medium; **10**, OMVs from *P. ruminicola* in Hobson's M2 medium; **11**, OMVs from *P. ruminicola* in TM buffer; **12**, ultracentrifuged OMV-free pellet from neat Hobson's M2 medium.

Zymograms

Zymograms are used for detection of enzymes activity (Heussen and Dowdle, 1980; d'Avila-Levy *et al.*, 2012). Zymograms were prepared with substrates to check enzymatic activity against gelatine, CMC or starch.

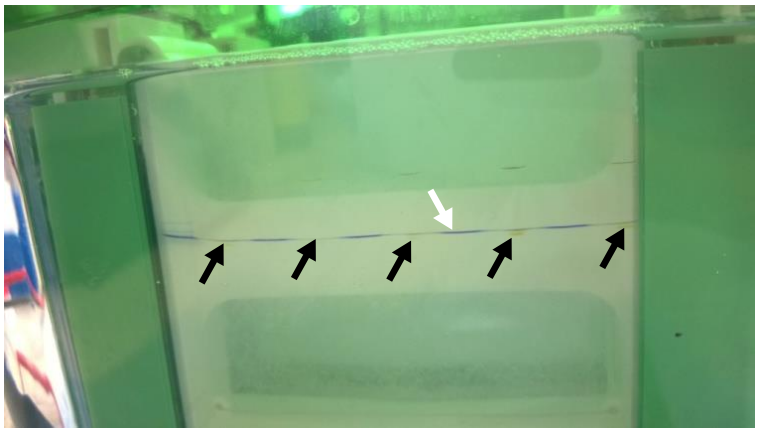


Fig. III.12. Mini-protean apparatus used for SDS-PAGE gels and zymograms. Dark arrows indicate a yellow faint line. White arrow indicate the bromophenol blue line.

The gel substrate, the electrophoresis voltage, the electrode buffer temperature, the detergent of the renaturating buffer, the composition of the developing buffer, the pH, the period of incubation and temperature in the developing buffer, among others, will influence enzyme activation (d'Avila-Levy *et al.*, 2012). Although rumen pH can range from 4.5 to 7.0 (Hungate, 1966), it was decided to set pH of developing buffer to 6.8, as it is the optimum pH for *P. ruminicola* protease activity (Wang and Hsu, 2005). Also, gels were soaked in developing buffer for 18- to 20-h at 39°C (Wang and Hsu, 2005).

There is evidence that temperature can inactivate OMVs enzymes (Elhenawy *et al.*, 2014), thus all zymograms were carefully prepared to prevent heating. No sonication was used as the components of the loading buffer and the triton X-100 wash might are enough for OMV lysis (Vanhove *et al.*, 2015).

Proteolytic activity using samples from P. ruminicola incubated in Hobson's M2 medium and TM buffer with different protease inhibitors added to samples

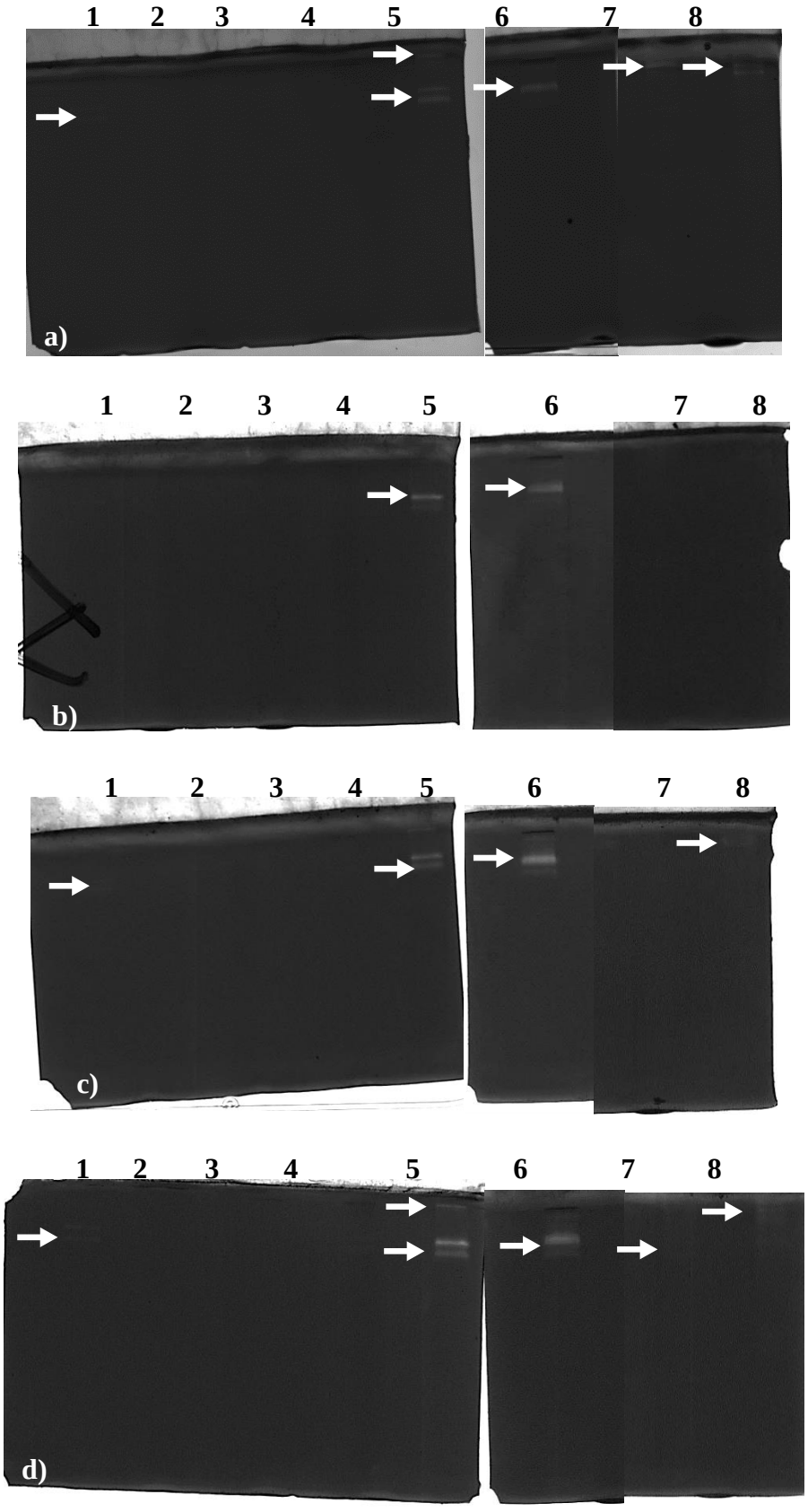
To explore protease classes, protease inhibitors were individually added to samples and samples were loaded on gelatine zymograms. Four broad spectrum protease inhibitors were added individually to samples: EDTA as a metalloproteinase inhibitor (5 mM); E64 as a cysteine proteinase inhibitor (5 µM); PMSF as a serine protease inhibitor (0.5 mM); or pepstatin A as an aspartic protease inhibitor (1 µM) (Fig. III.13) (Wang and Hsu, 2005). Few bands were observed on the gels. Lanes 1, 5 and 6 had clear bands with ~150 kDa, and lanes 5, 7 and 8 had bands with ~250 kDa.

Discrete activity against gelatine can be observed on lanes 1, but not lane 2. These 2 lanes correspond to *P. ruminicola* in HOM and TM buffer, respectively (Fig. III.13).

Lanes 3 and 4 had no or very little activity against substrate, and lanes 5-8 all had activity against substrate. Lanes 5 and 6 had samples of OMVs in HOM and TM buffer, respectively, and clear distinct bands can be observed. Thus, proteolytic activity might be selectively secreted on OMVs. There is evidence that a culture of *P. ruminicola* 23 in late stationary phase will have most of protease activity secreted (65%), and a small part will be associated with the cell surface (18%) (Wang and Hsu, 2005). Indeed, some acidic glycosidases and proteases are preferentially packaged into *Bacteroides* OMVs and show *in vitro* activity (Elhenawy *et al.*, 2014). Furthermore, proteases, phosphatases and other hydrolases are packaged on *M. xanthus* OMVs (Evans *et al.*, 2012). Outer membrane vesicles have been described as part of the secretory system for bacteria (Kulp and Kuehn, 2010) and proteolytic activity was predominately seen on lanes 5, 6, which had OMV samples. *P. ruminicola* first degradation mechanism could be cell-associated (Hazlewood *et al.*, 1981), and as nutrient becomes unavailable, protease is secreted. Outer membrane vesicles offers an interesting mechanism for secretion of proteases, as luminal content is protected against host proteases (Kulp and Kuehn, 2010). It is noteworthy that *P. ruminicola* is non-motile bacterium (Shah and Collins, 1990), thus a secretory mechanism involving OMVs is an interesting way to explain how a non-motile bacterium degrades distant substrates, with a probable release of nutrient in the biofilm, benefiting the whole community.

Gelatine substrate degradation on zymogram was similar for most of the protease inhibitors used (Fig. III.13). Overall, only resolution of bands was affected, which could be consequence of gel preparation. On gel b and lane 1, EDTA was added to sample of *P. ruminicola* in HOM, and no degradation can be observed. Also, on gels b and c, lane 7, EDTA and E64 were added to samples, respectively and no activity was detected compared to the same lane on the other gels. Serine and metalloprotease inhibitors might inhibit protease activity on *P. ruminicola* (Wang and Hsu, 2005), which was the case for gel b, but not for gel d. Using *in vitro* assays, proteolytic activity was decreased by 51.2% with PMSF, 64.7% with pepstatin A, 42.4% with EDTA, and increased by 23.1% with iodoacetate, which is a cysteine protease inhibitor (Wang and Hsu, 2005). However, these studies were not performed on zymograms. Zymograms gels were incubated in renaturating buffer for at least 18 h, but proteases from *P. ruminicola* can be active for 50 h and more (Wang and Hsu, 2005). Thus, gels could be more sensitive if left in developing buffer for longer time. Also, there is evidence that protease activity

is reduced by 50% when aerobic condition was used (Hazlewood *et al.*, 1981), but this condition was not used on the present project.



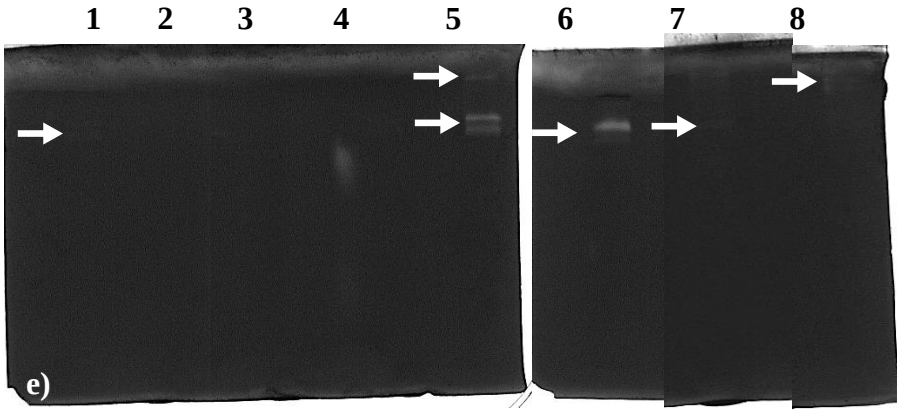


Fig. III.13. Gelatine zymogram with samples from *P. ruminicola* incubated in Hobson's M2 medium and TM buffer, with addition of protease inhibitors to samples: **a**, no protease inhibitor; **b**, EDTA (5 mM); **c**, E-64 (5 µM); **d**, PMSF (0.5 mM); **e**, pepstatin (1 µM).

1, whole culture of *P. ruminicola* in Hobson's M2 medium; **2**, whole culture of *P. ruminicola* in TM buffer; **3**, washed *P. ruminicola* in Hobson's M2 medium; **4**, washed *P. ruminicola* in TM buffer; **5**, OMVs from *P. ruminicola* in Hobson's M2 medium; **6**, OMVs from *P. ruminicola* in TM buffer; **7**, ultracentrifuged supernatant from *P. ruminicola* in Hobson's M2 medium; **8**, ultracentrifuged supernatant from *P. ruminicola* in TM buffer.

It should be noted that although proteolytic activity can be observed, the nitrogen source is a key component when evaluating proteases degradation (Griswold and Mackie, 1997; Wang and Hsu, 2005). *P. ruminicola* proteases will have greater affinity for peptone compared to casein or ammonia (Wang and Hsu, 2005); and *P. bryantii* proteases will have greater affinity for casein or soybean-based substrate (Griswold and Mackie, 1997). Difference in enzymatic activity due to nitrogen source could be related to size of the amino acid residue (Chen *et al.*, 1987) and ruminal bacteria have a preference for cleaving dipeptides rather free amino acids (Wallace *et al.*, 1990).

Carboxymethylcellulase activity using samples from P. ruminicola incubated in Hobson's M2 medium and TM buffer

No activity against the substrate was detected on zymogram gel with 0.1% of CMC (Fig. III.14). It is not clear the sensitivity of the gel for the enzymes, as there is evidence that *P. ruminicola* have CMCase activity, although no quantitative information is described (Avguštin *et al.*, 1997). The β-1,4-endoglucanase from *P. bryantii*, has activity against CMC and barley glucan in *in vitro* assays, but not xylan or mannan

(Gardner *et al.*, 1995). *P. bryantii* was grown with different carbon sources added to medium, cell were harvested and washed, and loaded on zymograms with CMC as substrate (Gardner *et al.*, 1995). Endoglucanases with activity against CMC was observed, but it was dependent on the carbon source added to the medium (Gardner *et al.*, 1995).

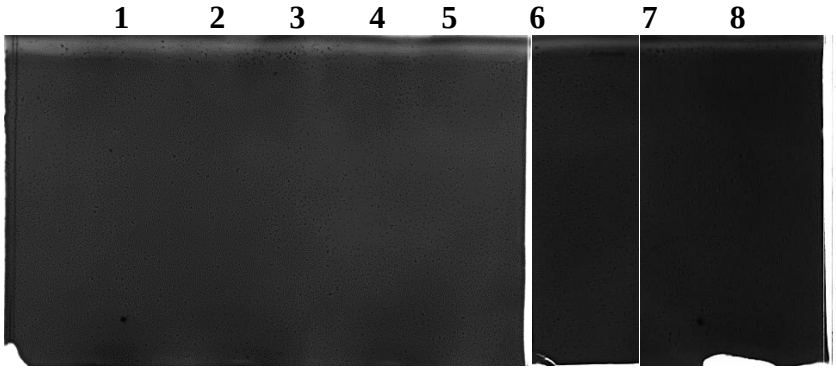


Fig. III.14. CMC zymogram with samples from *P. ruminicola* incubated in Hobson's M2 medium and TM buffer.

1, whole culture of *P. ruminicola* in Hobson's M2 medium; **2**, whole culture of *P. ruminicola* in TM buffer; **3**, washed *P. ruminicola* in Hobson's M2 medium; **4**, washed *P. ruminicola* in TM buffer; **5**, OMV from *P. ruminicola* in Hobson's M2 medium; **6**, OMV from *P. ruminicola* in TM buffer; **7**, ultracentrifuged supernatant from *P. ruminicola* in Hobson's M2 medium; **8**, ultracentrifuged supernatant from *P. ruminicola* in TM buffer.

Amylase activity using samples from P. ruminicola incubated in Hobson's M2 medium and TM buffer

Although *P. ruminicola* are known to be utilise starch to generate succinate (Wang and Hsu, 2005), which is converted to propionate and absorbed the rumen wall, no activity was detected on the zymograms, for all samples (Fig. III.15). Again, the sensitivity of the gel is not known for starch.

Outer membrane vesicles implications

Microbial population is affected by a myriad of factors, thus data generated using axenic culture is important at it removes disturbing factors and unveils specific behaviour. To our knowledge, this is the first study to evaluate enzymatic cargo in OMVs using a ruminal bacterium, as enzymatic activity on the study from Forsberg *et al.* (1981) is bacterial and secretome, and not OMV-specific. The OMV isolation protocol was successful when working with axenic culture of *P. ruminicola*. There was a diverse

range of OMV diameter when OMVs were isolated from the stationary phase. On zymograms, OMVs had degradative activity against gelatine, but not against starch or CMC. Several factors influence enzymes activity and under different conditions the results could be different. Nonetheless, there is now evidence that OMVs from an axenic culture from an important rumen bacterium has degradative action against gelatine. This further supports the accepted proteolytic activity of *P. ruminicola*. Furthermore, data from this experiment suggest that most activity from *P. ruminicola* against gelatine was secreted into OMVs. Understanding the fundamentals of bacteria is an important step to comprehend rumen microbiome interactions.

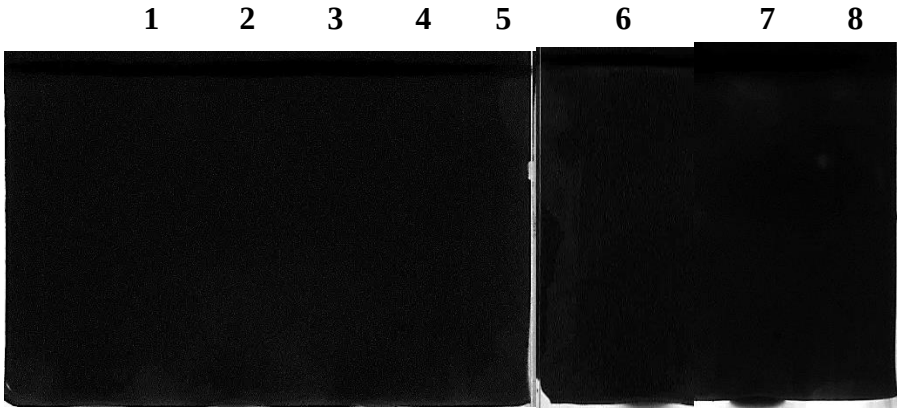


Fig. III.15. Starch zymogram with samples from *P. ruminicola* incubated in Hobson's M2 medium and TM buffer.

1, whole culture of *P. ruminicola* in Hobson's M2 medium; **2**, whole culture of *P. ruminicola* in TM buffer; **3**, washed *P. ruminicola* in Hobson's M2 medium; **4**, washed *P. ruminicola* in TM buffer; **5**, OMV from *P. ruminicola* in Hobson's M2 medium; **6**, OMV from *P. ruminicola* in TM buffer; **7**, ultracentrifuged supernatant from *P. ruminicola* in Hobson's M2 medium; **8**, ultracentrifuged supernatant from *P. ruminicola* in TM buffer.

EXPERIMENTAL PROCEDURES

Bacterial strain

The axenic culture of *P. ruminicola* ATCC 19189 was recovered from the collection that is kept at -80°C at the Institute of Biological, Environmental and Rural Sciences at Aberystwyth University, Penglais, Aberystwyth, Ceredigion, SY23 3DA, United Kingdom.

Media preparation

Hobson's M2 medium (Hobson, 1969) was prepared as suitable medium for *P. ruminicola* growth. The medium was prepared by combining 2.0 g of glucose, 2.0 g of maltose, 4.0 g of sodium hydrogen carbonate, 10.0 g of peptone, 2.5 g of yeast extract, and 2.0 g of cellobiose; 150 ml of mineral solution I (3.0 g of dipotassium hydrogen, in 1 l of distilled water); 150 ml of mineral solution II (3.0 g of potassium dihydrogen orthophosphate anhydrous, 6.0 g of ammonium sulphate, 6.0 g of sodium chloride, 0.6 g of magnesium sulphate heptahydrate, and 0.6 g of calcium chloride dihydrate, in 1 l of distilled water); 200 ml of clarified ruminal liquid; 10 ml of sodium lactate 60%; and 1 ml of 0.1% resazurin solution (w/v); and volume was completed to 1 l with distilled water. To prepare clarified rumen fluid, rumen fluid was strained through two layers of muslin, centrifuged at 13,000 *g* for 25 min at 4°C, and stored at 4°C. The medium was heated in microwave twice, until bubbling was visible, to remove oxygen. On a magnetic stirrer plate and under carbon dioxide gassing, the medium was left to cool and 1 g of L-cysteine hydrochloride was added as reducer agent. Under carbon dioxide gassing, the medium was dispensed (9 ml per Hungate tube, and 450 ml per Duran bottle), and sealed. Bottles and tubes were autoclaved at 121°C for 15 min at 15 lb/in^2. Several Hungate tubes were reserved for sterility evaluation using the "Most-probable-number" technique (Dehority *et al.*, 1989).

TM buffer was used as maintenance medium, which consisted of 50 mM of Tris and 10 mM of magnesium sulphate, with adjusted pH to 6.8. The volume was microwaved, gassed, dispensed (450 ml into 500 ml Duran bottles), and autoclaved as above.

P. ruminicola growth curve

A Hungate tube containing a frozen *P. ruminicola* ATCC 19189 culture was left at room temperature until the content was homogeneous. The culture was sub-cultured into 4 Hungate tubes, containing HOM, and incubated at 39°C. Samples were taken at 0 h, 0.5 h, and every hour until stationary phase, and then 2 h onwards until 24 h, when monitoring was halted. One ml aliquot was withdrawn with 1 ml disposable syringes and 23 gauge needles (25 mm). A drop of ethanol was dispensed on the stopper and the top was flamed to prevent contamination. Absorbance was measured at 600 nm using a Pharmacia Biotech Ultrospec 4000 UV:visible (Amersham Pharmacia Biotech, United States) and data was plotted.

Isolation of OMVs from P. ruminicola incubated in Hobson's M2 medium

To better understand the experimental procedure, a flow chart with step-by-step procedures was prepared (Fig. III.16). A Hungate tube containing frozen *P. ruminicola* ATCC 19189 culture was left at room temperature until the culture was homogeneous. Then, the culture was sub-cultured in 5 Hungate tubes containing HOM until early stationary phase at 39°C. The content of the 5 viable Hungate tubes was used to inoculate a 500 ml Duran bottle containing 450 ml of HOM, and incubated at 39°C. At early stationary phase, aliquots were taken and observed in haemocytometer chamber with an optical microscope at 40 x magnification to evaluate contamination. At early stationary phase, 50 ml of the culture was aliquoted to represent the whole culture and was stored at 4°C. The remaining volume was centrifuged in a GSA Sorvall rotor (Du Pont Instruments, United States) at 13,000 *g* for 20 min at 4°C in a Sorvall RC-5B Refrigerated Superspeed Centrifuge (Du Pont Instruments, United States) to pellet bacteria. At the end of the centrifugation, the pellet was quickly resuspended in TM buffer to complete 50 ml, which was further inoculated in a 500 ml Duran bottle containing 450 ml of TM buffer, and incubated at 39°C. The supernatant from the first centrifugation was recentrifuged with the same conditions to pellet remaining bacteria. The pellet was discarded and the supernatant was filtered with 0.45 µm and 0.22 µm Supor® Acrodisc®, polyethersulphone, sterile, syringe filter membrane disk (Pall Life Sciences, United States). One ml was aliquoted and the "Most-probable-number" technique (Dehority *et al.*, 1989) was used to check sterility of the outer membrane vesicles. Briefly, serial dilutions were prepared (neat, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) and were inoculated in Hungate tubes, in triplicate, using same conditions for culturing *P. ruminicola*. The ultrafiltered volume was divided in polycarbonate vials, placed in a 70.1 Ti rotor (Beckman Coulter, United States) and centrifuged at 115,000 *g* for 120 min at 4ºC in an Optima L-100xp (Beckman Coulter, United States), in order to pellet the OMVs. The pellet was resuspended in 100 µl TM buffer and stored at 4°C for further analysis. OMV-free supernatant was stored for further concentration. After 18-h, the *P. ruminicola* culture incubated in TM buffer was processed as above, with the exception of no further inoculation.

Outer membrane vesicle-free supernatant concentration

The supernatant was concentrated to a final volume of 2 ml using 50 ml of OMV-free supernatant aliquots. The volume was centrifuged at 4,000 *g* for 30 min at 4°C in an

ALC PK-131R (ALC International, Italy) in Amicon Ultra 15 ml tubes (10 kDa – Merck Millipore; United States). Throughout the results and discussion section this fraction will be addressed as supernatant.

Washed cells preparation

To wash away the supernatant from the cells, 10 ml of the whole culture aliquot was centrifuged at 13,000 *g* for 1 min at 4°C (Biofuge Fresco Heraeus Instruments, DE). The supernatant was discarded and the pellet resuspended in 900 µl of TM buffer (pH 6.8). This procedure was repeated 4 times. Throughout the results and discussion fraction this fraction will be addressed as washed cells.

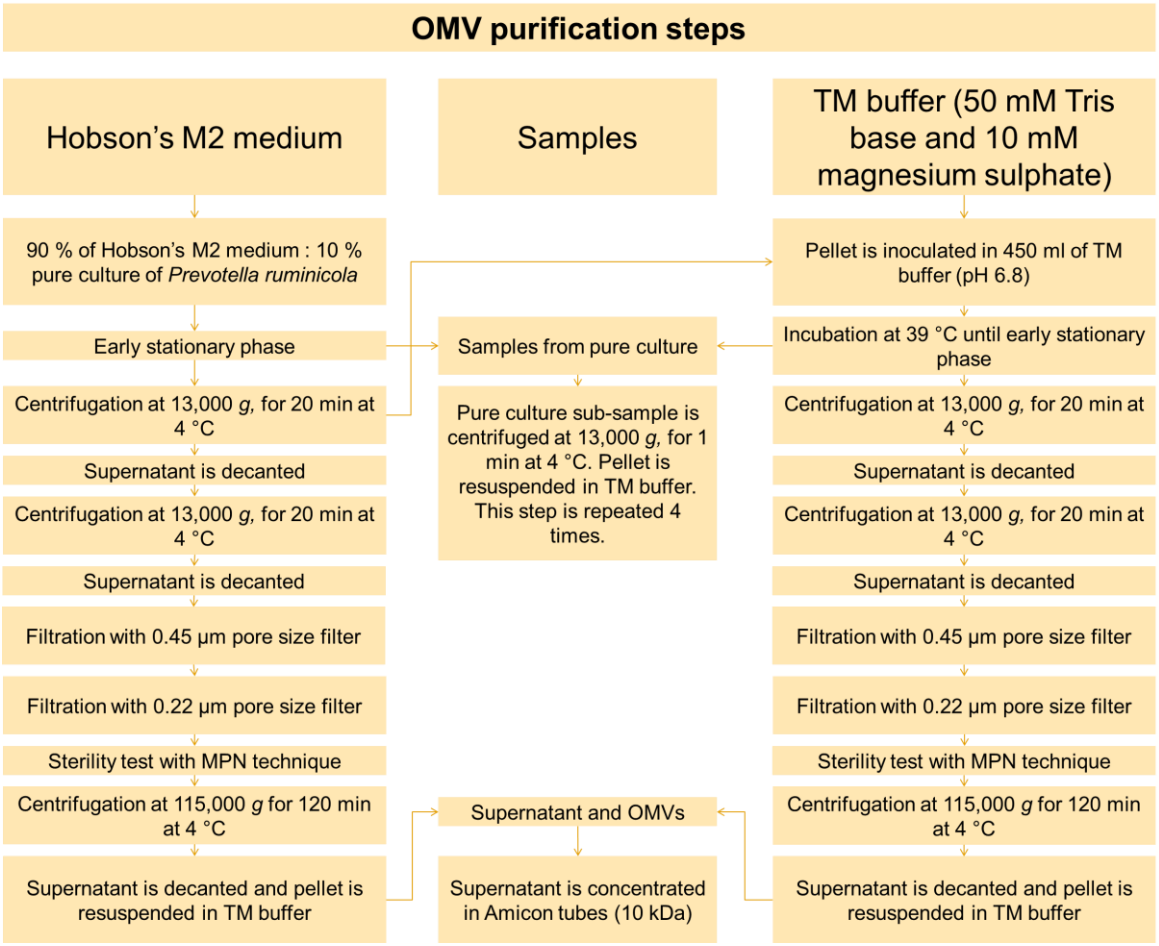


Fig. III.16. Flow chart of the experimental scheme for OMV isolation from *P. ruminicola* culture in Hobson's M2 medium or TM buffer.

Protein concentration determination

Bradford protein assay (Bradford, 1976) was adapted for microplate assay. A standard curve was built using BSA as standard with a 3-parameter polynomial equation. To estimate protein concentration, 5 µl of sample was mixed with 250 µl of 1x Dye Reagent (Bio-Rad, United States) and incubated at room temperature for 10 min. Absorbance was measured at 595 nm using a Synergy H1 (Biotek, Switzerland) with Gen5 Data Analysis Software (Biotek).

Sample preparation for SDS-PAGE gels

For sample preparation for SDS-PAGE, 10 % of protease inhibitor cocktail (Sigma Aldrich, United States) was added to samples aliquots (v/v) in 1.5 ml eppendorfs. Then, samples were mixed with 4x Laemmli buffer (Laemmli, 1970), which consisted of 2% sodium dodecyl sulphate (w/v), 10 % glycerol (v/v), 62.5 mM Tris-hydrochloride, and 0.0025% bromophenol blue (w/v). Samples were centrifuged at 13,000 *g* for 2 min at 4°C (Biofuge Fresco Heraeus Instruments, DE). Supernatant was decanted and pellet was discarded. Then 10% β-mercaptoethanol (v/v) was added to samples, and samples were heated in heated block (Techne Dri-Block DB-2D, Cambridge, UK) at 95°C for 5 min. Samples were centrifuged at 13,000 *g* for 2 min at 4°C (Biofuge Fresco Heraeus Instruments, DE). Supernatant was decanted and pellet was discarded. Each individual lane was loaded with 2.9 µg of protein.

SDS-PAGE gels preparation

SDS-PAGE gels were prepared (Silhavy *et al.*, 1984) using 12.5% separating gel (3.5 ml of distilled water, 2.5 ml of 40% acrylamide and bis-acrylamide solution, 2.0 ml of running buffer (1.5 M Tris base, 10% sodium dodecyl sulphate – w/v, and pH adjusted to 8.5), 8 µl of TEMED, and 80 µl of 10% ammonium persulphate solution – w/v). After casting the separating gel, a small layer of distilled water was poured to even the gel surface. A 4% stacking gel (2.6 ml of distilled water, 0.4 ml of 40% acrylamide and bis-acrylamide solution, 1.0 ml of stacking buffer (0.5 M Tris base, 10% sodium dodecyl sulphate – w/v, and pH adjusted to 6.8), 4 µl of TEMED, and 40 µl of 10% ammonium persulphate solution – w/v) was poured and 1.0 mm combs were inserted to form wells. Electrode buffer was prepared using 25 mM of Tris, 192 mM of glycine and 0.1% sodium dodecyl sulphate (w/v). 5 µl of pre-stained dual colour (Bio-Rad, United States) was used as molecular masses of marker proteins. Vertical electrophoresis was

performed in mini-gels using Bio-rad Mini Protean II (Bio-rad, United States) and voltage was set to 180 V. The electrophoresis was allowed to progress until the bromophenol blue line reached 2 mm above the base of the gel (normally 60 min). Protein bands were stained overnight on orbital shaker in protein staining solution (1.25 g of Coomassie blue G, 180 ml of methanol, 40 ml of acetic acid and 280 ml of distilled water). Background stain was removed with solution of 25% methanol (v/v); 7% of acetic acid (v/v); in distilled water. Gels were scanned in a GS-800 densitometer (Bio-Rad, United States). Gels were also stained using silver stain (Morrissey, 1981) to improve band detection. Briefly, gels were washed in 50% methanol solution (v/v) with distilled water for 10 min, washed in distilled water for 10 min, soaked in 0.02% sodium thiosulfate solution (w/v) for 1 min, rinsed in distilled water twice for 1 min each time, soaked in 0.1% silver nitrate solution (w/v) at 4°C for 20 min, rinsed in distilled water twice for 10 s each time, and finally developed in 2% sodium carbonate (w/v) and 0.04% formalin (v/v) solution.

Zymogram sample preparation

Samples were mixed with zymogram loading buffer with no β-mercaptoethanol (2% sodium dodecyl sulphate – w/v, 10% glycerol – v/v, 62.5 mM Tris-hydrochloride, and 0.0025% bromophenol blue – w/v). For protease class distinction, either 5 µM of E-64; 5 mM of EDTA; 0.5 mM of PMSF; or 1 µM of pepstatin was added to samples. Protease inhibitor concentrations were selected according to Sigma-Aldrich (United States) recommendations. Samples were centrifuged at 13,000 *g* for 2 min at 4°C (Biofuge Fresco Heraeus Instruments, DE). Supernatant was decanted and pellet was discarded. Each individual lane was loaded with 2.71 µg of protein.

Zymogram substrate solutions

Gelatine solution was prepared by heating distilled water to 35°C on a hot-plate magnetic stirrer and adding 1% gelatine (w/v). Carboxymethylcellulose solution was prepared by heating distilled water to 80°C on hot-plate magnetic stirrer and adding 1% CMC (w/v). Starch solution was prepared by adding 1% starch (w/v) to distilled water on a magnetic stirrer plate.

Zymograms preparation

Zymogram gels were prepared using 10% separating gel (3.12 ml of distilled water, 2.0 ml of 40% acrylamide and bis-acrylamide solution, 2.0 ml of running buffer (1.5 M Tris base, 10% sodium dodecyl sulphate – w/v, and pH adjusted to 8.5), 0.88 ml of one of the zymogram substrate solutions, 8 µl of TEMED, and 80 µl of 10% ammonium persulphate – w/v). After casting the separating gel, a small layer of distilled water was poured to even the gel surface. A 4% stacking gel (2.6 ml of distilled water, 0.4 ml of 40% acrylamide and bis-acrylamide solution, 1.0 ml of stacking buffer (0.5 M Tris base, 10% sodium dodecyl sulphate – w/v, and pH adjusted to 6.8), 4 µl of TEMED, and 40 µl of 10% ammonium persulphate solution – w/v) was poured and 1.0 mm combs were inserted to form wells. Electrode buffer was prepared using 25 mM of Tris, 192 mM of glycine and 0.1% sodium dodecyl sulphate (w/v). 5 µl of pre-stained dual colour (Bio-Rad, United States) was used as molecular masses of marker proteins and zymogram loading buffer was added to empty lanes as negative control. Trypsin from bovine pancreas, α-amylase from porcine pancreas, or cellulase from *Aspergillus niger*, all acquired from Sigma Aldrich (United States), were used as positive controls. Vertical electrophoresis was performed in mini-gels using Bio-rad Mini Protean II (Bio-rad, United States) and voltage was set to 150V. The electrophoresis was allowed to progress until the bromophenol blue line reached 2 mm above the base of the gel (normally 80 min). Gels were washed 4 times in 2.5% Triton X-100 solution (v/v) for 15 min at room temperature. Then, gels were completely soaked in developing buffer (50 mM of Tris base, 200 mM of sodium chloride, 0.0007% of zinc chloride – w/v, 5 mM of calcium chloride dihydrate, and 0.02% of sodium azide – w/v) described by Troeberg and Nagase (2004) and placed in incubator at 39°C overnight. Gels with gelatine as substrate were soaked in substrate stain solution (1.25 g of Coomassie blue G, 36% of methanol – v/v, 8% of acetic acid – v/v, and 36% distilled water) overnight and destained with solution of 25% methanol (v/v), 7% of acetic acid (v/v), and 68% of distilled water (v/v). Gels with CMC as substrate were stained in solution of 1.43 mM of Congo red, 99 ml of water and 1 ml ethanol for one hour and destained with solution of 1 M of sodium chloride for 30 min. Gels with starch as substrate were stained in iodine solution (40 mM of iodine and 300 mM of potassium iodide) for two hours. All gels were scanned in a GS-800 densitometer (Bio-Rad, United States).

Transmission electron microscopy

Outer membrane vesicle samples were imaged using transmission electron microscopy. One ml of sample was mixed with 1 ml of fixative (2.5% of glutaraldehyde – v/v, and 0.1 M sodium cacodylate - pH 7.2). A 2% ultra-low gelling temperature agarose solution (w/v) was prepared and filtered with a 0.22 µm syringe filter (Whatman, United States). Then, solution was dissolved at 50°C, cooled and kept at 25°C. After 30 min fixation, the samples were centrifuged at 13,000 *g* for 1 min and the supernatant discarded. The pellets were resuspended in 2 ml fresh fixative as above. After 30 min fixation, the previous step was repeated but resuspended in 2 ml 0.1 M sodium cacodylate wash buffer (pH 7.2). The samples were centrifuged at 13,000 *g* for 1 min and the supernatant discarded. The samples were resuspended in 100 µl agarose solution at 25°C. After mixing, 10 µl drops were pipetted onto a new polished glass microscope slide and placed in a refrigerator at 4°C. After gelling, samples were transferred into 2 ml 0.1 M sodium cacodylate wash buffer (pH 7.2) in 5 ml glass vials with push-on lids at 4°C. After 30 min the solutions were changed for fresh. Samples then progressed through an alcohol wash series: 30%, 50%, 70%, 95% and 3 changes of 100% for at least an hour each at 4°C. Samples were transferred to a 1:2 mixture of ethanol/LR White -hard grade resin (London Resin Company, United Kingdom), then a 2:1 mixture, and finally 100% resin overnight at 4°C. Samples were then placed in size 4 gelatine moulds (Agar Scientific, United Kingdom) and polymerised overnight at 60°C in an oven. Ultrathin 60–80 nm sections were cut on a Reichert-Jung Ultracut E Ultramicrotome (Vienna, Austria) with a Diatome Ultra 45 diamond knife (Diatome, Switzerland) and collected on Gilder GS2X0.5 3.05 mm diameter nickel slot grids (Gilder Grids, United Kingdom) float-coated with Butvar B98 polymer (Agar Scientific, United Kingdom) films. Samples were observed using a JEOL JEM1010 transmission electron microscope (JEOL Ltd, Japan) at 80 kV.

Transmission electron microscopy using two-step immunolabel indirect method

Aldehyde quenching was achieved by room-temperature incubation in standard saline citrate with 0.015 % Tween-20 (SSC-T) for 30–60 min. Non-specific binding was reduced by immersion at room temperature in casein–thiomersal buffer (**CTM-T**) for 30–60 min. Anti-bacterial alkaline phosphatase (**BAP**) antibody produced in mouse, and sheep polyclonal antibody to tyrosinase antisera were applied at a ratio of 1:200 in CTM-T overnight at 4 °C. After washing in carbon dioxide-free distilled reverse-

osmosis water using microwave irradiation, and re-blocking with CTM-T, sections were incubated at room temperature in EMDAS.15 (EM donkey anti-sheep IgG:15 nm gold and EMGAMM.5 goat anti-mouse IgG 5 nm gold - British Biocell, United Kingdom) at a ratio of 1:50 in CTM-T for 1–2 h and microwave-washed four times. As a result, BAP protein was labelled with 5 nm gold particles. All sections were double-stained with uranyl acetate (Agar Scientific, United Kingdom) and Reynold's lead citrate (TAAB Laboratories Equipment Ltd, United Kingdom) and observed using a JEOL JEM1010 transmission electron microscope (JEOL Ltd, Japan) at 80 kV.

Scanning electron microscopy with cryo-stage

Samples from OMV isolated in HOM, or TM buffer, were imaged by SEM with cryo-stage. A drop of sample was inserted into a double-slotted rivet and rapidly frozen at -196°C in a rivet holder under an argon flush, and the assembly transferred to the precooled (-186°C) stage of an EMScope SP2000A sputter cryo-system (EMScope, United Kingdom). The holder was then transferred under vacuum to the cold stage where ice crystals on the surface of the specimen were removed by sublimation at 70°C, and then visualized by JEOL 840A high-performance scanning electron microscope (Jeol Ltd, United Kingdom) with accelerating voltage of up to 10 kV and connected to SEMAPHORE image grabber software (Jeol Ltd, United Kingdom) to record the images in digital format.

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IV. Membrane vesicles from the rumen contain proteases, cellulases and amylases

(Normas: Environmental Microbiology)

SUMMARY

Membrane vesicles production is a ubiquitous process for all the bacteria investigated to date. Membrane vesicles are also produced by archaea and eukaryotes. The aim of this study was to assess the potential importance of membrane vesicles isolated from the rumen fluid to degradation of plant nutrients. Samples were aliquoted from fistulated cows and membrane vesicles were isolated. Membrane vesicles sample from only one cow was imaged with negative staining TEM. Enzymatic activity on zymograms with gelatine, casein, CMC or starch was diverse and several clear bands could be observed. Fractions from the same type of sample, but from a different cow had different enzymatic activity, as observed by different zymogram profile. These results provide evidence from the enzymatic richness loaded in ruminal membrane vesicles. Membrane vesicles in the rumen have been overlooked and their contribution for degradation processes may be greater than the contribution of the attached microbes themselves. Understanding the fundamentals of rumen microbiome is key to improve feed use by ruminants.

RUNNING TITLE: Membrane vesicles from rumen microbiome.

INTRODUCTION

The rumen is a rich ecosystem where Gram-negative, Gram-positive, archaea, protozoa, fungi and viruses interact (Hobson and Stewart, 1997). The host provides a fermentation chamber and continuous plant material, and in turn microbes break down plant cells, providing nutrients for the host.

Apart from viruses, all other organisms in rumen can secrete vesicle-like structures (Soler *et al.*, 2008; Kulp and Kuehn, 2010; Gurung *et al.*, 2011; Rodrigues *et al.*, 2013). Outer membrane vesicles are produced by Gram-negative (Kulp and Kuehn, 2010), MVs are produced by Gram-positive (Gurung et al., 2011), and archaea (Soler et al., 2008), and EVs are produced by eukaryote (Rodrigues et al., 2013). Vesicles are an ubiquitous physiological phenomenon (Schwechheimer *et al.*, 2013). Their diameter can range from 15 to 250 nm (Lee *et al.*, 2007; Soler *et al.*, 2008; Ellen *et al.*, 2009; Kulp

and Kuehn, 2010; Wolf *et al.*, 2012; Olaya-Abril *et al.*, 2014). Vesicles are found when bacteria are in the planktonic state (Biller *et al.*, 2014), in biofilms (Schooling and Beveridge, 2006), in infected tissues (Shah *et al.*, 2012), in laboratory cultures (Kulp and Kuehn, 2010), riverbeds, domestic water drains, sewage and freshwater fish aquarium (Schooling and Beveridge, 2006). Several functions are implicated to vesicles e.g. concentration and activation of toxic components, prey competing bacteria, secretion and delivery of soluble and insoluble components, degradation of tissues, provoking inflation, share antibiotic resistance, promoting biofilm strength, decoying for antimicrobial peptides and bacteriophage, relieve membrane stress, and others (MacDonald and Kuehn, 2012).

In the human pathogenic bacteria *Pseudomonas aeruginosa*, OMV protein profiles were different when compared with planktonic and biofilm bacteria (Schooling and Beveridge, 2006). It has also been demonstrated that the occurrence of OMVs is greater in biofilms than in planktonic environment (Remis *et al.*, 2014), which suggests that the ruminal biofilms could potentially host a large number of these structures. Nonetheless, most membrane vesicle studies are performed using axenic cultures from human pathogens (Kulp and Kuehn, 2010). Rumen metagenomic studies provide evidence for the diversity of rumen microorganism, and have shown that only 11% of rumen bacteria are culturable (Edwards *et al.*, 2004; Kim *et al.*, 2011). Thus, sampling rumen liquid and particulate matter, and isolating vesicles can provide evidence of the rich hydrolytic cargo from the vesiculome, even if the bacteria remains unculturable. The aim of this study was to assess the enzymatic capacity of MVs from the rumen microbiome in order to investigate their potential importance to nutrient availability to the animal

RESULTS AND DISCUSSION

Membrane vesicles isolation scheme

It was decided to sample rumen fluid from 3 different cows to have a broader idea of the heterogeneity of the vesicle isolated cargo. Furthermore, there is evidence that the medium influence OMV quantity and quality (Choi *et al.*, 2014; Fulsundar *et al.*, 2014). Thus, it was decided to sample the rumen fluid with particulate matter, and the bacterial pellet resulting from a 13,000 *g* centrifugation was used to be inoculated in TM buffer with anaerobic conditions. Samples were fractionated into liquid-associated bacteria (**LAB**) and solid-attached bacteria (**SAB**) (Merry and McAllan, 1983). At the end, samples were divided on liquid-associated bacteria from rumen fluid (**RF-LAB**), solid-

attached bacteria samples from rumen fluid (**RF-SAB**), liquid-associated bacteria samples from TM buffer (**TM-LAB**), and solid-attached bacteria samples from TM buffer (**TM-SAB**) (Fig. IV.1).

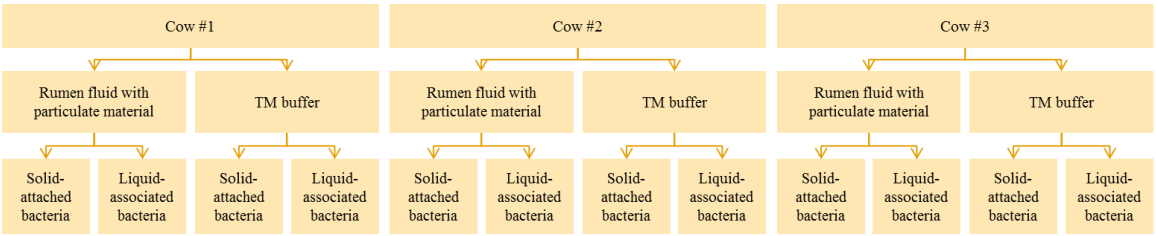


Fig. IV.1. Ruminal sample fractioning scheme.

Isolation of vesicles from the rumen microbiome

Ruminal fluid and particulate matter were sampled at the same time, from the 3 different cows. Centrifugations to remove protozoa and bacteria were performed in less than 1 h after animal handling. Solid-attached bacteria obtained from the rumen were incubated in TM buffer, which had no solid material (Fig. IV.10).

The amount of 0.45 µm syringe filters used to filter the supernatant after bacterial removal was greater when compared to axenic culture of *P. ruminicola*. Typically, two to four 0.22 µm pore size syringe filter (32 mm diameter) were used for *P. ruminicola*. However, a 5-times more syringe filters were needed to filter these samples. Instead of using several filters to completely remove bacteria, it was decided to increase the quantity of 13,000 *g* centrifugations, until no clear pellet was visible. For ruminal fractions, 10 centrifugations were performed, and for TM fractions, 6 centrifugations were performed. It was decided to filter ruminal fractions with an increased number of disk filters on a vacuum apparatus, to completely remove bacterial contamination. Although this involved more steps, less material was used. While samples could have been concentrated prior to filtering, this typically involves heating, which we wanted to avoid. In Fig. IV.2, pigmentation can be observed on a 0.22 µm pore size, PVDF membrane filter after filtration of a ruminal fraction. This pigmentation was typical for all the fractions (data not shown). At the end of ultracentrifugations, vesicle yield after ultracentrifugation was 1.63% for RF-LAB, 1.62% for RF-SAB, 1.68% for TM-LAB, and 1.46% for TM-SAB. Individual cows had vesicle yield of 1.61%, 1.56%, and 1.63%, for cow#1, cow#2, and cow#3, respectively.

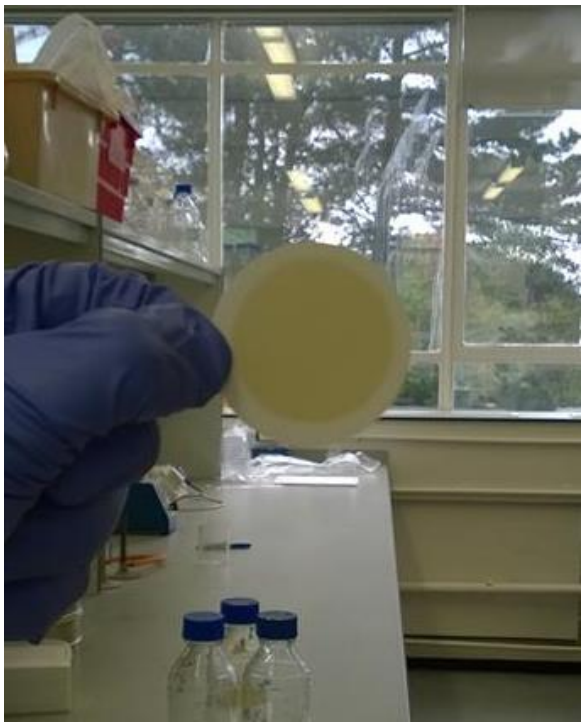


Fig. IV.2. Image of a Durapore, 0.22 µm pore size, PVDF membrane filter (Millipore, United States) after filtration in vacuum apparatus. Note pigmentation on the filter.

Negative staining TEM

There were 12 vesicle samples in total, thus it was decided to image all the vesicle samples from cow# 1 only (Fig. IV.3). The majority of vesicles had a small diameter size compared to those produced by *P. ruminicola*, but some had a diameter close to 200 nm. The diameter size for Gram-negative, Gram-positive, archaea, and fungi range from 20 to 250 nm (Lee *et al.*, 2007; Soler *et al.*, 2008; Ellen *et al.*, 2009; Kulp and Kuehn, 2010; Wolf *et al.*, 2012; Olaya-Abril *et al.*, 2014). Some vesicles were not spherical, but rather elongate. Although this could be result of negative-staining dehydration, elongated and elliptical OMVs have been reported (Fulsundar *et al.*, 2014).

There was a low level of bacterial contamination within the RF-SAB sample from cow# 1 (Fig. IV.3). The filtrations steps included a 2 juxtaposed 0.45 µm pore size filter, a single 0.45 µm pore size filter, 2 juxtaposed 0.22 µm pore size, a single 0.22 µm pore size, a single 0.45 µm pore size syringe filter, and a single 0.22 µm pore size syringe filter. Yet, there was some contamination. It should be noted that samples were imaged 2 weeks after filtrations, although samples were kept at 4°C in-between.

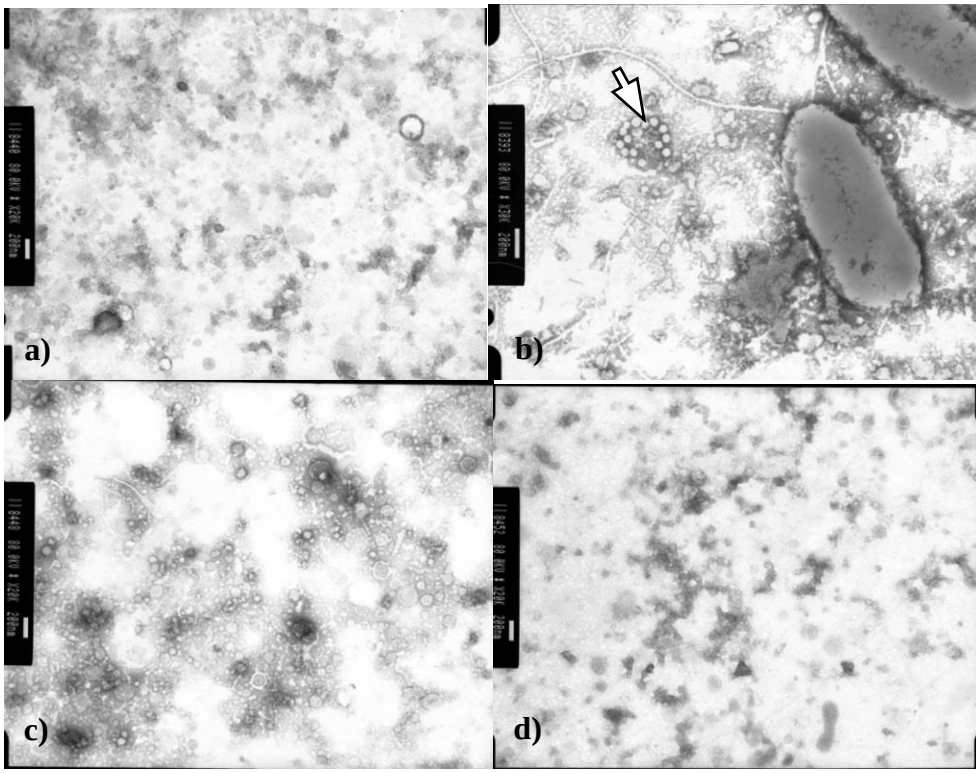


Fig. IV.3. Methylamine tungstate negative staining TEM micrograph of purified vesicles from: a) liquid-associated bacteria from rumen fluid; b) solid-attached bacteria from rumen fluid; c) liquid-associated bacteria from TM buffer; d) solid-attached bacteria from TM buffer. White arrow with black edge indicates an aggregate of vesicle. Bars, 200 nm.

Despite the low level of contamination, the image does allow visualisation of cell-to-cell interaction. A chain-like structure, with spheres ~20 nm in diameter can be observed (Fig. IV.4). Recently *Myxococcus xanthus* were reported to form outer membrane vesicle chains with ~40-60 nm of diameter, which could have implications on message signalling and OM protein transfer (Palsdottir *et al.*, 2009; Berleman *et al.*, 2014; Remis *et al.*, 2014). Further studies to evaluate these structure in the rumen should be implemented, as intra- and inter-specific interaction might occurs in the rumen environment.

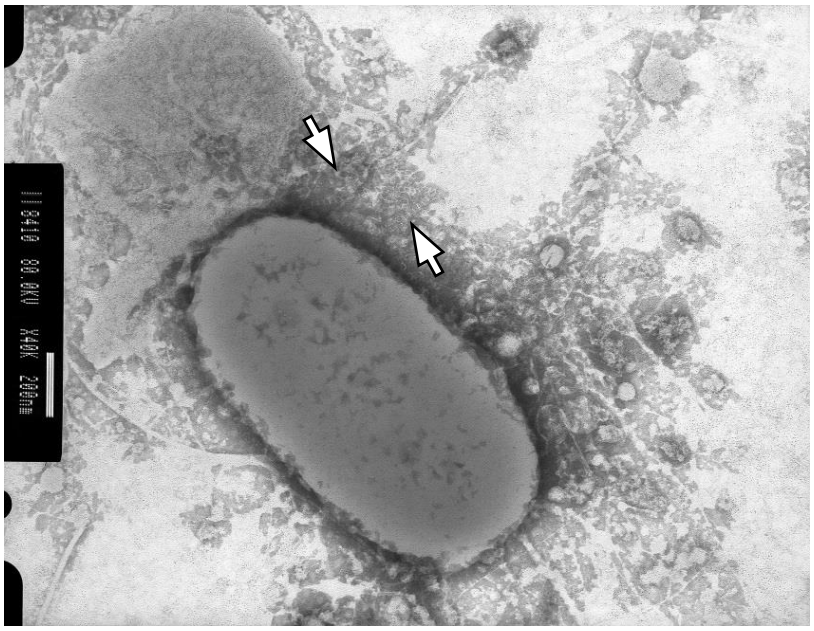


Fig. IV.4. Methylamine tungstate negative staining TEM micrograph of purified vesicles from solid-attached bacteria from rumen fluid. White arrows with black edge indicate vesicle-like chain. Bar, 200 nm.

Low-temperature SEM of purified vesicles from solid-attached bacteria

Similarly to what was observed with *P. ruminicola*, a homogenous pattern can be observed on the micrograph from SEM (Fig. IV.5). Also, the micrographs suggest a vesicle dense sample, which further supports the vesicle quantity observed on Fig. IV.3. Interestingly, no contamination was observed on SEM micrographs, as observed on Fig. IV.4. The diameter size of the vesicles is accordingly to the diameter size observed on TEM micrographs (Fig. IV.3).

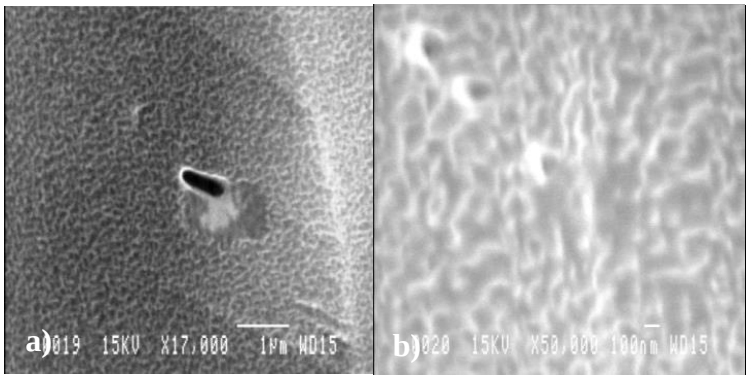


Fig. IV.5. Low-temperature scanning electron microscopy micrographs showing morphology of purified vesicles from solid-attached bacteria from rumen fluid. Bar, a) 1 µm, b) 100 nm.

Protein content

Protein content was estimated using a Bradford assay (Table IV.1). As expected, protein estimated from washed microorganisms was higher than whole microorganisms. Although washed microorganisms samples were supernatant free, the pellet was concentrated 10-fold. Results from samples from TM were lower than samples from RF. Again, this was expected as samples were diluted on TM buffer.

Table IV.1. Protein concentration estimation

Sample	µg / µl
RF-LAB cow#1 Whole Microorganisms	1.29
RF-LAB cow#2 Whole Microorganisms	1.82
RF-LAB cow#3 Whole Microorganisms	2.05
RF-SAB cow#1 Whole Microorganisms	2.29
RF-SAB cow#2 Whole Microorganisms	1.74
RF-SAB cow#3 Whole Microorganisms	1.56
TM-LAB cow#1 Whole Microorganisms	0.99
TM-LAB cow#2 Whole Microorganisms	1.40
TM-LAB cow#3 Whole Microorganisms	1.30
TM-SAB cow#1 Whole Microorganisms	0.90
TM-SAB cow#2 Whole Microorganisms	0.96
TM-SAB cow#3 Whole Microorganisms	0.97
RF-LAB cow#1 Washed Microorganisms	4.94
RF-LAB cow#2 Washed Microorganisms	6.19
RF-LAB cow#3 Washed Microorganisms	5.68
RF-SAB cow#1 Washed Microorganisms	5.53
RF-SAB cow#2 Washed Microorganisms	5.75
RF-SAB cow#3 Washed Microorganisms	2.54
TM-LAB cow#1 Washed Microorganisms	2.90
TM-LAB cow#2 Washed Microorganisms	4.38
TM-LAB cow#3 Washed Microorganisms	4.56
TM-SAB cow#1 Washed Microorganisms	1.90
TM-SAB cow#2 Washed Microorganisms	3.07
TM-SAB cow#3 Washed Microorganisms	2.64
RF-LAB cow#1 Supernatant	3.32
RF-LAB cow#2 Supernatant	2.59
RF-LAB cow#3 Supernatant	2.83
RF-SAB cow#1 Supernatant	1.08
RF-SAB cow#2 Supernatant	1.50
RF-SAB cow#3 Supernatant	3.63
TM-LAB cow#1 Supernatant	1.05
TM-LAB cow#2 Supernatant	1.60
TM-LAB cow#3 Supernatant	1.66
TM-SAB cow#1 Supernatant	1.40

TM-SAB cow#2 Supernatant	1.64
TM-SAB cow#3 Supernatant	1.86
RF-LAB cow#1 Vesicles	1.69
RF-LAB cow#2 Vesicles	2.00
RF-LAB cow#3 Vesicles	1.09
RF-SAB cow#1 Vesicles	0.88
RF-SAB cow#2 Vesicles	1.20
RF-SAB cow#3 Vesicles	1.44
TM-LAB cow#1 Vesicles	0.50
TM-LAB cow#2 Vesicles	1.10
TM-LAB cow#3 Vesicles	0.52
TM-SAB cow#1 Vesicles	0.22
TM-SAB cow#2 Vesicles	0.46
TM-SAB cow#3 Vesicles	0.40

SDS-PAGE using rumen samples

No clear bands can be observed for washed microorganisms and whole microorganisms (Fig. IV.6). There is a dark background, which can be observed for the RF-LAB whole microorganisms, for the 3 cows, which is brighter than RF-SAB whole microorganisms. It should be noted that RF-SAB samples were washed twice on saline solution, which might have diluted some of the rumen components. After incubation on TM buffer, the background is again brighter. Again, this is probably from rumen soluble components being diluted in TM buffer (Schneider *et al.*, 2012). Furthermore, vesicles band profile is discrete, but some bands can be observed.

Protease activity using rumen samples

Several gelatine zymograms were prepared to evaluate proteolytic activity of vesicle samples (Fig. VI.2 and appendices). Outer membrane vesicles seemed to degrade more gelatine than washed microorganisms, whole organisms or supernatant. Numerous bands with activity against the substrate can be observed for all the samples, but each sample had individual characteristics (i.e. different bands, bands with different sizes, bands with same sizes but different intensity). Also, whole microorganisms, washed microorganisms, and vesicle fractions have different degradation band profile. Furthermore, protein bands with activity against the substrate grouped on the upper part of the gel, with the exception of the samples from TM-SAB, which has 4 distinct bands on the lower part of the gel. The marker protein band indicated with the arrow had a 37 kDa size, thus proteins with proteolytic activity are bigger than this size.

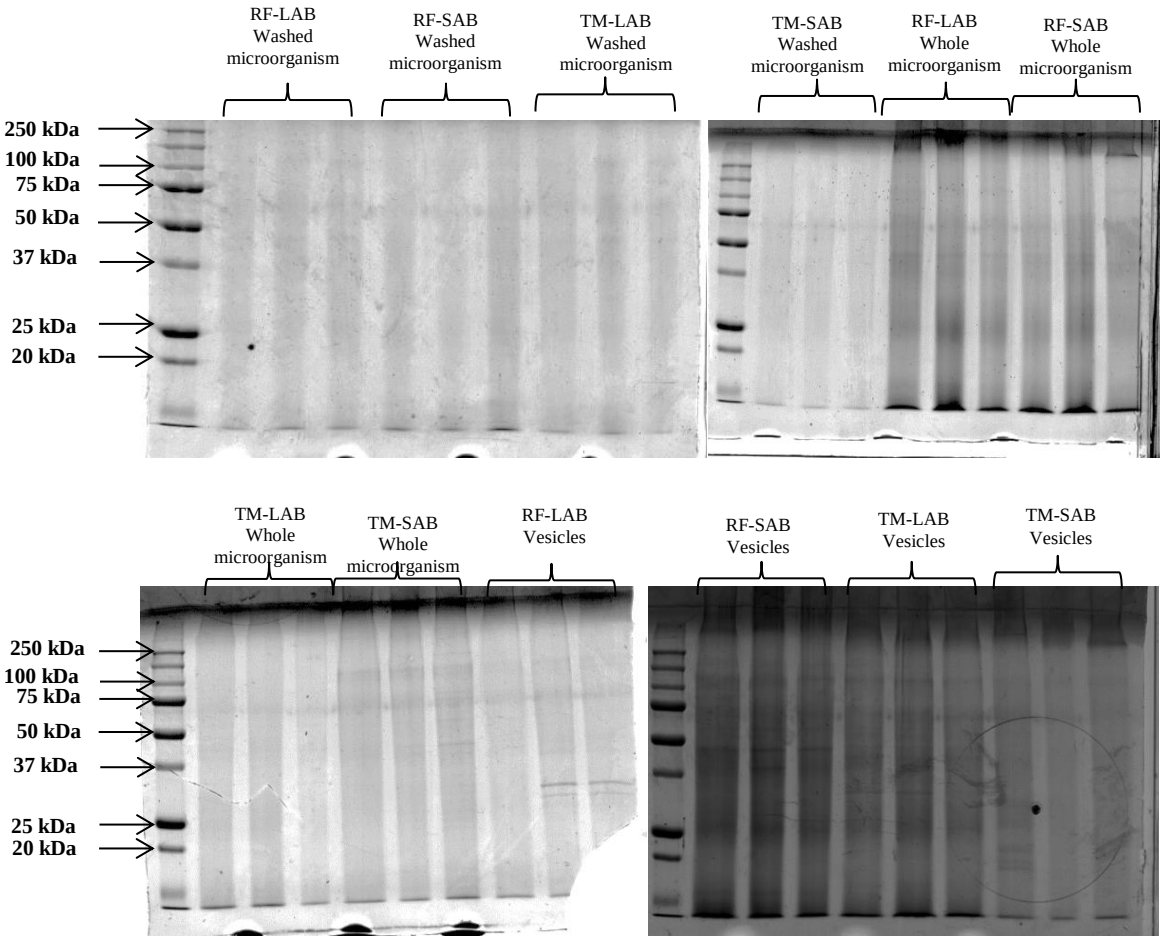
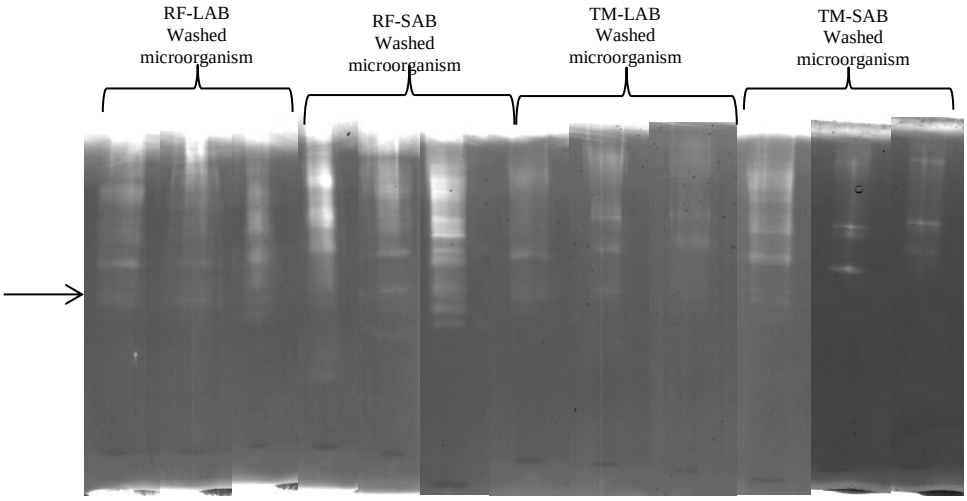


Fig. IV.6. SDS-PAGE stained with Coomassie blue of protein marker with RF-LAB washed microorganisms, RF-SAB washed microorganisms, TM-LAB washed microorganisms, TM-SAB washed microorganisms, RF-LAB whole microorganisms, RF-SAB whole microorganisms, TM-LAB whole microorganisms, TM-SAB whole microorganisms RF-LAB vesicles, RF-SAB vesicles, TM-LAB vesicles, TM-SAB vesicles. Cow order is cow#1, cow#2 and cow#3.

When samples are compared between cows, again, a different profile can be observed. Same fractions, sampled in different animals, treated exactly the same way, had a diverse response. Also, cows were fed the same diet, had similar weight and same handling. Although diet was similar for all the cows, microorganism population diversity can be different (Huws *et al.*, 2013), thus responses can be different. The different band pattern observed in SABs and LABs samples might indicate a specific vesicle-enrichment, as the hydrolytic cargo is different across species (Kulp and Kuehn, 2010; Avila-Calderón *et al.*, 2015).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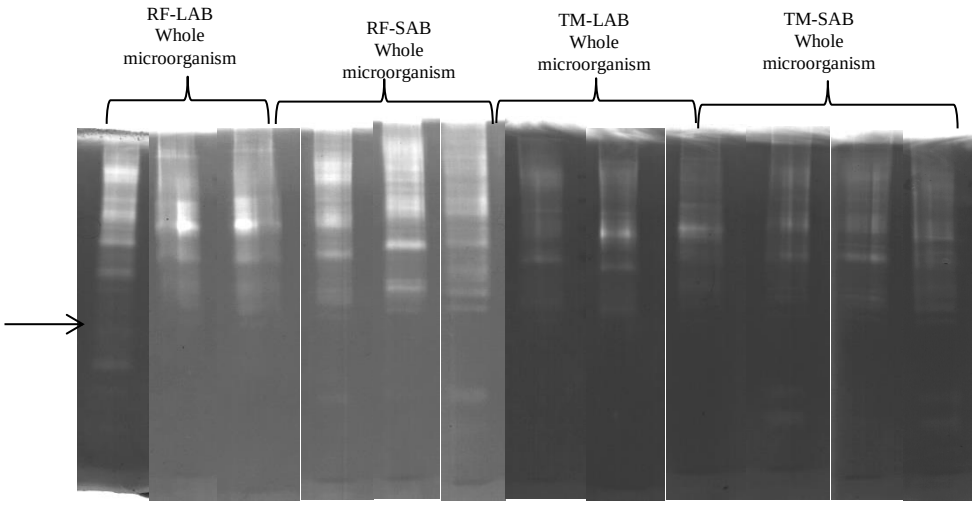
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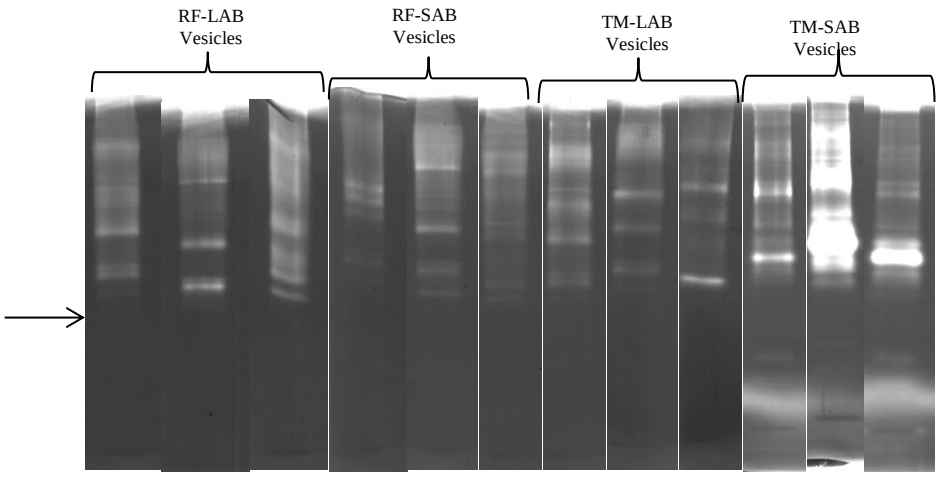
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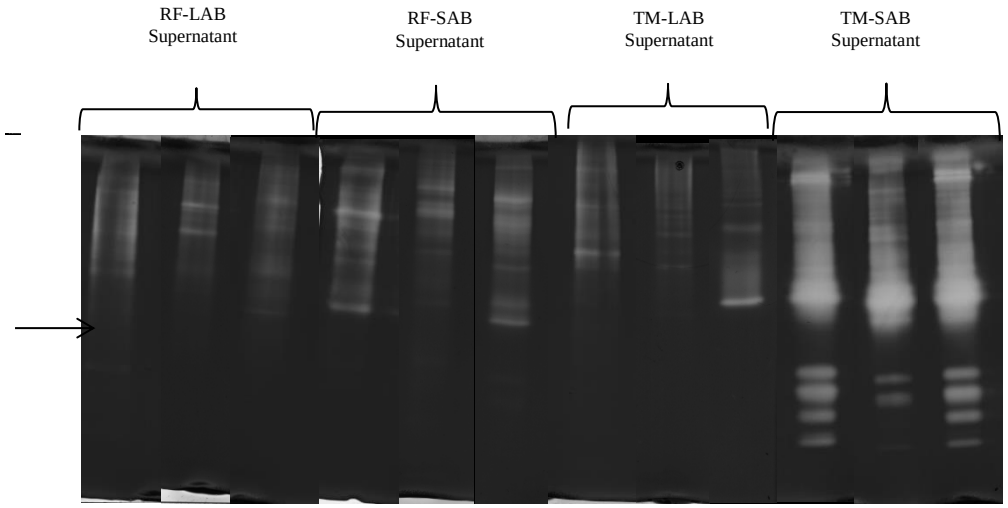
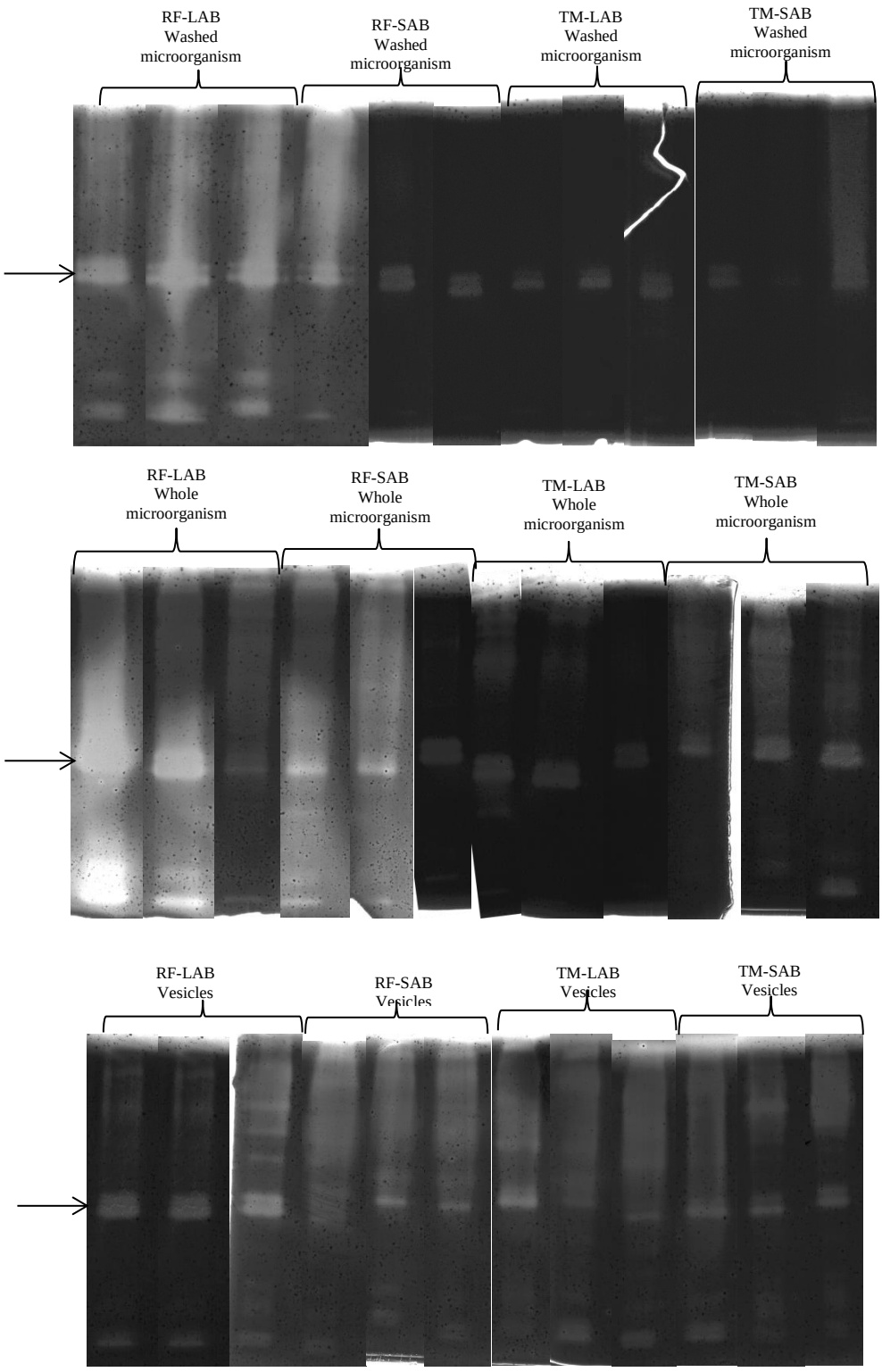


Fig. IV.7. Gelatine zymogram. RF-LAB washed microorganisms, RF-SAB washed microorganisms, TM-LAB washed microorganisms, TM-SAB washed microorganisms, RF-LAB whole microorganisms, RF-SAB whole microorganisms, TM-LAB whole microorganisms, TM-SAB whole microorganisms, RF-LAB vesicles, RF-SAB vesicles, TM-LAB vesicles, TM-SAB vesicles, RF-LAB supernatant, RF-SAB supernatant, TM-LAB supernatant, TM-SAB supernatant. Cow order is cow#1, cow#2 and cow#3. Arrow indicates 37 kDa.

Carboxymethylcellulase activity using rumen samples

Some bands can be observed on CMC zymograms (Fig IV.8.). Temperature, pH, solution in which gel is soaked overnight, time of incubation, anaerobic conditions, are some of the factors that affect the optimum activity of enzymes (Wang and Hsu, 2005). Therefore, as a first attempt, conditions were set to mimic rumen environment, i.e. 39°C and pH 6.8. Samples from vesicle and whole microorganisms had richer diversity of bands, compared to washed microorganisms and supernatant. The diversity of bands from the whole microorganisms is probably from vesicles, as washed microorganisms and supernatant showed few bands. It seems that the microorganisms sample had two distinct bands in the middle of the gel, and two bands on the lower part of the gel. Similar bands in the middle of the gel were also observed for all the samples, thus this is probably a well-conserved enzyme. In *Bacteroides fragilis* fucosidase activity was detected exclusively in OMV. The bacteria was cultivated in media with glucose or fucose as carbon source, and OMV in fucose media was enriched by 7-fold in fucose media (Elhenawy *et al.*, 2014).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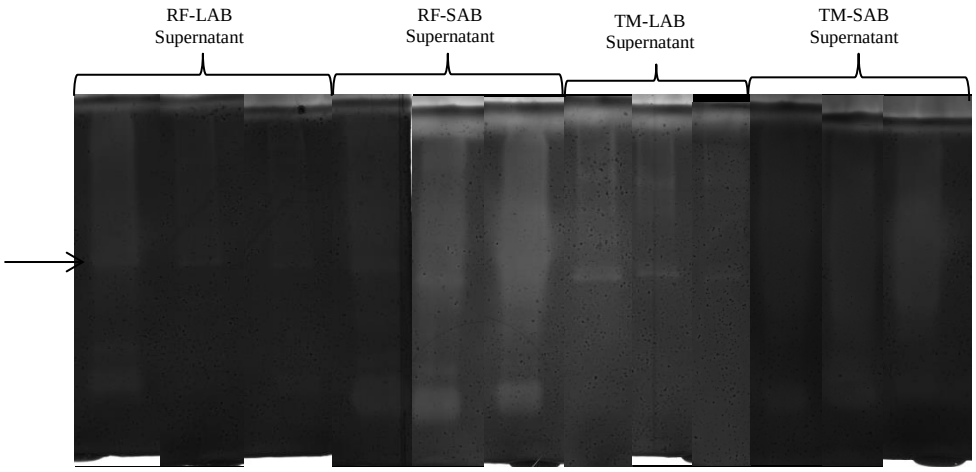
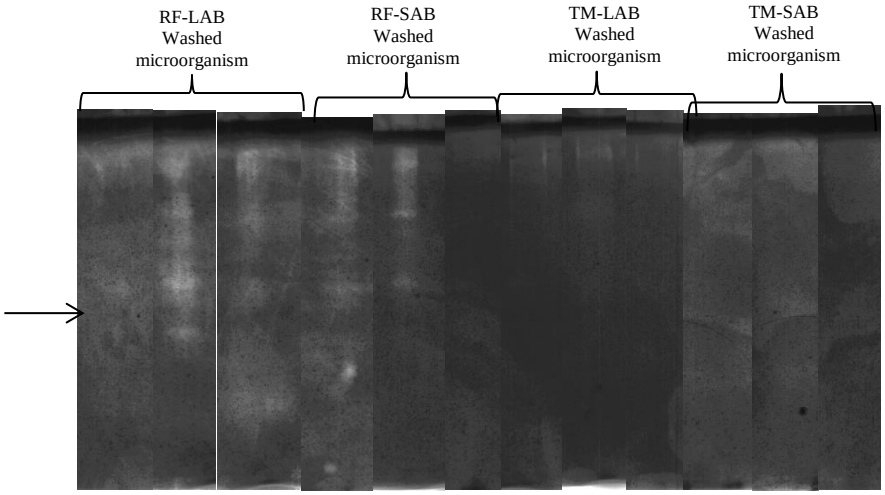


Fig. IV.8. Carboxymethylcellulose zymogram. RF-LAB washed microorganisms, RF-SAB washed microorganisms, TM-LAB washed microorganisms, TM-SAB washed microorganisms, RF-LAB whole microorganisms, RF-SAB whole microorganisms, TM-LAB whole microorganisms, TM-SAB whole microorganisms, RF-LAB vesicles, RF-SAB vesicles, TM-LAB vesicles, TM-SAB vesicles, RF-LAB supernatant, RF-SAB supernatant, TM-LAB supernatant, TM-SAB supernatant. Cow order is cow#1, cow#2 and cow#3. Arrow indicates 37 kDa.

Amylase activity using rumen samples

Similarly to what was observed with CMC zymograms, the location of enzymes to degrade starch seem to be located in the vesicles, although fewer bands can be observed. Using a ruminal bacterial extract Kopecny and Wallace (1982) found no amylase activity. On the other hand, (Elhenawy *et al.*, 2014) found proteins annotated as "starch utilization system" in *B. fragilis* OMVs.



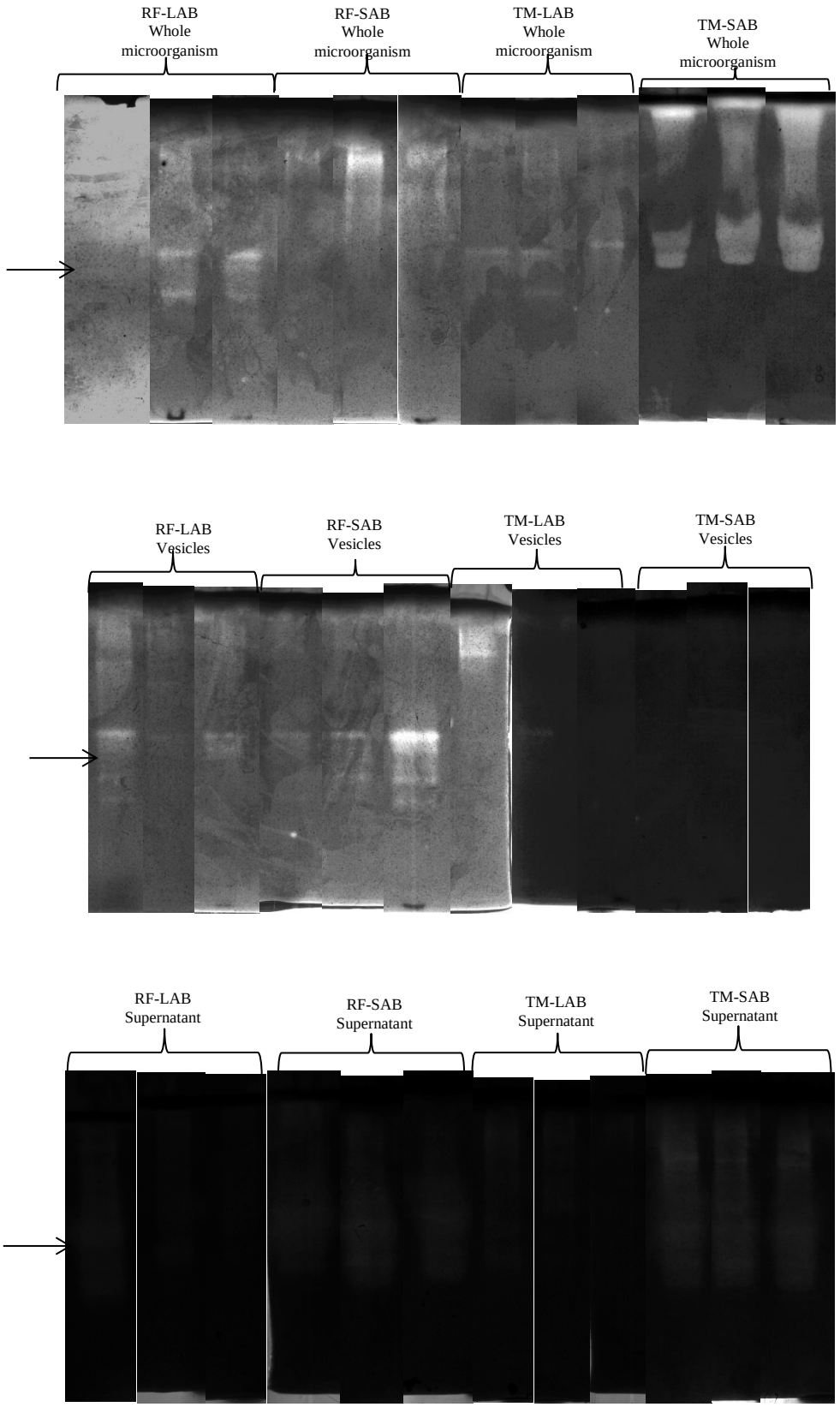


Fig. IV.9. Starch zymogram. RF-LAB washed microorganisms, RF-SAB washed microorganisms, TM-LAB washed microorganisms, TM-SAB washed microorganisms, RF-LAB whole microorganisms, RF-SAB whole microorganisms, TM-LAB whole microorganisms, TM-SAB whole microorganisms, RF-LAB vesicles, RF-SAB vesicles,

TM-LAB vesicles, TM-SAB vesicles, RF-LAB supernatant, RF-SAB supernatant, TM-LAB supernatant, TM-SAB supernatant. Cow order is cow#1, cow#2 and cow#3. Arrow indicates 37 kDa.

Vesicle implications for rumen system

Future studies should evaluate vesicle capacity to degrade plant cell wall. The products from substrate degradation could serve as 'goods' for bacterial common use (Whitworth, 2011). The vast hydrolytic activity of vesicles might not benefit the vesicle-producer bacteria, but rather the community. Such mechanism would be more efficient if nutrient would to be released on a controlled environment, like a biofilm. Also, vesicles upkeep bacterial population by improving biofilm stability (Yonezawa *et al.*, 2009).

Membrane vesicles might also benefit the bacterial community by serving as substrate. *Prochlorococcus* membrane vesicles size and concentration are stable for at least 17 days in sterile seawater and can support growth of *Alteromonas*, an heterotrophic organism, compared to an organic carbon mix (Biller *et al.*, 2014). It is very likely that rumen vesicles could have a similar role on the rumen and support microorganism growth.

Outer membrane vesicles are implicated on horizontal gene transfer in bacteria (Kulp and Kuehn, 2010). Outer membrane vesicles from *Ruminococcus* spp. were responsible to transform a mutant strain (Klieve *et al.*, 2005). *Ruminococcus flavefaciens* were cultivated in cellobiose as carbon source and lost 4 to 5-fold the degradation capacity of cotton after 15-18 subcultures (Stewart *et al.*, 1990) and two cultures lost the ability to degrade cotton. When cotton was introduced as carbon source, high cotton degrading activity was obtained after 7-15 subcultures, and a strain which had lost the ability to degrade cotton, regained such ability (Stewart *et al.*, 1990). On rumen, this could be an important mechanism to cope with stressful situations, like the introduction of synthetic antibiotics.

Up to date, the general consent is that bacteria will attach to the plant cell wall, secrete hydrolases and start colonization. Vesicles propose a different insight, as vesicles could be responsible for a first attack to the cell wall for a further bacteria attachment, with subsequent biofilm formation. After several decades of ruminant research, it is still not known as why bacteria do not use the feed particle at 100 %, since the rumen is such a competitive environment. As metagenomics studies point as

how complex is the ruminal microbiome, vesicles add a bit more of complexity. Proteomics studies will shed light on interesting areas, as different functions will be implied for new roles of rumen vesicles. Therefore, studies with the complete ruminal vesicles OMVs/MV/EVs, should enrich knowledge on the rumen environment and their interactome.

EXPERIMENTAL PROCEDURES

TM buffer preparation

TM buffer was used as maintenance medium, which consisted of 50 mM of Tris and 10 mM of magnesium sulphate, with adjusted pH to 6.8. The medium was heated in microwave twice, until bubbling was visible, to remove oxygen. The medium was left to cool on a magnetic stirrer plate and under carbon dioxide gassing. Under carbon dioxide gassing, the medium was dispensed in 450 ml per Duran bottle, and sealed. Bottles were autoclaved at 121°C for 15 min at 15 lb/in^2. Several Hungate tubes were reserved for sterility evaluation using the "Most-probable-number" technique (Dehority *et al.*, 1989).

Animal housing

In order to isolate vesicles from the rumen, samples were collected from the rumen of 3 healthy nonlactating multiparous Holstein x Friesian cows with rumen cannula, 1 h after morning feeding. Experiments were conducted with the authority of licences under the United Kingdom Animal Scientific Procedures Act, 1986. Animals were fed a diet consisting of silage of *Lolium perenne*, mineral block *ad libitum*, and 1 kg of dairy concentrate (16.0% of crude protein, 13.8% of moisture, 13.5% of crude fibre, 7.5% of crude ash, and 4.7% of crude oil – Wynnstay Group, United Kingdom).

Ruminal vesicle isolation scheme

A flow chart with step-by-step procedures used for vesicles isolation using rumen fluid was prepared (Fig. IV.10). Specific methodology will be further discussed.

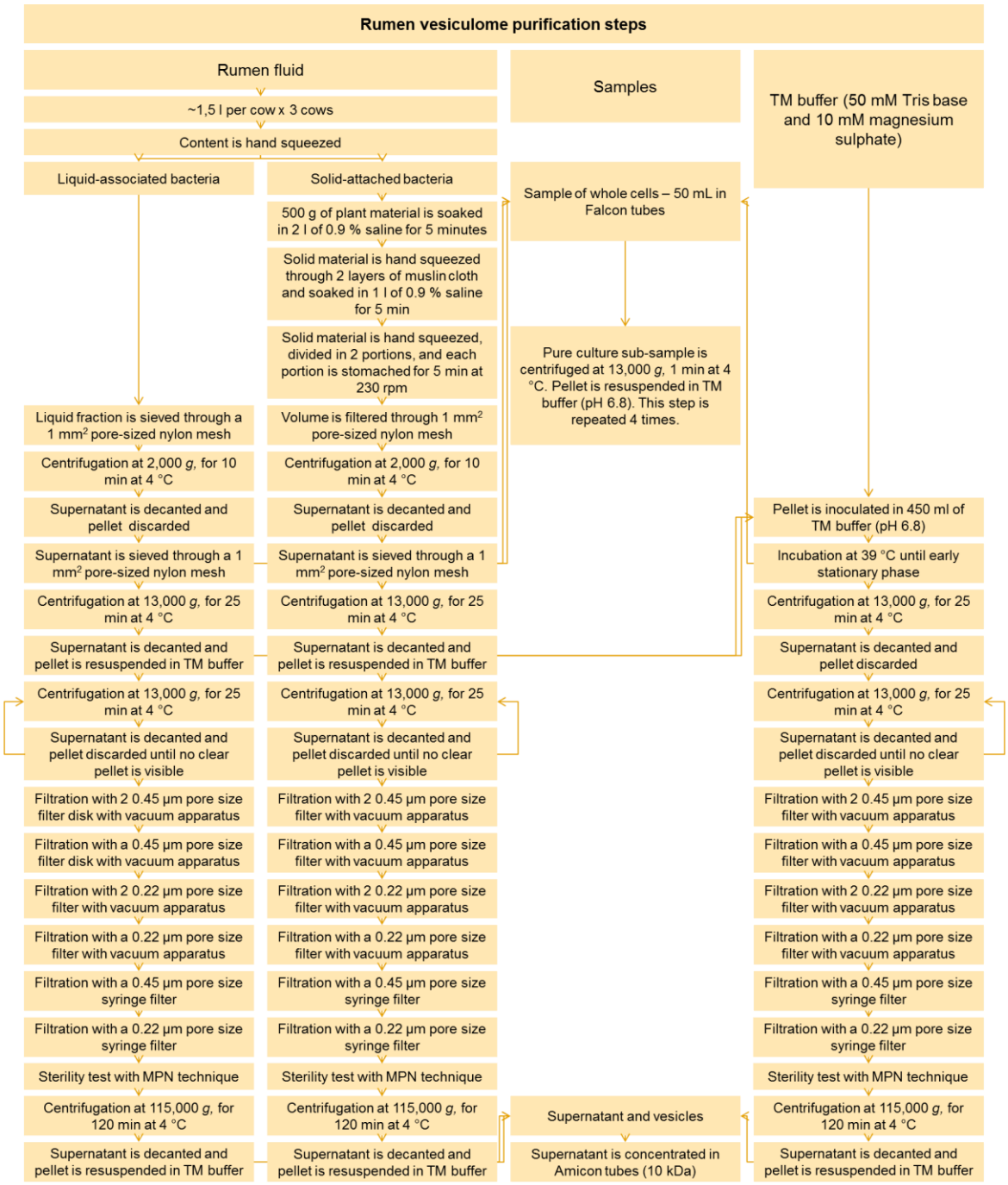


Fig. IV.10. Flow chart of experimental scheme for vesicle purification from rumen fluid.

Liquid-associated and solid-attached bacterial fractioning

Liquid-associated and solid-attached bacterial fractions were processed according to Merry and McAllan (1983). Rumen content (~1.5 l) was sieved through a 1 mm^2 pore-sized nylon mesh and the solid fraction was separated from the liquid fraction. Approximately 500 g of solid material was placed in 2 l of 0.9% saline solution (w/v)

for 5 min. Then, solid material was hand squeezed and placed in 1 l of 0.9% saline solution (w/v) for another 5 min. The solid material was hand squeezed one more time and divided in 2 portions. Each portion was stomached (Stomacher® 400 Circulator, United Kingdom) for 5 min at 230 rpm to detach bacteria.

Cell removal and incubations

The liquid and solid fraction were sieved separately through a 1 mm^2 pore-sized nylon mesh and centrifuged at 2,000 *g* for 10 min at 4°C in a JLA-8.100 rotor (Beckman Coulter, United States) in an Avanti J-26 XP centrifuge (Beckman Coulter, United States). The pellet was discarded and the supernatant was sieved through a 1 mm^2 pore-sized nylon mesh. Fifty ml of the volume was aliquoted and stored at 4°C, and throughout the results and discussion section this fraction will be addressed as whole microorganisms. The remaining volume was centrifuged in a GSA Sorvall rotor (Du Pont Instruments, United States) at 13,000 *g* for 25 min at 4º C in a Sorvall RC-5B Refrigerated Superspeed Centrifuge (Du Pont Instruments, United States). At the end of the centrifugation, the pellet from each fraction was quickly resuspended in TM buffer to complete 50 ml, which was inoculated in individual 500 ml Duran bottles containing 450 ml of TM buffer, and incubated at 39°C. Then the supernatant was centrifuged at 13,000 *g* for 25 min at 4°C and the pellet was discarded. This step was repeated until no clear pellet was visible. For the TM buffer incubation, after 18-h, 50 ml of the volume was aliquoted to represent the whole microorganisms and stored at 4°C. The remaining volume was centrifuged at 13,000 *g* for 25 min at 4°C and pellet was discarded. This step was repeated until no clear pellet was visible.

Complete cell removal using a series of filtrations

Several filtrations were used to completely remove cells. Filtration using membrane filters was performed with a vacuum apparatus. The cell-free supernatant was filtered through 2 juxtaposed membrane (GF/A, 47 mm, glass microfiber filter, binder free – Whatman, United States) with 0.45 µm pore size; a single membrane (GF/A, 47 mm, glass microfiber filter, binder free – Whatman, United States) with 0.45 µm pore size; a 2 juxtaposed membrane (47 m, Durapore, PVDF membrane filter – Millipore, United States) with 0.22 µm pore size; a single membrane (47 mm, Durapore, PVDF membrane filter – Millipore, United States) with 0.22 µm pore size; a Supor® Acrodisc®, polyethersulphone, sterile, syringe filter membrane disk (Pall Life Sciences,

United States) with 0.45 µm pore size; and a Supor® Acrodisc®, polyethersulphone, sterile, syringe filter membrane disk (Pall Life Sciences, United States) with 0.25 µm pore size. At the end of the filtrations, all cells should be removed.

Sterility and vesicles isolation

After the last filtration, 1 ml was aliquoted and the "Most-probable-number" technique (Dehority *et al.*, 1989) was used to check sterility of the membrane vesicles. Briefly, serial dilutions were prepared (neat, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) and were inoculated in Hungate tubes containing HOM, in triplicate. Bacterial growth was monitored during 120 h. The ultrafiltered volume was divided in polycarbonate vials, placed in a 70.1 Ti rotor (Beckman Coulter, United States) and centrifuged at 115,000 *g* for 120 min at 4ºC in an Optima L-100xp (Beckman Coulter, United States), in order to pellet the vesicles. The vesicle pellet was resuspended in 100 µl TM buffer and stored in fridge for further analysis. Vesicles-free supernatant was stored for further concentration.

Vesicle-free supernatant concentration

Supernatant was concentrated to a final volume of 2 ml using 50 ml of vesicle-free supernatant aliquots. The volume was centrifuged at 4,000 *g* for 30 min at 4°C in an ALC PK-131R (ALC International, Italy) in Amicon Ultra 15 ml tubes (10 kDa – Merck Millipore; United States). Throughout the results and discussion section this fraction will be addressed as supernatant.

Washed microorganisms preparation

To wash away the supernatant from the microorganisms, 10 ml of the whole culture aliquot was centrifuged at 13,000 *g* for 1 min at 4°C (Biofuge Fresco Heraeus Instruments, DE). The supernatant was discarded and the pellet resuspended in 900 µl of TM buffer (pH 6.8). This procedure was repeated 4 times. Throughout the results and discussion section this fraction will be addressed as washed microorganisms.

Protein concentration determination

Bradford protein assay (Bradford, 1976) was adapted for microplate assay. A standard curve was built using BSA as standard with a 3-parameter polynomial equation. To estimate protein concentration, 5 µl of sample was mixed with 250 µl of 1x Dye Reagent (Bio-Rad, United States) and incubated at room temperature for 10 min.

Absorbance was measured at 595 nm using a Synergy H1 (Biotek, Switzerland) with Gen5 Data Analysis Software (Biotek).

Sample preparation for SDS-PAGE gels

For sample preparation for SDS-PAGE, 10 % of protease inhibitor cocktail (Sigma Aldrich, United States) was added to samples aliquots (v/v) in 1.5 ml eppendorfs. Then, samples were mixed with 4x Laemmli buffer (Laemmli, 1970), which consisted of 2% sodium dodecyl sulphate (w/v), 10% glycerol (v/v), 62.5 mM Tris-hydrochloride, and 0.0025% bromophenol blue (w/v). Samples were centrifuged at 13,000 *g* for 2 min at 4°C (Biofuge Fresco Heraeus Instruments, DE). Supernatant was decanted and pellet was discarded. Then 10% β-mercaptoethanol (v/v) was added to samples, and samples were heated in heated block (Techne Dri-Block DB-2D, Cambridge, UK) at 95°C for 5 min. Samples were centrifuged at 13,000 *g* for 2 min at 4°C (Biofuge Fresco Heraeus Instruments, DE). Supernatant was decanted and pellet was discarded. Each individual lane was loaded with 2.9 µg of protein.

SDS-PAGE gels preparation

SDS-PAGE gels were prepared (Silhavy *et al.*, 1984) using 12.5% separating gel (3.5 ml of distilled water, 2.5 ml of 40% acrylamide and bis-acrylamide solution, 2.0 ml of running buffer (1.5 M Tris base, 10% sodium dodecyl sulphate – w/v, and pH adjusted to 8.5), 8 µl of TEMED, and 80 µl of 10% ammonium persulphate solution – w/v). After casting the separating gel, a small layer of distilled water was poured to even the gel surface. A 4% stacking gel (2.6 ml of distilled water, 0.4 ml of 40% acrylamide and bis-acrylamide solution, 1.0 ml of stacking buffer (0.5 M Tris base, 10% sodium dodecyl sulphate – w/v, and pH adjusted to 6.8), 4 µl of TEMED, and 40 µl of 10% ammonium persulphate solution – w/v) was poured and 1.0 mm combs were inserted to form wells. Electrode buffer was prepared using 25 mM of Tris, 192 mM of glycine and 0.1% sodium dodecyl sulphate (w/v). 5 µl of pre-stained dual colour (Bio-Rad, United States) was used as molecular masses of marker proteins. Vertical electrophoresis was performed in mini-gels using Bio-rad Mini Protean II (Bio-rad, United States) and voltage was set to 180 V. The electrophoresis was allowed to progress until the bromophenol blue line reached 2 mm above the base of the gel (normally 60 min). Protein bands were stained overnight on orbital shaker in protein staining solution (1.25 g of Coomassie blue G, 180 ml of methanol, 40 ml of acetic acid and 280 ml of distilled

water). Background stain was removed with solution of 25% methanol (v/v); 7% of acetic acid (v/v); in distilled water. Gels were scanned in a GS-800 densitometer (Bio-Rad, United States).

Zymogram sample preparation

Samples were mixed with zymogram loading buffer with no β-mercaptoethanol (2% sodium dodecyl sulphate – w/v, 10% glycerol – v/v, 62.5 mM Tris-hydrochloride, and 0.0025% bromophenol blue – w/v). For protease class distinction, either 5 µM of E-64; 5 mM of EDTA; 0.5 mM of PMSF; or 1 µM of pepstatin was added to samples. Protease inhibitor concentrations were selected according to Sigma-Aldrich (United States) recommendations. Samples were centrifuged at 13,000 *g* for 2 min at 4°C (Biofuge Fresco Heraeus Instruments, DE). Supernatant was decanted and pellet was discarded. Each individual lane was loaded with 2.71 µg of protein.

Zymogram substrates solutions

Casein solution was prepared by heating 0.1 M sodium hydroxide solution to 35°C on a hot-plate magnetic stirrer and adding 1% casein (w/v). Gelatine solution was prepared by heating distilled water to 35°C on a hot-plate magnetic stirrer and adding 1% gelatine (w/v). Carboxymethylcellulose solution was prepared by heating distilled water to 80°C on hot-plate magnetic stirrer and adding 1% CMC (w/v). Starch solution was prepared by adding 1% starch (w/v) to distilled water on a magnetic stirrer plate.

Zymograms preparation

Zymogram gels were prepared using 10% separating gel (3.12 ml of distilled water, 2.0 ml of 40% acrylamide and bis-acrylamide solution, 2.0 ml of running buffer (1.5 M Tris base, 10% sodium dodecyl sulphate – w/v, and pH adjusted to 8.5), 0.88 ml of one of the zymogram substrate solutions, 8 µl of TEMED, and 80 µl of 10% ammonium persulphate – w/v). After casting the separating gel, a small layer of distilled water was poured to even the gel surface. A 4% stacking gel (2.6 ml of distilled water, 0.4 ml of 40% acrylamide and bis-acrylamide solution, 1.0 ml of stacking buffer (0.5 M Tris base, 10% sodium dodecyl sulphate – w/v, and pH adjusted to 6.8), 4 µl of TEMED, and 40 µl of 10% ammonium persulphate solution – w/v) was poured and 1.0 mm combs were inserted to form wells. Electrode buffer was prepared using 25 mM of Tris, 192 mM of glycine and 0.1% sodium dodecyl sulphate (w/v). 5 µl of pre-stained dual colour (Bio-

Rad, United States) was used as molecular masses of marker proteins and zymogram loading buffer was added to empty lanes as negative control. Trypsin from bovine pancreas, α-amylase from porcine pancreas, or cellulase from *Aspergillus niger*, all acquired from Sigma Aldrich (United States), were used as positive controls. Vertical electrophoresis was performed in mini-gels using Bio-rad Mini Protean II (Bio-rad, United States) and voltage was set to 150V. The electrophoresis was allowed to progress until the bromophenol blue line reached 2 mm above the base of the gel (normally 80 min). Gels were washed 4 times in 2.5% Triton X-100 solution (v/v) for 15 min at room temperature. Then, gels were completely soaked in developing buffer (50 mM of Tris base, 200 mM of sodium chloride, 0.0007% of zinc chloride – w/v, 5 mM of calcium chloride dihydrate, and 0.02% of sodium azide – w/v) described by Troeberg and Nagase (2004) and placed in incubator at 39°C overnight. Gels with gelatine, or casein as substrate were soaked in substrate stain solution (1.25 g of Coomassie blue G, 36% of methanol – v/v, 8% of acetic acid – v/v, and 36% distilled water) overnight and destained with solution of 25% methanol (v/v), 7% of acetic acid (v/v), and 68% of distilled water (v/v). Gels with CMC as substrate were stained in solution of 1.43 mM of Congo red, 99 ml of water and 1 ml ethanol for one hour and destained with solution of 1 M of sodium chloride for 30 min. Gels with starch as substrate were stained in iodine solution (40 mM of iodine and 300 mM of potassium iodide) for two hours. All gels were scanned in a GS-800 densitometer (Bio-Rad, United States).

Transmission electron microscopy using negative staining

A 2% solution was prepared using methylamine tungstate, and pH was adjusted to 7.0 with 1 M potassium hydroxide. Samples were mixed with 2% solution in equal volumes. A drop was placed in a formvar grid held by tweezers. After 20 s, the excess of solution was removed using a filter paper. Samples were observed using a JEOL JEM1010 transmission electron microscope (JEOL Ltd, Japan) at 80 kV.

Scanning electron microscopy with cryo-stage

Samples from vesicles isolated from rumen were imaged by SEM with cryo-stage. A drop of sample was inserted into a double-slotted rivet and rapidly frozen at -196°C in a rivet holder under an argon flush, and the assembly transferred to the precooled (-186°C) stage of an EMScope SP2000A sputter cryo-system (EMScope, United

Kingdom). The holder was then transferred under vacuum to the cold stage where ice crystals on the surface of the specimen were removed by sublimation at 70°C, and then visualized by JEOL 840A high-performance scanning electron microscope (Jeol Ltd, United Kingdom) with accelerating voltage of up to 10 kV and connected to SEMAPHORE image grabber software (Jeol Ltd, United Kingdom) to record the images in digital format.

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V. CONCLUSIONS

Methodology to isolate and purify outer membrane vesicle was successfully employed on axenic culture of *Prevotella ruminicola*. Transmission electron microscopy micrographs provide evidence of sterility of the outer membrane vesicle preparation and sodium dodecyl sulphate polyacrylamide gel electrophoresis further support a different protein profile of outer membrane vesicles. Furthermore, outer membrane vesicles have proteolytic activity against gelatine on zymograms. No activity was detected on carboxymethylcellulose and starch zymograms, for none of the samples evaluated.

When used for rumen vesicle isolation, methodology to isolate outer membrane vesicle was used with extra steps added to ensure a cell-free vesicle preparation. Transmission electron microscopy supported sterility of the preparation, although samples from solid-attached bacteria from the rumen had minor bacterial contamination. Enzymatic activity of the solid-attached and liquid-associated fractions from rumen fluid and TM buffer provide evidence of rich hydrolytic activity of vesicles isolated from the rumen. Enzymatic activity on zymograms with gelatine, casein, carboxymethylcellulose, or starch further support the importance that vesicles probably play in the rumen environment.

VI.APPENDICES

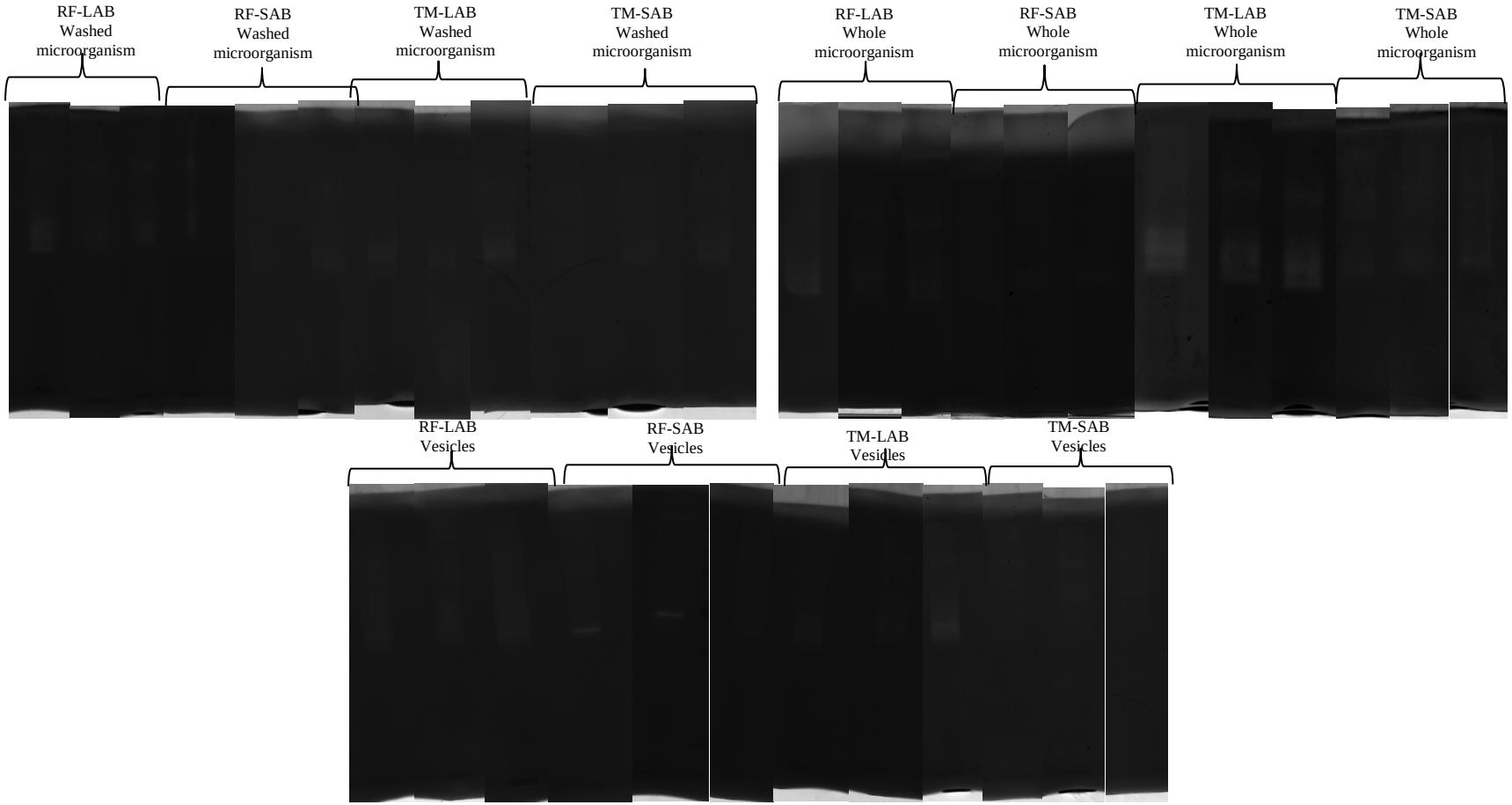

Fig. VI.1. Casein zymogram of protein marker with RF-LAB washed microorganisms, RF-SAB washed microorganisms, TM-LAB washed microorganisms, TM-SAB washed microorganisms, RF-LAB whole microorganisms, RF-SAB whole microorganisms, TM-LAB whole microorganisms, TM-SAB whole microorganisms, RF-LAB vesicles, RF-SAB vesicles, TM-LAB vesicles, TM-SAB vesicles. Cow order is cow#1, cow#2 and cow#3.

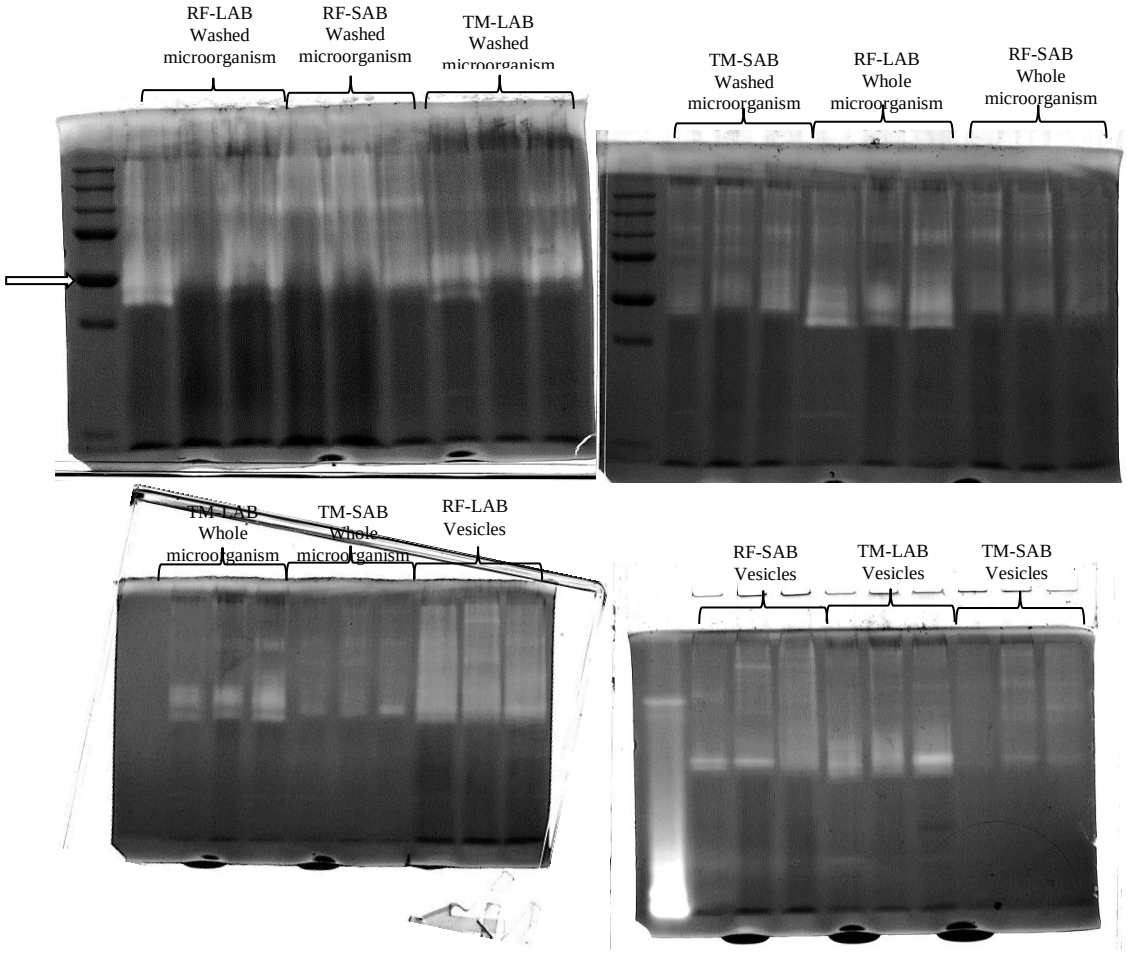

Fig. VI.2. Gelatine zymogram of protein marker with RF-LAB washed microorganisms, RF-SAB washed microorganisms, TM-LAB washed microorganisms, TM-SAB washed microorganisms, RF-LAB whole microorganisms, RF-SAB whole microorganisms, TM-LAB whole microorganisms, TM-SAB whole microorganisms RF-LAB vesicles, RF-SAB vesicles, TM-LAB vesicles, TM-SAB vesicles. Cow order is cow#1, cow#2 and cow#3. White arrow with black edge indicate 37 kDa protein marker band.

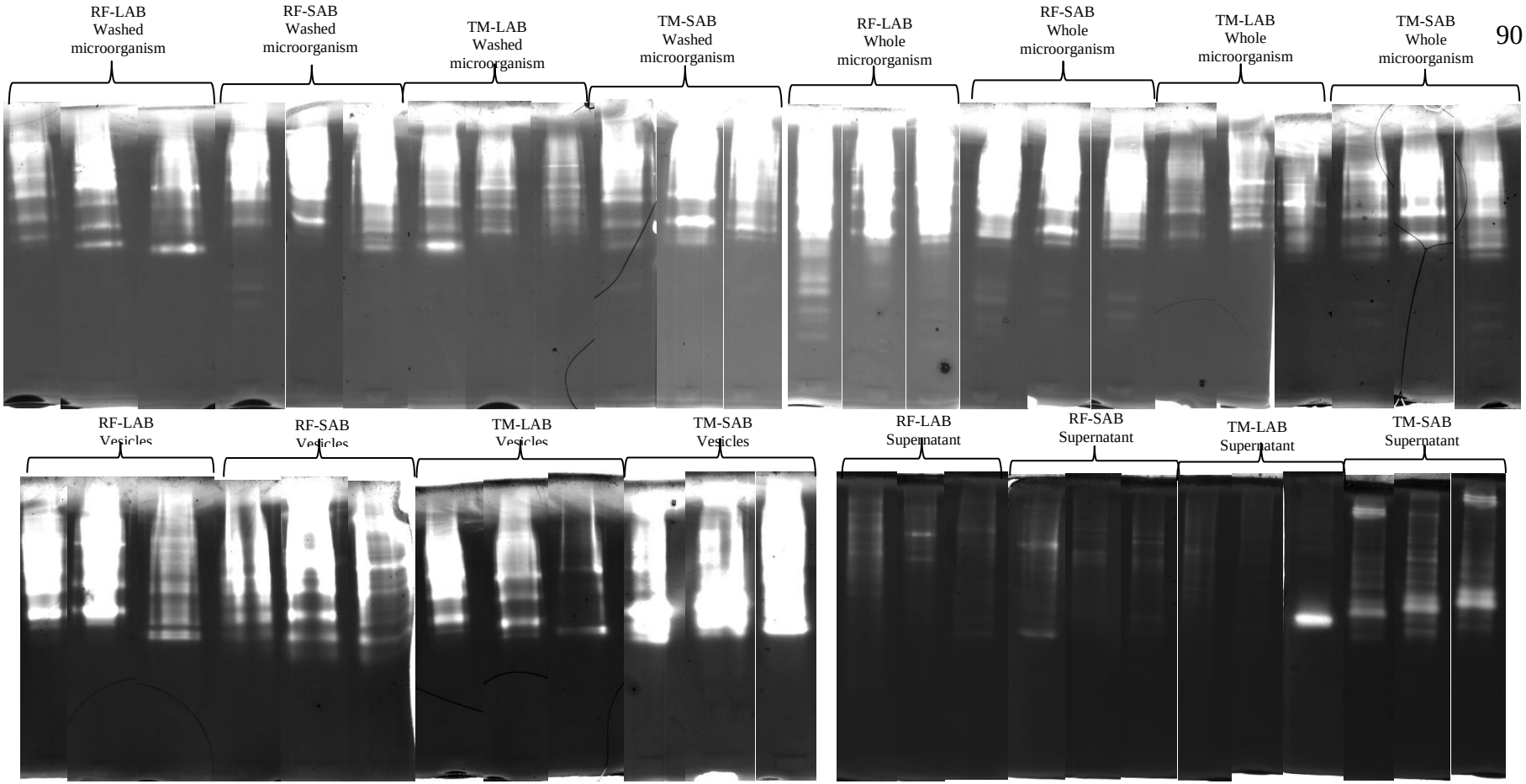

Fig. VI.3. Gelatine zymogram with EDTA (5 mM) on samples. RF-LAB washed microorganisms, RF-SAB washed microorganisms, TM-LAB washed microorganisms, TM-SAB washed microorganisms, RF-LAB whole microorganisms, RF-SAB whole microorganisms, TM-LAB whole microorganisms, TM-SAB whole microorganisms, RF-LAB vesicles, RF-SAB vesicles, TM-LAB vesicles, TM-SAB vesicles, RF-LAB supernatant, RF-SAB supernatant, TM-LAB supernatant, TM-SAB supernatant. Cow order is cow#1, cow#2 and cow#3.

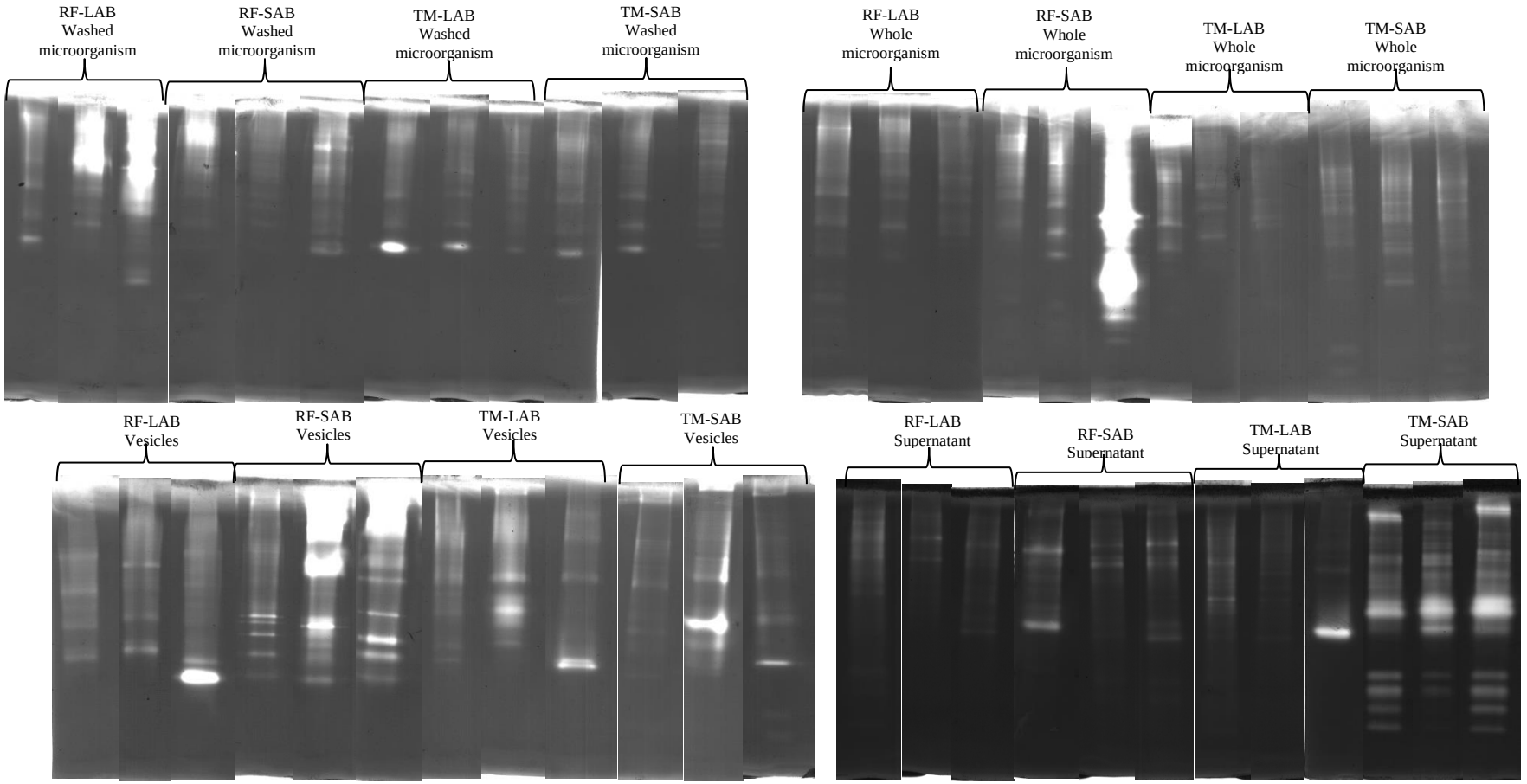

Fig. VI.4. Gelatine zymogram with E-64 (5 µM) on samples. RF-LAB washed microorganisms, RF-SAB washed microorganisms, TM-LAB washed microorganisms, TM-SAB washed microorganisms, RF-LAB whole microorganisms, RF-SAB whole microorganisms, TM-LAB whole microorganisms, TM-SAB whole microorganisms, RF-LAB vesicles, RF-SAB vesicles, TM-LAB vesicles, TM-SAB vesicles, RF-LAB supernatant, RF-SAB supernatant, TM-LAB supernatant, TM-SAB supernatant. Cow order is cow#1, cow#2 and cow#3.

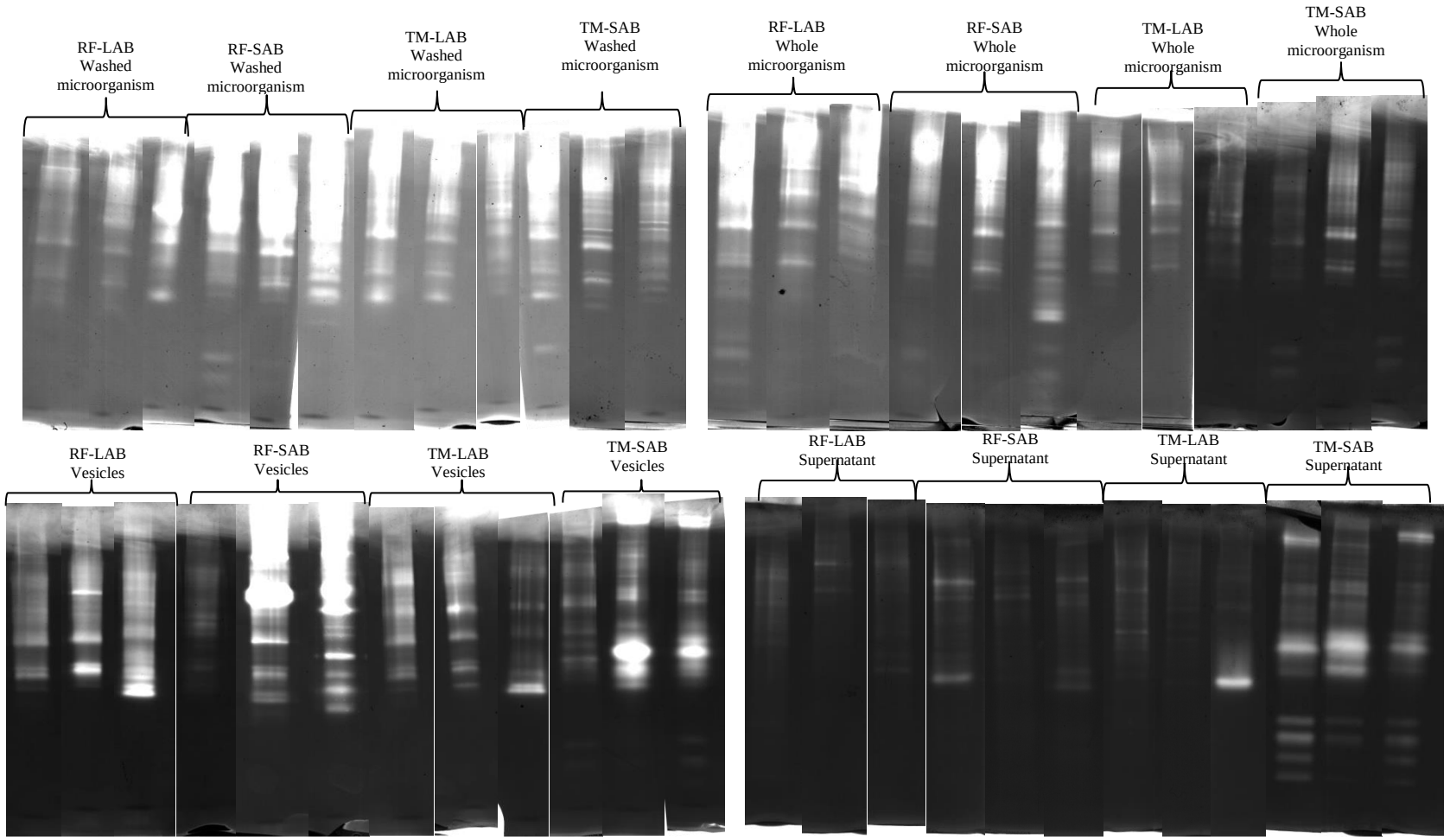

Fig. VI.5. Gelatine zymogram with pepstatin (1 µM) on samples. RF-LAB washed microorganisms, RF-SAB washed microorganisms, TM-LAB washed microorganisms, TM-SAB washed microorganisms, RF-LAB whole microorganisms, RF-SAB whole microorganisms, TM-LAB whole microorganisms, TM-SAB whole microorganisms, RF-LAB vesicles, RF-SAB vesicles, TM-LAB vesicles, TM-SAB vesicles, RF-LAB supernatant, RF-SAB supernatant, TM-LAB supernatant, TM-SAB supernatant. Cow order is cow#1, cow#2 and cow#3.

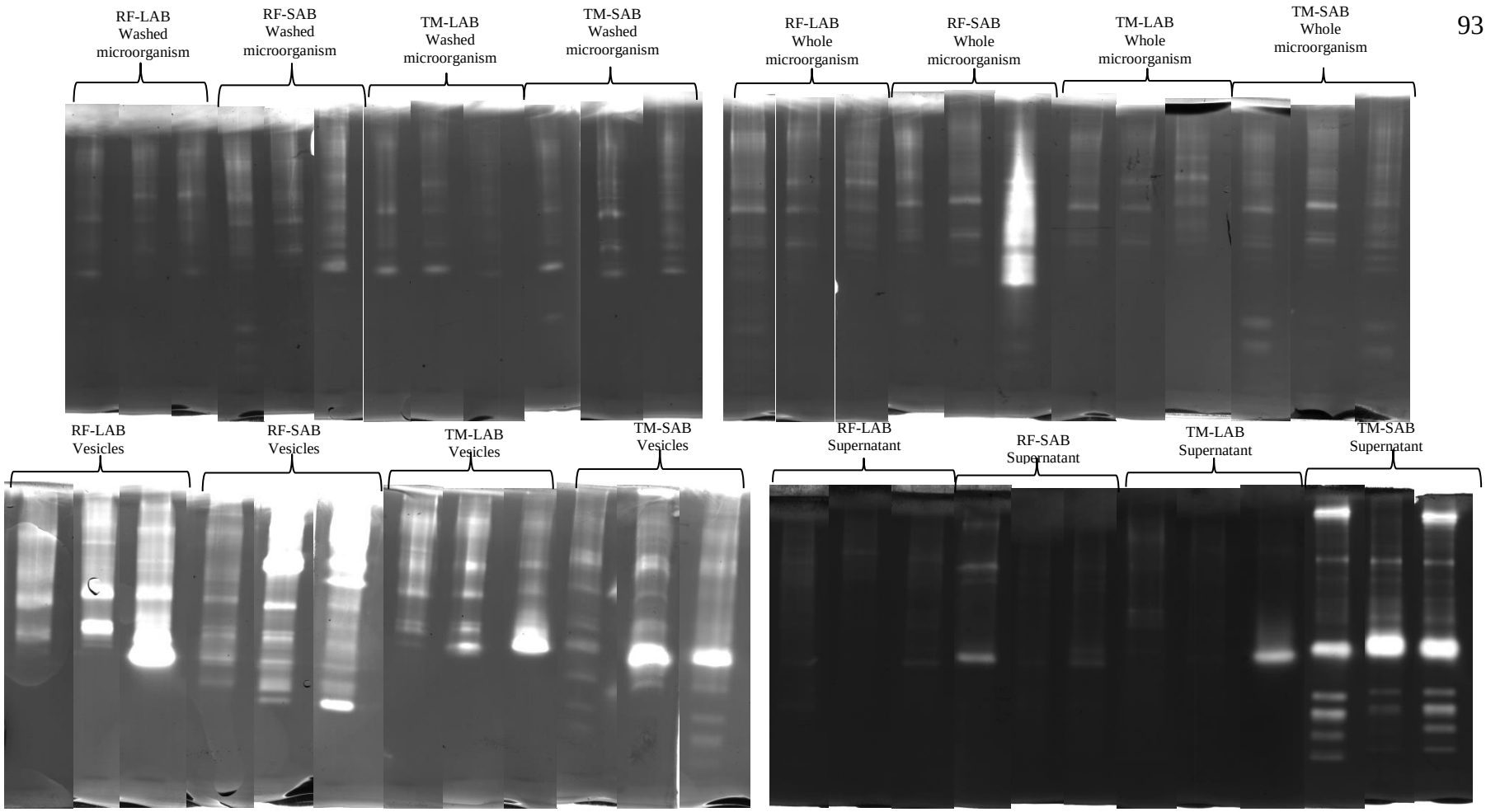

Fig. VI.6. Gelatine zymogram with PMSF (0.5 mM) on samples. RF-LAB washed microorganisms, RF-SAB washed microorganisms, TM-LAB washed microorganisms, TM-SAB washed microorganisms, RF-LAB whole microorganisms, RF-SAB whole microorganisms, TM-LAB whole microorganisms, TM-SAB whole microorganisms, RF-LAB vesicles, RF-SAB vesicles, TM-LAB vesicles, TM-SAB vesicles, RF-LAB supernatant, RF-SAB supernatant, TM-LAB supernatant, TM-SAB supernatant. Cow order is cow#1, cow#2 and cow#3.