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PROSPECÇÃO POR ENZIMAS DA MICROBIOTA RUMINAL CAPAZES DE AUMENTAR A CAPACIDADE ANTIOXIDANTE

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(Prospecting enzymes from the rumen microbiota with the ability to increase antioxidant capacity)

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"Tese apresentada como parte das exigências para obtenção do título de DOUTOR EM ZOOTECNIA, no Programa de Pós-Graduação em Zootecnia da Universidade Estadual de Maringá – Área de concentração Produção Animal"

MARINGÁ
Estado do Paraná
Abril – 2018

Dados Internacionais de Catalogação na Publicação (CIP)
(Biblioteca Central - UEM, Maringá, PR, Brasil)

Durman, Thomer
D963p Prospecção por enzimas da microbiota ruminal capazes de aumentar a capacidade antioxidante / Thomer Durman. -- Maringá, 2018.
104 f. : il. color., figs., tabs.

Orientador: Prof. Dr. Geraldo Tadeu dos Santos.
Coorientadora: Profa. Dra. Sharon Ann Huws.
Tese (doutorado) - Universidade Estadual de Maringá, Centro de Ciências Agrárias, Programa de Pós-Graduação em Zootecnia, 2018.

1. Rúmen. 2. Secoisolariciresinol diglicosídeo. 3. Linhaça. 4. Microbiota ruminal. I. Santos, Geraldo Tadeu, orient. II. Huws, Sharon Ann, coorient. III. Universidade Estadual de Maringá. Centro de Ciências Agrárias. Programa de Pós-Graduação em Zootecnia. IV. Título.

CDD 21.ed.636.2
ECSL-1202/9

"In the field of observation, chance favors only the prepared mind"

Louis Pasteur

Às pessoas que sempre depositaram
amor incondicional, coragem irrefutável,
força inenarrável, companheirismo inefável e sentido
na minha trajetória,
minha família e amigos

DEDICO

AGRADECIMENTOS/ACKNOWLEDGMENTS

À Universidade Estadual de Maringá e Aberystwyth University, instituições que proporcionaram excelente estrutura para desenvolvimento da pesquisa;

Ao Programa de Pós-Graduação em Zootecnia (PPZ/UEM) que me proporcionou todas as oportunidades vivenciadas no período da pós-graduação;

À Coordenação de Aperfeiçoamento Pessoal de Nível Superior (CAPES) pela concessão de bolsa no Brasil e de doutorado sanduíche no Reino Unido por meio do Projeto Pesquisador Visitante Especial (PVE) - PROC. 88881.030408/2013-01/CAPES; CSF-PVE - 88887.115008/2015-00;

Ao Prof. Dr. Geraldo Tadeu dos Santos por depositar confiança no meu trabalho, por supervisionar e amparar os projetos, e por todo crescimento profissional e pessoal que a orientação me proporcionou;

To Dr. Sharon Ann Huws, for allowing this great experience to take place in my life. For hosting me and advising me during the whole project. For the irrefutable kindness and knowledge;

To Prof Dr. Paul Shaw for all the support to make this project possible;

To Dr. Barbara for all the patience in teaching me how to use lab equipment and read/understand results;

To everyone involved in this project, from application to conclusion, especially Dr. Ana Schogor and Dr. Rodolpho Martin Do Prado for the attention in advising me and sharing forms/data when I needed the most; And to all lab mates helping me in all assays and top notch tea time breaks, especially Dr. Linda, Dr. Lucy, and Cate Williams;

To all IBERS staff, students and friends that somehow helped me in the project and were part of all the good times in Aberystwyth;

To the lab technicians, who were always willing to help, especially Pauline and Terry;

To Dr.Christopher Creevey and all the Bioinformatic team that helped a lot with data analysis, essential to this project;

À Maithê, que sempre esteve disposta a ajudar quando precisei, por participar dos melhores momentos e melhores memórias. Por ter sido essencial no desenvolvimento deste projeto, do início ao fim. Por tudo, gratidão imensurável;

Ao corpo docente do Programa de Pós-Graduação em Zootecnia, pelos ensinamentos e por sempre estarem dispostos a contribuir para o desenvolvimento dos trabalhos, em especial à Profª Drª Eliane Gasparino, coordenadora do programa durante a execução do projeto, por não medir esforços na confecção de documentação necessária para minha participação na pesquisa;

Aos colegas de programa, grupo NUPEL, amigos e todos aqueles que de alguma forma participam da minha vida e que são essenciais para minha felicidade;

Aos membros da banca, que se dispuseram a contribuir para com o crescimento do trabalho;

À minha família, em especial Marcelo, Solânia e Liz Durman, peças vitais da minha felicidade e combustível para seguir em frente, por todo apoio e amor irrefutável. Sou grato.

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LISTA DE ABREVIAÇÕES

A	adenine
Aβ	β-amyloid
ABCG2	ATP-binding cassete trasporter G2
ATP	adenosine triphosphate
BLAST	basic local alignment search tool
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
C	cytosine
CAT	catalase
cDNA	complementary DNA
CFU	colony forming units
DM	dry matter
DNA	deoxyribonucleic acid
DGGE	denaturing gradient gel electrophoresis
ED	enterodiol
EL	enterolactone
FM	flax meal
G	guanine
GSSG	glutathione disulphide
GSH	glutathione
GSHp	glutathione peroxidase
GSHr	glutathione reductase
G6PD	glucose-6-phosphate-dehydrogenase
HOCl	hypochlorous acid
HPC	high-performance computing
HMG	hydroxymethylglutarate
HMGA	hydroxymethyl glutaric acid
HPLC	high-performance liquid chromatography
LC-MS	liquid chromatograpgy-mass spectrometry
MPO	neutrophil myeloperoxidase
NGS	next-generation sequencing
NADPH	nicotinamide adenine dinucleotide phosphate
ORFs	open reading frames
PCR	polymerase chain reaction
PUFA	polyunsaturated fatty acids
ROS	reactive oxygen species
RNA	ribonucleic acid
SECO	secoisolariciresinol
SDEV	standard deviation
SDG	secoisolariciresinol diglucoside
SOD	enzyme superoxide dismutase
T	thymine

RESUMO

A linhaça (*Linum usitatissimum*) é amplamente estudada por seu valor nutricional excepcional e benefícios dos seus compostos na saúde humana e animal. Dentre eles, as lignanas podem ser destacadas, capazes de afetar positivamente a capacidade antioxidante do consumidor. Ainda, são capazes de serem transferidas aos produtos de origem animal, promovendo benefícios à saúde de quem os consome; seus efeitos estão ligados ao controle eficiente da oxidação, com papel principal na prevenção de diversas doenças, como doenças cardiovasculares, *diabetes*, vários tipos de câncer, dentre outras. O rúmen é comprovadamente um ambiente eficiente no metabolismo de lignanas vegetais, convertendo-as à enterolignanas, as quais possuem capacidade antioxidante potencializada, sendo superior a boa parte dos antioxidantes já conhecidos, como a vitamina E. Entretanto, uma porção pequena das espécies ruminais pode ser cultivada em condições de laboratório, limitando os estudos da biologia de diversas espécies. Portanto, o uso de técnicas metagenômicas para prospecção por enzimas no rúmen é crucial para o avanço no conhecimento na área de microbiologia do rúmen. Desta forma, foi proposto estudar os genes ligados ao metabolismo de lignanas da linhaça. Ainda, se estudou a capacidade de degradação de lignanas vegetais e seus produtos da degradação. Para alcançar os objetivos deste estudo foram realizados dois ensaios experimentais. Primeiramente, foi preparada uma biblioteca metagenômica de 11.500 clones, com inserções de DNA de microrganismos ruminais alocadas em *Escherichia coli* hospedeiras. Em seguida, foi realizada a análise genômica dos clones com atividade positiva (144 clones) para degradação de secoisolariciresinol diglicosídeo, a qual foi avaliada frente à análise por cromatografia. Ainda, um metatranscriptoma de microrganismos ruminais gerado a partir de incubações de líquido ruminal com ou sem adição de secoisolariciresinol diglicosídeo, a fim de estudar os transcritos e possíveis genes envolvidos com a degradação

da lignana vegetal. Como conclusão, não foram identificados produtos da degradação de secoisolariciresinol diglicosídeo (enterodiol e enterolactona), bem como possíveis genes envolvidos na degradação de lignanas vegetais nos clones positivos da biblioteca, o que sugere que as inserções de DNA bacteriano ruminal não continham informações genômicas que codificassem para enzimas capazes de aumentar a capacidade de degradação do composto, ou que o método de análise adotado não foi eficiente na recuperação dos genes. Em adição, o metatranscriptoma revelou sequências super-representadas que podem revelar genes diferencialmente expressos da microbiota ruminal quando em contato com secoisolariciresinol diglicosídeo.

Palavras-chave: rúmen, secoisolariciresinol diglicosídeo, metatranscriptoma, linhaça, microbiota ruminal

ABSTRACT

The flaxseed (*Linum usitatissimum)* is widely studied by its exceptional nutritional value and benefits of its compounds on animal and human health. Among them, the lignans can be highlighted, capable to affect effectively the antioxidant capacity of the consumer, as well as being able to be transferred to animal products, which can provide health benefits to the consumer. It effects are linked to the effective oxidative stress control, with main role in the prevention of many diseases, such as cardiovascular diseases, diabetes, many types of cancer, and others. The rumen is proven to be an efficient environment to the metabolism of plant lignans, converting them to enterolignans, which have their antioxidant capacity enhanced, being higher than great part of the well-known antioxidants, such as vitamin E. However, just a little portion of all rumen microorganisms can be cultivated in laboratory conditions, limiting the studies of many rumen species and their biology. Therefore, the use of metagenomic techniques for rumen prospection is crucial for the knowledge advance on the rumen microbiota field. Thus, it was proposed in this project, to assess the genes linked to flaxseed lignans metabolism. As well as, it was explored the capacity of flaxseed lignans breakdown and its degradation products. In order to achieve the aims of the study two experimental assays were carried out. Firstly, a metagenomic library, with rumen microbes DNA insertions allocated in *Escherichia coli* host bacteria was prepared, followed by genome analysis of clones with positive activity on secoisolariciresinol diglucoside breakdown, assessed by chromatography analysis. Secondly, a metatranscriptome of rumen microbes, based on rumen fluid incubations with and without secoisolariciresinol diglucoside was studied regarding the transcripts and putative genes linked to the plant lignan breakdown. As conclusion, it was not identified secoisolariciresinol diglucoside breakdown products, as well as putative genes linked to plant lignans breakdown in positive clones of the library. This suggests that the rumen bacteria DNA inserts did not contain genomic information that codes

to enzymes capable of enhancing the compound breakdown capacity, or the analysis method was not effective on the gene recovery. In addition, the metatranscriptome showed overrepresented sequences which can reveal differentialy expressed genes from the rumen microbiota when in contact with secoisolariciresiniol diglucoside.

Key-words: rumen, secoisolariciresinol diglucoside, metatranscriptome, flaxseed, rumen microbiota

I LITERATURE REVIEW

I.1 Reactive Oxygen Species (ROS) and oxidative stress

Reactive Oxygen Species (ROS) are essential for many processes in the cell, such as programmed cell death, which is crucial for both plant and animal tissue development and homeostasis of cells (Kerr et al., 1972). However, ROS can be dangerous when there is an imbalance between their production and their inhibition, delay or removal. The substances capable of tackling oxidative damage can do it by direct scavenging ROS or indirect acting up-regulating antioxidant defences (Halliwell, 2007; Khlebnikov et al., 2007).

The mitochondria are essential cellular organelles that have the prevailing role of producing adenosine triphosphate (ATP) and metabolic regulation through cellular respiration. However, this vital function fosters ROS formation as an unavoidable by-product of the process (Donald et al., 2006). This process can be increased in case of oxidative stressed mitochondria, leading to a drop in ATP production and higher induction of programmed cell death (Levine et al., 1994; Levine et al., 1996; Tiwari et al., 2002). According to Mittler (2002), the chemical reaction is triggered by atmospheric oxygen (O_2), forming singlet oxygen (O_2^1) by O_2 excitation or by electrons transfer forming superoxide radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) or a hydroxyl radical (HO^-) (Figure 1).

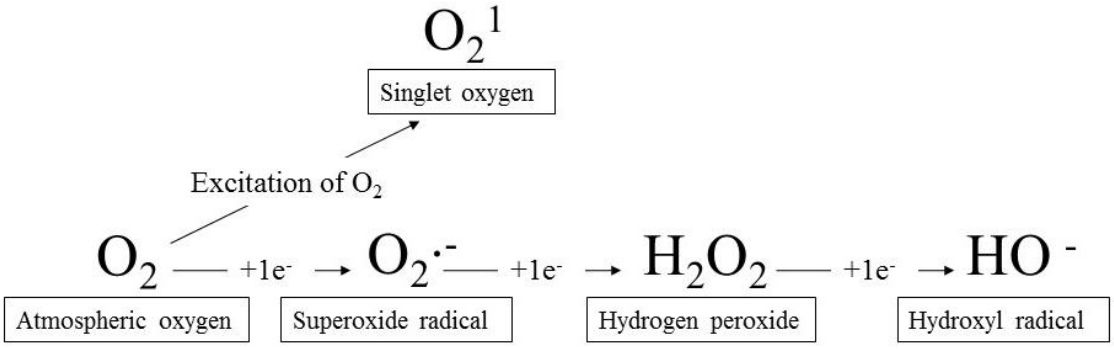

Figure 1. Atmospheric oxygen triggering reactive oxygen species formation.
Source: The author

I.2 Effect of ROS on human health

Free radicals are linked to the aging and cellular degenerative process and are associated with several metabolic disorders (Table 1), such as cancer, cardiovascular disease, immune-system decline, brain dysfunction, endocrine functions, cataracts and others (Ames et al., 1993; Christen, 2000; Hitchon and El-Gabalawy, 2004; Vincent et al., 2004; Nunomura et al., 2006; Wood-Kaczmar et al., 2006; Carreau et al., 2008). Therefore, in order to prolong animal and human life expectancy and improve quality of life, understanding of free radicals, oxidation process and antioxidants must keep advancing.

Table 1. Oxidative stress implicating metabolic disorders

Disorder	**ROS implication**	**Reference**
Alzheimer's disease	Oxidation of Nerve cells	Christen (2000); Nunomura et al. (2006); Gella and Durany (2009); Deibel et al. (1996); Manczak et al. (2006); Wang et al. (2014)
Atherosclerosis	Oxidation of endothelial cells, activation of matrix metalloproteinases, altered vasomotor activity	Harrison et al. (2003);Rajendran et al. (2014)
Cancer	Somatic mutations and neoplastic transformation	Fang et al. (2009); Reuter et al. (2010)
Cardiovascular disease	Drop in production of nitric oxide by endothelial cells	Münzel et al. (2010)
Cataracts	Oxidation of lens cells	Spector (1995)
Huntington's disease	Oxidative phosphorylation in occipital cortex	Jenkins et al. (1993)
Immune-system decline	Oxidation of Immune system cells	De la Fuente, 2002
Parkinson's disease	Oxidation of Dopaminergic neurons	Wood-Kaczmar et al. (2006); Nakabeppu et al. (2007); Pope et al. (2008); Brown and Neher (2010); Nikolova (2012); Hwang (2013)
Pulmonary disease	Increase in lung concentrations of elastase	Repine et al. (1997)
Rheumatoid arthritis	Autoimmune phenomena in Rheumatoid synovitis	Vasanthi et al. (2009); Stamp et al. (2012)
Stroke	Neuronal death	Alexandrova et al. (2004)
Type 2 Diabetes	Induced development of insulin resistance	Urakawa et al. (2003); Vincent et al. (2004)

Oxidative stress can lead to several diseases linked to cellular degenerative process, which might be delayed if the oxidation balance is controlled. For instance, the Alzheimer's disease, an age-related neurodegeneration causing progressive dementia, which might be delayed if antioxidant defence mechanism slows down radical production of ROS (Gella and Durany, 2009). Neurons have a high oxygen demand and a prominent metabolic rate;

moreover, the neuronal components as lipids, protein and nucleic acids can be oxidized due to mitochondrial dysfunction, increased metal levels, inflammation, or β-amyloid (Aβ) peptides (Deibel et al., 1996; Manczak et al., 2006; Wang et al., 2014). In addition, the brain cells are susceptible to lipid oxidation (due to large amount of polyunsaturated fatty acids) and have low levels of glutathione, a potent antioxidant (Pocernich and Butterfield, 2012; Skoumalová and Hort, 2012). These factors, when put together, make the human brain very vulnerable to oxidative stress. Thus, studies aiming to enhance the antioxidant defence mechanism can be the most effective solution to prevent Alzheimer's disease (Chen and Zhong, 2014).

Parkinson's disease is another progressive neurodegenerative disorder, which is related to the demise of neurons in the midbrain area and it affects normal movements due to resting tremor, muscular rigidity, brasykinesia, and postural imbalance. The pathological mechanism can have idiopathic or genetic explanations but the cause is most likely linked to oxidative stress as well (Hwang, 2013). The disease affects the nigral region of the human brain, resulting in selective loss of dopaminergic neurons, followed by modifications that compromise cell integrity. Moreover, mitochondrial dysfunction is also one possible cause of disorder, since apoptosis that can be triggered by peroxidation of cardiolipin (mitochondrial lipid) and by cytochrome c being released to the cytosol. Furthermore, neuroinflammatory responses can be responsible for nitric oxide and superoxide production, free radicals, which role is to remove cell debris and pathogens, however, unusual response can potentially cause imbalance on cellular oxidation (Nakabeppu et al., 2007; Pope et al., 2008; Brown and Neher, 2010). The multifactorial etiology of the neurodegenerative movement disorder challenges the ability to treat it; however, oxidative control seems to be a valid target to keep aiming towards it (Nikolova, 2012).

Other diseases incidences can be controlled by oxidation regulation, such as rheumatoid arthritis and many different cancers. The first one is a chronic and systemic disease, where cells of immune system release pro-inflammatory mediators, which can cause cartilage and bone damage in case of inordinate responses. The autoimmune phenomena rheumatoid synovitis can be triggered by oxidant stress (Vasanthi et al., 2009; Stamp et al., 2012). In a similar stressed microenvironment, where ROS are being generated progressively, cell structure and functions was affected, inducing somatic mutations and neoplastic transformation which leads to genome instability and cell proliferation, characterizing the cancer etiology (Fang et al., 2009; Reuter et al., 2010).

Oxidative stress is a potential trigger and aggravates several diseases with different etiology. Indeed, in human and animal health, studies aiming to increase antioxidant capacity, in order to maintain a favourable balance between ROS production and antioxidant defence mechanisms, are an effective tool for preventing a wide range of metabolic disorders and hence, improving expectancy and quality of life.

I.3 Reducing ROS through use of antioxidants

By virtue of the wide range of metabolic disorders that can be triggered or aggravated by intense and unbridled free radical production, the use of antioxidants has been explored for decades as a way of oxidative control. Antioxidant compounds act in different pathways, which ensure an efficient defence system. The molecular mechanisms (Figure 2) are various, four of which were highlighted by Cadenas (1997), as underlying the antioxidant activities.

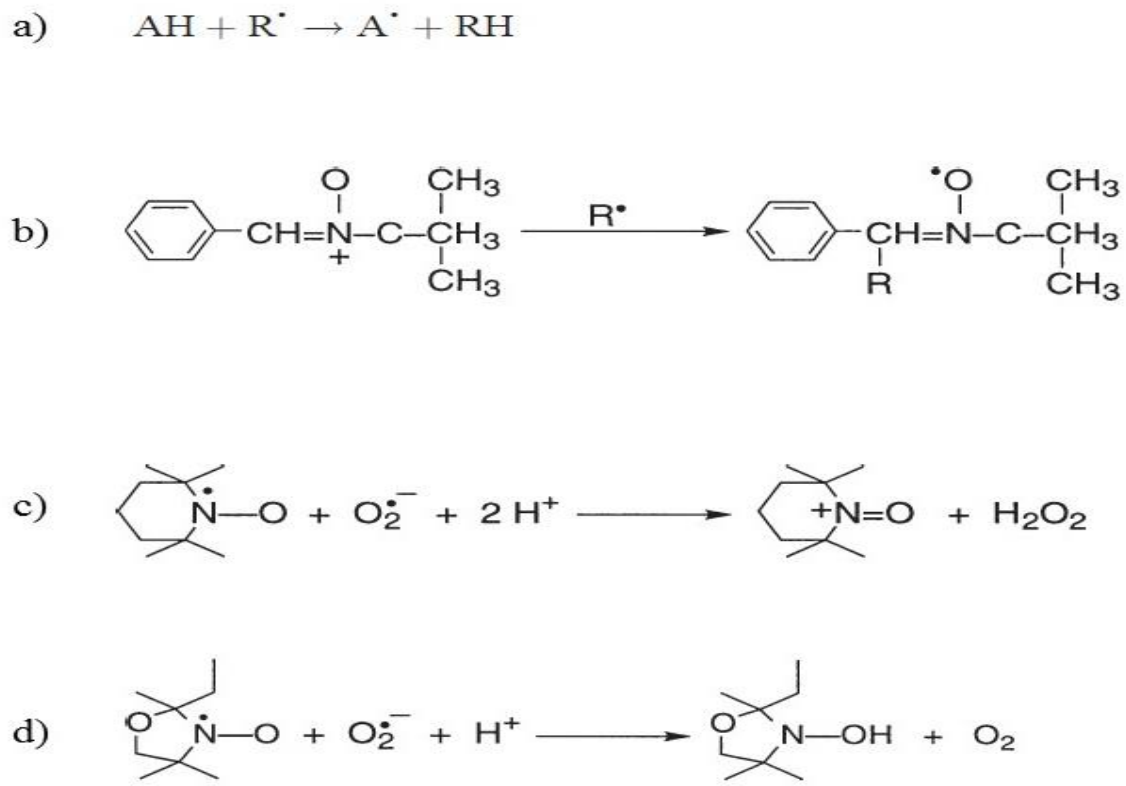

Figure 2. Antioxidant mechanisms for oxidation control. a) transfer of radical; b) antioxidant-derived radical formation; c) antioxidants molecules; d) enzymatic-mimetic activity.
Adapted from Cadenas (1997)

First, the process of transfer of radical is characterized by the formation of a reactive antioxidant-derived radical (a). The reaction is illustrated in Figure 2 as a compound (AH) involved on a donation of a single electron to a free radical species (R·), comprehending a redox transition (reduction potentials are listed in Table 2), moreover, the radical is transferred to the antioxidant, forming its derived radical (A·). However, the radical generated is not inert and might be implicated in cellular toxicity, as the alkylperoxy radical ROO for instance, the major lipid radical at normal oxygen pressure, is an oxidising agent, reduced and converted to a hydroperoxide by an electron donor, or to a hydroperoxide by a hydrogen donor. Furthermore, in a similar mechanism, the transfer of the radical is followed by the formation of a stable or inert antioxidant-derived radical (b), being limited in reactivity, preventing further reactions. For instance, the nitrone radical traps, such as phenyl-tert-butyl nitrone reacting with a variety of free radical species forming a stable radical adduct. In addition, other possible mechanisms to tackle oxidative stress are small antioxidants molecules (c) (nitroxides or aminoxyls) involved in the dismutation of $O^{\cdot -2}$ to H_2O_2. Nitroxides also catalyze the dismutation of O^{-2} to O_2 with enzymatic-mimetic activities such as superoxide dismutase and glutathione peroxidase mimics (d) (Cadenas, 1997). Alternatively, oxidants other than $O^{\cdot -2}$, such as semiquinones with a suitable reduction potential or metal ions can also accomplish reaction (Gordon, 1990).

Table 2. Antioxidant reduction potential

Species	E^o/V
Hydroxyl radical	2.18
Alkoxyl radical	1.60
Oxoferryl complex	1.40
Peroxynitrite	1.40
Hydroperoxyl radical	1.06
Lipid Peroxyl radical	1.00
Protein Trp radical	0.64 – 1.00
Protein Tyr radical	0.78 – 0.91
β-carotene cation radical	0.50 – 1.06
Zeaxanthin cation radical	0.54 – 1.03
Lycopene cation radical	0.98
Nitric dioxide	0.87
Thiyl radical	0.85
Disulfide anion radical	0.65
Lipid alkyl radical	0.60
Caffeic acid	0.54
Uric acid radical	0.52
α-tocopheroxyl radical	0.48
Ubisemiquinone	0.35
Ascorbyl radical	0.28
Protein Cys radical	0.13 – 0.27

Adapted from Cadenas (1997) and Griffiths (2015)

The oxidation defence mechanism can be diverse, where antioxidant substances (Figure 3) can act in different pathways, directly or indirectly, in order to repress damage caused by the products of aerobic metabolism. Most of the antioxidants can be found in nature, some produced by the organism itself (endogenous antioxidants), others can be obtained by dietary sources (exogenous antioxidants), and the intake of antioxidant rich foods is essential to boost the defensive system against ROS. Moreover, antioxidants such as BHT (butylated hydroxytoluene) and BHA (butylated hydroxyanisole) can be synthetically developed and incorporated into food to prevent oxidation, especially fatty acids (Sen et al., 2010; Carocho and Ferreira, 2013).

Antioxidants can be grouped according to their composition or function. The two major clusters are enzymatic and non-enzymatic antioxidants, with enzymic antioxidants being substances endogenously produced and most of the non-enzymatic antioxidants being exogenous substances. The levels of both types of antioxidants is dependent on the dietary intake, with some exceptions as metabolic antioxidants such as coenzyme Q10, vitamin A, glutathione, uric acid and enterolignans (enterodiol and enterolactone), produced in the gastrointestinal tract.

With respect to antioxidant activity, the mechanisms are diverse, for instance: inhibiting free radical oxidation or the formation of lipid radicals; as chain breaking antioxidants, interruption autoxidation chain reaction; supressing singlet oxygen; as oxygen scavenger and reducing agents; chelating metals; and inhibiting pro-oxidative enzymes. Moreover, antioxidants might present multiple functions (Gordon, 1990; Carocho and Ferreira, 2013). Thus, antioxidant analyses are complex to be evaluated separately, considering that, possible synergic interactions might occur.

The two main groups of antioxidants (enzymatic and non-enzymatic) also have subdivisions dependent on their characteristics. The enzymatic antioxidants are divided into primary defence (glutathione peroxidase, catalase and superoxide dismutase), which are responsible for preventing the formation or neutralizing free radicals, and secondary defence antioxidants such as glutathione reductase and glucose-6-phosphate dehydrogenase, which do not neutralize free radicals directly, but have supporting roles in recycling and regenerating other endogenous antioxidants. They are therefore essential for the continuity of the defence mechanisms. On the other hand, the non-enzymatic antioxidants are divided in several groups, mainly, regarding their composition (Rahman, 2007).

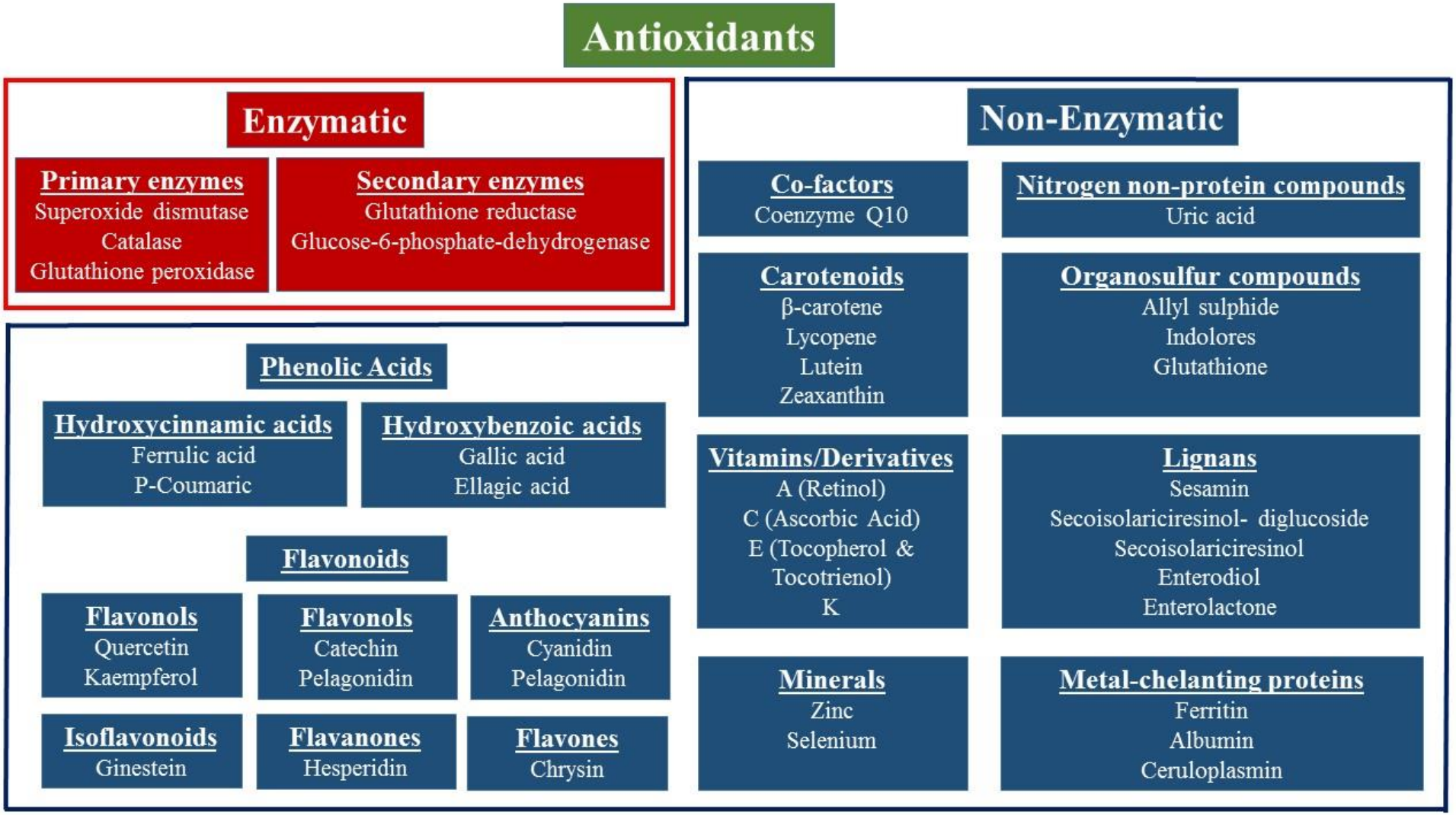

Figure 3. Natural antioxidants characterization. Adapted from Jacob (1995); Sen et al. (2010); Touré and Xueming (2010); Carocho and Ferreira (2013).

I.3.1 Enzymatic antioxidants as ROS scavengers

The enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSHp), glutathione reductase (GSHr), and glucose-6-phosphate-dehydrogenase (G6PD) have an essential role in the enzymatic antioxidant defence system (Figure 4), with respect to oxygen free radical control. In the presence of a transition metal, mostly ferrous iron (Fe^{++}), through a Fenton reaction involving hydrogen peroxide (H_2O_2), can produce hydroxyl radical ($OH\cdot$), a potent oxidant, which might also be generated in a Haber-Weiss reaction between superoxide anion ($O_2\cdot^-$) and H_2O_2. Alternatively, H_2O_2 and a chloride ion can generate hypochlorous acid (HOCl), a toxic oxidant, formed when the reaction is catalysed by neutrophil myeloperoxidase (MPO) (Halliwell, 1996). Therefore, the major role of this system is to avoid the formation of those oxidant compounds as much as possible.

Through a dismutation reaction, SOD can detoxify superoxide anions to hydrogen peroxide, which acts as a substrate to CAT, converting the hydrogen peroxides into water and molecular oxygen. In addition, GSHp competes with CAT for the same substrate, and through a reaction involving reduced monomeric glutathione (GSH), reduces H_2O_2 to glutathione disulphide (GSSG) and water, reducing glutathione from its oxidized to its reduced form. GSSG is either converted to GSH by GSHr through a reaction that obtain electrons from the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH). Finally, NADPH needs to be regenerated to $NADP^+$ in order to guarantee the sustainability of further reactions, thus, G6PD mechanism takes place through the pentose phosphate pathway, regenerating this useful cofactor (Gordon, 1990; Halliwell, 1996; Rahman, 2007; Carocho and Ferreira, 2013).

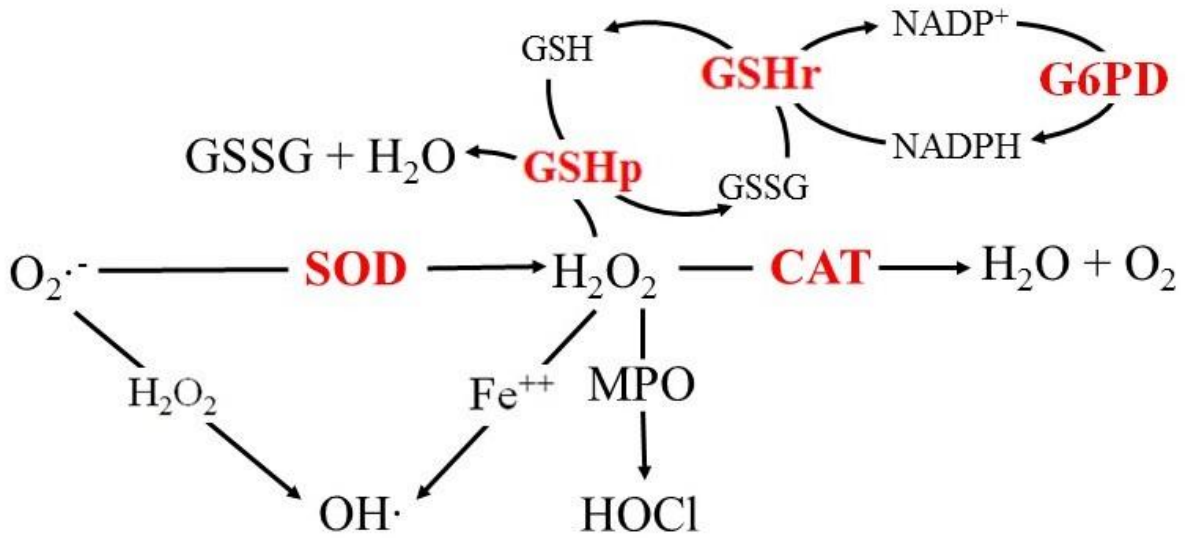

Figure 4. Role of enzymatic antioxidants on the oxygen radicals control. In red, the antioxidant substances. SOD: Superoxide dismutase; CAT: catalase; GSHp: glutathione peroxidase; GSHr: glutathione reductase; G6PD: glucose-6-phosphate-dehydrogenase. Adapted from Repine et al. (1997); Carocho and Ferreira (2013).

I.3.1.1 Superoxide dismutase

The knowledge surrounding SOD have evolved in the past 50 years since its discovery by McCord and Fridovich in the 1960's, and its purification from bovine erythrocytes (McCord and Fridovich, 1969). Moreover, in the 1980's, the studies regarding the comprehension of the role of superoxide dismutase in basic biology of cells and tissues metabolism began to take firm hold. Furthermore, the development of molecular biology techniques in the 90's allowed a new field to broaden knowledge. Since then, the studies regarding determining the enzyme's kinetics and mechanisms, as well as the adverse oxidative reactions showed an exponential growth on the comprehension of redox biology (Buettner, 2011).

Through conversion of superoxide anions into dioxygen and hydrogen peroxide, SOD is known as one of the most effective intracellular enzymatic antioxidant (Rahman, 2007). According to Landis and Tower (2005), there are three forms of this enzyme: Cu/Zn-SOD in the cytoplasm and outer mitochondrial space; Mn-SOD in the inner mitochondrial space; and Cu/Zn-SOD present in extracellular space. The different isoforms vary in nature of active metal centre, amino acid composition, co-factors, molecular weight, chemical structure, compounds affinity, and other features (Fridovich, 1995; Rahman, 2007).

The necessity to detoxify $O_2^{\cdot -}$ in the compartment of origin explains the distinct forms of SODs in the cytosol. The superoxide anion should not easily cross biological membranes, with the exception of those rich in anion channels. However, O_2^- might also be originated in the extracellular compartment, for instance, due to ultraviolet irradiation of water (Fridovich, 1995). Therefore, in order to guarantee an efficient ROS control, particularly superoxide anion radicals, SODs must be present in intra and extracellular compartments.

According to Matés et al. (1999), CuZn-SOD has two subunits with a molecular weight of 32 kDa, containing a dinuclear metal cluster (copper and zinc ions) as active site, which is correlated with a signature motif related shared between CuZn-SOD genes (Landis and Tower, 2005). On the other hand, Mn-SOD, has an unrelated active site, and is a complex made up of four identical subunits, containing one manganese atom per subunit, exclusively of the mitochondrial spaces. The molecular weight is 96 kDa, and it cycles in a two-step dismutation of superoxide. Finally, SOD of extracellular compartment, is a tetrameric secretary glycoprotein, containing copper and zinc and having high affinity for specific glycosaminoglycans, however, the regulation in mammalian tissues occurs primarily coordinated by cytokines, rather than oxidative stress response (Matés et al., 1999; Landis and Tower, 2005; Rahman, 2007).

I.3.1.2 Catalase

Catalase, an intracellular enzyme, is present in both animals and plants, mostly in aerobic cells, in mammalian and non-mammalian, with a few exceptions. In 1900, Loew first reported the presence of CAT in plant and animal's cells. Moreover, the purification of a haemoglobin-free erythrocyte catalase was reported more than a century ago by Wolff and De Stoecklin in 1910, and the activity has been intensively studied since then (Deisseroth and Dounce, 1970; Kirkman and Gaetani, 2007). The genomic analysis tools have also allowed novel perspectives of research regarding CAT regulatory mechanisms, such as gene polymorphisms and mutations (Góth et al., 2016).

The enzyme catalase has a great turnover, allowing just one molecule to convert six billion molecules of hydrogen peroxide into water and molecular oxygen (Carocho and Ferreira, 2013). Mammalian CAT is formed from four monomers, and which, contains an iron heme (porphyrin) group bond to the catalytic site. The subunits of CAT have four domains: non-globular amino terminal arm; anti-parallel β barrel; exterior wrapping domain; and a final α-helix structure (Góth et al., 2004). In addition, the H_2O_2 selectivity is aid by

non-polar pocket with narrow hydrophobic channels, and also provide protection to the heme group (Day, 2009).

I.3.1.3 Glutathione peroxidase and glutathione reductase

Discovered in 1957 by Gordon C. Mills, glutathione peroxide catalyses the reduction of hydrogen peroxide or organic hydroperoxides to water or corresponding alcohols. The enzyme is involved besides the H_2O_2 removal, in the metabolism of lipid peroxides (Wendel, 1980). Moreover, there are two forms of GSHp, one selenium-dependent, which acts in association with GSH (H_2O_2 reduction) and the other one, is selenium-independent, which is active only with organic hydroperoxides (Matés et al., 1999). Regarding the evolutionary rates, the complex relationship of this gene family suggest that basal GSHp classes, have originated from gene duplication, gene losses, lateral gene transfer among invertebrates and vertebrates or plants, or other independent evolutionary event (Margis et al., 2008).

The secondary enzymatic system acts indirectly on the ROS scavenging, and is essential to maintain the constancy of the antioxidant defence system. For instance, GSHr, which occurs in both prokaryotic and eukaryotic organisms, and belongs to the family of NADPH-dependent oxidoreductase. This enzyme is located in chloroplasts, cytosol, and mitochondria (Edwards et al., 1990), being important for maintaining the reduced pool of GSH trough mechanisms cited above. Considering it importance, genetically engineered plants for overexpressing of selected GSH and GSHr genes have been developed as a strategy for improving performance (Gill et al., 2013).

Furthermore, playing an important role in keeping the constant flow of NAPH available to the antioxidant defence system and production of ribose-5-phosphate, the enzyme G6PD is essential for a cellular reduced environment (Yoshida and Beutler, 1986). In addition, because of lack of mitochondria in erythrocytes, G6PD is the only source of NADPH in red blood cells (Luzzatto L, 2001), therefore, a deficiency in this enzyme can lead to several dysfunctions. Indeed, at least 400 million people worldwide carry the gene for G6PD deficiency, thus, even 85 years since the discovery of the enzyme by Warburg and Christian in 1931, it is important to keep advancing knowledge in this enzymatic pathway (Yoshida and Beutler, 1986; Cappellini and Fiorelli, 2008).

I.3.2 Non-enzymatic antioxidants as ROS scavengers

The animal body is not self-sufficient in antioxidant compounds, with a dependency on dietary antioxidants to complete the defence system against the oxidation process. In humans for instance, during the evolution process, uric acid replaced the ascorbic acid as the main biological fluids' water-soluble antioxidant. Thus, humans lack the endogenous ability to synthesize ascorbic acid, even having an expressive requirement (Nishikimi and Yagi, 1991). Therefore, the needs for ascorbic acid (vitamin C), as well as most of non-enzymatic antioxidants (vitamin E, thiols carotenoids, polyphenols, flavonoids, etc.) can only be met depending on the intake of dietary sources of antioxidants, mostly plants, rather than animal origin foods. (Ratnam et al., 2006; Rahman, 2007; Sikora et al., 2008).

The mechanisms of the non-enzymatic compounds against ROS and oxidation implications are diverse. Vitamin E (α-tocopherol), which is strongly connected with the prevention of cancer and degenerative diseases, has a main role on lipid peroxidation control, by the donation of a labile hydrogen to a lipid or lipid peroxyl radical. This compound might be synergic to vitamin C (ascorbic acid), which is connected with several antioxidants pathways and largely used in canned or bottled products for its oxygen scavenging activity.

Also, regenerating α-tocopherol from α-tocopherol radicals in membranes and lipoproteins, as well as increasing intracellular glutathione level, thiol antioxidants as tripeptide glutathione, are a multifunctional intracellular antioxidant, with important role in ROS scavenging as described before. In addition to the group, carotenoids are plants pigments containing double bonds which ease delocalizing unpaired electrons and physical quenching of singlet oxygen, being lycopene the most effective compound. Those compounds also are responsible to prevent lipophilic compartments by scavenging peroxyl radical. Furthermore, flavonoids are other plant component capable of scavenging peroxyl radicals, inhibiting lipid peroxidation, and chelating redox-active metals, preventing the Fenton reaction (H_2O_2 producing OH·). Finally, minerals such as selenium and zinc also play an expressive role in the antioxidant defensive system and being intensively studied overtime. The bioactivity of selenium is connected with GSHp, metalloenzymes and thioredoxin reductase, playing a role similar to vitamin E. Zinc is also important in many pathways, inhibiting NADPH oxidases, inducing the production of metallothionein (H_2O_2 scavenger), competing with copper for binding to the cell (reducing H_2O_2 production), and is present in the enzyme SOD, an important enzyme as discussed previously. Many others compounds and substances with antioxidant activity have been studied overtime, however, facing the great

impact in animals and human health much effort should still be given to advance the knowledge in the field (Gordon, 1990; Knekt et al., 1994; Halliwell, 1996; Ratnam et al., 2006; Rahman, 2007; Sikora et al., 2008; Carocho and Ferreira, 2013).

The plant components are crucial for defence against oxidative stress and the activity remains after intake, thus, benefiting the animal who consumed it. Antioxidant supplementation is possible, although, might not be as effective as a complex diet rich in those components (vegetables, fruits and herbs), which contains a mix of several antioxidants that works as a continuous chain, whilst the supplementation might not enable completely the antioxidant chain, since it is usually given using one or two substances (Mittler, 2002; Sen et al., 2010; Rafieian-Kopaei et al., 2013).

Therefore, it is important to change our feeding behaviour in order to enhance the amount of antioxidants being consumed. Although, according to Halliwell (2012), provided one is not deficient in vitamins, the endogenous synthesis of antioxidant defences is far more important than benefits from larger intakes of vitamins, carotenoids, flavonoids, etc. Consequently, agents that challenge our defence system and enhance endogenous antioxidant defences, and other protective systems may end up being more protective against oxidative damage in cells and tissues than the intake of large amounts of vitamins. Thus, it is relevant to not only evaluate the effects of antioxidant compounds separately, but also assess the physiological interactions and real impact in health.

I.3.2.1 Lignans and flaxseed

Lignans, non-enzymatic antioxidants, can be found in many plants, such as flaxseed (the richest source), pumpkin, sunflower, poppy, sesame, rye, oats, barley, wheat, beans, berries, and vegetables (Meagher and Beecher, 2000; Adlercreutz, 2007).

Lignans, such as secoisolariciresinol diglucoside (SDG), enterodiol (ED) and enterolactone (EL), have been proven to be effective at lowering lipid peroxidation in different media systems (Kitts et al., 1999; Matumoto-Pintro et al., 2011). In addition, both mammalian lignans (ED and EL) show great efficacy in reducing deoxyribose oxidation and DNA strand breakage, as well as, none SDG, ED or EL demonstrate indirect prooxidant activity in Fenton reagents (Kitts et al., 1999). Indeed, the lignans are a powerful ROS scavenger. The hydroxyl radical (OH·) scavenging activity of SDG, ED and EL at the concentration of 10μM for non-site-specific is 4.20, 58.53, 60.83% respectively. Moreover,

for the same flax lignans, the site-specific scavenging activity is 6.74, 23.45, 31.93% respectively (Kitts et al., 1999).

The metabolism of lignans might vary according to the host studied; therefore, unreproducible results might complicate comparison between species. For instance, 7-hydroxymatairesinol is metabolized to 7-hydroxyenterolactone in humans rather to enterolactone as reported to happen in rats (Heinonen et al., 2001). The differences in intestinal microflora might explain the different paths in lignans metabolism between species. In addition, differences in structure or even mild variance in composition might lead to completely different pathways. For example, isolariciresinol ($C_{20}H_{24}O_6$), which is not metabolized to enterolactone or enterodiol as secoisolariciresinol ($C_{20}H_{26}O_6$) is. The possible explanation is the rigid fused ring structure protecting the lignan from reactions (Heinonen et al., 2001).

Linum usitatissimum, the Latin name of the flaxseed, which means "very useful", has been proven to have an accurate nomenclature. Firstly, being one of the oldest cultivated plants, it was applied to produce fibre for clothing, and currently employed for several products as linen production, linoleum, stain, paints and others. In addition, widely studied for it outstanding nutritional value and benefit of its compounds in animals and human health, preventing many diseases (Muir and Westcott, 2003; Adolphe et al., 2010; Goyal et al., 2014).

Flaxseed composition (Table 3) is high in ω-3 fatty acid: α-linolenic acid, short chain polyunsaturated fatty acids, soluble and insoluble fibres, proteins, as well as, is a rich source of antioxidants, as the phytoestrogenic lignans (e.g. SDG) (Choo et al., 2007; Goyal et al., 2014). Although, the composition might change according to the flaxseed maturity stage, as described by Herchi et al. (2014), showing difference in composition when comparing immature, half-mature, pre-mature, and mature flaxseeds (7, 21, 42 and 56 age days respectively). In addition, there are several extraction methods being used, as well as results expressed in diverse units of measure. The lack of standardization on analysis complicates accurate comparisons.

Table 3. Compilation of composition data in different Flaxseed Products.

Item	Flaxseed products		
	Hull	Oil	Flaxseed Meal
Dry matter (g/100g)	93.64	n.e	n.e
Total carbohydrate (g/100g)	100	n.e	29
Dietary fibre (g/100g)		n.e	28.0
Neutral detergent fibre (g/100g)	19.4	n.e	25.6
Acid detergent fibre	14.3	n.e	17.7
Energy (kcal/100g)	460.90	n.e	n.e
Crude protein (g/100g)	17.21–23.5	n.e	20-36.3
Albumin (g/100g)	1.40	n.e	n.e
Globulin (g/100g)	0.54	n.e	n.e
Prolamin (g/100g)	0.22	n.e	n.e
Glutelin (g/100g)	0.35	n.e	n.e
Fatty acids (g/100g)	20.32-29.8	n.e	41
C16:0 (Palmitic acid)	7.76[1]	4.90-8[2]	2.1[2]
C18:0 (Stearic acid)	3.95[1]	2.24-4. 59[2]	1.3[2]
C18:1 (Oleic acid)	20.54[1]	13.44-19.39[2]	7.3[2]
C18:2 (Linoleic acid)	17.70[1]	12.25-17.44[2]	5.9[2]
C18:3 (α-Linolenic acid)	48.95[1]	39.90-60.42[2]	22.8-23.0[2]
∑ PUFA (%GC area)	66.65	n.e	n.e
Ω3/ Ω6 (% GC area)	2.76	n.e	n.e
Lignans (mg/100g)	n.e	n.e	35-70
SDG	0.99[1] / 32.0[3]	n.e	6.1-30[4]
SECO (g/100g)	n.e	n.e	0.3699
Ascorbic acid (mg/100g)	n.e	n.e	0.50
Thiamin (mg/100g)	n.e	n.e	0.53
Riboflavin (mg/100g)	n.e	n.e	0.23
Niacin (mg/100g)	n.e	n.e	3.21
Pyridoxin (mg/100g)	n.e	n.e	0.61
Pantothenic acid (mg/100g)	n.e	n.e	0.57
Folic acid (mg/100g)	n.e	n.e	112.0
Biotin (mg/100g)	n.e	n.e	6.0
α-Tocopherol (mg/100g)	n.e	n.e	7.0
δ-Tocopherol (mg/100g)	n.e	n.e	10.0
γ-Tocopherol (mg/100g)	n.e	n.e	552.0
Ash (g/100g)	2.37	n.e	n.e
Calcium (mg/100g)	n.e	n.e	236.0
Copper (mg/100g)	n.e	n.e	1.0
Magnesium (mg/100g)	n.e	n.e	431.0
Manganese (mg/100g)	n.e	n.e	3.0
Phosphorus (mg/100g)	n.e	n.e	622.0
Potassium (mg/100g)	n.e	n.e	831.0
Sodium (mg/100g)	n.e	n.e	27.0
Zinc (mg/100g)	n.e	n.e	4.0

SDG: secoisolariciresinol diglucoside; SECO: secoisolariciresinol; PUFA: Polyunsaturated fatty acids
1: % GC area; 2: g/100g; 3: mg/g; 4: nmol/mg; n.e: not evaluated
Adapted from Johnsson et al. (2000); Raffaelli et al. (2002); Muir and Westcott (2003); Côrtes et al. (2008); Côrtes et al. (2012); Bernacchia et al. (2014); Goyal et al. (2014); Herchi et al. (2014); Lima et al. (2016).

A wide variety of lignans might be found in the outer layer (hull) of the flaxseed: secoisolariciresinol diglucoside (SDG); secoisolariciresinol (SECO); isolariciresinol; pinoresinol; and matairesinol, and all of these compounds except isolariciresinol could be enterediol (ED) and enterolactone (EL) precursors (Heinonen et al., 2001). SDG is the main phytoestrogen of flaxseed, and the concentration will differ depending on the flax produt (9-30 mg/g of defatted flax meal; 6.1-13.3 mg/g in whole flaxseeds) (Meagher et al., 1999; Johnsson et al., 2000; Muir and Westcott, 2003; Côrtes et al., 2008).

As a complex, the flax lignans are connected with hydroxymethylglutarate (HMG). SDG, for instance, is linked to this complex by the glucose, through an ester bond (Figure 5). This lignan might not be readily hydrolysed by β-glucosidase, however, β-glucuronidase can readily cleave the sugars, as well as being capable to hydrolyse mammalian lignan conjugates and positively correlate with plant lignan intake and urinary excretion of ED and EL. Although, the results concerning the activity of this enzyme are inconstant and remains unclear if β-glucuronidase is capable of releasing significant amounts of SECO and which sources of enzyme activity is responsible for it release from flaxseed (Jenab and Thompson, 1996; Muir and Westcott, 2003).

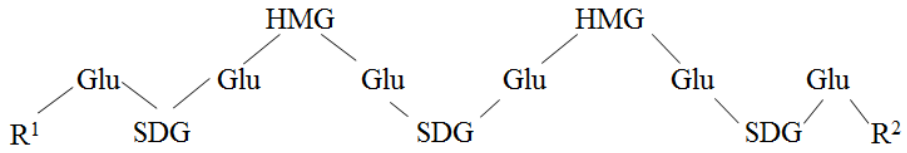

Figure 5. SDG ester-linked via hydroxymethylglutarate (HMG)
SDG: secoisolariciresinol diglucoside; Glu: glucose
Source: The author

Despite of all nutritional benefits of flax products consumption, there are some nutritional compounds present in flaxseed reported as negatively influence in health, as cyanogenic glycosides and linatine, antiopyridoxine factors. However, those substances might be in a risky level considering a daily basis of intake in other foods containing those compounds, but not in flaxseed (Touré and Xueming, 2010).

The enterolignans (ED and EL) are not present in plant material and need to be converted (Figure 6) by the microbial community within the host that is consuming the compounds. The conversion of plant SDG into mammalian lignans in humans is described by the following catalytic reactions: Firstly, SDG passes through a process of O-deglycosylation forming SECO. The next process is O-demethylation, which form the intermediate metabolic 2,3-bis(3,4-dihydroxybenzyl)-butane-1,4 diol. Moreover, a dihydroxylation process takes

place, converting the compound into the enterolignan ED, which in finally converted to EL after a dehydrogenation of the molecule (Wang et al., 2000b; Clavel et al., 2006).

In humans, most of the biological activities of SDG depend upon bacterial transformations occurring in the colon, since dietary plant lignans reaches the colon poorly digested. The first step of the transformation is dependent of gut microbiota, being the removal of the glucose moieties from SDG performed by strains producing β-glucosidases (Quartieri et al., 2016).

The health beneficial effects of the flax intake have been shown with respect to prevention of many diseases, such as cardiovascular disorders (coronary heart disease and cardiovascular disease), diabetes, several types of cancer and others. Since the first isolation of ED and EL, increasing research efforts have been focussed on understanding their mechanisms of action, especially in humans and rat. (Wang, 2002; Peterson et al., 2010). Buck et al. (2010), used meta-analyses based on 24 publications to assess the association between lignans and breast cancer risk. They observed that high plant-lignan intake was associated with a risk decrease for breast cancer. The mechanism of action of phytoestrogens in protection against breast cancer is linked to the attraction of the lignans to estrogen receptors, a hormone which is associated to cellular proliferation, resulting in reduced hormone activity in the presence of antioxidants (Carreau et al., 2008). However, overall, lignans were not significantly inversely linked with breast cancer risk. The effect of lignans on tumour development, regarding estrogen receptors, remains unclear. In summary, consumption of flaxseed and it products are positively associated with reduced breast cancer risk, especially in postmenopausal women (Buck et al., 2010; Lowcock et al., 2013). Although, further studies concerning possible interactions with diverse variation factors might be necessary to explain inconsistence results.

In another review regarding the beneficial effects of flaxseed consumption in animals and human health, Adolphe et al. (2010), observed the same issues concerning comparison between results with different hosts. Animal studies using rat, mice and rabbit models showed that SDG supplementation offers protection against the development of chronic diseases such as cardiovascular diseases, cancer, and diabetes. However, the outcomes of these studies are variable depending on gender, age and host species. Therefore, direct correlations cannot be made between different species regarding the health effects. In addition, it was recently shown that, the phytoestrogen SDG might also have radioprotective properties, as shown by Velalopoulou et al. (2015) in murine lung cells, as SDG demonstrated capability of preventing DNA damage and enhancing the antioxidant capacity

of normal lung cells. Indeed, the health effects of lignans are gradually increasing, and therefore, more studies concerning the intake of antioxidants must be carried out.

I.4 Rumen

I.4.1 Rumen microbiome

The ruminants' competitive advantages over other species on feed digestibility can be widely explained by their ability to digest fibre materials, not because they are efficient in digesting fibre, but rather because they are efficient in hosting a wide range of microorganisms that can do it for them and provide the degradation products (eg. volatile fatty acids) for absorption (Russell et al., 1992).

The rumen metabolism has been explored for more than 100 years (Lindsay, 2006), however, its population diversity and microbial ecosystem was first studied only in the 1950s by Robert Hungate, one of the pioneers on rumen microbiology studies. By means of the anaerobic culture techniques developed, anaerobic ecosystems such as the rumen could then be assessed (Puniya et al., 2015).

Although all advances in material and methods to grow rumen microbes, most of the species (>90%) are yet to be cultured (Flint et al., 2008; Kim et al., 2011). This is due to the unique conditions found in the rumen (Table 4), and once the microbial community (Table 5) is adapted to the physical conditions and nutrients available in the rumen, growing the microbes in *in vitro* conditions is both a challenge and opportunity to advance the knowledge on their biology.

Many rumen microbial populations can gain or lose dominance in the rumen depending on the composition of the diet (Stiverson et al., 2011; Carberry et al., 2012; Huws et al., 2012); (Friedman et al., 2017), age (Jami et al., 2013), host animal breed (King et al., 2011), host animal species (Qi et al., 2011; Baraka, 2012) (eg. muskoxen rumen), feed efficiency (Jami et al., 2014; Shabat et al., 2016), and other factors. However, a core microbiome can be found across individuals within a wide geographical range (Jami and Mizrahi, 2012; Henderson et al., 2015).

Table 4. Rumen ecosystem characteristics

Physical properties	
Dry matter	10 - 18%
Osmolarity	250 - 350 mOsmol/Kg^{-1}
pH	5.5 - 6.9
Redox potential	-350 to -400 mV
Temperature	38 - 41ºC
Chemical properties	
Amino acids and oligopeptides	<1 mmol L^{-1} (2 - 3h post feeding)
Ammonia	2-12 mmol L^{-1}
Dietary (cellulose, hemicelluloses, pectin) component	Always present
Endogenous (mucopolysaccharides)	Always present
Growth factors	Branched-chain fatty acids, long-chain fatty acids, purines, pyrimidines
Lignin	Always present
Minerals	Always present
Non-volatile acids	Lactate <10mmol L^{-1}
Soluble carbohydrates	<1mmol L^{-1} (2 - 3h post feeding)
Trace elements/vitamins	Always present; high B vitamins
Acetate	60-90 mmol L^{-1}
Propionate	15-30 mmol L^{-1}
Butyrate	10-25 mmol L^{-1}
Branched chain and higher	2-5 mmol L^{-1}
CO_2	65%
CH_4	27%
N_2	7%
O_2	0.6%
H_2	0.2%
H_2S	0.1%
CO	0.1%
Microbiological properties	
Bacteria (40-50% of microbial mass)	10^{9-11} g^{-1} (>300 species)
Ciliate protozoa (40-50% of microbial mass)	10^{4-6} g^{-1} (25 genera, 40 species)
Anaerobic fungi (3-4% of microbial mass)	10^{3-5} g^{-1} (6 genera, 30 species)
Archaea (2-3% of microbial mass)	10^{6-8} g^{-1} (6 species)
Bacteriophage (<0.1% of microbial mass)	10^{7-9} g^{-1}

Adapted from McAllister and Cheng (1996) Stewart (1997), Dehority (2003), Castro-Montoya et al. (2011); Puniya et al. (2015).

In addition, Puniya et al. (2015) numbered several other factors affecting rumen bacterial community, such as antibiotic usage, health of the host animal, geographic location, season, photoperiod, stress level, environment, and feeding regimen (intake and frequency). Moreover, the manipulation techniques, such as dietary intervention and use of suitable chemicals, probiotics, and bioactive plant secondary metabolites could be an efficient tool to modify rumen fermentation and enhance the host animal performance, mainly by reducing energy loss by mean of diminishing methane production, and by increasing feed utilization and VFA production by mean of rumen microorganisms selection.

Table 5. Major rumen microbial community

Microbial species	% of total isolates	Secondary substrates[a]	Net product in mixed culture[b]	Nutrient requirement[c]
Hemicellulolytic/Cellulolytic				
Bacteroides succinogenes	5 a 9	ST, P	A, PS, CO_2	VFA, V, NH_3, biotin, PAB
Ruminococcus albus	3 a 5		A, H_2, CO_2	VFA, NH_3, biotin, PAB
Ruminococcus flavefacients	3 a 5		A, PS, H_2, CO_2	VFA, NH_3, biotin, PAB
Fibrobacter succinogenes	n.e			
Eubacterium cellulosolvens	n.e			
Prevotella sp.	50%			
Lipolytic				
Anaerovibrio lipolytica	n.e			
Amylo and Dextrinolytic				
Bacteroides amylophilus	1 a 10	P, PR	A, PS, CO_2	NH_3
Streptococcus bovis	0 a 20	SS, PR	A, L, CO_2(H_2)	AA, biotin
Succinimonas amylolytica	1 a 3		A, PS, CO_2	VFA
Succinivibrio dextrinosolvens	1 a 13	P	A, PS, L, CO_2(H_2)	AA
Saccharolytic				
Bacteroides ruminicola	10 a 19	ST, P, PR	A, P, CO_2(H_2)	VFA
Butyrivibrio fibrisolvens	8 a 12	C, CT, PR	A, B, L, CO_2(H_2)	NH_3, AA, biotin, folic acid, pyridoxal
Megasphera elsdenii	0 a 1	L, PR	A, P, B, V, H_2, CO_2	AA
Selenomonas ruminantium	4 a 12	ST, L	A, P, L, H_2, CO_2	VFA, MET
Succinivibrio sp.	n.e			
Lactobacillus sp.	n.e			
Bifidobacterium sp.	n.e			
Hydrogen utilizers				
Methanobrevibacter sp.	0 a 1		CH_4	VFA, NH_3
Methanobacterium formicicum	n.e			
Vibrio succionogenes	0 a 1		PS, NH_3	NH_3
Protozoa				
Isotricha, Epidinium		ST, SS	A, B, L, H_2, CO_2	
Dasytricha, Diplodinium sp.		ST, SS	A, B, L, H_2, CO_2	(Bacterial cells)
Entodinium sp.		ST	A, P, B, L, CO_2(H_2)	
Bacteriophages				
Methanobacterium phage Ψ (M1, M10, M100, M2)	n.e			
Methanothermobacter phage Ψ M100	n.e			

Source: Adapted from Baldwin and Allison (1983),Pfister et al. (1998),Luo et al. (2001), Sirohi et al. (2012), Kumar et al. (2014)

n.e: not evaluated

[a]Coding for substrates is C for hemicellulose+cellulose, ST for starch, SS for soluble sugars, P for pectin , PR for protein and L for lactate

[b]Coding for products is A for acetate, P for propionate, B for butyrate, V for valerate and longer chained fatty acids and L for lactate. Listing H_2, CO_2, indicates organism has a hydrogenase and procuces hydrogen while CO_2 (H_2) indicates organisms producing formate that is converted to CO_2 + H_2 by another organism. PS codes for succinate that is converted to proprionate and CO_2 by other organisms in mixed culture.

[c]Coding for nutrients is VFA for C_4 – C_5 (branched chain) volatile fatty acids, V for valerate, AA for amino acids, MET for methionine, PAB for para-aminobenzoate.

Bacterial communities may also be specific and adapted to the harbouring environment, as showed by Liu et al. (2015), comparing the compositional differences

between bacterial microbiota associated with the ruminal content, ruminal epithelium and faeces. Significant dissimilarities were observed between all the portions. An abundance of the predominant phyla Bacteroidetes was lower in the ruminal epithelium than in ruminal content, while an abundance of Proteobacteria was observed in the epithelium rather than the ruminal content. The faeces were prominent in abundance of Firmicutes and Verrucomicrobia compared with ruminal content, which contained a greater abundance of Bacteroidetes and Tenericutes. In addition, *Butyrivibrio*, *Campylobacter*, genera *Turicibacter* and *Clostridium* also showed significant difference between the bacterial communities analysed.

According to Lindsay (2006), reviewing the ruminant metabolism studies in a 100 years period, microbial ecology studies have got a massive boost with the introduction and development of molecular biology techniques. The 16S/18S DNA analyses supported the development of phylogenetic base data, allowing the identification of the rumen microbiota members. The molecular biology applied to the rumen studies have resulted in three main lines. First line, linked to cloning techniques, allowing several genes from rumen micro-organisms to be cloned (mainly into *Escherichia coli* cells), being the genes of interest mostly related to polysaccharide degradation enzymes (Kobayashi and Onodera, 1999), such as active beta-glucosidases (Del Pozo et al., 2012), carbohydrate-active enzymes (Wang et al., 2013), lignocellulosic enzymes (Colombo et al., 2016), and others. Secondly, the analysis of rumen species, enabling precise identification and quantification of the microbes present in the rumen. Results can be shared between researchers in online databases, such as the Hungate 1000, a catalogue of reference genomes from the rumen microbiome (http://genome.jgi.doe.gov/TheHunmicrobiome/TheHunmicrobiome.info.html). This information is essential for the development of novel primers for polymerase chain reaction (PCR) assays. The third line of study is the introduction of enzymes into rumen microbes, in order to transform them, aiming to enhance rates of digestion (Lindsay, 2006).

Real time PCR (qPCR) is still the main method of identification of rumen bacteria. Many pairs of primers have been published to detect rumen microbes. For instance: *Anaerovibrio lipolytica*, *Butyrivibrio fibrisolvens*, *Eubacterium ruminantium*, *Prevotella albenbacter*, *P. brevis*, *P. bryantii*, *P. ruminicola*, *Ruminobacter amylophilus*, *Selenomonas ruminantium*, *Streptococcus bovis*, *Succinivibrio dextrinisolvens*, *Treponema bryantii*, Genus *Prevotella*. In addition, methanogens have also been isolated and quantified using qPCR assays (Zhou et al., 2010; Carberry et al., 2012; Li et al., 2012) showing some microorganism that can be identified and quantified in the rumen followed by primers and product size (Table 6).

Table 6. Rumen microbes identified by means of qPCR

Target taxon	rRNA	Primer (5´-3´) Forward	Reverse	Product size (bp)
16S V3	16S	CCTACGGGAGGCAGCAG	ATTACCGCGGCTGCTGG	194
Entodinium	18S	GAGCTAATACATGCTAAGGC	CCCTCACTACAATCGAGATTTAAGG	317
Fibrobacter succinogenes	16S	GTTCGGAATTACTGGGCGTAAA	CGCCTGCCCCTGAACTATC	121
Anaerobic fungi	18S	GAGGAAGTAAAAGTCGTAACAAGGTTTC	CAAATTCACAAAGGGTAGGATGATT	120
Prevotella spp.	16S	GGTTCTGAGAGGAAGGTCCCC	TCCTGCACGCTACTTGGCTG	121
Prevotella brevis	16S	GGTTTCCTTGAGTGTATTCGACGTC	CTTTCGCTTGGCCGCTG	219
Protozoa	18S	GCTTTCGWTGGTAGTGTATT	CTTGCCCTCYAATCGTWCT	223
Ruminococcus albus	16S	TGTTAACAGAGGGAAGCAAAGCA	TGCAGCCTACAATCCGAACTAA	75
Ruminococcus flavefaciens	16S	CGAACGGAGATAATTTGAGTTTACTTAGG	CGGTCTCTGTATGTTATGAGGTATTACC	132

Source: Adapted from Carberry et al. (2012)

Although efficient, qPCR is still limited in terms of rumen prospection for phylogenetic analysis of microbial community, especially regarding the low approach range, and that is why other techniques had to be developed to keep advancing the knowledge in the rumen microbial population and their biology. The NGS sequencing techniques allow the development of those analyses, linked to the discovery of novel enzymes, especially on full-length reads. The approach of small-subunit ribosomal RNA gene (16S rRNA gene) for studies in microbial ecology has been vastly changed by next generation sequencing technologies. The main goal is to minimize the disadvantages of PCR-based estimates of microbial diversity, and take a central role in studies of microbial ecology, especially with regard to culture-independent methods based on molecular phylogenies of the 16S rRNA gene (Myer et al. 2016).

I.4.2 Rumen microbiome contribution to the antioxidant content of ruminant products

Rumen microorganisms can efficiently convert SDG to SECO, then to enterolignans (ED, and then to EL) (Côrtes et al., 2008; Petit and Gagnon, 2009a, b; Zhou et al., 2009; Schogor et al., 2014; Lima et al., 2016). Zhou et al. (2009), infused 1mg/kg body weight SDG into the rumen of goats and reported an increase in the level of SDG, ED and EL in both rumen fluid and serum. Moreover, Côrtes et al., (2008) demonstrated that, after ruminal fluid and faecal incubation of flaxseed products, the lignans were converted by the microorganisms present in the inoculum. The activity of β-glucuronidase has also been reported in dairy cows, with five-fold higher activity in faecal material than in rumen fluid, suggesting that this enzyme might play a more effective role of deconjugation on the intestinal level than in the rumen (Gagnon et al., 2009). Moreover, supplementation with flax hulls in diet might increase faecal β-glucuronidase activity (Côrtes et al., 2013). However, when introduced directly in the rumen, flax hulls might have no effect on faecal β-glucuronidase activity (Gagnon et al., 2009), indicating that the intake of the flax hulls and chewing movements may contribute to greater breakdown of seed coat and greater release of flax lignans from seeds.

The gastrointestinal environment can also cause variation in β-glucuronidase activity. Gagnon et al. (2009) showed that flax oil supplementation in the rumen caused a decrease in the microbial β-glucuronidase, indicating a negative effect of the n-3 fatty acids on the enzyme activity. Furthermore, when introduced in the small intestine, by means of abomasal flax oil infusion, β-glucuronidase activity also decrease, however, with no effect on the absorption of EL. In addition, the lower activity of the enzyme in the rumen might not interfere on the conversion of flax lignans into EL in the rumen (Côrtes et al., 2013). Although, Lima et al. (2016), also supplied flaxseed oil through abomasal infusion, and conversely showed no effect on microbial activity of β-glucuronidase in ruminal fluid and faeces and EL concentrations, suggesting that n-3 fatty acids did not interfere with flax lignans breakdown and absorption of mammalian lignans in dairy cows.

The environment of the rumen may also be affected by exposure of lignans. Changes in the ruminal metabolism might occur due to SDG exposure for long periods, such as decreases in pH value and NH_3-N (ammonia–N), increase of total volatile fatty acids and ratio of acetate and proprionate, and enhancement of rumen bacterial protein synthesis and microbial crude protein, as well as possible changes in the microbial community (Zhou et al., 2009; Côrtes et al., 2013). However, a recent study (Lima et al., 2016) showed that dietary

flax meal did not affect ruminal ammonia N and total VFA, although a small reduction on molar proportion of acetate was observed, as well as, significant increase of propionate and decrease of isovalerate. In addition, the acetate to proprionate ratio was lower in cows fed flax meal than in those fed the control diet. Consequently, SDG may facilitate the anabolic metabolism of carbohydrates and alter the fermentation pattern, therefore, studies regarding the effects of lignans in the rumen may present inconstant results, depending on the period of exposition of lignans to the ruminal microbiota, or the methods of incubation (in vivo or in vitro), demanding attention in data comparison.

According to Zhou et al. (2009), the SDG may be released gradually, since the concentration in the rumen fluid increased slowly after SDG infusion. A possible reason for this observation is due the SDG being part of a macromolecule in which it is connected through the linker molecule hydroxymethyl glutaric acid (HMGA). In addition, the same study illustrated denaturing gradient gel electrophoresis (DGGE) of polymerase chain reaction (PCR) products from rumen samples of goats being treated with SDG for different period of time (7 and 14 days), showing that the ruminal community might change composition depending on the exposure time to the plant lignan. Those results showed that SDG supplement stimulates the growth of *Ruminococcus gnavus*, bacteria which activity in the rumen is linked to glucuronidase. Therefore, different incubation time might be considered when comparing lignans conversion results.

Studies concerning the elucidation of the microorganisms and reactions linked to the plants lignans conversion processes are also necessary to understand the metabolism of those compounds and to broaden knowledge in the area. Wang et al. (2000b) in a study investigating the intestinal human microflora, in order to spot the bacteria connected to each process, demonstrated that in the first conversion process of O-deglycosylation (SDG to SECO), the microorganisms involved were: *Bacteroides distasonis*, *Bacteroides fragilis*, *Bacteroides ovatus*, *Clostridium cocleatum*, *Clostridium ramosum* and *Clostridium saccharogumia*. Moreover, in the O-demethylation process (SECO to 2,3-bis(3,4-dihydroxybenzyl)-butane-1,4 diol or didemethyl-SECO), the following bacteria were involved: *Eubacterium limosum* and *Blautia* spp. Furthermore, the dihydroxylation process (didemethyl-SECO to ED) was linked to the activity of *Eggerthella lenta*, and finally, the dehydrogenation (ED to EL) was attributed to the *Lactonifactor longoviformis* activity. In a similar study, Clavel et al. (2006), showed high initial rate of SDG O-deglycosylation for the strain *Clostridium* sp. SDGMt85-3Db, followed by O-demethylation activity in *Peptostreptococcus productus*, demethylating the lignans pinoresinol, lariciresinol and

matairesinol, and finally, *E. lenta* catalysing the reduction of pinoresinol and lariciresinol. Zhu et al. (2014), used in vitro assays with human intestinal bacteria and observed conversion activity of SDG into ED. The strains implicated in the conversion were *Bacteroides uniformis*, *Eubacterium limosum* and *E. lenta*. *Eubacterium limosum* species was observed to possess the activities of O-demethylation, while *E. lenta* had the activities of dihydroxylation. Schogor et al. (2014) also assessed the effects of inclusion of flax meal in the diet of dairy cows on the ruminal microbiota using terminal restriction fragment length polymorphisms (T-RFLP), PCR-DGGE and Q-PCR. DGGE bands potentially linked with SDG conversion to SECO, ED and EL were sequenced. This study (Figure 6) demonstrated that *Prevotella* spp. (*Prevotella bryantii, Prevotella albensi, Prevotella ruminicola, Prevotella brevis*), have a high potential activity in SDG conversion in SECO, as well as other bacteria (*Peptostreptococcus anaerobius, Butyrivibrio fibrosolvens, Fibrobacter succinogens, Ruminococcus albus, Eubacterium ruminantium, Butyrivibrio proteoclasticus, Ruminococcus flavefaciens*) having the same capacity, however, with lower conversion level. Moreover, through taxonomic identification (DGGE bands), considering a nearest match (accession number, maximum % sequence similarity) and Ribosomal Database Project Classification (Cole et al., 2005), microorganisms potentially associated with EL production in ruminal fluid, were identified as being: genus *Prevotella*, *Succinivibrio*, *Fibrobacter*, *Anaerovorax*, unclassified Succinivibrionaceae, unclassified ‘‘Lachnospiraceae’’, unclassified Alphaproteobacteria, unclassified ‘‘Bacteroidales’’, unclassified ‘‘Prevotellaceae’’. Most matched to as yet uncultured rumen bacteria, showing that metagenomic approaches might be a reliable tool to provide elucidation of the microorganisms involved in metabolic processes, considering the limitation of pure culture assays. Even though culture-based studies should continue identifying novel species linked to lignans metabolism, as demonstrated recently by Gaya et al. (2017), showing *Bifidobacterium adolescentis* INIA P784 as the first probiotic bacterium capable of producing enterodiol from lignan extracts.

In livestock, supplementation with antioxidants is intensively studied for its potential to increase defensive system of the animal and improving the content of antioxidants in final products (e.g. beef and milk), which offers a product with beneficial properties for human health, as well as, increasing shelf life. α-tocopherol is one of the most studied antioxidants in ruminants, and has been shown to be effective in its purpose. Vitamin E is used to increase blood concentration for improvement in metabolic processes and for reducing lipid oxidation in the final product. In beef cattle, vitamin E supplementation can improve the utilization efficiency of dietary nitrogen (Wei et al., 2016), and increase vitamin E content in beef,

providing lipid oxidation protection (Gobert et al., 2010). In addition, in dairy cows' vitamin E content in plasma and milk might also be increased by vitamin E supplementation (Lindqvist et al., 2011). Therefore, with a similar premise, the supplementation with flax products to increase lignans levels in blood and milk have also been target of study, since the presence of SDG, SECO, ED and EL might offer higher antioxidant capacity (lower amount of oxygen species generated) compared to vitamin E (1.27, 4.86, 5.02 and 4.35%, respectively) (Prasad, 2000). Indeed, recent published data shows that supplementation of flax product in the diet of dairy cows effectively increase the EL concentration in milk (Matumoto-Pintro et al., 2011; Côrtes et al., 2012; Côrtes et al., 2013; Lima et al., 2016).

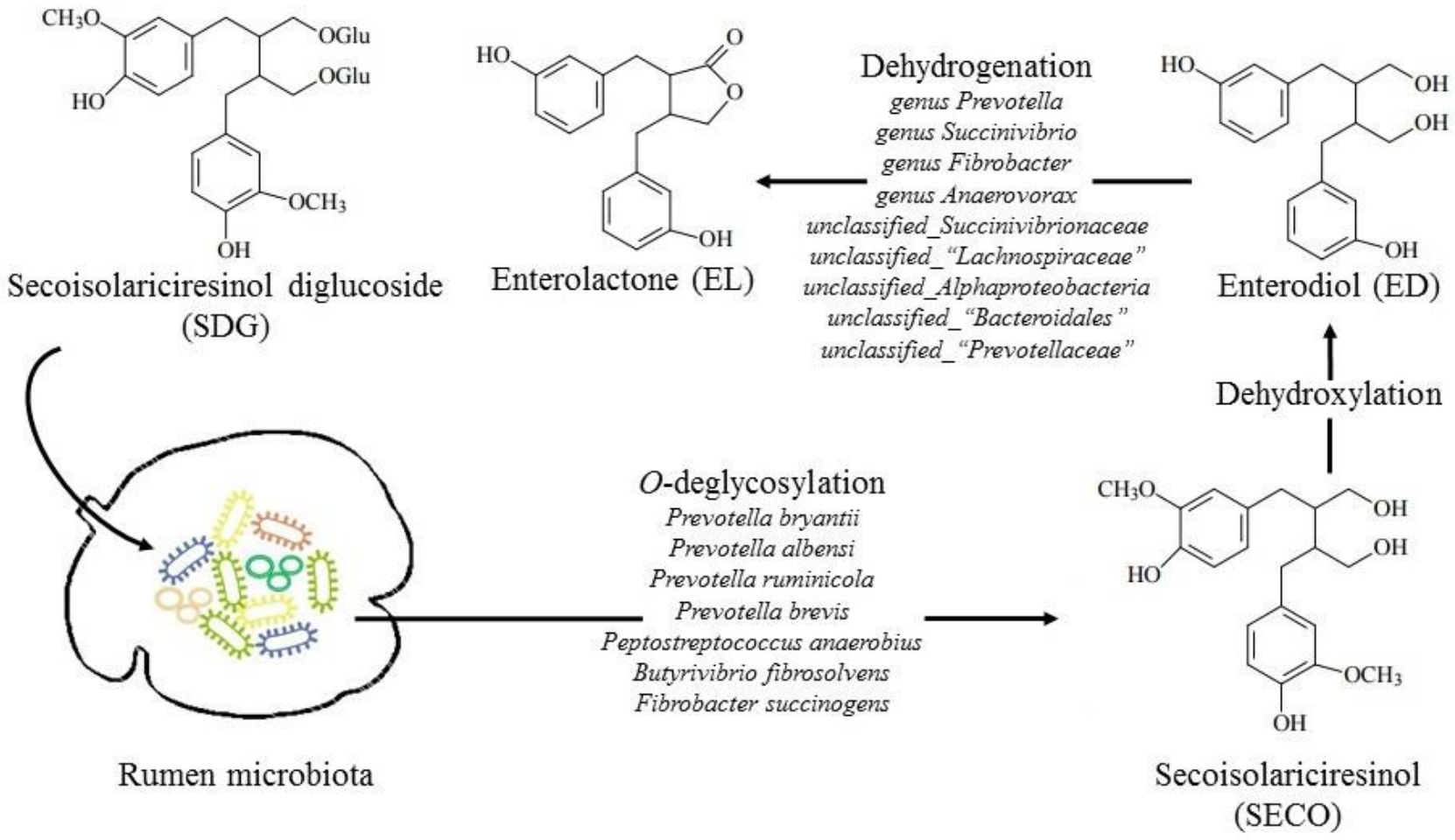

Figure 6. Selected pure cultures associated with conversion of secoisolariciresinol diglucoside (SDG) into secoisolariciresinol (SECO) and taxonomic identification (DGGE bands) potentially associated with enterolactone production in ruminal fluid. Adapted from Wang et al. (2000b) and Schogor et al. (2014).

Matumoto-Pintro et al. (2011) also showed that the use of synthetic and purified EL added to milk (0.20mg EL/kg) significantly protected the product against lipid oxidation, which was not observed when increasing the mammalian lignan in milk (0.18 mg EL/kg) by means of up to 15% of flax meal supplementation. Moreover, Côrtes et al. (2012), supplementing 15.9% flax hulls in the diet (dry matter), observed EL concentration in milk of 0.39, 0.35 and 0.33 µmol/l after three level of infusion of flax oil in the abomasum (0, 250 and 500 g/d, respectively), where the abomasal infusion was not a variation factor. Enterolignans antioxidant activity also improve physiological indexes in ruminants. For instance, it was observed in recent published data, the positive correlation between intrafollicular EL concentrations and estradiol concentrations in preovulatory follicles in dairy cows (Zachut, 2015). Hence, supplementation with flax antioxidants have been proven as an effective tool with the potential of enhancing the milk quality and physiological processes in ruminants, nonetheless, the knowledge surrounding this application should keep advancing.

Enterolactone might also have other very useful appliance, regarding the ability to reduce the transfer of drugs (e.g. antibiotics) into milk. The mechanism is connected to the potential linkage of EL and ATP-binding cassete trasporter G2 (ABCG2) (Miguel et al., 2014), being the G2 a transporter member of the ABC protein superfamily, with representative expression in mammary gland and connected to the active secretion of some endogenous and exogenous substances (van Herwaarden et al., 2007). Otero et al. (2016), demonstrated that enterolactone inhibited in vitro ABCG2-mediated transport of the drug mitoxantrone, as a model substrate. In addition, the same study assessed the effects of the Y581S polymorphism of the ABCG2, showing that this genetic variant affects positively the accumulation of antioxidants as EL, and thus, demonstrating a potential tool for improvement of lignans content in milk. Therefore, is irrefutable that several factors might interfere in milk proprieties, and should be consider for further approaches to enhance milk quality.

Therefore, supplementation of flaxseed and flaxseed products in livestock can be used as strategy in order to increase mammalian lignans levels of animal products as the compounds are effectively transfer to physiological fluids. Flaxseed, flaxseed meal or clover grass silages supplementation in dairy cows have been shown to increase EL concentration in ruminal fluid, plasma, urine and milk (Steinshamn et al., 2008; Gagnon et al., 2009; Petit et al., 2009; Lima et al., 2016). For instance, Matumoto-Pintro et al. (2011), supplying up to 15% of flax meal to cows increased EL concentration in milk to 0.18 mg/kg.

I.5 Rumen meta-omics

Microbial cells are well spread in all kinds of environments on Earth, being mainly prokaryotes individuals and most of them uncultured species. Therefore, the meta-omics technologies (metagenomics, metatranscriptomics, metaproteomics and metabolomics), are a powerful tool to bypass the need for isolation or cultivation in laboratory conditions is essential to compare and explore the ecology of microorganisms (Handelsman, 2004; Ferrer et al., 2009; Simon and Daniel, 2011). Direct isolation of nucleic acids from environmental samples is followed by metagenomic approaches, but the assay will depend on final aim of the gene mining (Figure 7).

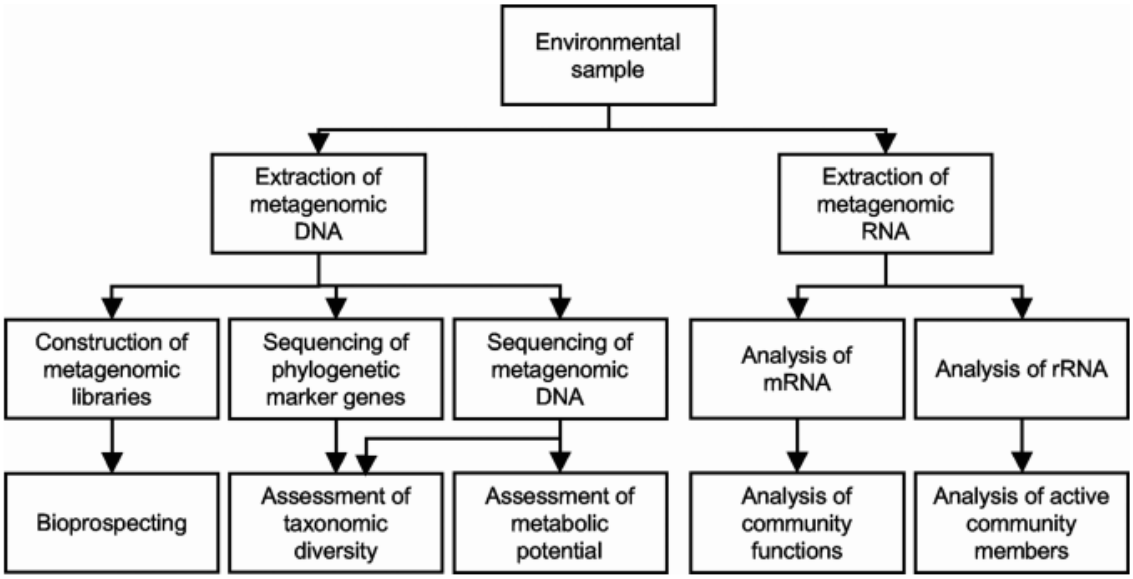

Figure 7. Metagenomic analysis of environmental microbial communities based on nucleic acids.

Source: Simon and Daniel (2011)

The metabolic and functional capacity of a microbial community can be assessed by means of metagenomics (DNA-based), metaproteomics and metabolomics. However, DNA-based analyses cannot differentiate between expressed and non-expressed genes, and therefore, do not reflect the actual community metabolic activity, which can be assessed by metatranscriptomics (RNA-based). Moreover, the community immediate catalytic potential is assessed by metaproteomics (Shrivastava. et al., 2015).

NGS techniques are becoming more affordable methods overtime, allowing large-scale analysis of metagenomics studies, such as comparative metagenomics,

metatranscriptomics and mass spectrometry (Chistoserdova, 2010; Grada and Weinbrecht, 2013).

Microbial communities can be assessed based on nucleic acids from several different environments, such as soil (Delmont et al., 2011), groundwater (Uchiyama et al., 2005), hotspring (Rhee et al., 2005), glacier ice (Simon et al., 2009), buffalo rumen (Duan et al., 2009), deer rumen (Jarvis et al., 1999) sheep rumen (Hobson and Mann, 1961; Prins et al., 1975; Hazlewood and Dawson, 1979), cattle rumen (Ferrer et al., 2005; Cirne et al., 2006; Hess et al., 2011), human gut (Glasner, 2017) and many others. In addition, from the microbial environmental communities, different approaches (e.g bioprospecting, assessment of taxonomic diversity, assessment of metabolic potential, analysis of community functions, analysis of active community members) can be carried out by extracting and isolating DNA or RNA.

Bioprospecting environmental samples regard the construction of metagenomic libraries, which comprise the cloning of environmental small-insert or large-insert DNA into a bacterial host (most cases *Escherichia coli)* for further screening (Simon and Daniel, 2010). The two main approaches to recover novel biomolecules are: function-based, which do not necessarily requires sequence information prior to DNA insertion, can be applied as activity-driven screen with targeted genes encoding a bacterial enzyme in the host cell (e.g cloning bacterial β-D-glucuronidases in *E. coli* cells deficient in this enzyme (Gloux et al., 2011)); and sequence-based screening, which comprise the design of DNA probes or primers derived from already-known genes (Handelsman, 2004; Daniel, 2005; Ferrer et al., 2009; Simon and Daniel, 2011).

Assessment of taxonomic diversity studies are based on conserved marker genes, such as 16S rRNA genes and data-bases of reference sequences can provide rRNA gene-based classification information to assess the microbiota diversity. The taxonomic diversity studies can be carried out in large scale due to the introduction of NGS platforms, such as the Genome Analyzer of Illumina (Bentley, 2006). In addition, the novel technologies allowed a greater depth on the analysis, for example, shotgun sequencing (direct sequencing of metagenomic DNA) and pyrosequencing of 16S rRNA gene amplicons compared to other assays such as DGGE, T-RFLP, or Sanger sequening (Simon and Daniel, 2011). Moreover, following the sequencing data, the microorganisms are clustered in phylogenetic groups (binning step), based on their taxonomic origins (ranging from the kingdom to the genus level). Sequence homology are then assessed by similarity-based approaches to classify DNA fragments by means of search on reference database and use of tools such as Basic Local

Alignment Search Tool (BLAST) and similarity-based binning tools (Simon and Daniel, 2011). Although, the major limitation for taxonomic diversity is that the analysis relies on reference sequences, making the result biased toward already known and cultivable microorganisms, representing only up to 10% of an ecosystem diversity, making most of the metagenomic data set as unidentified due to lack of reference sequences (Huson et al., 2009).

Metagenomics are indeed a reliable tool to explore a microbiome, however, it still have some limitations, which can be supressed by use of metatranscriptomic approaches, which has distinct characteristics when compared to metagenomics (Simon and Daniel, 2011). Briefly, the metatranscriptomics analysis identifies most transcribed genes, whereas metagenomics identifies the most dominant genes. The main issue is that a numerically prevalent group of microbes not always play active role in some metabolism activity. For instance, as much as 60% of total bacteria in rumen are represented by *Prevotella* sp., however, this group of bacteria play no active role in recalcitrant cellulose digestion (Stevenson and Weimer, 2007; Kong et al., 2010; Purushe et al., 2010). In addition, the prevalence is variable between ruminant species as well and each metatranscriptome may vary in the community microbes linked to the transcript genes (eg. muskoxen rumen (Figure 8)).

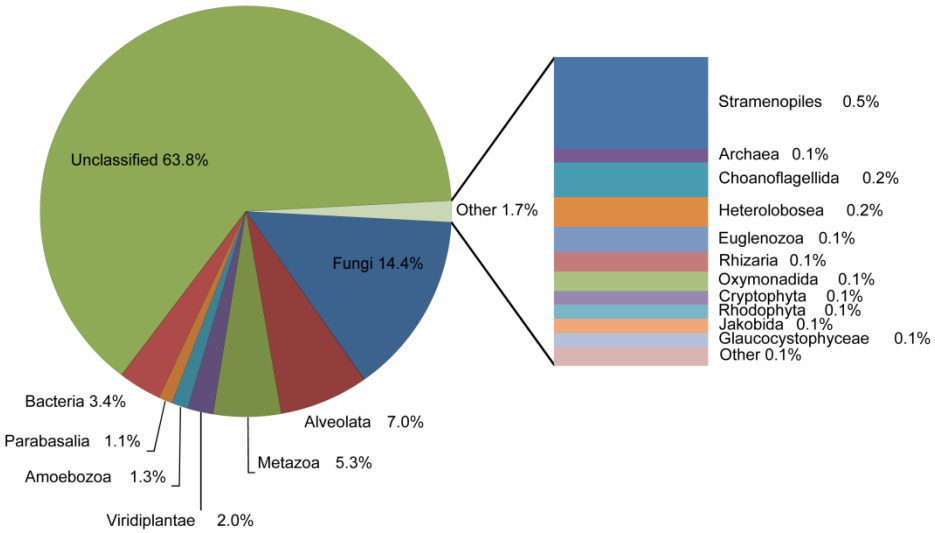

Figure 8. Phylogenetic distribution of muskoxen rumen metatranscriptome putative protein encoding reads. The percentages of the major phylogenetic groups were indicated.
Source: Qi et al. (2011).

Successful rumen metatranscriptome has proven that it is a reliable tool to explore the rumen in novel relevant genes discovery. Reverse transcription on glycoside hydrolases from celF, xynD, and cel3 from *Fibrobacter succinogenes* S85 in cow rumen (Wang et al., 2011), and exploring degradation patterns, such as plant cell wall polysaccharide in cow rumen (Dai et al., 2015) and genes coding for potentially valuable lignocellulolytic enzymes in muskoxen rumen (Qi et al., 2011).

One of the fastest-developing research areas at the moment, meta-omics technologies have, in addition to improved DNA isolation methods, cloning strategies, and screening techniques, allowed the assessment and exploiting of various microbial environments with great potential to novel enzymes and functions findings, such as the rumen microbial community. Those finding can then be explored to commercial application, like many enzymes from various animals with commercial relevance (Table 7).

Table 7. Enzymes from different animal sources and their commercial application

Enzyme	Source	Application
Aldolases	Liver and muscle	Fructose digestion
Alkaline phosphatase	Calf intestine/kidney	Diagnostic (indicator in ELISA)
Ancrod	Snake venom	Anticoagulant
Catalase	Liver	Food industry
Chymotrypsin	Pancreas	Leather industry
Lipase	Pancreas	Food industry
Pepsin	Porcine stomach	Body fortifying agents
Proteases	Bovine and porcine pancreas	Digestive enzymes, anti-inflammatory agents, health food additives
Rennet (chymosin)	Ruminant abomasum	Cheese manufacture
Trypsin	Pancreas	Leather industry
Urokinase	Urine	Thrombolytic agent

Soure: Adapted from Puniya et al., (2015)

I.5.1 Rumen plasmidome

Plasmids are extrachromosomal (Figure 9) circular double-stranded DNA molecules, linked to enhancement of fitness and changes in microbial population. They are key elements in the dissemination of a variety of traits by means of their mobilization between different hosts, providing advantages to adverse environments, being essential to the survival of the organisms, especially prokaryotic cells. The plasmids are self-replicating, which means that they can replicate independently of chromosomal DNA (Funnell and Phillips, 2004).

These DNA molecules are present in both Gram-Positive and Gram-Negative bacteria, and sometimes present in archaea and eukaryotic organism (Kado, 1998). They are an essential factor in microbial population change, once they can propagate virulence, antimicrobial agents resistance, enhanced fitness, ability of metabolism of substances, and others. Plasmid, therefore, can carry genes that provide some survival benefit to the host organism, and this lateral gene transfer, can strongly drive the evolution of a species in microbioal environments (Kado, 1998; Funnell and Phillips, 2004; Koonin and Wolf, 2008).

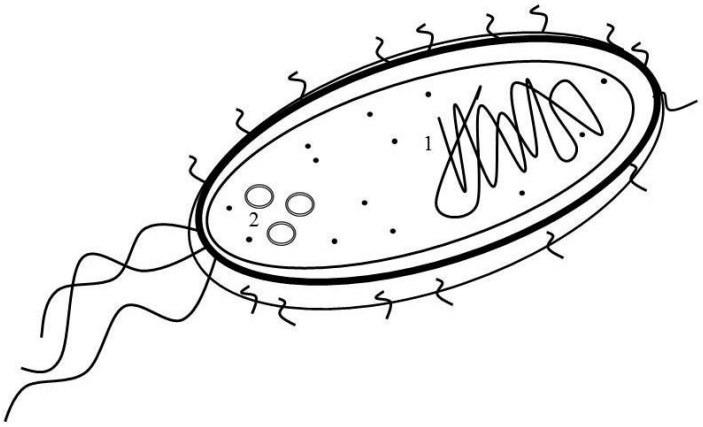

Figure 9. Bacteria genetic material, [1]chromosomal DNA and [2]plasmid DNA, not to scale.
Source: The author

Briefly, the characteristic of the independent replication of plasmid is duo to a stretch of DNA that can act as origin of replication. The structure of the origin of replication (typically assigned names containing "ori") varies between species, but they share some common properties, as high AT content (adenine and thymine are easier to separate than guanine and cytosine) (Yakovchuk et al., 2006). The origin of replication binds a complex (pre-replication), protein complex that recognizes, unwinds, and begins the copy of DNA. The segment of DNA that is copied starting from each unique replication origin is called a replicon (Funnell and Phillips, 2004).

Not all plasmids have the same characteristic, for instance, a wide range of plasmid sizes can be found, among those, the ones that are relatively small (300-400bp) usually do not contain open reading frames (ORFs), not coding for proteins. Although, those might contain a replication origin for plasmids (Kado, 1998).

I.5.2 Fosmid libraries

Metagenomic fosmid libraries can be used to access plasmid DNA information from the rumen bacteria, including the uncultivated which are in greater diversity compared to the cultivated, and thus, prospect new enzymes.

DNA has been isolated from several microbial communities in many different environments, such as soil and sea water, due to their microbial concentration (5,000-40,000 species/g soil), and diversity in biological activities and products (Daniel, 2005; DeLong, 2005). In addition, studies have been focusing also in the animals' microbiota as well, with novel biocatalysts been discovered (Quartieri et al., 2016). Many studies aimed to assess the animals gut microbiota (Frank and Pace, 2008). The rumen, one of the most complex natural environments has also been the target of gene prospection studies. Metagenomic datasets can provide novel, and even unexpected insights into community structure and function. The animal-associated communities studies, for example, resulted in many genes/molecules been discovered, such as hydrolases, laccases, and xylanases, antibiotic resistance genes, and inter/intraspecies communication molecules (Healy et al., 1995; Diaz-Torres et al., 2006; Guan et al., 2007).

Direct cloning from environmental DNA was first proposed by Pace et al. (1985). However, the first successful function-driven screening of metagenomic libraries was conducted only a decade later (Healy et al., 1995). The technique is rapidly been improved and used in large-scale in order to assess novel biomolecules (Handelsman, 2004; Daniel, 2005; Ferrer et al., 2009; Steele et al., 2009; Simon and Daniel, 2010).

E. coli is the most used species as recombinant host bacteria in metagenomic libraries. The reasons why include: culture in cheap media and reduction of protease activity. Moreover, *E. coli* can accumulate heterologous proteins up to 50% of its dry cell weight. (Williams, 2007). On the other hand, *E. coli* function-based screening can be problematic owing to insufficient expression of foreign genes (Uchiyama and Miyazaki, 2009). However, only 40% of the enzymatic activities in an bacterial community assessed may be detected by random cloning using *E. coli* cells (Gabor et al., 2004). In order to expand the range of

detectable activities in the community screening different species can be employed as host bacteria, such as *Streptomyces spp*. (Wang et al., 2000a), *Thermus thermophiles* (Angelov et al., 2009), *Sulfolobus solfataricus* (Albers et al., 2006), and various Proteobacteria (Craig et al., 2010).

According to Simon and Daniel (2011), metagenomic libraries can be constructed using different cell components, depending on the desired insert size. Can be used as vectors: bacterial artificial chromosomes (40 kb); fosmids and cosmids (up to 40kb); and plasmids (up to 15 kb). The choice of the vector is driven by the DNA quality, targeted genes, and screening strategy. Small-insert libraries are recommended for identification of single gene or small operon novel biocatalysts, and large-insert libraries are recommended for recover complex pathways from large gene clusters (Daniel, 2005).

Library construction, screening and sequencing are followed by different approaches, such as genome hunting and data mining. The first, based on prospecting for ORFs in the genome and sequences annotated as putative enzymes are then conducted to cloning, over-expression and activity screening assays. On the other hand, data mining is based on homology alignment between sequences find in databases. Using bioinformatics tools such as BLAST, conserved regions are identified for further characterization (Adrio and Demain, 2014; Liu et al., 2015).

Fosmid libraries can be efficient tools to prospect novel enzymes. As showed by Colombo et al. (2016), applying functional metagenomics to search for novel lignocellulosic enzymes in a microbial consortium derived from a thermophilic composting phase of sugarcane bagasse and cow manure. From a fosmid library, 182 clones had the ability to hydrolyse carbohydrate (9 xylanases, 9 cellulases). Sequencing of 30 fosmids resulted in 12 contigs encoding 34 putative carbohydrate-active enzymes belonging to 17 glycosyl hydrolase families. One third of the putative proteins belong to the glycosyl hydrolase 3 family, which includes β-glucosidase enzymes known to be important in the cellulose deconstruction. In addition, metagenomic libraries have resulted in many other novel biocatalysts identification, such as cellulases (Healy et al., 1995; Duan et al., 2009), chitinases (Hjort et al., 2009), DNA polymerases (Simon et al., 2009), proteases (Waschkowitz et al., 2009), antibiotics (Riesenfeld et al., 2004).

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II. GENERAL OBJECTIVES

Unculturable rumen species are a bottleneck in rumen prospection for novel genes, therefore, metagenomic approaches must be used in order to access to genomic information regarding rumen metabolism.

To prospect genes and enzymes from the rumen microbiota linked to SDG breakdown a fosmid library was constructed using *Escherichia coli* as bacterial host and DNA inserts from the rumen microbiome and SDG lignan metabolism was screened.

Moreover, the transcripts and differentially expressed genes linked to SDG metabolism were assessed by means of the isolation of RNA from SDG and rumen fluid incubations.

III. Assessing secoisolariciresinol diglucoside metabolism in the rumen by means of a fosmid library

(Normas: Environmental Microbiology)

SUMMARY

Secoisolariciresinol diglucoside, a lignan found in many plants, predominantly in flaxseed. The plant lignan can play active role enhancing antioxidant capacity, however, it need to be metabolized into enterolignans, process which the gut microbiota is responsible. The rumen is a known efficient environment for lignan metabolism, which comprises the breakdown of SDG into secoisolariciresinol, then to enterodiol and enterolactone. The product enterolactone increases concentration in the rumen as flax is added in the diet of ruminants and the compound can be transferred to milk, benefiting human health by the functional food intake, preventing many oxidative-related disorders such as cardiovascular diseases, atherosclerosis, Alzheimer's disease, breast-cancer and others. Although the knowledge on lignans metabolism in the rumen, little information is available regarding the microorganisms linked to secoisolariciresinol diglucoside breakdown. The main bottleneck is that just few species of the rumen microbiome can be cultured in laboratory conditions, and thus, metagenomic approaches are needed to access the rumen ecology by-passing the *in vitro* culture. Therefore, a fosmid library was constructed using rumen microbes DNA inserts from the rumen of cows fed up to 15g/100g (dry matter basis) of flax meal, resulting on an 11,520 fosmid clones library, using *Escherichia coli* strain EPI300 as host bacteria. The library was screened for secoisolariciresinol diglucoside breakdown capacity using high-performance liquid chromatography and positive clones followed to plasmid DNA purification, deep sequencing and bioinformatic data analysis. Two out of 30 library plates showed effective secoisolariciresinol diglucoside breakdown, however, no metabolism products were identified and no putative gene linked to direct secoisolariciresinol diglucoside breakdown was identified from clones genome. To our knowledge this is the first fosmid library constructed to prospect rumen microorganisms with active role in plant lignans metabolism, thus, more studies regarding the subject must be carried out to broaden genes and enzymes involved in the process.

Key-words: lignans, metagenomic, ruminants, ruminal microbes

RUNNING TITLE: SDG breakdown prospected by a fosmid library

INTRODUCTION

Oxidative disorders, such as Alzheimer's disease, Parkinson's disease, cardiovascular disease, atherosclerosis and others may be caused by an imbalance between reactive oxygen species (ROS) formation and its control (Gella and Durany, 2009; Münzel et al., 2010; Hwang, 2013; Rajendran et al., 2014). Plant antioxidants are highly recommended to prevent oxidative-related diseases, and between them, the lignans, found in great concentration in plants such as flaxseed (*Linum usitatissimum*) (Choo et al., 2007; Goyal et al., 2014). The plant phytoestrogenic compounds, such as secoisolariciresinol diglucoside (SDG) and secoisolariciresinol (SECO) can be metabolized into enterolignans, such as enterodiol (ED) and enterolactone (EL) by the consumer gut microbiota implying in the formation of more effective antioxidant compounds (Wang et al., 2000; Clavel et al., 2006). Lignans have been proven as powerful ROS scavenger lowering lipid peroxidation, reducing deoxyribose oxidation, DNA strand breakage and other oxidation damages (Kitts et al., 1999; Matumoto-Pintro et al., 2011), due to lignans antiestrogenic, antioxidant, anti-inflammatory, anticarcinogenic, and cardioprotective activities (Adolphe et al., 2010; Imran et al., 2015). Furthermore, with oxidative control capacity up to 5% higher than compounds such as vitamin E (α-tocopherol), known by its exceptional antioxidant ability (Prasad, 2000). In addition, the phytoestrogens can also prevent oestrogen-induced cancers, such as breast cancer (Buck et al., 2010; Lowcock et al., 2013).

Lignans can also be found in ruminants products, such as milk, improving the beneficial effects of its consumption and improving the animals health status. Flaxseed supplementation in ruminants have been shown to increase enterolactone concentration in the rumen fluid, plasma, urine and milk (Gagnon et al., 2009; Petit and Gagnon, 2009a; Petit et al., 2009; Matumoto-Pintro et al., 2011; Lima et al., 2016). In addition, as showed by Ghedini et al. (2017), the mammalian lignan EL is absorbed by new-borns fed enterolatone-enriched milk, resulting in high plasma concentration of the enterolignans. Therefore, human-specific studies are challenging due to the limitations of using animals as model for humans, however, based on the pharmacokinetics data of EL, humans should consume EL-enriched milk in combination with other lignan-rich sources to enhance potential health benefits (Ghedini et al., 2017).

The rumen microbiome is such a complex community which can provide an enormous range of novel discoveries, such as cellulases (Dai et al., 2015), lipases/esterases (Privé et al., 2015), antimicrobial peptides (Oyama et al., 2017), and others. Rumen

microorganisms can efficiently convert SDG to SECO, then to enterolignans (ED, and then to EL) (Côrtes et al., 2008; Petit and Gagnon, 2009a, b; Zhou et al., 2009; Schogor et al., 2014; Lima et al., 2016). It is been proven that the rumen environment is efficient in converting plant lignans into enterolignans, however, the microbes responsible for the conversion and the genes/enzymes activated to carry the breakdown are not clear yet. The main bottle neck regarding the rumen community ecology is that it cannot be mimic efficiently on laboratory conditions (in vitro) for most of the species (>90%) (Flint et al., 2008; Kim et al., 2011). Therefore, the meta-omics technologies (metagenomics, metatranscriptomics, metabolomics and metaproteomics), are powerful tools to bypass the need for isolation or cultivation in laboratory conditions (Ferrer et al., 2009; Simon and Daniel, 2011). A reliable tool to assess the rumen community genes involved on the metabolism of plant lignans are metagenomic fosmid libraries. Thus, technology have been used to help scientists to discover and identify novel biocatalysts/genes/enzymes linked to many biological processes. For instance, cellulases (Healy et al., 1995; Duan et al., 2009), chitinases (Hjort et al., 2009), DNA polymerases (Simon et al., 2009), proteases (Waschkowitz et al., 2009), antibiotics (Riesenfeld et al., 2004) and lignocellulosic enzymes (Colombo et al., 2016).

This work combines the need for further findings on lignans metabolism and the complex rumen community yet to be deeply explored in the SDG breakdown pathways. Therefore, a metagenomic library using fosmid inserts from the rumen was constructed and screened.

RESULTS AND DISCUSSION

SDG incubation

The rumen microorganisms play active role in flaxseed lignans (Côrtes et al., 2008; Petit and Gagnon, 2009a, b; Zhou et al., 2009; Schogor et al., 2014; Lima et al., 2016). Schogor et al. (2014) showed that after 24h incubation of rumen fluid with SDG, the remaining SDG content was 2.1% and 46.6% of SECO was produced based on initial SDG amount. Therefore, it was decided to produce a fosmid library with random DNA inserts from the rumen microbes in order to identify possible clones with capacity to breakdown SDG. The assessment of SDG breakdown was carried out by serial incubations (quadruplicates) of SDG (0.364 nM), for 24h incubation (total volume 1000 µl). The remaining SDG content was calculated based on HPLC analysis and standard curve (Table 1).

Table 1. Whole plate (384 wells) SDG remaining after 24 hours 0.364 nM SDG incubation

Plate number	SDG (nM)	STDEV
1	0.348	0.02
2	0.364	0.03
3	0.364	0.002
4	0.357	0.01
5	0.364	0.004
6	0.364	0.003
7	0.353	0.002
8	0.261	0.06
9	0.345	0.009
10	0.325	0.07
11	0.364	0.02
12	0.356	0.007
13	0.355	0.007
14	0.317	0.0007
15	0.364	0.03
16	0.364	0.004
17	0.364	0.002
18	0.364	0.02
19	0.364	0.003
20	0.364	0.003
21	0.363	0.005
22	0.320	0.07
23	0.364	0.01
24	0.364	0.009
25	0.364	0.006
26	0.364	0.0007
27	0.359	0.01
28	0.364	0.003
29	0.353	0.01
30	0.354	0.003
Control 1	0.364	0.005
Control 2	0	0
EPI300	0.364	0.001

Control 1: no clone added; Control 2: no SDG added; SDG: secoisolariciresinol diglucoside; STDEV: standard deviation.

The SDG breakdown capacity of each plate was ranked and the plates which presented greater SDG degradation (two (8 and 14) out of 30 plates) were divided in eight sessions and screened again for plant lignan breakdown capacity (Table 2).

Table 2. Sessions of selected plates (8 and 14) after 24 hours 0.364 nM SDG incubation

Plate session	SDG (nM)	STDEV
8R1	0.351	0.01
8R2	0.356	0.01
8R3	0.356	0.01
8R4	0.243	0.002
8R5	0.341	0.001
8R6	0.342	0.03
8R7	0.353	0.01
8R8	0.364	0
14R1	0.333	0.01
14R2	0.364	0
14R3	0.364	0
14R4	0.364	0
14R5	0.364	0
14R6	0.364	0
14R7	0.364	0
14R8	0.364	0
Control 1	0.364	0
Control 2	0	0

Control 1: no clone added; Control 2: no SDG added. SDG: secoisolariciresinol diglucoside; STDEV: standard deviation.

High-Performance Liquid Chromatography (HPLC) and Liquid Chromatograpgy-Mass spectrometry (LC-MS)

According to HPLC lignans standard curves (Appendices), the retention time (minutes) of the lignans analyzed was: 9.3; 11.4; 12.9; and 13.2 for SDG, SECO, ED and EL respectively.

The rumen is a known efficient environment for flaxseed lignan metabolism (SDG converted into SECO, ED and EL) and the products can be transferred to plasma, meat and milk (Gagnon et al., 2009; Petit and Gagnon, 2009a; Petit et al., 2009; Matumoto-Pintro et al., 2011; Lima et al., 2016). Thus, SDG breakdown products identification was also carried out. The SDG concentration after incubation (Figure 1) was calculated based on SDG pure standard curve. Additional peaks were assessed for the presence of the SDG breakdown products using HPLC and LC-MS (Figure 2). However, despite of a SDG peak (7.26 retention time), none of the lignans products (SECO, enterodiol and enterolactone) were identified (Figure 2). The data was similar to Schogor et al. (2014), that showed that even

with efficient SDG breakdown of *Prevotella bryantii* (2.1% of remaining SDG after 24 h incubation) degradation products such as ED might not be detected with spectrometry.

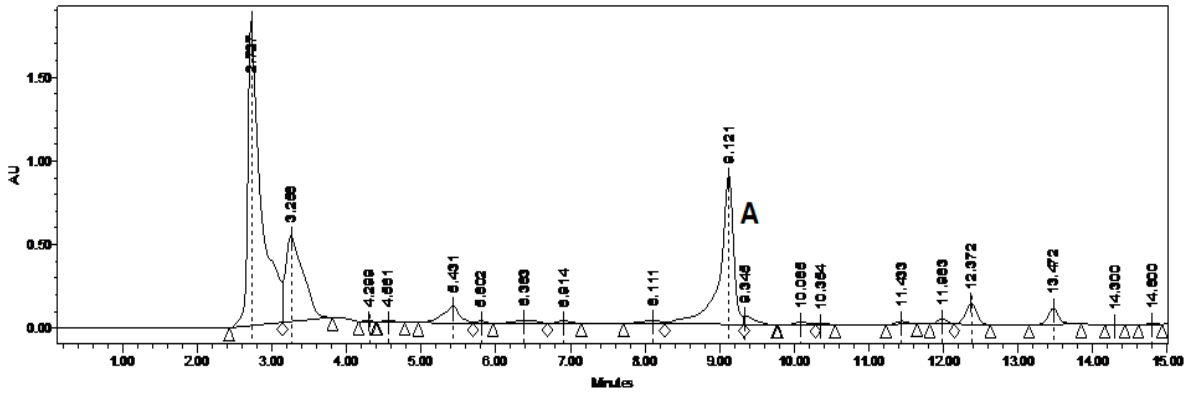

Figure 1. HPLC chromatogram after secoisolariciresinol diglucoside (SDG) incubation (fosmid library screening); A: SDG peak

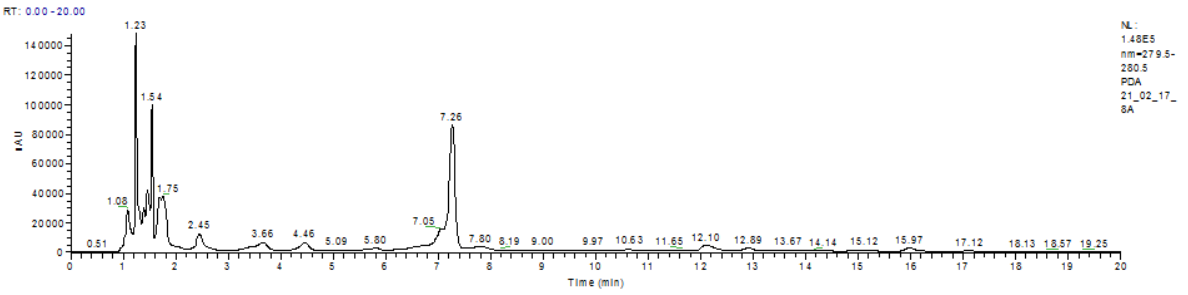

Figure 2. LC-MS chromatogram after secoisolariciresinol diglucoside (SDG) incubation (Fosmid library screening); peak at 7.26 minutes of retention time: SDG peak.

Sequencing data

Three sessions of the selected plated were identified as possible positives for SDG degradation activity (8R4, 8R5 and 14R1). The fosmid clones selected by means of SDG breakdown screening were purified in order to sequence high quality plasmid DNA. The purification was carried on using the QIAprep Spin Miniprep Kit (QIAGEN, UK), following the manufacturer instructions. The plasmid DNA was eluted in Buffer EB (10 mM Tris-Cl, pH 8.5) and kept in the freezer at -80ºC until being sent to sequence in a conventional whole-genome resequencing to 15× depth Hiseq sequencing system (Illumina®), as recommended by other studies using fosmid libraries (Colombo et al., 2016; Maruthamuthu et al., 2016; Lewin et al., 2017).

The library contained clones with inserts of ~43 kb average size and the three sessions were sequenced, yielding approximately 300 Megabits of total cloned genomic DNA. Basic statistics for fosmid DNA quality (Conventional base calls; Ecoding Sanger / Illumina 1.9):
8R4: Total sequences: 434168; Sequences flagged as poor quality: 0 (zero); Sequence length (bp): 101; %GC (guanine-cytosine content): 52.

8R5: Total sequences: 401587; Sequences flagged as poor quality: 0 (zero); Sequence length (bp): 101; %GC (guanine-cytosine content): 53.

14R1: Total sequences: 787511; Sequences flagged as poor quality: 0 (zero); Sequence length (bp): 101; %GC (guanine-cytosine content): 52.

Gene Annotation stats:

8R4: median coverage depth : 2.936585, final graph has 11870 nodes and n50 of 498, max 6322, total: 3537395, using: 847496 out of 867642 reads (kmer length: 21).

8R5: median coverage depth: 1.520000, final graph has 15860 nodes and n50 of 68, max 580, total: 560521, using 284484/802638 reads (max kmer length: 31).

14R1: median coverage depth: 8.177429, final graph has 3030 nodes and n50 of 6830, max 44909, max 44909, total: 4424666, using 1555262 out of 1572732 reads (kmer length: 21).

MULTIPLE GENOME ALIGNMENTS – MAUVE

Mauve employs algorithmic techniques that scale well in the lengths of sequences being aligned (Darling et al., 2010). In the fosmid analysis for instance, each pool fosmid sample (8R4, 8R5 and 14R1) was compared with a reference genome from *Escherichia coli k-12* (Blattner et al., 1997) (high similarity to *E.coli* EPI 300) using Mauve (Figure 3, 4 and 5).

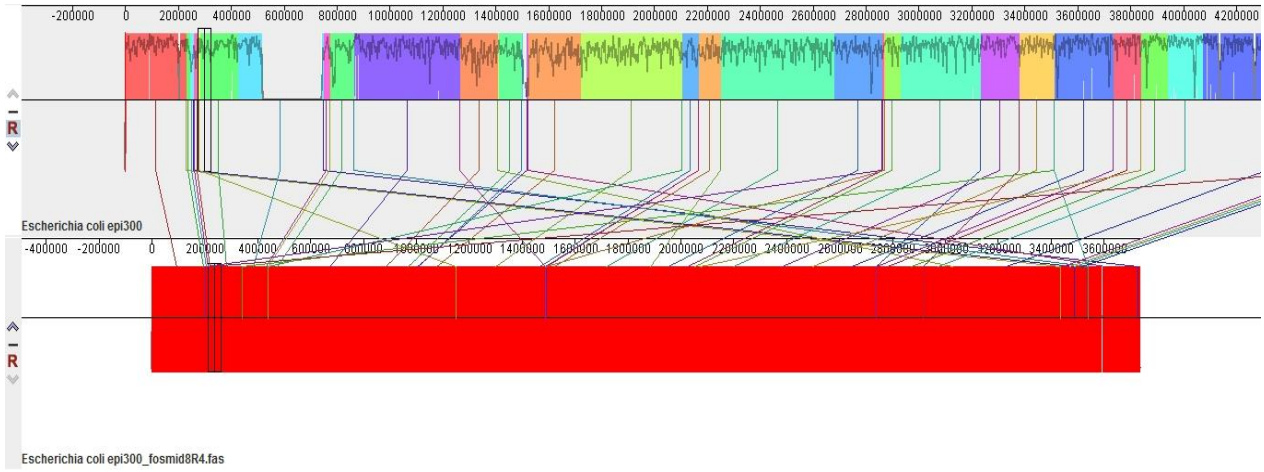

Figure 3. Fosmid library 8R4 clone genome (red bar bellow) aligned with *E. coli* reference genome (above).

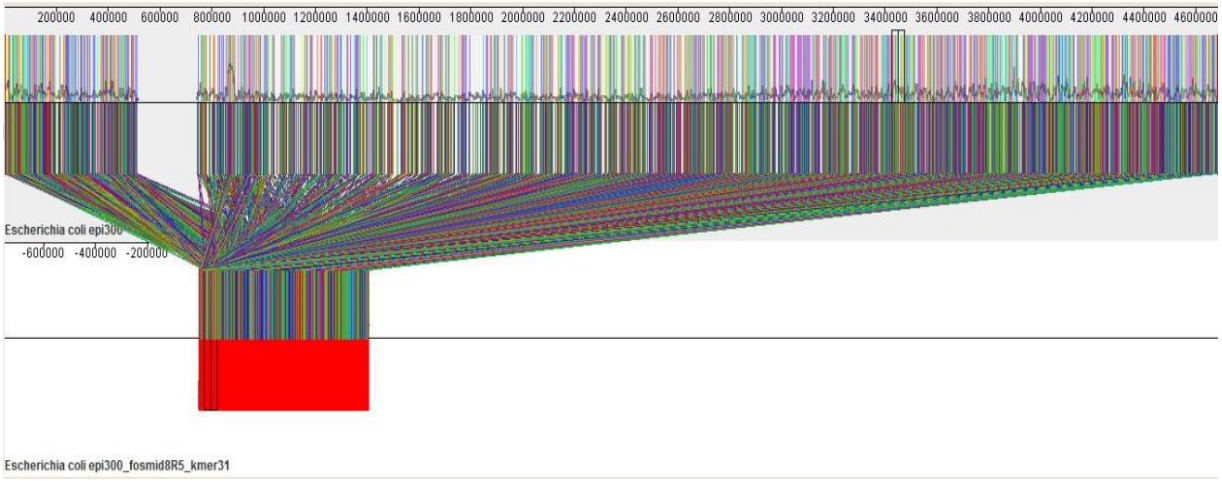

Figure 4. Fosmid library 8R5 clone genome (red bar bellow) aligned with *E. coli* reference genome (above).

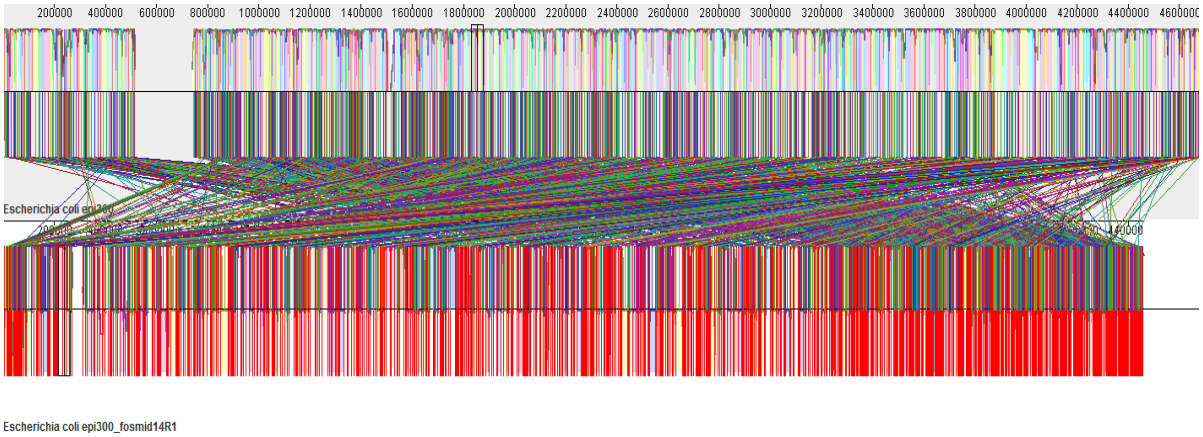

Figure 5. Fosmid library 14R1 clone genome (red bar bellow) aligned with *E. coli* reference genome (above).

It is possible to observe that most of the genes from the fosmid genome relates with some part of the reference genome. However, a clear gap is shown in the reference genome. Those genes are not found in the fosmids genome and are listed in Table 3. All genes were analysed for their biologic function on a digging for annoteded genes (GenBank database) and enzymes linked to lignans metabolism, such as glucosidases and cellulases (Renouard et al., 2010; Quartieri et al., 2016). However, no genes related to direct plant lignans metabolism were identified. Genes playing active role on SDG breakdown may not be annoteded previously or no gene from the DNA inserts of the rumen microbes is directly linked to the observed degradation. According to some studies, using *E. coli* function-based, screening can be problematic owing to insufficient expression of foreign genes (Uchiyama and Miyazaki, 2009). In addition, only 40% of the enzymatic activities in a bacterial community assessed may be detected by random cloning using *E. coli* cells (Gabor et al., 2004). Moreover, another possible issue is that the DNA inserts may be recruited from a numerically prevalent group of microbes (Kong et al., 2010), excluding microbes from less prevalent groups that could play active role on lignan metabolism. Therefore, other methods might be developed to enhance the potential of recovery of putative genes from the rumen for SDG metabolism.

Furthermore, according to Kanehisa and Goto (2000), many biological functions of the living cells are a result of many interacting molecules and it cannot be attributed to just a single gene or a single molecule. Therefore, the SDG breakdown pathway might be the result of interacting genes as a complex mechanism.

232 Table 3. Genes from reference genome that do not relate to fosmid clones

Gene	Locus_tag	Product	Gene	Locus_tag	Product
	DCHBEMND_00495	Hypothetical protein		DCHBEMND_00529	Hypothetical protein
	DCHBEMND_00496	Hypothetical protein		DCHBEMND_00530	Hypothetical protein
	DCHBEMND_00497	Hypothetical protein	entD_1	DCHBEMND_00531	Enterobactin synthase component D
	DCHBEMND_00498	Hypothetical protein	fepA_1	DCHBEMND_00532	Ferrienterobactin receptor
OmpD_1	DCHBEMND_00499	Outer membrane porin protein OmpD	fes_1	DCHBEMND_00533	Enterochelin esterase
	DCHBEMND_00500	Hypothetical protein	ybdZ_1	DCHBEMND_00534	Enterobactin biosynthesis protein YbdZ
rrrD_1	DCHBEMND_00501	Lysozyme RrrD	entF_1	DCHBEMND_00535	Enterobactin synthase component F
	DCHBEMND_00502	Hypothetical protein	fepE_1	DCHBEMND_00536	Ferric enterobactin transport protein FepE
	DCHBEMND_00503	Hypothetical protein	fepC_1	DCHBEMND_00537	Ferric enterobactin transport ATP-binding protein FepC
	DCHBEMND_00503	Hypothetical protein	fepG_1	DCHBEMND_00538	Ferric enterobactin transport system permease protein FepG
	DCHBEMND_00504	Hypothetical protein	fepD_1	DCHBEMND_00539	Ferric enterobactin transport sytem permease protein FepD
	DCHBEMND_00505	Hypothetical protein	entS_1	DCHBEMND_00540	Enterobactin exporter EntS
	DCHBEMND_00506	Hypothetical protein	fepB_1	DCHBEMND_00541	Ferrienterobactin-binding periplasmic protein
tfaE_1	DCHBEMND_00507	Prophage tail fiber assembly protein TfaE	entC_1	DCHBEMND_00542	Isochorismate synthase EntC
	DCHBEMND_00508	Hypothetical protein	entE_1	DCHBEMND_00543	Enterobactin synthase component E
	DCHBEMND_00509	Hypothetical protein	entB_1	DCHBEMND_00544	Enterobactin synthase component B
appY_1	DCHBEMND_00510	HTH-type transcriptional regulator AppY	entA_1	DCHBEMND_00545	2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase
ompT_1	DCHBEMND_00511	Protease 7	entH_1	DCHBEMND_00546	Proofreading thioesterase EntH
gadX_1	DCHBEMND_00512	HTH-type transcriptional regulator Gadx	cstA_1	DCHBEMND_00547	Carbon starvation protein A
	DCHBEMND_00513	Hypothetical protein		DCHBEMND_00548	Hypothetical protein
nfrA_1	DCHBEMND_00514	Bacteriophage adsorption protein A	ybdH_1	DCHBEMND_00549	Putative oxireductase YbdH
	DCHBEMND_00515	Hypothetical protein	ybdL_1	DCHBEMND_00550	Methionine aminotransferase
cusS_1	DCHBEMND_00516	Sensor kinase CusS	noc_1	DCHBEMND_00551	Nucleoid occlusion protein
cusR_1	DCHBEMND_00517	Transcriptional regulatory protein CusR	cysH_1	DCHBEMND_00552	Phosphosulfate reductase
cusC_1	DCHBEMND_00518	Cation efflux system protein CusC	ybdO_1	DCHBEMND_00553	Putative HTH-type transcriptional regulator YbdO
cusF_1	DCHBEMND_00519	Cation efflux system protein CusF	dsbG_1	DCHBEMND_00554	Thiol:disulphide interchange protein DsbG
cusB_1	DCHBEMND_00520	Cation efflux system protein CusB	ahpC_1	DCHBEMND_00555	Alkyl hydroperoxide reductase subunit C
cusA_1	DCHBEMND_00521	Cation efflux system protein CusA	ahpF_1	DCHBEMND_00556	Alkyl hydroperoxide reductase subunit F
pheP_1	DCHBEMND_00522	Phenylalanine-specific permease	uspG_1	DCHBEMND_00557	Universal stress protein UP12
ybdG_1	DCHBEMND_00523	Miniconductance mechanosensitive channel YbdG		DCHBEMND_00558	Putative zinc-binding alcohol dehydrogenase
nfsB_1	DCHBEMND_00524	Oxygen-insensitive NAD(P)H nitroreductase		DCHBEMND_00559	Hypothetical protein
	DCHBEMND_00525	Hypothetical protein	rnk_1	DCHBEMND_00560	Regulator of nucleoside diphosphate kinase
	DCHBEMND_00526	Hypothetical protein	rna_1	DCHBEMND_00561	Ribonuclease I
ybdK_1	DCHBEMND_00527	Putative glutamate-cysteine ligase 2	citT_1	DCHBEMND_00562	Citrate carrier
	DCHBEMND_00528	Hypothetical protein	citG_1	DCHBEMND_00563	2-(5”-triphosphoribosyl)-3’-dephosphocoenzyme-Asynthase

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Gene	Locus_tag	Product	Gene	Locus_tag	Product
citX_1	DCHBEMND_00564	Apo-citrate lyase phosphoribosyl-dephospho-CoA transferase	esiB_2	DCHBEMND_00597	Secretory immunoglobulin A-binding protein EsiB
citF_1	DCHBEMND_00565	Citrate lyase alpha chain		DCHBEMND_00598	Hypothetical protein
citE_1	DCHBEMND_00566	Citrate lyase subunit beta		DCHBEMND_00599	Hypothetical protein
citD	DCHBEMND_00567	Citrate lyase acyl carrier protein	hscC_1	DCHBEMND_00600	Chaperone protein HscC
citC_1	DCHBEMND_00568	[Citrate [pro-3S]-lyase] ligase	rihA_1	DCHBEMND_00601	Pyrimidine-specific ribonucleoside hydrolase RihA
dpiB_1	DCHBEMND_00569	Sensor histidine kinase DpiB	qlnQ_1	DCHBEMND_00602	Glutamine transport ATP-binding protein GlnQ
dpiA_1	DCHBEMND_00570	Transcriptional regulatory protein DpiA	gltK_1	DCHBEMND_00603	Glutamate/aspartate import permease protein GltK
		Putative cryptic C4-dicarboxylate transporter	glnM_1	DCHBEMND_00604	Putative glutamine ABC transporter permease protein GlnM
dcuD_1	DCHBEMND_00571	DcuD			
pagP_1	DCHBEMND_00572	Lipid A palmitoyltransferase PagP	gltI_1	DCHBEMND_00605	Glutamate/aspartate import solute-binding protein
cspE_1	DCHBEMND_00573	Cold shock-like protein CspE		DCHBEMND_00606	Hypothetical protein
crcB_1	DCHBEMND_00574	Putative fluoride ion transporter CrcB	ompD_2	DCHBEMND_00607	Outer membrane porin protein OmpD
	DCHBEMND_00575	Hypothetical protein		DCHBEMND_00608	Hypothetical protein
	DCHBEMND_00576	Hydrolase	rrrD_2	DCHBEMND_00609	Lysozyme RrrD
tatE_1	DCHBEMND_00577	Sec-independent protein translocase protein TatE		DCHBEMND_00610	Hypothetical protein
lipA_1	DCHBEMND_00578	Lipoyl synthase		DCHBEMND_00611	Hypothetical protein
ybdO_2	DCHBEMND_00579	Putative HTH-type transcriptional regulator YbdO		DCHBEMND_00612	Hypothetical protein
lipB_1	DCHBEMND_00580	Octanoyltransferase		DCHBEMND_00613	Hypothetical protein
	DCHBEMND_00581	Hypothetical protein		DCHBEMND_00614	Hypothetical protein
dacA_1	DCHBEMND_00582	D-alanyl-D-alanine carboxypeptidase DacA	tfaE_2	DCHBEMND_00615	Prophage tail fiber assembly protein TfaE
rlpA_1	DCHBEMND_00583	Endolytic peptidoglycan transglycosylase RlpA		DCHBEMND_00616	Hypothetical protein
mrdB_1	DCHBEMND_00584	Peptidoglycan glycosyltransferase MrdB		DCHBEMND_00617	Hypothetical protein
mrdA_1	DCHBEMND_00585	Peptidoglycan D,D-transpeptidase MrdA	appY_2	DCHBEMND_00618	HTH-type transcriptional regulator AppY
rlmH_1	DCHBEMND_00586	Ribosomal RNA large subunit methyltransferase H	ompT_2	DCHBEMND_00619	Protease 7
rsfS_1	DCHBEMND_00587	Ribosomal silencing fator RsfS	gadX_2	DCHBEMND_00620	HTH-type transcriptional regulator GadX
cobC_1	DCHBEMND_00588	Adenosylcobalalamin/alpha-ribazole phosphatase		DCHBEMND_00621	Hypothetical protein
nadD_1	DCHBEMND_00589	Nicotinate-nucleotide adenyltransferase	nfrA_2	DCHBEMND_00622	Bacteriophage adsorption protein A
holA_1	DCHBEMND_00590	DNA polymerase III subunit delta		DCHBEMND_00623	Hypothetical protein
lptE_1	DCHBEMND_00591	LPS-assembly lipoprotein LptE	cusS_2	DCHBEMND_00624	Sensor kinase CusS
leuS_1	DCHBEMND_00592	Leucine-tRNA ligase	cusR_2	DCHBEMND_00625	Transcriptional regulatory protein CusR
	DCHBEMND_00593	Hypothetical protein	cusC_2	DCHBEMND_00626	Cation efflux protein CusC
esiB_1	DCHBEMND_00594	Secretory immunoglobulin A-binding protein Esib	cusF_2	DCHBEMND_00627	Cation efflux system protein CusF
	DCHBEMND_00595	Hypothetical protein	cusB_2	DCHBEMND_00628	Cation efflux system protein CusB
	DCHBEMND_00596	Hypothetical protein	cusA_2	DCHBEMND_00629	Cation efflux system protein CusA

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Gene	Locus_tag	Product	Gene	Locus_tag	Product
pheP_2	DCHBEMND_00630	Phenylalanine-specific permease	ahpC_2	DCHBEMND_00663	Alkyl hydroperoxide reductase subunit C
ybdG_2	DCHBEMND_00631	Miniconductance mechanosensitive channel YbdG	ahpF_2	DCHBEMND_00664	Alkyl hydroperoxide reductase subunit F
nfsB_2	DCHBEMND_00632	Oxygen-insensitive NAD(P)H nitroreductase	uspG_2	DCHBEMND_00665	Universal stress protein UP12
	DCHBEMND_00633	Hypothetical protein		DCHBEMND_00666	Putative zinc-binding alcohol dehydrogenase
	DCHBEMND_00634	Hypothetical protein		DCHBEMND_00667	Hypothetical protein
ybdK_2	DCHBEMND_00635	Putative glutamate-cysteine ligase 2	rnk_2	DCHBEMND_00668	Regulator of nucleoside diphosphate kinase
	DCHBEMND_00636	Hypothetical protein	rna_2	DCHBEMND_00669	Ribonuclease I
	DCHBEMND_00637	Hypothetical protein	citT_2	DCHBEMND_00670	Citrate carrier
	DCHBEMND_00638	Hypothetical protein	citG_2	DCHBEMND_00671	2-(5”-triphosphoribosyl)-3’-dephosphocoenzyme-Asynthase
entD_2	DCHBEMND_00639	Enterobactin synthase component D	citX_2	DCHBEMND_00672	Apo-citrate lyase phosphoribosyl-dephospho-CoA transferase
fepA_2	DCHBEMND_00640	Ferrienterobactin receptor	citF_2	DCHBEMND_00673	Citrate lyase alpha chain
fes_2	DCHBEMND_00641	Enterochelin esterase	citE_2	DCHBEMND_00674	Citrate lyase subunit beta
ybdZ_2	DCHBEMND_00642	Enterobactin biosynthesis protein YbdZ	citD_2	DCHBEMND_00675	Citrate lyase acyl carrier protein
entF_2	DCHBEMND_00643	Enterobactin synthase component F	citC_2	DCHBEMND_00676	[Citrate [pro-3S]-lyase] ligase
fepE_2	DCHBEMND_00644	Ferric enterobactin transport protein FepE	dpiB_2	DCHBEMND_00677	Sensor histidine kinase DpiB
			dpiA_2	DCHBEMND_00678	Transcriptional regulatory protein DpiA
fepC_2	DCHBEMND_00645	Ferric enterobactin transport ATP-binding protein FepC			
fepG_2	DCHBEMND_00646	Ferric enterobactin transport system permease protein FepG	dcuD_2	DCHBEMND_00679	Putative cryptic C4-dicarboxylate transporter DcuD
fepD_2	DCHBEMND_00647	Ferric enterobactin transport system permease protein FepD	pagP_2	DCHBEMND_00680	Lipid A palmitoyltransferase PagP
entS_2	DCHBEMND_00648	Enterobactin exporter EntS	cspE_2	DCHBEMND_00681	Cold shock-like protein CspE
fepB_2	DCHBEMND_00649	Ferrienterobactin_binding periplasmic protein	crcB_2	DCHBEMND_00682	Putative fluoride ion transporter CrcB
entC_2	DCHBEMND_00650	Isochorismate synthase EntC		DCHBEMND_00683	Hypothetical protein
entE_2	DCHBEMND_00651	Enterobactin synthase component E		DCHBEMND_00684	Hydrolase
entB_2	DCHBEMND_00652	Enterobactin synthase component B	tatE_2	DCHBEMND_00685	Sec-independent protein translocase protein TatE
entA_2	DCHBEMND_00653	2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase	lipA_2	DCHBEMND_00686	Lipoyl synthase
entH_2	DCHBEMND_00654	Proofreading thioesterase EntH	ybdO_4	DCHBEMND_00687	Putative HTH-type transcriptional regulator YbdO
cstA_2	DCHBEMND_00655	Carbon starvation protein A	lipB_2	DCHBEMND_00688	Octanoyltransferase
	DCHBEMND_00656	Hypothetical protein		DCHBEMND_00689	Hypothetical protein
ybdD_2	DCHBEMND_00657	Putative oxidoreductase YbdH	dacA_2	DCHBEMND_00690	D-alanyl-D-alanine carboxypeptidase DacA
ybdL_2	DCHBEMND_00658	Methionine aminotransferase	rlpA_2	DCHBEMND_00691	Endolytic peptidoglycan transglycosylase RlpA
noc_2	DCHBEMND_00659	Nucleoid occlusion protein			
			mrdB_2	DCHBEMND_00692	Peptidoglycan glycosyltransferase MrdB
cysH_2	DCHBEMND_00660	Phosphosulfate reductase	mrdA_2	DCHBEMND_00693	Peptidoglycan D,D-transpeptidase MrdA
ybdO_3	DCHBEMND_00661	Putative HTH-type transcriptional regulator YbdO	rlmH_2	DCHBEMND_00694	Ribosomal RNA large subunit methyltransferase H
dsbG_2	DCHBEMND_00662	Thiol:disulphide interchange protein DsbG	rsfS_2	DCHBEMND_00695	Ribosomal silencing fator RsfS

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Gene	Locus_tag	Product	Gene	Locus_tag	Product
cobC_2	DCHBEMND_00696	Adenosylcobalalamin/alpha-ribazole phosphatase		DCHBEMND_00706	Hypothetical protein
nadD_2	DCHBEMND_00697	Nicotinate-nucleotide adenyltransferase		DCHBEMND_00707	Hypothetical protein
holA_2	DCHBEMND_00698	DNA polymerase III subunit delta	hscC_2	DCHBEMND_00708	Chaperone protein HscC
lptE_2	DCHBEMND_00699	LPS-assembly lipoprotein LptE	rihA_2	DCHBEMND_00709	Pyrimidine-specific ribonucleoside hydrolase RihA
leuS_2	DCHBEMND_00700	Leucine-tRNA ligase	qlnQ_2	DCHBEMND_00710	Glutamine transport ATP-binding protein GlnQ
	DCHBEMND_00701	Hypothetical protein	gltK_2	DCHBEMND_00711	Glutamate/aspartate import permease protein GltK
esiB_3	DCHBEMND_00702	Secretory immunoglobulin A-binding protein Esib	glnM_2	DCHBEMND_00712	Putative glutamine ABC transporter permease protein GlnM
	DCHBEMND_00703	Hypothetical protein	gltI_2	DCHBEMND_00713	Glutamate/aspartate import solute-binding protein
	DCHBEMND_00704	Hypothetical protein		DCHBEMND_00714	Hypothetical protein
esiB_4	DCHBEMND_00705	Secretory immunoglobulin A-binding protein EsiB	Int	DCHBEMND_00715	Apolipoprotein N-acyltransferase

EXPERIMENTAL PROCEDURES

Library starting sample

As a starting sample for the production of the fosmid library, a pool of DNA, extracted from rumen content samples from an early study (Schogor et al., 2014) was used. The rumen fluid was sampled from eight lactating multiparous Holstein cows fitted with rumen cannulas averaging 686 ± 35 kg of body weight and 112 ± 21 days in milk. The animals were assigned to four treatments in a double 4x4 Latin Square design with four 21-d periods (14-d adaptation and 7-d sampling). Cows were fed a total mixed ration with no flax meal (FM) (control), or diets containing, dry matter (DM) basis, 5% FM, 10% FM and 15% FM (Table 4). The starting sample pool was composed by the genetic material from the rumen of the cows fed 10 or 15% DM, which pooled together represented enough genetic material to construct the library. Samples from cows fed with control diet and 5% FM were not included in the pooled DNA starting sample because of possible less prevalence of species linked to FM breakdown. The presence of the pooled nucleic acids was checked by means of a gel electrophoresis run in 1% agarose gel with control DNA (45kb) (CopyControl Fosmid Control DNA – Epicentre) and size marker ladder (Lambda DNA MonoCut Mix - BioLabs®), and checked in UV scan before size-selection (Figure 11). The sample concentration was 220ng/µL (22,000 ng in 100µL).

Table 4. Ingredients and chemical composition of experimental diets

	Control[1]	5FM[1]	10FM[1]	15FM[1]
Ingredients (DM basis)				
Corn silage	29.22	29.08	28.98	29.06
Grass silage	31.53	31.57	31.75	31.39
Ground corn	21.13	20.23	19.16	18.90
Soya meal	10.76	7.45	4.14	2.04
Top Suplement[2]	1.74	1.74	1.79	0.85
Beet pulp	3.43	2.96	2.50	1.61
Calcium carbonate	0.55	0.55	0.54	0.58
Mineral and vitamins[3]	1.64	1.63	1.63	1.53
Flax meal	0	4.79	9.53	14.06
Chemical analysis[4]				
DM%	37.7±1.60	37.9±1.60	37.6±1.60	38.1±1.60
Crude protein, % of DM	17.0±0.15	17.4±0.15	17.6±0.15	17.9±0.15
Acid detergent, % of DM	18.3±0.34	18.5±0.34	19.2±0.34	19.3±0.34
Neutral detergent fiber	28.4±0.31	28.6±0.31	29.5±0.31	29.6±0.31
Ether extract, % of DM	2.4±0.07	2.4±0.07	2.4±0.07	2.4±0.07

[1]Control diet with no flax meal (FM) or a diet with 5%, 10% and 15% FM (DM basis).
[2]Contained 20% of canola meal, 30% of corn gluten meal, 20% of soybean meal, and 30% of brewer's corn.
[3]Contained 9.2% Ca; 4.79% P; 4.78% Mg; 1.52% S; 13.72% Na; 1.37% K; 19.5 mg/kg Se; 23 mg/kg Fe; 1068 mg/kg Cu; 1796 mg/kg Mn; 57 mg/kg Co; 265 mg/kg FI; 442000 IU vitamin A; 56670 IU/kg vitamin D; and 2630 IU/kg vitamin E.
[4]Values with standard errors of the mean.
Source: Schogor et al. (2014).

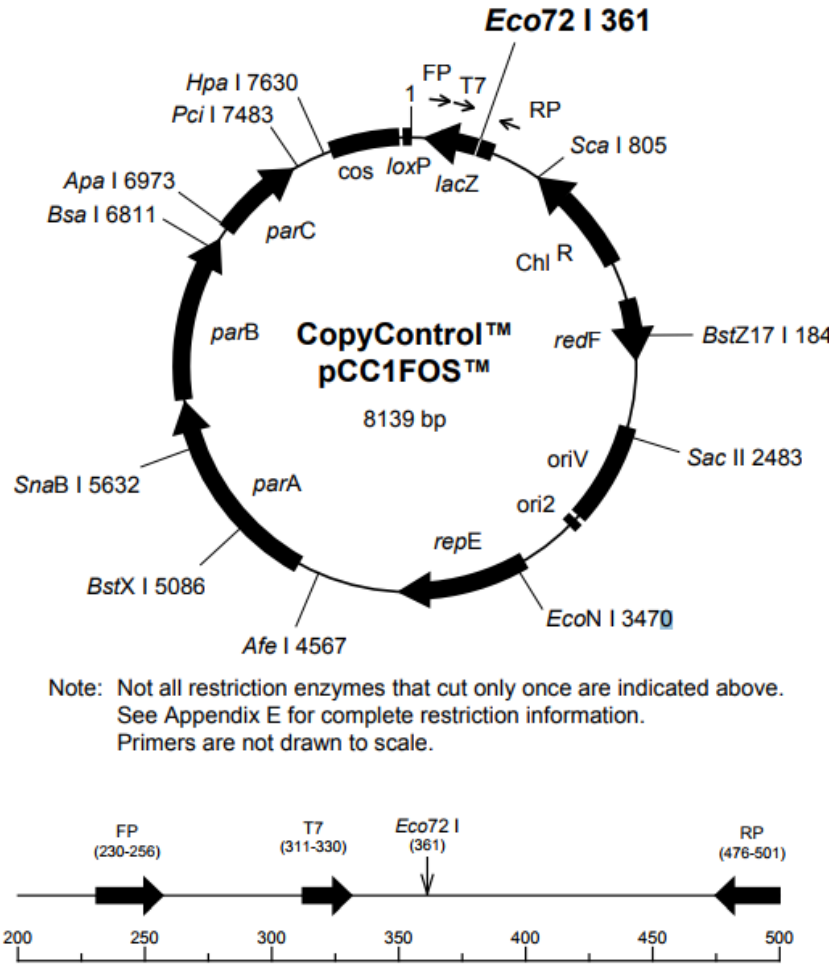
Fosmid Library Production

A fosmid library was produced using CopyControl™ Fosmid Library Production Kit with pCC1FOS™ Vector (EPI300™, Epicentre, USA) following the manufacturer instructions (Epicentre, 2012) and Brady (2007) recommendations. Such host strain has been successfully used in cloning assays in many studies (Rhee et al., 2005; Diaz-Torres et al., 2006; Kazimierczak et al., 2008). Briefly (Figure 6), the pooled DNA starting sample was size fractionated to 30 to 40kb and inserted into bacteriophages vectors (Figure 7 and 8).

Phage-resistant *E. coli* cells (EPI300-T1R phage T1-resistant *E. coli* hostTM, Epicentre, USA) were infected to provide chloramphenicol resistance and a rumen bacterial fosmid insertion to the infected clones (Figure 9). Clones were spread on LB plates + 12.5µg ml^{-1} chloramphenicol and incubated at 37ºC for 24 hours. Each colony forming unit was picked manually and added into a well of a 384-well (Figure 10) plate containing LB Broth + 12.5µg ml^{-1} chloramphenicol. This process was repeated until 30 plates were filled (11,520 clones). The fosmid library was then duplicated and stored at -80ºC.

Figure 6. Fosmid Library workflow.

Source: The author



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297 Figure 7. pCC1FOS™ Fosmid Vector Map.

298 Source: Epicentre (2012).

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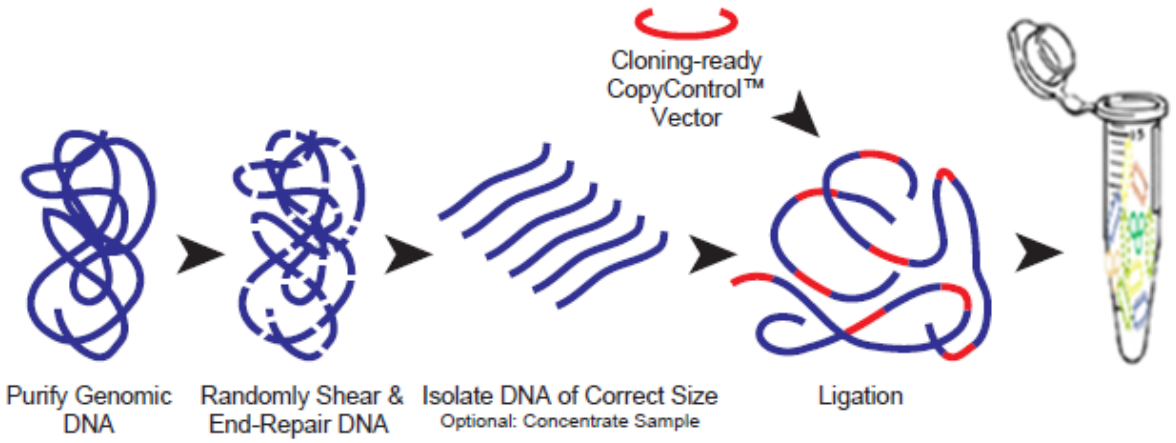

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302 Figure 8. Preparing insert of rumen bacteria DNA for Fosmid Library production.

303 Adapted from Epicentre (2012).

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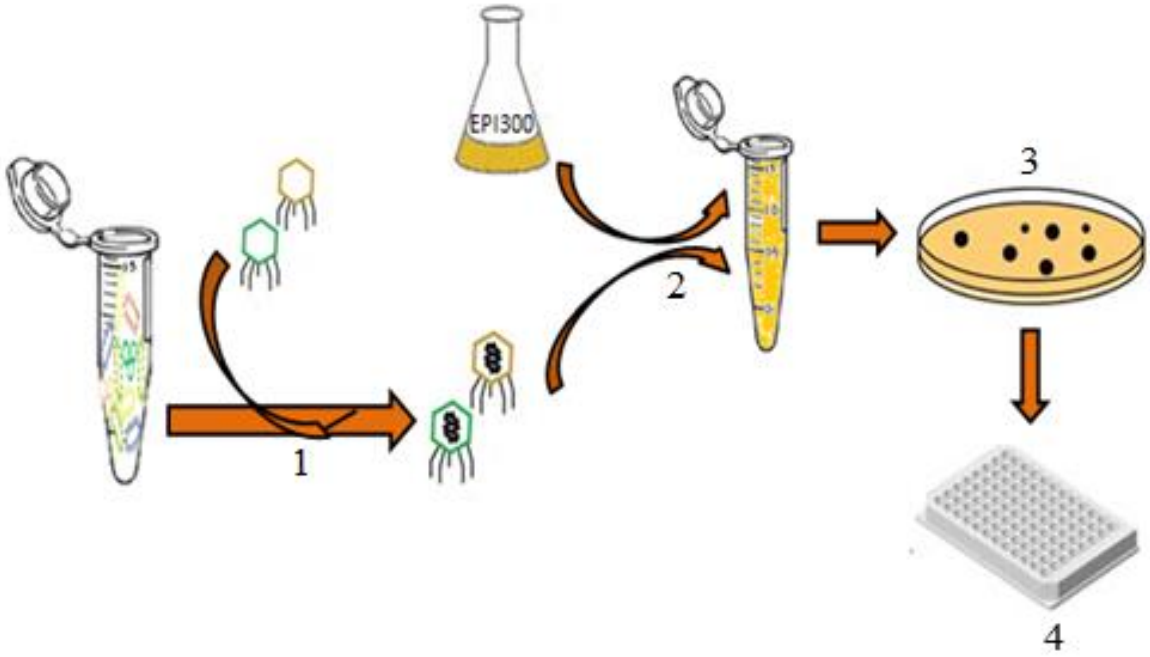

Figure 9. Fosmid Library Production overview; 1: fosmid vector and size-selected plasmid DNA insertion + bacteriophages; 2:EPI300 + packaged bacteriophages; 3: Clones grew in LB agar and chloramphenicol plates; 4: Picked clones in 384 – well plate.

Source: The author

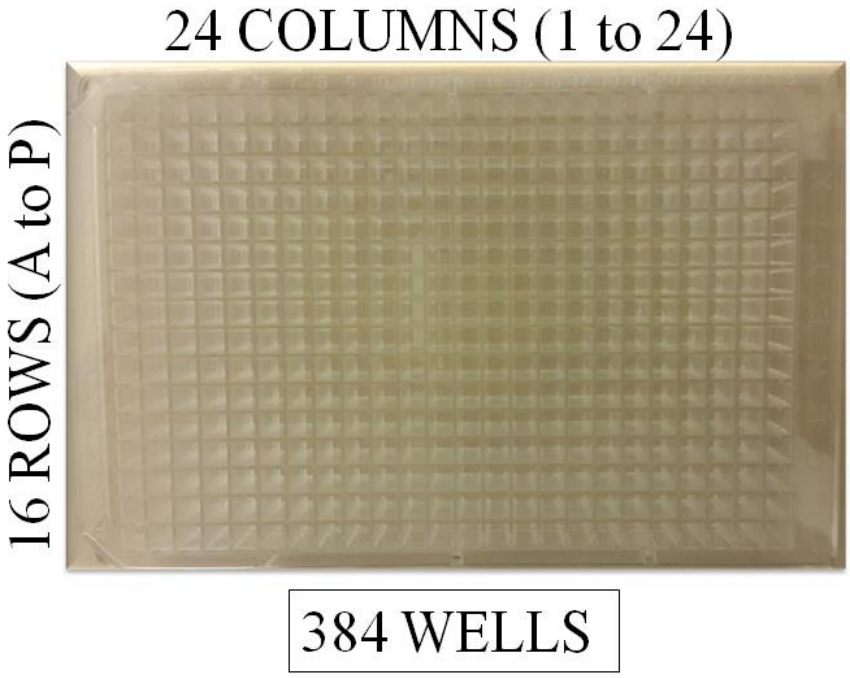

Figure 10. Fosmid Library organization (384 wells plate).

Source: The author

Size selection of insert plasmid DNA

In order to size select the sample (Figure 4), the DNA insert was previously treated with an End-Repair of the Insert DNA procedure, this step (Table 5) generates blunt-ended, 5′-phosphorylated DNA.

The pooled sample was then run on a 20 cm long 1% LMP agarose gel in TBE buffer (Figure 11) at 30V for 12 hours. A size marker (Lambda DNA Mono Cut Mix – BioLabs) and a Fosmid Control DNA supplied with the Epicentre kit was used. The gel was cut (Figure 12) between 30 to 45kb, which is the size able to be inserted into the bacteriophage vector. By means of GELase procedure (β-agarase) the plasmid DNA was recovered for vector packaging use.

Table 5. End-repair of the insert DNA

Product	Quantity
10X End-Repair Buffer	10 µl
2.5 mM dNTP Mix	10 µl
10 mM ATP	10 µl
Sheared insert DNA	65 µl (22 µg DNA)
End-repair enzyme mix	5 µl
Total	**100µL**

The DNA concentration was checked right after size-fractioned DNA recovery. It was used read tubes in Qubit® Fluorometer, following the manufacturer instructions. The DNA concentration was 58.5 µg/mL (0.0585µg/µL). The total amount of DNA was 0.2925µg (0.0585µg/µL x 5µL). For a ligation reaction, 0.25µg of DNA is needed, therefore, an additional size-selection DNA recovery protocol was carried out to end up with a safe amount of genetic material before ligation reaction. The second protocol DNA concentration was 9.59 µg/mL. All samples were stored at -80ºC until next step.

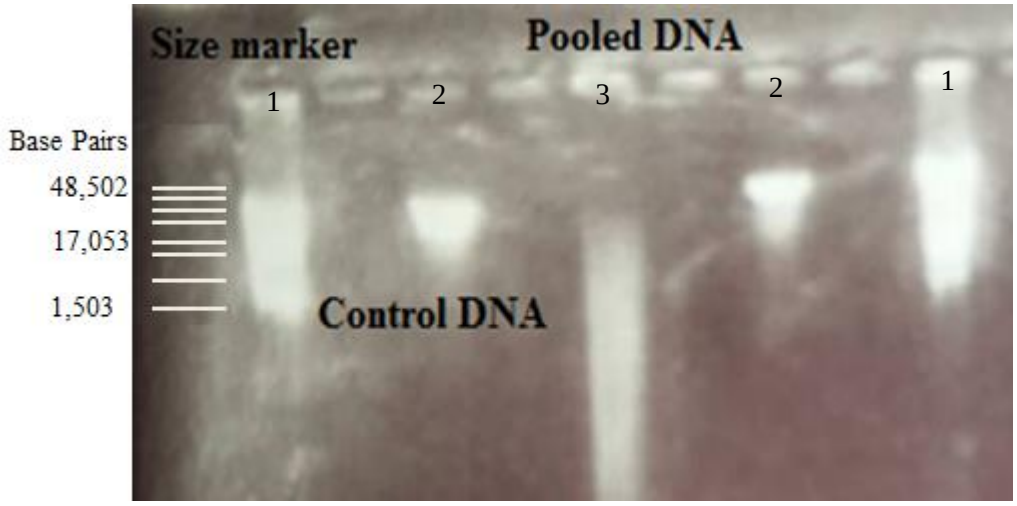

Figure 11. DNA to be size-selected (Pooled DNA from treatments 10% and 15% of flax meal intake (DM basis). Control DNA = 40 kb. Wells: 1: Size marker ladder; 2: Control DNA; 3: Pooled DNA.

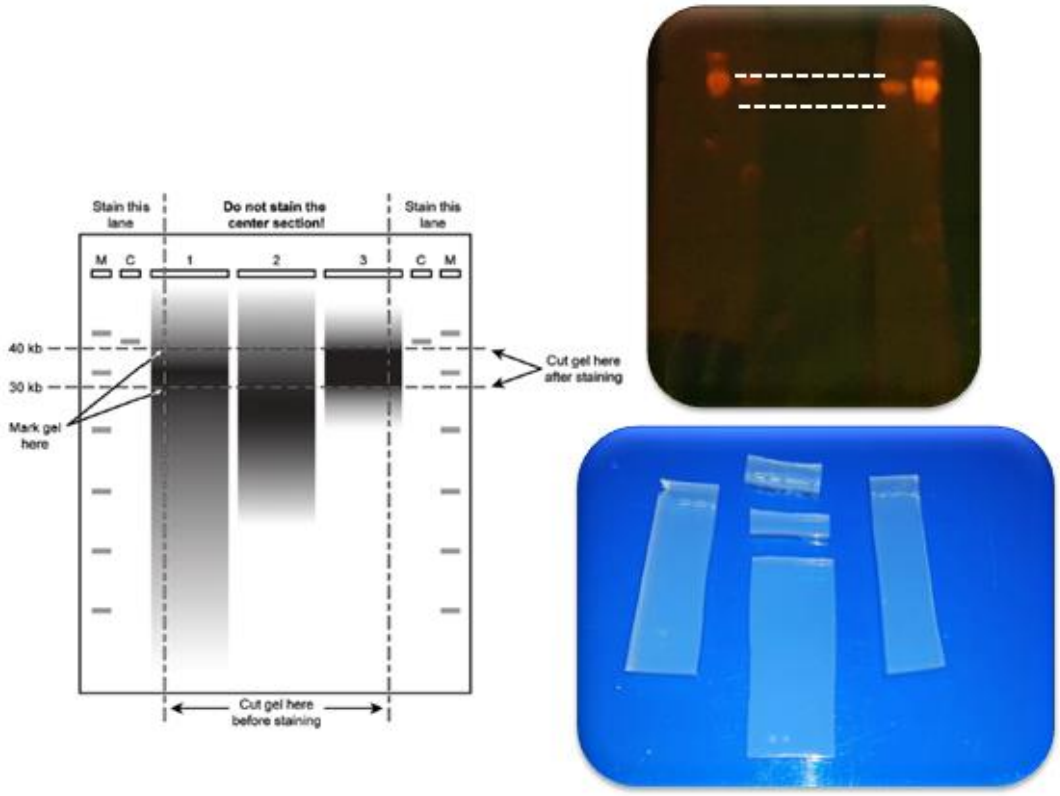

Figure 12. Shearing the insert size of the starting DNA sample.

Ligation reaction

The MaxPlax kit was used to ligate insert DNA, following manufacture instruction (Epicentre, USA) (Table 6).

Table 6. Ligation reaction

Product	Quantity
Sterile water	1 µl
10X Fast-Link Ligation Buffer	1 µl
10 mM ATP	1 µl
CopyControl pCC1FOS Vector (0.5 µg/µl)	1 µl
Size-fractionated DNA	5 µl
Fast-Link DNA ligase	1 µl
Total	10 µl

Packing phage

Briefly, the size-fractionated DNA was inserted into MaxPlax Lambda bacteriophages (10 µl ligation reaction solution + 25 µl MaxPlax Lambda Packing Extract), kept in phage dilution buffer and stored at -80ºC in several aliquots.

Plating infected clones

LB broth + 10 mM $MgSO_4$ + 0.2% Maltose was inoculated with a culture of EPI300 (phage resistant *E.coli*) grown until reaching 1.0 of optical density at wavelength of 600 nm (OD600) as recommended by Brady (2007). The *E. coli* cells at 1.0 OD600 were infected with the phages vectors at 30ºC for 2 hours and spread on LB plates + 12.5µg/mL chloramphenicol, which was used as selection marker. The plated was carried out in different dilution in order to select a proper assay (10^{-3} dilution) to get as many colonies forming units (CFU) as possible, although, apart from each other enough to pick them individually (Figure 13).

Picking infected clones

Each CFU was added manually into a well in a 384-well plate containing LB broth + chloramphenicol, the process was repeated until 30 were filled, representing 11,520 clones with chloramphenicol resistance and a ruminal insert DNA (Figure 14).

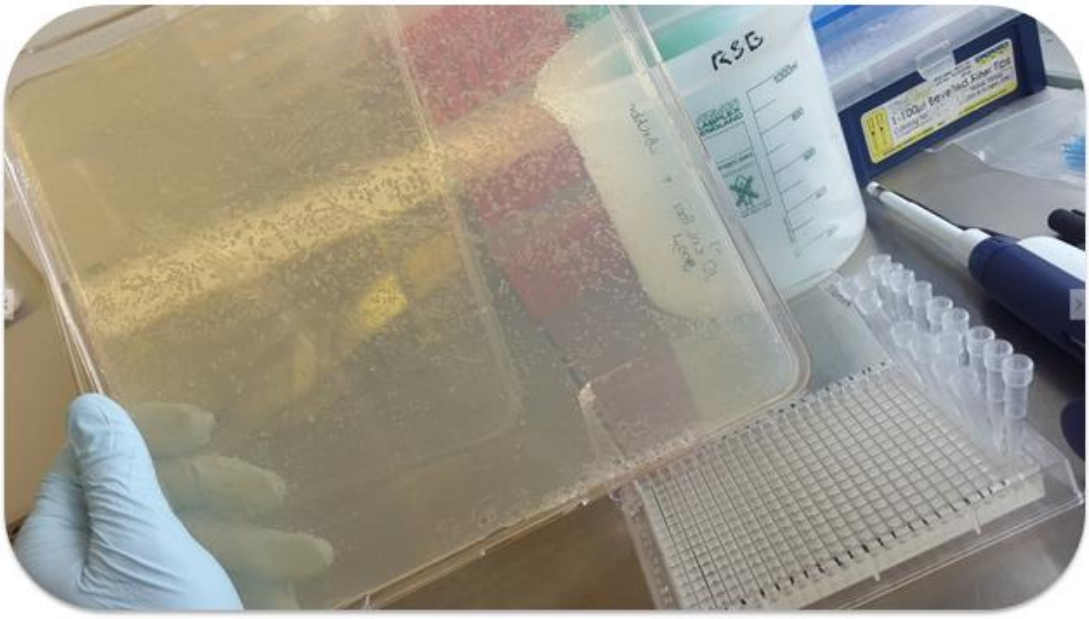

Figure 13. LB agar + chloramphenicol plate with approximately 5,000 CFU of packed EPI300 fosmid clones.

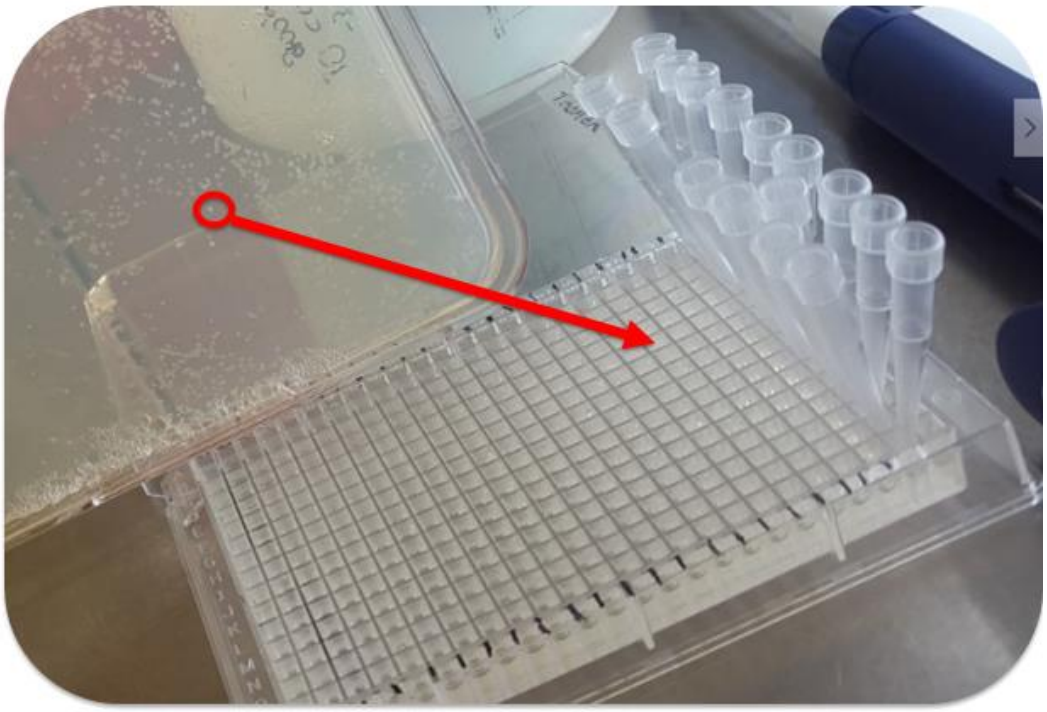

Figure 14. Picking procedure for the infected fosmid clones into a 384-well plate.

Duplicating the Fosmid Library

In order to replicate the study in the future or prospect the effect of novel compounds in the fosmid library, the 30 plates were duplicated and stored at -80ºC. The method was based on preparing 30 384-well plates with fresh LB broth + chloramphenicol and using a replicator pin all the wells were duplicated at once into the new plate, the copied library followed to a 24 hours growth at 37ºC and addition of 50:50 glycerol into the wells before storing the plates at 4ºC for four hours, -20ºC for 12 hours and definitive storage at -80ºC.

Average insert size

Seven clones were randomly selected, grown in LB broth, plasmid DNA was purified using QIAprep Spin Miniprep Kit – (QIAGEN GmbH), digested with restriction enzyme (NotI – NEB) and run on a 0.4% agarose gel in 1X TAE buffer at 35V for 15 hours (Figure 15). The average size of inserts was obtained comparing the fosmid clones, control DNA (40 kb) and size ladder (Lambda DNA – MonoCut – NEB).

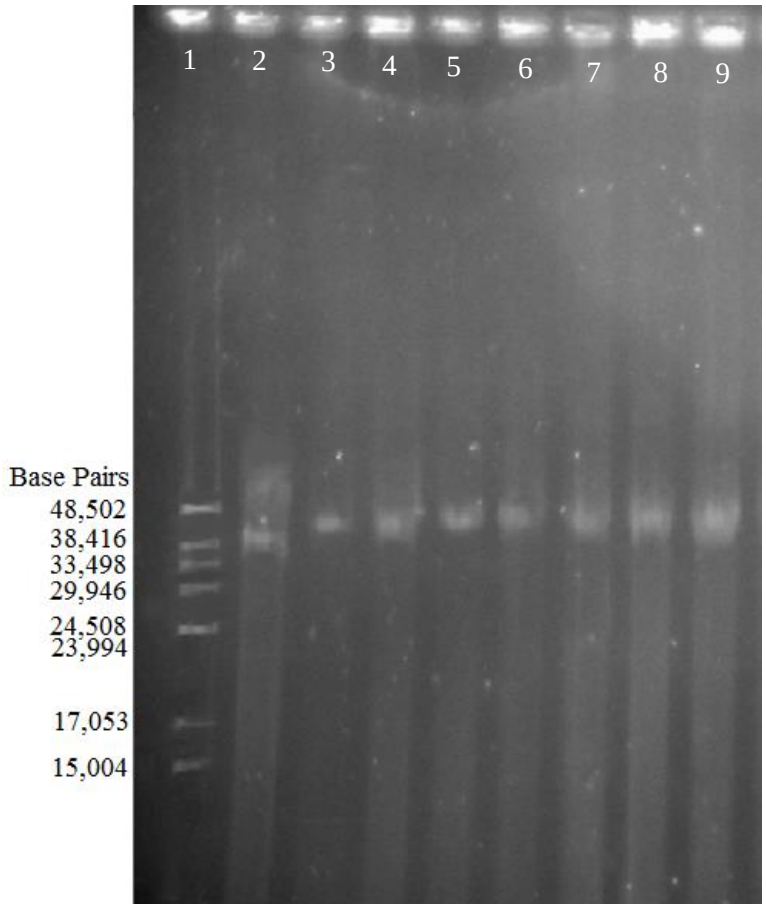

Figure 15. Fosmid clones average insert size (43,500 base pairs). Well: 1: size ladder; 2: Control DNA (40kb); 3 to 9: plasmid DNA extracted from random clones in the library.

Screening the Fosmid Library

The SDG breakdown capacity (Table 1) was assessed by the difference of initial and final SDG concentration in incubations of the flaxseed lignan and the fosmid clones.

The content from each plate was printed on a clear plate (Figure 16), pooled in 1000µL and incubated (Figure 17) with 0.25 mg of standard SDG (≥97%-HPLC) (SIGMA, UK) at 37ºC for 24h to assess the SDG breakdown. Negative controls with bacteria but without SDG and positive controls with SDG without any bacteria were also analyzed. Subsequently, incubations were centrifuged (1 min, 13,000 x g) and the supernatant was purified on C18 cartridges. Samples were concentrated at 50ºC and re-suspended in 200 µl of methanol. The presence of SDG was detected by reverse-phase HPLC on a Waters system with a 996 Photodiode Array Detector (PDA; Waters Ltd., United Kingdom) and a Waters C18 Nova-Pak radial compression column (4 mm, 8 mm, 6100 mm) equilibrated with 95% solvent A (5% acetic acid) at a flow rate of 2 ml/min. The sample injection volume was typically 50 µl, and compounds were eluted by linear gradient to 70% solvent B (100% methanol) over 15 min and monitored from 240 to 400 nm, and the concentration was quantified using a pure standard curve. In addition, it was also checked the presence of the SDG degradation products (secoisolariciresinol, enterodiol and enterolactone) on HPLC and Liquid Chromatography-Mass Spectrometry.

Mass spectrometry was performed on a Thermo Finnigan LC-MS system (Thermo Electron Corp, Waltham, MA, USA) comprising a Finnigan PDA Plus detector, a Finnigan LTQ linear ion trap with ESI source and a Waters C18 Nova-Pak column (3.9 100 mm, particle size 4 µm), with column oven temperature maintained at 30º C. The PDA scan range was set to 240–400 nm, and injection volume was typically 10 µl. The mobile phase consisted of water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). The column was equilibrated with 95% solvent A at a flow rate of 1 ml/min (20µL typical injection volume), with 10% going to the mass spectrometer, and the percentage of solvent B increased linearly to 65% over 60 min. MS parameters were as follows: sheath gas 30, auxiliary gas 15 and sweep gas zero (arbitrary units), spray voltage -4.0 kV in negative and 4.8 kV in positive ionisation mode, capillary temperature 320 ºC, capillary voltage -1.0 V and 45 V, respectively, tube lens voltage -68 (negative mode) and 110 V (positive mode), respectively, and normalised collision energy typically 35%.

Four screening assays were carried out (2 in October, 2016 and 2 in February, 2017), and the plates were ranked from the best breakdown (0.261 nM final SDG concentration) to

the worst (0.364 nM final SDG concentration) the SDG content was calculated based on the amount incubated (0.364 nM SDG) and the final concentration based on the predicted concentration, which was obtained on the SDG peak area and by standard curve equation (Y=1E+07X-6874; R^2=0.99). The plates considered effective in SDG breakdown were divided in eight sessions (every two rows of the plate) (Figure 18) and reassessed for SDG breakdown.

Figure 16. 384-well plate content printed on clear petri dish plate.

Figure 17. SDG incubation: Fosmid clones pool + SDG + LB broth + Chloramphenicol.

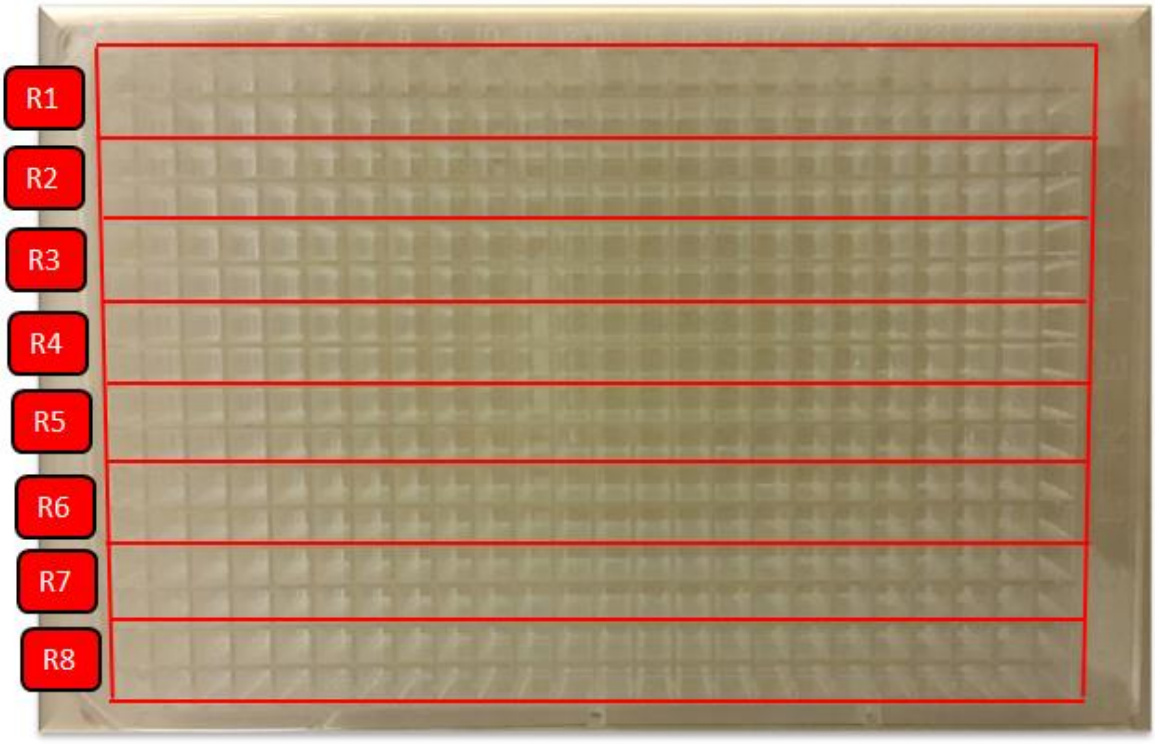

Figure 18. Sessions on selected 384-well plate

R1: Row A and B; R2: Row C and D; R3: Row E and F; R4: Row G and H; R5: Row I and J; R6: Row K and L; R7: M and N; R8: Row O and P.

Purifying fosmid clones to sequencing

The fosmid clones selected by means of SDG breakdown screening (8R4, 8R5 and 14R1) were purified in order to sequence high quality plasmid DNA. The purification was carried on using the QIAprep Spin Miniprep Kit (QIAGEN, UK), following the manufacturer instructions. The plasmid DNA was eluted in Buffer EB (10 mM Tris-Cl, pH 8.5) and kept in the freezer at -80ºC until being send to sequence in a Hiseq sequencing system (Illumina®).

The final plasmid DNA concentration was 79.65 ng/µL (8R4), 113.55ng/µL (8R5), and 158.55 ng/µL (14R1).

Bioinformatics

The bioinformatics analysis of the samples fosmid library data was performed at the Aberystwyth University between the 11th June and 09th July, 2017. Data analysis of the fosmid library was performed in an UNIX platform, using High-Performance Computing (HPC), which consists of a master node, a login node, 11 compute nodes and two storage nodes. The combined compute capacity of the HPC is: 544 CPU cores, 4.2TB RAM and 43TB storage capacity and 11TB of fast access disk. This is backed by a 1/4 PB storage array for storage of sequencing data.

FASTQC

NGS can generate tens of millions of sequences in a single run. Before analysing this sequence to draw biological conclusions, some simple quality control checks should be performed to ensure that the raw data looks good and there are no problems or biases in the data. Most sequencers generate a QC report as part of their analysis pipeline, but this is usually only focused on identifying problems which were generated by the sequencer itself. FastQC aims to provide a QC report which can spot problems which originate either in the sequencer or in the starting library material (Schmieder and Edwards, 2011).

Per base sequence quality

All samples (3 fosmid pool samples) from the fosmid library were similar in quality, therefore, one of the samples (8R4, read 1 (R1 - forward)) was used as example for the quality check.

The background of the graph (Figure 19) divides the y axis (quality score) into very good quality calls (green), calls of reasonable quality (orange), and calls of poor quality (red). The x axis represents the position in read (bp).It is possible to observe that all the reads bigger than 5bp were in excellent quality condition (green background).

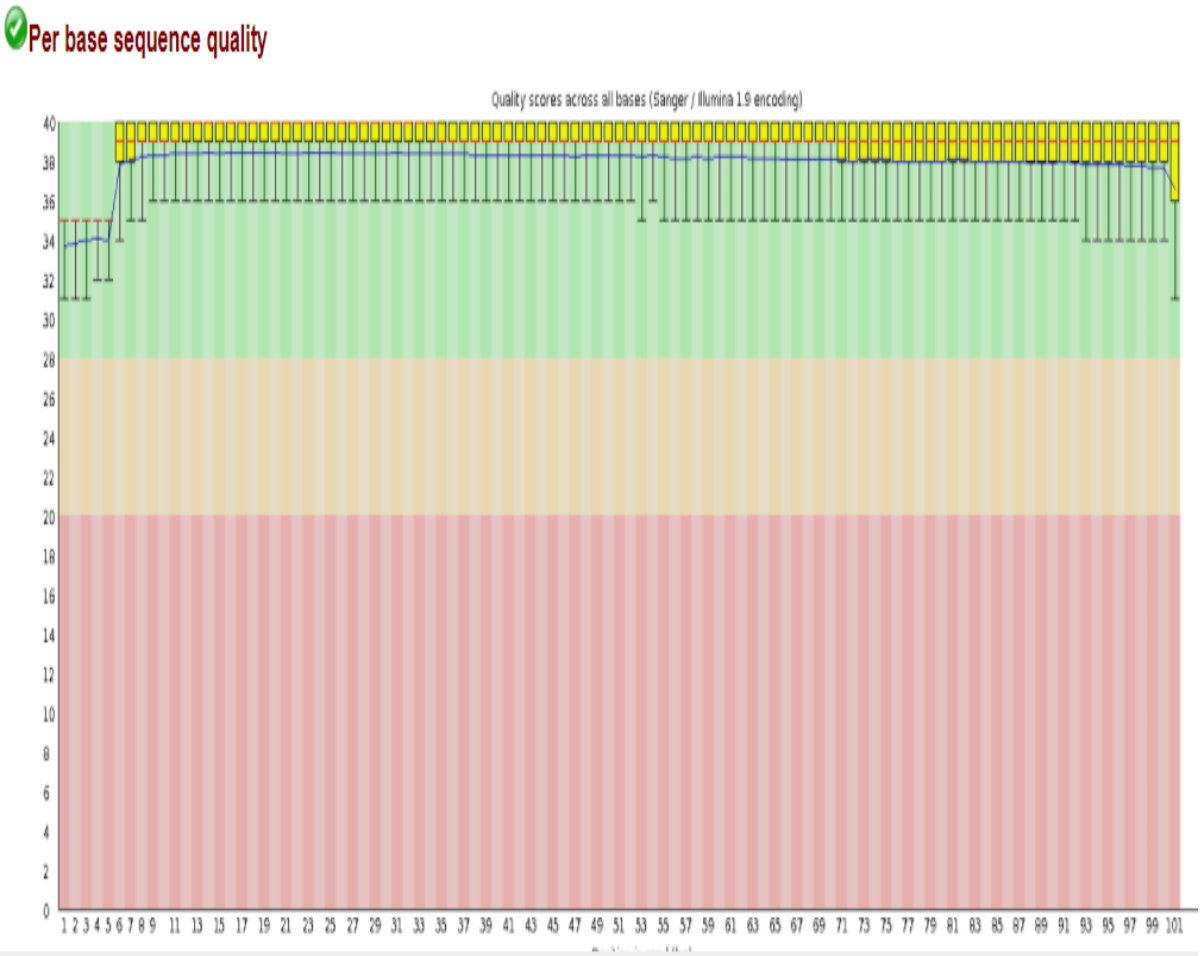

Figure 19. Per base sequence quality of the 8R4, forward read.

Per sequence quality scores

The per sequence quality score report allows to see if a subset of the sequences have universally low quality values (Schmieder and Edwards, 2011). It is possible to observe (Figure 20) that the major portion of the reads presented high quality scores (above 36, in a scale from 0 to 40).

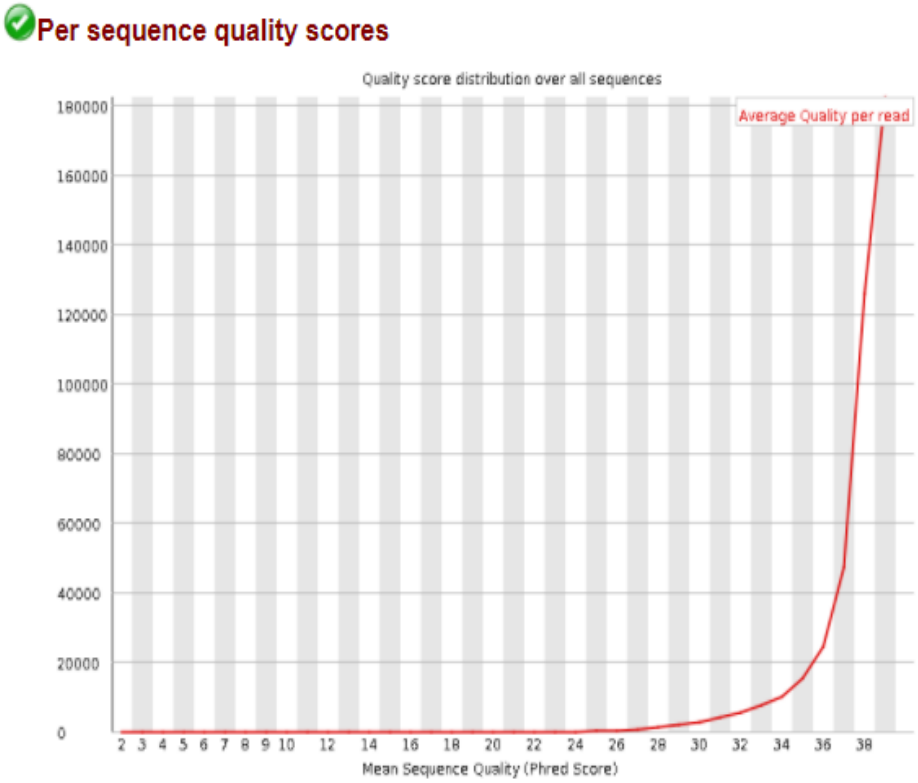

Figure 20. Per sequence quality score of the 8R4, forward read.

Per base sequence content

In a normal library it is expected that there would be little to no difference between the different bases of a sequence run, so the lines in the plot should run parallel with each other. The graph (Figure 21) plots thymine (T) in red; cytosine (C) in blue; adenine (A) in green; and guanine (G) in black. It is possible to observe that the nitrogen bases have a little fluctuation up to 5bp reads; the different bases of the run present no difference within the rest of the run.

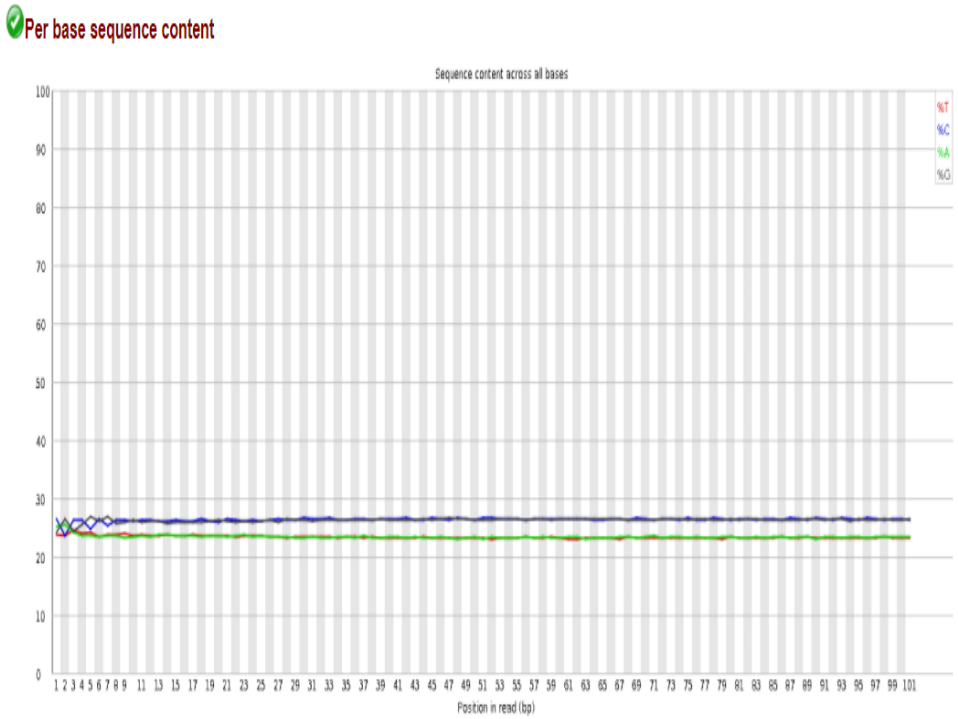

Figure 21. Per base sequence content of 8R4, forward read.

Overrepresented Sequences

A normal high-throughput library will contain a diverse set of sequences, with no individual sequence making up a tiny fraction of the whole. Finding that a single sequence is very overrepresented in the set either means that it is highly biologically significant, or indicates that the library is contaminated, or not as diverse as you expected. It is possivel to observe (Figure 22) that an Illumina (TruSeq) adapter was found with overrepresentation in the run. The following sequence contaminate the analysis and should be excluded from the reads. This can be done in the next step, trimming the reads.

Overrepresented sequences

Sequence	Count	Percentage	Possible Source
GATCGGAAGAGCACACGTCTGAACTCCAGTCACATTCAGAAATCTCGTAT	889	0.1128873120502443 8	TruSeq Adapter, Index 13 (97% over 38bp)

Figure 22. Overrepresented sequences from 8R4, forward read.

TRIMMOMATIC

Trimmomatic is a fast, multithreaded command line tool that can be used to trim and crop Illumina (FASTQ) data as well as to remove adapters (Bolger et al., 2014).

The following trimming steps were used:

- ILLUMINACLIP: Cut adapter and other illumina-specific sequences from the read
- HEADCROP: Cut the specified number of bases from the start of the read
- MINLEN: Drop the read if it is below a specified length

ILLUMINACLIP: removed Truseq Adapter; HEADCROP: cut 5bp from the start of the read; MINLEN: droped reads below 50 bases long.

FASTQC AFTER TRIMMING

After trimming unwanted parts of the run a new Fastqc was performed in order to check if the new result is reliable to further analysis (Figure 23). The new quality check results showed that the run was shortened up to 95bp and the beginning of the run was cut off. All runs were trimmed with the same protocol step with the same parameters.

Per base sequence quality after trimming

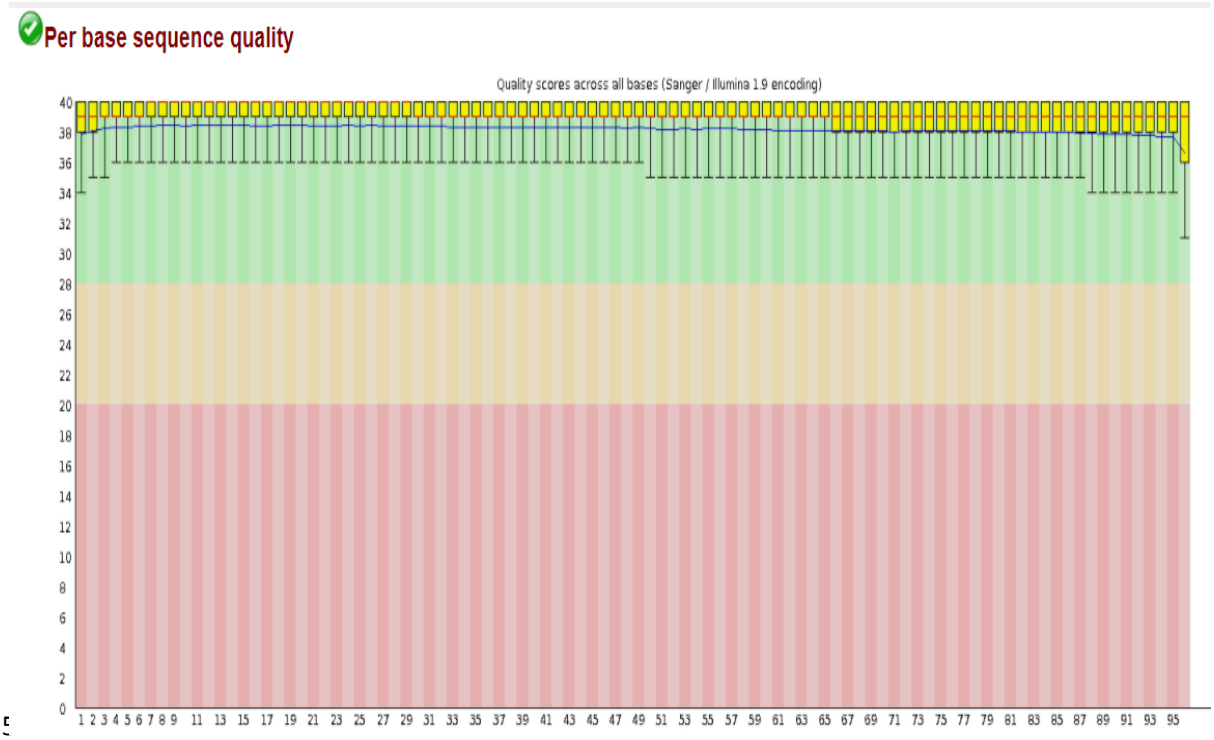

Figure 23. Per base sequence quality of the 8R4, forward read, after trimming.

Overrepresented sequences after trimming

The Illumina (TruSeq) adapter found with overrepresentation in the run was removed (Figure 24) from the read, leaving the data without overrepreented sequences.

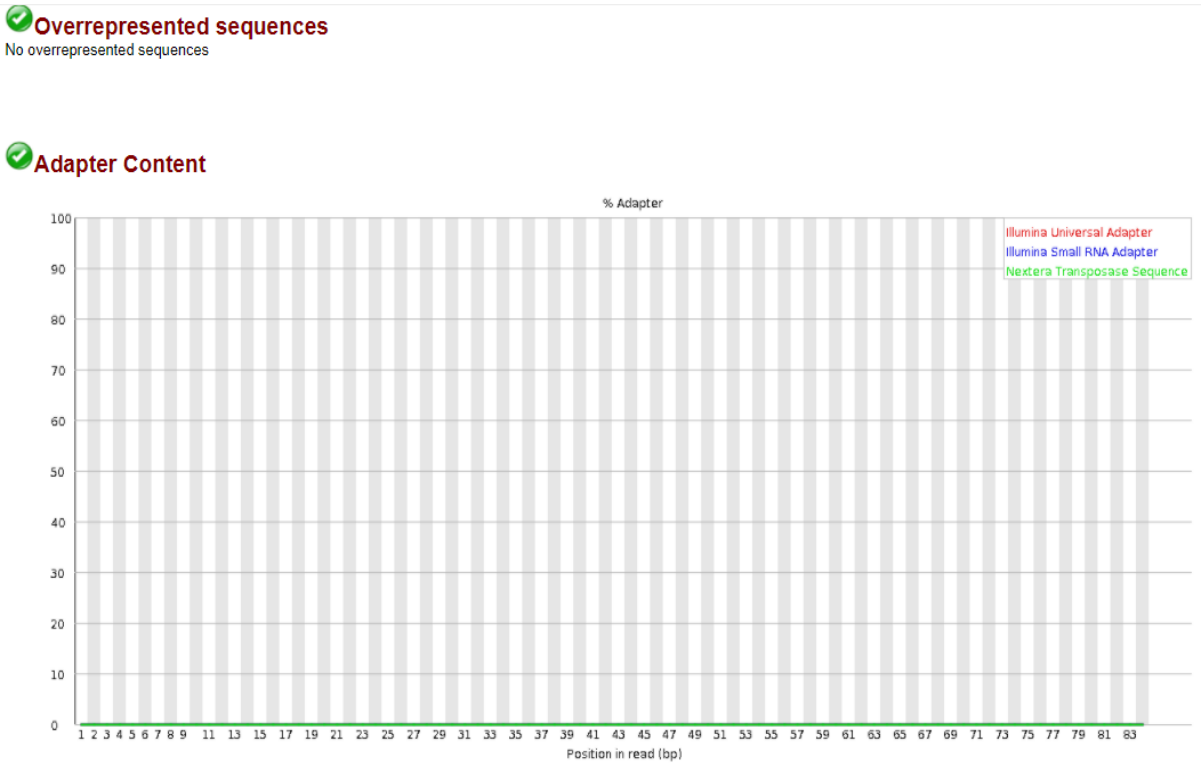

Figure 24. Overrepresented sequences removal from 8R4, forward read

ALIGNMENT – VELVET ASSEMBLER

Reads were assembled in a *de novo* approach using Velvet (Zerbino and Birney, 2008). The data followed with *de novo* genome assembly and short read sequencing alignment. Velvet is an algorithm package that was applied for dealing with the trimmed fastq files. The data was processed through the manipulation of De Bruijn graphs for genomic sequence assembly via the removal of errors and the simplification of repeated regions. This step prepares the data for genome annotation (Compeau et al., 2011).

The parameters chosen for this step were: 21k-mer coverage; fastq files type; shortPaired data type (velveth); and automatic settings for expected coverage and cutoff (exp cov to the length of median contig coverage and cov_cutoff to half that value) due to reasonably uniform coverage over the sample.

GENOMIC ANNOTATION – PROKKA

The final step was to annotate all relevant genomic features on those contigs. This step was performed using the module Prokka, a command line software tool to fully annotate a draft bacterial. It produces standards-compliant output files for further analysis or viewing in genome browser. (Seemann, 2014).

MULTIPLE GENOME ALIGNMENTS – MAUVE

In order to align the reference genome of an *E. coli k-12* str. K12 substr. DH10B, GenBank: CP000948.1, available online at the National Center for Biotechnology Information (NCBI, 2014) with the fosmid clones data (8R4, 8R5 and 14R1). The gbk files generated in the genomic annotation step were aligned using Mauve software following operation guidelines (Darling et al., 2010).

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IV. **Transcripts linked to secoisolariciresinol diglucoside metabolism in the rumen**

(Normas: Environmental Microbiology)

SUMMARY

The flaxseed (*Linum usitatissimum)*, the richest source of lignans among foods, mainly secoisolariciresinol diglucoside, is metabolized in the rumen into the enterolignans enterodiol and enterolactone, which are been pointed out as powerful antioxidant compounds. In addition, ruminants can transfer lignans produced in the rumen to physiological fluids (plasma) and animal products (milk), providing also increased mammalian lignan plasma concentration to the consumer. However, there is not much information regarding the rumen microbes responsible for secoisolariciresinol diglucoside breakdown and enterolignans production. Approaches that by-pass species isolation and culture are been recently used to study the rumen ecology and metabolic processes. Nonetheless, even metagenomic technologies have limitations. Analysis of RNA transcripts can overcome such obstacles and effective tools to find novel genes with active role in the rumen metabolism. Therefore, a metatranscriptome was carried out using rumen fluid as inoculum and secoisolariciresinol diglucoside in *in vitro* incubations. The total RNA from incubations was successfully extracted and sent to deep sequencing. RNA sequencing data was generated and overrepresented sequences listed as possible high biological significance, which can be used to find differentially expressed genes in order to find genes and enzymes linked to secoisolariciresinol diglucoside breakdown and lignans products.

KEY-WORDS: lignan, metatranscriptome, RNA, rumen, SDG
RUNNING TITLE: overrepresented sequences in SDG rumen metabolism

INTRODUCTION

Oxidative imbalance can lead to excessive free radicals production, caused by insufficient inhibition, delay or removal of those (antioxidant capacity). The substances capable of tackling oxidative damage can do it by direct scavenging reactive oxygen species or indirect acting up-regulating antioxidant defences (Halliwell, 2007; Khlebnikov et al., 2007). Such disorder in oxidative defences is linked to the aging and cellular degenerative process and

also associated with several metabolic disorders, such as cancer, cardiovascular disease, immune-system decline, brain dysfunction, endocrine functions, cataracts and others (Ames et al., 1993; Christen, 2000; Hitchon and El-Gabalawy, 2004; Vincent et al., 2004; Nunomura et al., 2006; Wood-Kaczmar et al., 2006; Carreau et al., 2008).

There are many sources of antioxidants compounds, such as minerals, animal products, plants, and others (Carocho and Ferreira, 2013). Lignans are non-enzymatic antioxidants, found in plants such as flaxseed (*Linum usitatissimum)*, the richest source of lignans among foods, in greater concentration in the seed hull (Choo et al., 2007; Goyal et al., 2014). A wide variety of lignans might be found in the flaxseed: secoisolariciresinol diglucoside (SDG); secoisolariciresinol (SECO); isolariciresinol; pinoresinol; and matairesinol, and all of these compounds except isolariciresinol could be enterediol (ED) and enterolactone (EL) precursors (Heinonen et al., 2001).

Flaxseed intake is recommended by the lignans antiestrogenic, antioxidant, anti-inflammatory, anticarcinogenic and cardioprotective capacity (Adolphe et al., 2010; Imran et al., 2015) and the benefits can be extended to the consumer of animals products, for instance, lignans fed to dairy cows can be transferred to milk and meat (Gagnon et al., 2009; Petit and Gagnon, 2009a; Petit et al., 2009; Matumoto-Pintro et al., 2011; Lima et al., 2016), and further more can be transferred, for example, to the new-born serum after lignan-rich milk intake (Ghedini et al., 2017). This suggests that lignans-rich sources should be included in animals diet as functional food, with potential for disease prevention and health benefits.

The rumen microbial community is a complex environment, which can differ between animals, considering that the microbiome populations can gain or lose dominance in the rumen depending on the composition of the diet (Stiverson et al., 2011; Carberry et al., 2012; Huws et al., 2012; Friedman et al., 2017), age (Jami et al., 2013), host animal breed (King et al., 2011), host animal species (Baraka, 2012) feed efficiency (Jami et al., 2014; Shabat et al., 2016), and other factors, such as antibiotic usage, health of the host animal, geographic location, season, photoperiod, stress level, environment, and feeding regimen (intake and frequency) (Puniya et al., 2015). However, a core microbiome can be found across individuals within a wide geographical range (Jami and Mizrahi, 2012; Henderson et al., 2015). Such complexity allows many possibilities for exploring novel biocatalysts of the rumen ecology, although, the main issue is the few number of ruminal species that can be successfully cultivated in laboratory conditions (less than ten percent) (Flint et al., 2008; Kim et al., 2011).

Rumen microorganisms can efficiently convert SDG to SECO, then to enterolignans (ED, and then to EL) (Côrtes et al., 2008; Petit and Gagnon, 2009a, b; Zhou et al., 2009; Lima et al., 2016), and few ruminal species are pointed as efficient on lignans metabolism (*Prevotella bryantii, Prevotella albensi, Prevotella ruminicola, Prevotella brevis, Peptostreptococcus anaerobius, Butyrivibrio fibrosolvens, Fibrobacter succinogens, Ruminococcus albus, Eubacterium ruminantium, Butyrivibrio proteoclasticus, Ruminococcus flavefaciens)* (Schogor et al., 2014). Due to limitations on ruminal microbes growth in laboratory conditions, the assessment of many microbes species biology depends on methods that bypass the need for isolation or cultivation of microorganisms. Exploring the whole rumen microbiome activity by metagenomic approaches allows enhancing the range of microorganisms being assessed achieving the challenge of exploring complex microbial environments (Handelsman, 2004; Ferrer et al., 2009; Simon and Daniel, 2011).

Metagenomics are indeed a reliable tool to explore a microbiome, however, it still have some limitations, this can be supressed by use of metatranscriptomic approaches, which have distinct characteristics when compared to metagenomics (Simon and Daniel, 2011). Briefly, the metatranscriptomics analysis identifies most transcribed genes, whereas metagenomics identifies the most dominant genes. The main issue is that a numerically prevalent group of microbes not always play active role in some metabolism activity. For instance, as much as 60% of total bacteria in rumen are represented by *Prevotella* sp., however, this group of bacteria play no active role in recalcitrant cellulose digestion (Stevenson and Weimer, 2007; Kong et al., 2010; Purushe et al., 2010).

Successful rumen metatranscriptome has proven that it is a reliable tool to explore the rumen in novel relevant genes discovery. Reverse transcription on glycoside hydrolases from celF, xynD, and cel3 from *Fibrobacter succinogenes* S85 in cow rumen (Wang et al., 2011), and exploring degradation patterns, such as plant cell wall polysaccharide in cow rumen (Dai et al., 2015) and genes coding for potentially valuable lignocellulolytic enzymes in muskoxen rumen (Qi et al., 2011).

This work combines the need for further findings on lignans metabolism and the complex rumen community yet to be deeply explored in the SDG breakdown pathways. Therefore, a metatranscriptome using cow rumen inoculum and SDG *in vitro* incubations was carried out, the extracts were sequenced and data is ready for further analysis using bioinformatics tools for possible transcript genes involved on SDG metabolism.

RESULTS AND DISCUSSION

Sequencing data

All samples form metatranscriptome were deep sequenced (Hiseq – Illumina), yielding approximately 11 GB of total complementary DNA (cDNA) (via RNA reverse transcription) data. Basic statistics for cDNA quality (Conventional base calls; Ecoding Sanger / Illumina 1.9) are listed in Table 1.

Table 1. Basic statistics for cDNA quality

Sample	Total sequences	Sequences flagged as poor quality	Sequence length (bp)	%GC (guanine-cytosine content):
RC1H0	4399318	0	96	52
RC2H0	5342618	0	96	50
RC3H0	4583533	0	96	51
RC1H6	3975126	0	96	51
RC2H6	4089860	0	96	48
RC3H6	2842790	0	96	50
RC1H24	7170743	0	96	52
RC2H24	3924789	0	96	51
RC3H24	5097049	0	96	51
SRC1H6	3391018	0	96	51
SRC2H6	4243010	0	96	47
SRC3H6	4044657	0	96	49
SRC1H24	4982588	0	96	52
SRC2H24	2913291	0	96	51
SRC3H24	5589767	0	96	52

Coding for incubation samples: Rumen fluid (R); Cow (C); Hour (H); SDG (S).
Cow: 1, 2 and 3; Time-points: 0h, 6h and 24h.

Overrepresented Sequences

A normal high-throughput library will contain a diverse set of sequences, with no individual sequence making up a tiny fraction of the whole. Finding that a single sequence is very overrepresented in the set either means that it is highly biologically significant, or indicates that the library is contaminated (Bolger et al., 2014). Overrepresented sequences were identified using FastQC software, as possible high biological significance in the metatranscriptome data and are listed in Table 2. Sequences will be analysed for putative genes linked to SDG breakdown.

Sequencing data must be assembled and differentially expressed genes should be assessed in order to find genes and enzymes with active role in SDG breakdown in the rumen.

Table 2. Rumen metatranscriptome overrepresented sequences

Sample	Overrepresented Sequence	Count	Percentage
RC1H0	GAGTACCTTTTATCCTTTGAGCGATGCAGTTTCCATACACTTGCACCGGA	6178	0.140
	TTGTAACACGTGTGTAGCCCCGGACGTAAGGGCCGTGCTGATTTGACGTC	4548	0.103
	ATCCTGGCTCAGGATGAACGCTAGCTACAGGCTTAACACATGCAAGTCGA	13819	0.314
RC2H0	TCTTTCGTCCTTGATTAATGAAAACATCCTTGGCAAATGCTTTCGCATAC	7633	0.142
	ATCTTTCGTCCTTGATTAATGAAAACATCCTTGGCAAATGCTTTCGCATA	7561	0.141
	CTTTCGTCCTTGATTAATGAAAACATCCTTGGCAAATGCTTTCGCATACG	6763	0.126
	TTTGGTCTACAAGAGATTTCTGTTCTCTTTGAGCTCACCTTTGGACACCT	6644	0.124
	CTTCGGCCGCCAACGTTTCCAATTGGCTATTTGCTACTACCACCAAGATC	6433	0.120
	TTTCATCTTTCCTTCACAGTACTTGTTCGCTATCGGTCTCCCATCAATAT	5836	0.109
	TGATTAATGAAAACATCCTTGGCAAATGCTTTCGCATACGTTAGTCTTTA	5674	0.106
	AGTTACGGATCTATTTTGCCGACTTCCCTTATCTACATTATTCTATCGAC	5606	0.104
	ACTGTCTCTATTAACCGTAATTTAGAATTAACCAAGATTCTAAATACATT	5519	0.103
	CTATCGGTCTCCCATCAATATTTAGCTTTGGAAGATTCATATCTCCCGCT	5419	0.101
	ATTTAGGACCGAGTAACCCCTGACCAAATGCTGTTCGCAGGGAACCCTTC	5362	0.100
	TTGGTCTACAAGAGATTTCTGTTCTCTTTGAGCTCACCTTTGGACACCTG	5354	0.100
RC3H0	TTGTAACACGTGTGTAGCCCCGGACGTAAGGGCCGTGCTGATTTGACGTC	5543	0.120
	GAGTACCTTTTATCCTTTGAGCGATGCAGTTTCCATACACTTGCACCGGA	5155	0.112
	ATCCTGGCTCAGGATGAACGCTAGCTACAGGCTTAACACATGCAAGTCGA	10134	0.221
RC1H6	No overrepresented sequences	0	0
RC2H6	TTTGGTCTACAAGAGATTTCTGTTCTCTTTGAGCTCACCTTTGGACACCT	7735	0.189
	ATCTTTCGTCCTTGATTAATGAAAACATCCTTGGCAAATGCTTTCGCATA	7639	0.186
	AGTTACGGATCTATTTTGCCGACTTCCCTTATCTACATTATTCTATCGAC	7093	0.173
	TCTTTCGTCCTTGATTAATGAAAACATCCTTGGCAAATGCTTTCGCATAC	6327	0.154
	CTATACCCAAATTTGACGAGTGATTTGCACGTCAACACCGCTTCGAGCCT	6261	0.153
	ATTTAGGACCGAGTAACCCCTGACCAAATGCTGTTCGCAGGGAACCCTTC	6169	0.150
	ATCCTGAGGGAAACTTCAGAGGGAACCAGCTACTAGATGGTTCGATTAGT	6074	0.148
	ATTAGTCTTTCGCCCCTATACCCAAATTTGACGAGTGATTTGCACGTCAA	4106	0.100
	AGATCTGCACTAGATGCCGTTCGACCCAGGCTCACGCCAAAGGCTTCTCA	4105	0.100
RC3H6	TTTCATCTTTCCTTCACAGTACTTGTTCGCTATCGGTCTCCCATCAATAT	3143	0.110
	CTGCCTTCCGTAGATGTAGTAGCCGTTTCTCAGGCTCCTTCTCCAGAATC	3046	0.107
	AGTTACGGATCTATTTTGCCGACTTCCCTTATCTACATTATTCTATCGAC	2969	0.104
	CAGGTCCAGACATAGTAAGGATTGACAGATTGATAGCTCTTTCTTGATTC	3346	0.117
RC1H24	GAGTACCTTTTATCCGTTGAGCGATGGCCCTTCCATACAGAACCACCGGA	60095	0.838
	CTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTG	45971	0.641
	GCTTTCGCACCTGCTTGACTTGTCAGTCTCGCAGTTAAGCACGCTTATGC	42552	0.593
	TACATCTTCCGCGCAGGACGACTCGATCAGTGAGCTATTACGCTTTCTTT	35861	0.500
	TTACATCTTCCGCGCAGGACGACTCGATCAGTGAGCTATTACGCTTTCTT	32237	0.449
	TTCGTTTCCCACTTAGCCAATTTTAGGGACCTTAGCTGGCGGTCTGGGTT	30994	0.432
	GGTTCGCCTCAACAGCCTATGTATTCAGCTGTTGATACCCTTGCGGGTGG	29255	0.407
	CAATTCCTTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCAACT	28005	0.390
	AGTACCTTTTATCCGTTGAGCGATGGCCCTTCCATACAGAACCACCGGAT	27840	0.388
	CTGCCGCACTCCAGCCTTGCAGTCACAATGGCAGTTCCCAGGTTGAGCCC	26006	0.362
	CTTTCCGTCTTTCCGCGGGGAGATTGCATCATCACAAACATTTCAACTTC	25924	0.361
	TGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGA	25593	0.356
	TGATGAGTATTTAGCCTTGGAGGATGGTCCCCCCATGTTCAGACAGGGTT	24410	0.340
	CACATCGCACTATTGAGCGGTACAGGAATATTGACCTGTTTCCCATCAGT	24279	0.338
	TTCACCCCTATCCACAGCTCATCCGCTAATTTTGCAACACTAGTCGGTTC	24091	0.335
	GTCAAACTGCCTACCATGCACTGTCCCCGATCCCGATTAGGGACCCAGGT	22667	0.316
	ATACATTTTCGGCGCAGCGTCACTCGACCAGTGAGCTATTACGCACTCTT	15826	0.403
	GAGTACCTTTTATCCGTTGAGCGATGGCCCTTCCATACAGAACCACCGGA	13323	0.339
	CTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTG	13044	0.332
	GCTTTCGCATCTGCTCGACTTGTCAGTCTCGCAGTTAAGCACGCTTATGC	12162	0.309
	TACATTTTCGGCGCAGCGTCACTCGACCAGTGAGCTATTACGCACTCTTT	11710	0.298
	TACATCTTCCGCGCAGGACGACTCGATCAGTGAGCTATTACGCTTTCTTT	10777	0.274
	ATCGATTAGTATTCGTCAGCTCCATGTGTCACCACACTTCCACCTCGAAC	10759	0.274
	CAATTCCTTTGAGTTTCAACCTTGCGGCCGTACTCCCCAGGCGGTCAACT	10436	0.265

RC2H24	CATCGCACTATTGATCGGTACAGGAATATTGACCTGTTTCCCATCAGCTA	9973	0.254
	ATCGAATTAAACCACATCATCCACCGCTTGTGCGGGTCCCCGTCAATTCC	9808	0.249
	TTACATCTTCCGCGCAGGACGACTCGATCAGTGAGCTATTACGCTTTCTT	9644	0.245
	ATTCACTGCGGCTCTCATGCGCTTGCACGCTCAAGAGCACCCCTTCTCCC	9274	0.236
	TTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTC	9185	0.234
	ATCCTGGCTCAGATTGAACGCTGGCGGCATGCCTTACACATGCAAGTCGA	17101	0.435
	AATGCCTTGGCACTAGGAGCCGAAGAAGGACGGCACTAACACCGATATGC	15846	0.403
	TTTACCGAATTCAGACAAACTCCGAATGCCAGATATTTATACACGGGAGT	8207	0.209
RC3H24	GAGTACCTTTTATCCGTTGAGCGATGGCCCTTCCATACAGAACCACCGGA	53636	1.05
	CAATTCCTTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCAACT	47398	0.929
	TACATCTTCCGCGCAGGACGACTCGATCAGTGAGCTATTACGCTTTCTTT	43316	0.849
	GCTTTCGCACCTGCTTGACTTGTCAGTCTCGCAGTTAAGCACGCTTATGC	41861	0.821
	TTACATCTTCCGCGCAGGACGACTCGATCAGTGAGCTATTACGCTTTCTT	40650	0.797
	CTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTG	40437	0.793
	CTGCCGCACTCCAGCCTTGCAGTCACAATGGCAGTTCCCAGGTTGAGCCC	37062	0.727
	TCTGCCGCACTCCAGCCTTGCAGTCACAATGGCAGTTCCCAGGTTGAGCC	35043	0.687
	GGTTCGCCTCAACAGCCTATGTATTCAGCTGTTGATACCCTTGCGGGTGG	34510	0.677
	TTCGTTTCCCACTTAGCCAATTTTAGGGACCTTAGCTGGCGGTCTGGGTT	31284	0.613
	ATCCTGGCTCAGATTGAACGCTGGCGGCATGCCTTACACATGCAAGTCGA	68960	1.352
	AGTACAGCTTGGGAGACAGAGCACCGGGTGCTAACGTCCGGACTCAAGAG	23536	0.461
	GTATTACCTTCGGGGGTAGAGCACTGTTTTGGCTAGGGGGTCATGGCGAC	21711	0.425
SRC1H6	TTTGGTCTACAAGAGATTTCTGTTCTCTTTGAGCTCACCTTTGGACACCT	4039	0.119
	ATCTTTCGTCCTTGATTAATGAAAACATCCTTGGCAAATGCTTTCGCATA	3995	0.117
	ATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAACACATGCAAGTCGA	4377	0.129
	CAGGTCCAGACATAGTAAGGATTGACAGATTGATAGCTCTTTCTTGATTC	4014	0.118
SRC2H6	ATCTTTCGTCCTTGATTAATGAAAACATCCTTGGCAAATGCTTTCGCATA	16339	0.385
	TTTGGTCTACAAGAGATTTCTGTTCTCTTTGAGCTCACCTTTGGACACCT	15002	0.353
	TCTTTCGTCCTTGATTAATGAAAACATCCTTGGCAAATGCTTTCGCATAC	12323	0.290
	TGATTAATGAAAACATCCTTGGCAAATGCTTTCGCATACGTTAGTCTTTA	9001	0.212
	CTTCGGCCGCCAACGTTTCCAATTGGCTATTTGCTACTACCACCAAGATC	8819	0.207
	CAGGTCCAGACATAGTAAGGATTGACAGATTGATAGCTCTTTCTTGATTC	10052	0.236
	CCAGTGAAATACCACTACTTGAGACGTCGTCTTACTTATTCCGTTAATTA	8819	0.207
	ATTTGTTAAAGACTAACGTATGCGAAAGCATTTGCCAAGGATGTTTTCAT	8078	0.190
	AGTAAGATGACTCGCTGGACTTAAGCATATTATTAAGCGAAGGAAAAGAA	7098	0.167
	CTTTTCTTCTTAACATGTATGAGGCCTTGAAATTGGATTACCCAGAGAGA	6921	0.163
SRC3H6	ATCTTTCGTCCTTGATTAATGAAAACATCCTTGGCAAATGCTTTCGCATA	11154	0.275
	TTTGGTCTACAAGAGATTTCTGTTCTCTTTGAGCTCACCTTTGGACACCT	7922	0.195
	TCTTTCGTCCTTGATTAATGAAAACATCCTTGGCAAATGCTTTCGCATAC	7888	0.195
	AGTTACGGATCTATTTTGCCGACTTCCCTTATCTACATTATTCTATCGAC	5892	0.145
	CTTCGGCCGCCAACGTTTCCAATTGGCTATTTGCTACTACCACCAAGATC	5638	0.139
	TGATTAATGAAAACATCCTTGGCAAATGCTTTCGCATACGTTAGTCTTTA	5617	0.138
	TTTCATCTTTCCTTCACAGTACTTGTTCGCTATCGGTCTCCCATCAATAT	5104	0.126
	CTTTCGTCCTTGATTAATGAAAACATCCTTGGCAAATGCTTTCGCATACG	4388	0.108
	CAGGTCCAGACATAGTAAGGATTGACAGATTGATAGCTCTTTCTTGATTC	7581	0.187
	CCAGTGAAATACCACTACTTGAGACGTCGTCTTACTTATTCCGTTAATTA	6454	0.159
	ATTTGTTAAAGACTAACGTATGCGAAAGCATTTGCCAAGGATGTTTTCAT	4725	0.116
	CTTTTCTTCTTAACATGTATGAGGCCTTGAAATTGGATTACCCAGAGAGA	4621	0.114
	GTCTACCCTATATGCGAGTGTGCGAGTGGAGAAACTCATACGCGGAATGA	4407	0.108
SRC1H24	CAATTCCTTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCAACT	42489	0.852
	TACATCTTCCGCGCAGGACGACTCGATCAGTGAGCTATTACGCTTTCTTT	38773	0.778
	GGTTCGCCTCAACAGCCTATGTATTCAGCTGTTGATACCCTTGCGGGTGG	36637	0.735
	CTGCCGCACTCCAGCCTTGCAGTCACAATGGCAGTTCCCAGGTTGAGCCC	36330	0.729
	TTCGTTTCCCACTTAGCCAATTTTAGGGACCTTAGCTGGCGGTCTGGGTT	35856	0.719
	GAGTACCTTTTATCCGTTGAGCGATGGCCCTTCCATACAGAACCACCGGA	35794	0.718
	TTACATCTTCCGCGCAGGACGACTCGATCAGTGAGCTATTACGCTTTCTT	35638	0.715
	GCTTTCGCACCTGCTTGACTTGTCAGTCTCGCAGTTAAGCACGCTTATGC	35043	0.703
	CTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTG	34555	0.693
	TCTGCCGCACTCCAGCCTTGCAGTCACAATGGCAGTTCCCAGGTTGAGCC	31724	0.636
	GAGTCCGGACGTTAGCACCCGGTGCTCTGTCTCCCAAGCTGTACTCTTCG	28009	0.562
	TTTCGGGTCTACACCCAGCGACTCAAACGCCCTGTTCGGACTCGATTTCT	25838	0.518
	ATCCTGGCTCAGATTGAACGCTGGCGGCATGCCTTACACATGCAAGTCGA	65165	1.307
	AGTACAGCTTGGGAGACAGAGCACCGGGTGCTAACGTCCGGACTCAAGAG	26642	0.534
	GTATTACCTTCGGGGGTAGAGCACTGTTTTGGCTAGGGGGTCATGGCGAC	24070	0.483
	GATGAAAGCAGGGGACCCTCGGGCCTTGCGCTATCAGAGCGGCCGATGGC	23360	0.468
	AATACCGAAGAGTACAGCTTGGGAGACAGAGCACCGGGTGCTAACGTCCG	22775	0.457
	CCCAAAGCGTAACGGAGGAGTTCGAAGGTACGCTAGTTACGGTCGGACAT	21215	0.425
	ACTTACCAAACCAAGGCAAACTCCGAATACCGAAGAGTACAGCTTGGGAG	20646	0.4143

	ATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTTGTAAGACAGTG	17660	0.354
SRC2H24	CAATTCCTTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCAACT	18105	0.621
	TACATCTTCCGCGCAGGACGACTCGATCAGTGAGCTATTACGCTTTCTTT	16227	0.556
	GAGTACCTTTTATCCGTTGAGCGATGGCCCTTCCATACAGAACCACCGGA	13570	0.465
	CTTTCCGTCTTTCCGCGGGGAGATTGCATCATCACAAACATTTCAACTTC	13316	0.457
	TTCGTTTCCCACTTAGCCAATTTTAGGGACCTTAGCTGGCGGTCTGGGTT	13121	0.450
	GCTTTCGCACCTGCTTGACTTGTCAGTCTCGCAGTTAAGCACGCTTATGC	12317	0.422
	CTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTG	11977	0.411
	AGTACCTTTTATCCGTTGAGCGATGGCCCTTCCATACAGAACCACCGGAT	11662	0.400
	ATCCTGGCTCAGATTGAACGCTGGCGGCATGCCTTACACATGCAAGTCGA	26674	0.915
	AGTACAGCTTGGGAGACAGAGCACCGGGTGCTAACGTCCGGACTCAAGAG	10225	0.350
	CCCAAAGCGTAACGGAGGAGTTCGAAGGTACGCTAGTTACGGTCGGACAT	7136	0.244
	AATACCGAAGAGTACAGCTTGGGAGACAGAGCACCGGGTGCTAACGTCCG	5963	0.204
	GTATTACCTTCGGGGGTAGAGCACTGTTTTGGCTAGGGGGTCATGGCGAC	5629	0.193
SRC3H24	TACATCTTCCGCGCAGGACGACTCGATCAGTGAGCTATTACGCTTTCTTT	43861	0.784
	CTGCCGCACTCCAGCCTTGCAGTCACAATGGCAGTTCCCAGGTTGAGCCC	43439	0.777
	CAATTCCTTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCAACT	42291	0.756
	GCTTTCGCACCTGCTTGACTTGTCAGTCTCGCAGTTAAGCACGCTTATGC	37235	0.666
	TTCGTTTCCCACTTAGCCAATTTTAGGGACCTTAGCTGGCGGTCTGGGTT	37157	0.664
	GAGTACCTTTTATCCGTTGAGCGATGGCCCTTCCATACAGAACCACCGGA	34708	0.620
	CTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTG	34288	0.613
	GAGTCCGGACGTTAGCACCCGGTGCTCTGTCTCCCAAGCTGTACTCTTCG	0.569	0.569
	AGTACCTTTTATCCGTTGAGCGATGGCCCTTCCATACAGAACCACCGGAT	31590	0.565
	TGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGA	30636	0.548
	TTACATCTTCCGCGCAGGACGACTCGATCAGTGAGCTATTACGCTTTCTT	29558	0.528
	GGTTCGCCTCAACAGCCTATGTATTCAGCTGTTGATACCCTTGCGGGTGG	29312	0.524
	TCTGCCGCACTCCAGCCTTGCAGTCACAATGGCAGTTCCCAGGTTGAGCC	28676	0.513
	ATCCTGGCTCAGATTGAACGCTGGCGGCATGCCTTACACATGCAAGTCGA	78476	1.403
	AGTACAGCTTGGGAGACAGAGCACCGGGTGCTAACGTCCGGACTCAAGAG	32352	0.578
	CCCAAAGCGTAACGGAGGAGTTCGAAGGTACGCTAGTTACGGTCGGACAT	26139	0.467
	GTATTACCTTCGGGGGTAGAGCACTGTTTTGGCTAGGGGGTCATGGCGAC	25908	0.463
	GATGAAAGCAGGGGACCCTCGGGCCTTGCGCTATCAGAGCGGCCGATGGC	21926	0.392
	AATACCGAAGAGTACAGCTTGGGAGACAGAGCACCGGGTGCTAACGTCCG	21018	0.376
	ATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTTGTAAGACAGTG	19778	0.353
	AGCTTCGGGCTAATACCCTGGAGTCATGACGGTACCGTAAGAATAAGCAC	15265	0.273
	GAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGT	14436	0.258
	ACTTACCAAACCAAGGCAAACTCCGAATACCGAAGAGTACAGCTTGGGAG	14351	0.256
	CTTACCAAACCAAGGCAAACTCCGAATACCGAAGAGTACAGCTTGGGAGA	14346	0.256
	CCTTGCGCTATCAGAGCGGCCGATGGCAGATTAGGTAGTTGGTGGGATAA	13971	0.249
	GTGGCGAACGGGTGAGTAATACATCGGAACGTGCCTGGTAGTGGGGGATA	13643	0.244
	CGATAGTGCAATGGCATAAGCGTGCTTAACTGCGAGACTGACAAGTCAAG	13011	0.232
	ATATGGTGGATGCCTTGGCGATGATAGGCGAAGAAAGACGTGATAGCCTG	12850	0.229
	AGTTAACAAAACGGTCTGGAAAGGCCGGCCATAGTAGGTGATAGCCCTGT	12607	0.225
	AGTGGCGAACGGGTGAGTAATACATCGGAACGTGCCTGGTAGTGGGGGAT	12252	0.219
	CCTAAACGATGTCAACTGGTTGTTGGGGATTTATTTTCTCAGTAACGAAG	11765	0.210

In conclusion, RNA sequencing data was generated and now transcripts can be assessed in order to identify putative genes linked to SDG breakdown.

EXPERIMENTAL PROCEDURES

Mixed species metatranscriptome incubations

In order to assess the gene expression of the rumen microbiota when in contact to SDG a metatranscriptome was carried out, comparing the gene expression of rumen fluid solution in three different time points with or without SDG into the incubation solution.

First of all, rumen fluid was collected from three different cows and strained through two layers of cheese cloth. The rumen fluid was kept warm and taken to the laboratory for immediate use. Moreover, SDG and rumen fluid were added into the incubation tube inside an anaerobic workstation: 100µL of rumen fluid + 875µL of anaerobic medium (Table 3 and 4) (modified Van Soest medium (Theodorou et al., 1994)) + 25µL of SDG stock solution (0.25mg) (10mg/mL). The incubations were carried out in triplicates with a different cow in each repetition. Negative control incubations were carried out as following: 100µL of prepared pure culture + 900µL of anaerobic medium. The incubation times were: 0h; 6h; and 24h. Immediately after preparing the Hungate tubes, the incubations were transferred to an incubator at 39ºC.

The tubes were removed from the incubator according to each time point. The content (1 mL) was centrifuged at 13,000 rpm for 2 minutes, the supernatant was discarded and the remaining pellet was stored at -80ºC until RNA extraction procedure.

The incubations were carried out as following: Coding for incubation samples: Rumen fluid (R); Cow (C); Hour (H); SDG (S); Cow: 1, 2 and 3; Time-points: 0h, 6h and 24h. For instance, "RC1H0" stands for incubation of rumen fluid of cow 1 at 0 hour time point:

- RC1H0: SAMPLE 1a → Rumen fluid Cow 1 – 0h
- RC2H0: SAMPLE 1b → Rumen fluid Cow 2 – 0h
- RC3H0: SAMPLE 1c → Rumen fluid Cow 3 – 0h
- **RC1H6: SAMPLE 2a → Rumen fluid Cow 1 – 6h**
- **RC2H6: SAMPLE 2b → Rumen fluid Cow 2 – 6h**
- **RC3H6: SAMPLE 2c → Rumen fluid Cow 3 – 6h**
- RC1H24: SAMPLE 8a → Rumen fluid Cow 1 – 24h
- RC2H24: SAMPLE 8b → Rumen fluid Cow 2 – 24h
- RC3H24: SAMPLE 8c → Rumen fluid Cow 3 – 24h
- **SRC1H6: SAMPLE 9a → SDG + Rumen fluid Cow 1 – 6h**
- **SRC2H6: SAMPLE 9b → SDG + Rumen fluid Cow 2 – 6h**
- **SRC3H6: SAMPLE 9c → SDG + Rumen fluid Cow 3 – 6h**
- SRC1H24: SAMPLE 10a → SDG + Rumen fluid Cow 1 – 24h
- SRC2H24: SAMPLE 10b → SDG + Rumen fluid Cow 2 – 24h
- SRC3H24: SAMPLE 10c → SDG + Rumen fluid Cow 3 – 24h

Table 3. Anaerobic medium solutions for incubation

Product	Amount
Microminerals	
$CaCl_2 2H_2O$	13.2 g
$MnCl_2 4H_2O$	10 g
$CoCl_2 6H_2O$	1 g
$FeCl_2 6H_2O$	8 g
Distilled H_2O	100 ml
Solution A (Buffer)	
$NaHCO_3$	39.253 g
Distilled H_2O	1 L
Solution B (Macrominerals)	
Na_2HPO_4 (anhydrous)	9.45 g
KH_2PO_4 (anhydrous)	6.2 g
$MgSO_4$ $7H_2O$	0.6 g
Distilled H_2O	1 L
Solution C (Reducing Agent)	
Cystein HCl	0.625 g
1M NaOH	0.4 ml
Distilled H_2O	95 ml

Table 4. Working conditions for anaerobic medium

Solution	Amount
H_2O	550 ml
Solution A	220 ml
Solution B	220 ml
Solution C	40 ml
Resazurin (0.1%; w/v)	1 ml
Microminerals	100 µl
Total	1 L

Preparing RNA samples

Total RNA quantification

All the samples from the rumen microbiota metatranscriptome were submitted to RNA extraction, using FastRNA®Pro Soil – Direct Kit (MPBIO, UK), following the manufacturer instructions.

Total RNA was then quantified in duplicates using EPOCH® nucleic acid quantification. It was used 2µL of DEPC-H_2O as blank (Table 5). The quantification was double checked using alternative equipment, such as Nanodrop® and Qubit®.

Total RNA quantification

Total RNA available for sequencing (post purification and DNA depletion) was quantified in duplicates using EPOCH® nucleic acid quantification. It was used 2µL of DEPC-H_2O as blank. The quantification was double checked using alternative equipaments, such as Nanodrop® and Qubit®. According to Illumina (2014), all the samples were in satisfactory amounts (Total RNA 0.1 – 1 µg input).

Table 5. Mixed culture RNA nucleic acid quantification

Sample #	Total RNA (ng/µL)
RC1H0	18.97
RC2H0	16.81
RC3H0	13.53
RC1H6	12.57
RC2H6	11.61
RC3H6	16.89
RC1H24	22.41
RC2H24	25.05
RC3H24	46.57
SRC1H6	17.05
SRC2H6	22.65
SRC3H6	23.29
SRC1H24	32.97
SRC2H24	37.05
SRC3H24	38.81

Coding for incubation samples: Rumen fluid (R); Cow (C); Hour (H); SDG (S).
Cow: 1, 2 and 3; Time-points: 0h, 6h and 24h.

DNA depletion

All the RNA samples of mixed microbes from the rumen fluid metatranscriptome were submitted to a DNA depletion treatment (TURBO DNase Treatment, DNA-free Kit - Applied Biosystems, Austin,TX). Even though it was used a RNA extraction, the samples were still contaminated with DNA content, which would contaminate the sequencing information with unwanted data.

In order to check the 16S DNA content before and after the treatment (Figure 3) a 1% agarose gel was run to check 16s DNA content. It was used 2x MyTaqRed mix (Bioline, UK) as PCR amplification buffer and R1401/F969 as reverse and forward primers, respectively. The size ladder used was a 1kb ladder marker (Promega). In addition, it was checked the fungal contamination in all the samples by means of PCR amplification of EukF and EukR primers. It was identified no fungal contamination.

The rumen fluid metatranscriptome samples were stored at -80ºC until be sent to sequence in a Hiseq sequencing system (Illumina®), with 1µg Total RNA input, using a Truseq Adapter.

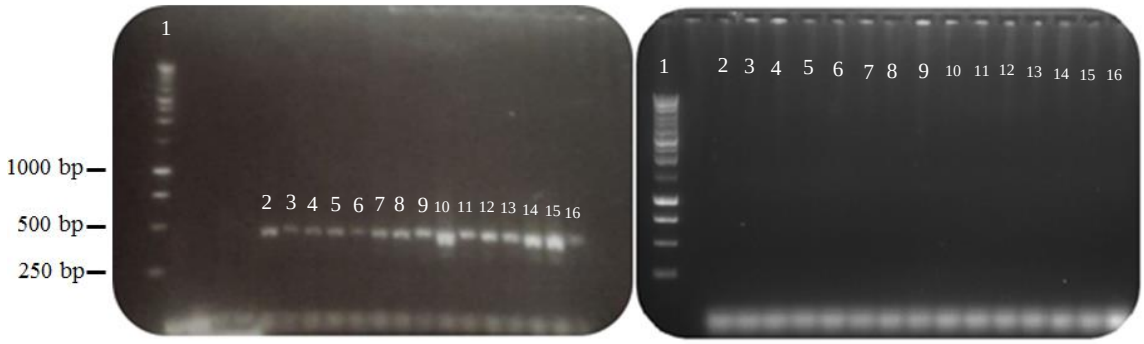

Figure 1. 16S DNA band detected in the RNA sample before DNase treatment (left) and 16S DNA band not detected in the RNA sample after the DNase treatment (right). Wells: size ladder; 2: RC1H0; 3: RC2H0; 4: RC3H0; 5: RC1H6; 6: RC2H6; 7: RC3H6; 8: RC1H24; 9: RC2H24; 10: RC3H24; 11: SRC1H6; 12: SRC2H6; 13: SRC3H6; 14: SRC1H24; 15: SRC2H24; 16: SRC3H24.

Bioinformatics

The bioinformatics analysis of the metatranscriptome data was performed in an UNIX platform, using High-Performance Computing (HPC), which consists of a master node, a login node, 11 compute nodes and two storage nodes. The combined compute capacity of the HPC is: 544 CPU cores, 4.2TB RAM and 43TB storage capacity and 11TB of fast access disk. This is backed by a 1/4 PB storage array for storage of sequencing data.

FASTQC

Before analysing the sequence to draw biological conclusions, some simple quality control checks were performed to ensure that the raw data looks reliable. FastQC was carried out in all data aiming to provide a QC report which can spot data problems (Schmieder and Edwards, 2011).

Per base sequence quality

All samples (15 samples) from the metatranscriptome were similar in quality, therefore, one of the samples (RC1H0, read 1 (R1 - forward)) was used as example for the quality check. Quality was checked using FastQC, followed by the trimming of unwanted data (ILLUMINACLIP: removed Truseq Adapter; HEADCROP: cut 5bp from the start of the read; MINLEN: droped reads below 50 bases long) using Trimommatic, according to (Bolger et al., 2014). After trimming, data quality was checked again (Figure 4).

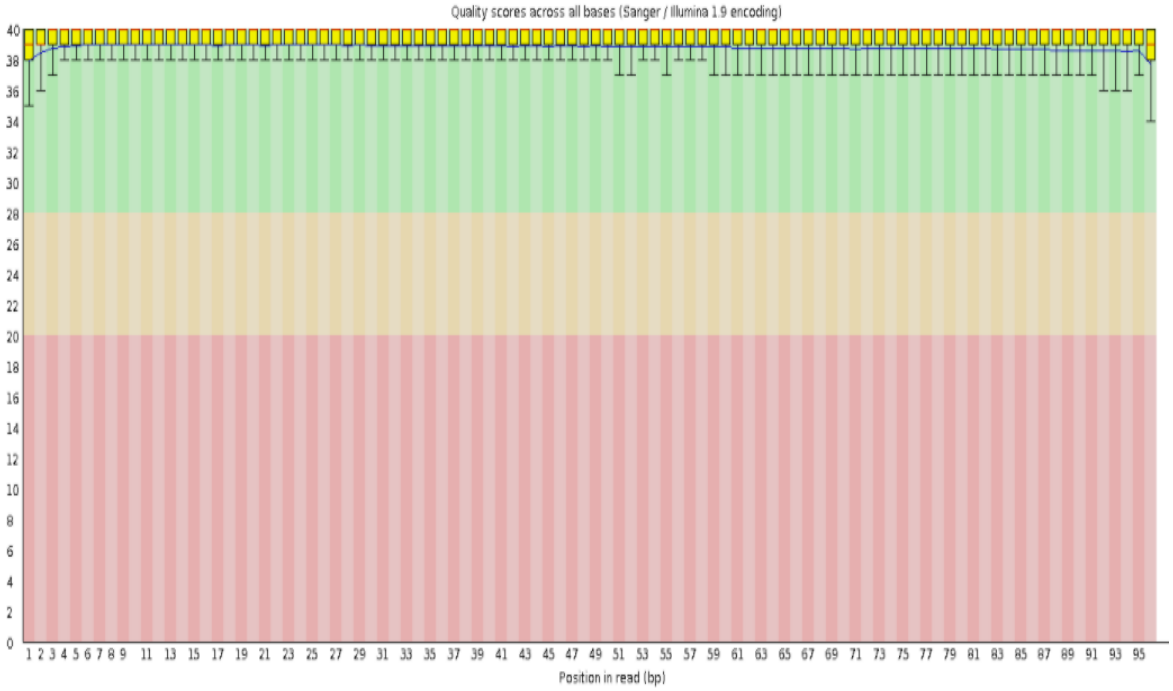

Figure 2. Per base sequence quality of the RC1H0, forward read.

Per sequence quality scores

The per sequence quality score report allows to see if a subset of the sequences have universally low quality values (Schmieder and Edwards, 2011). It is possible to observe (Figure 5) that the major portion of the reads presented high quality scores (above 36, in a scale from 0 to 40).

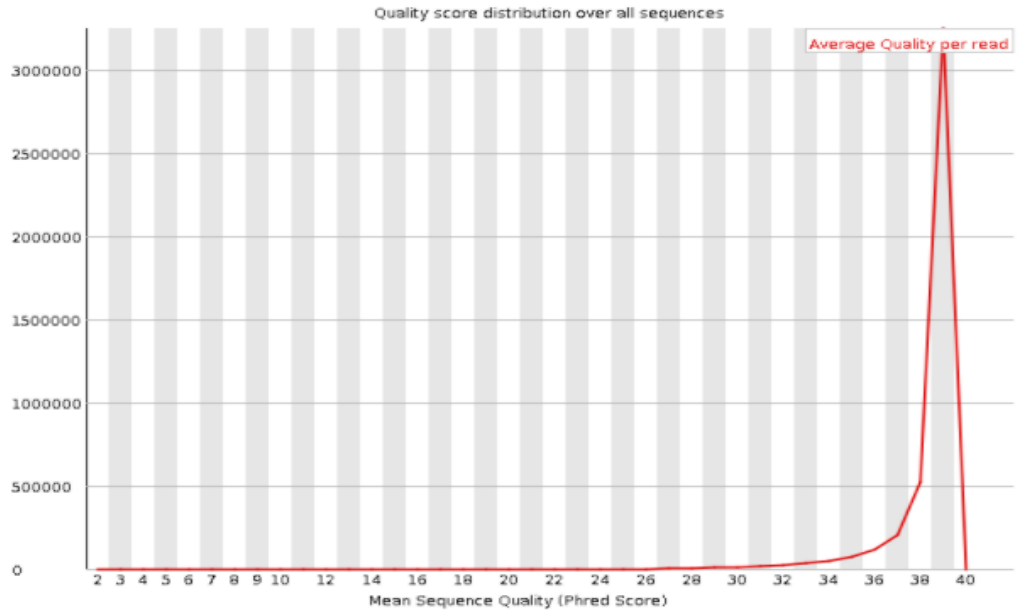

Figure 3. Per sequence quality score of the RC1H0, forward read.

Per base sequence content

It is expected to observe little or no difference between the different bases of a sequence run, however, as the nucleic acid were extracted from a mixed culture sample it was observed (Figure 6) a small fluctuation between bases (thymine (T) in red; cytosine (C) in blue; adenine (A) in green; and guanine (G) in black). (Schmieder and Edwards, 2011).

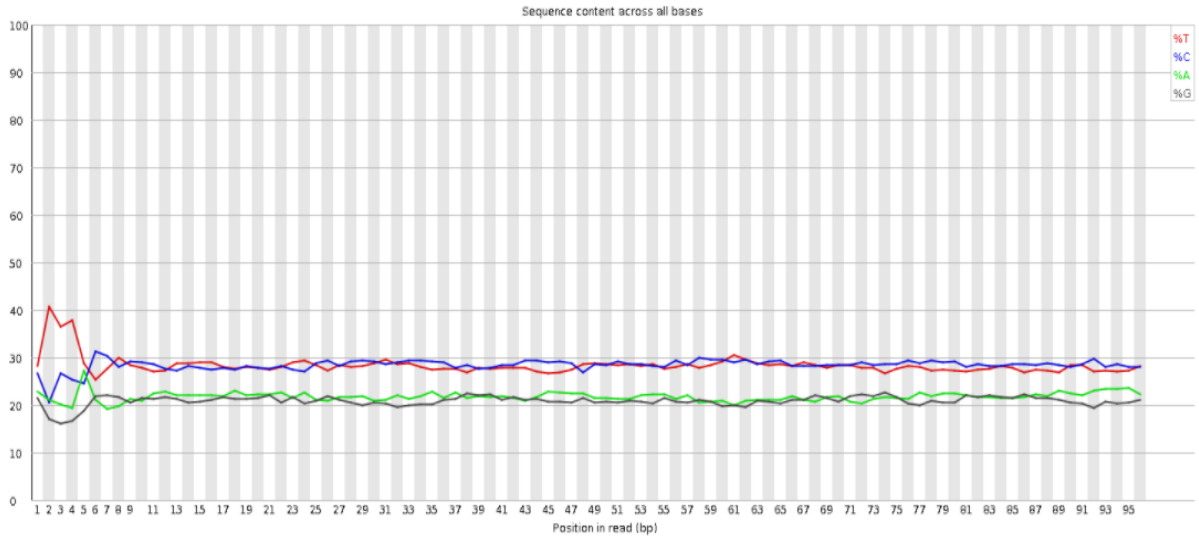

Figure 4. Per base sequence content of RC1H0, forward read.

Overrepresented Sequences

An Illumina (TruSeq) was observed adapter in most of the reads as overrepresentation in the run. The adapter was successfully removed from the read following trimming procedure using Trimmomatic (Bolger et al., 2014). Other overrepresented sequences were listed as possible high biological relevance.

RNA expression – Rnaseq data analysis

The metatranscriptome will be followed by genome annotation to map samples, using Tophat (Bowtie software) . TopHat is a program that aligns RNA-Seq reads to a genome in order to identify exon-exon splice junctions. It is built on the ultrafast short read mapping program Bowtie. TopHat is a bioinformatic sequence analysis package tool for fast and high throughput alignment of shotgun cDNA sequencing reads generated by transcriptomics technologies (e.g. RNA-Seq) using Bowtie first and then mapping to a reference genome to discover RNA splice sites *de novo* (Trapnell et al., 2009). The analysis will be followed by 4 steps using the following R software packages: GenomicAligments, Rsamtools, GenomicFeatures, DESeq2. Finally, the genes listed will follow to enrichment analysis to identify biological significance and pathways, it will be carried out using websites such as string-do.org; eggnogdb.de; uniprot.org.

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V. CONCLUSIONS

Flaxseed lignans are powerful antioxidants and their metabolism must be fully comprehended. As the rumen is an efficient environment for SDG conversion into enterolignans it need to be explored for the prospection of genes and enzymes linked to lignans metabolism. Nevertheless, as most of the microorganisms in the rumen cannot be cultured in laboratory conditions, metagenomic approaches must be used to assess genomic information associated with SDG breakdown.

A fosmid library, using *Escherichia coli* as bacterial host and DNA inserts from the rumen microbiome was successfully constructed, duplicated and screened for SDG breakdown activity. Positive clones for SDG breakdown did not present genes directly linked to SDG metabolism, showing that a fosmid library may not be an efficient method to recover those genes from the rumen microbiome.

Metatranscriptomic data obtained from serial incubations of rumen fluid and SDG, followed by RNA extraction was efficiently generated. Overrepresented sequences were identified as possible high biological significance and further studies must be carried out to list the differentially expressed genes and identify genes and enzymes with active role on SDG breakdown.

VI. APPENDICES

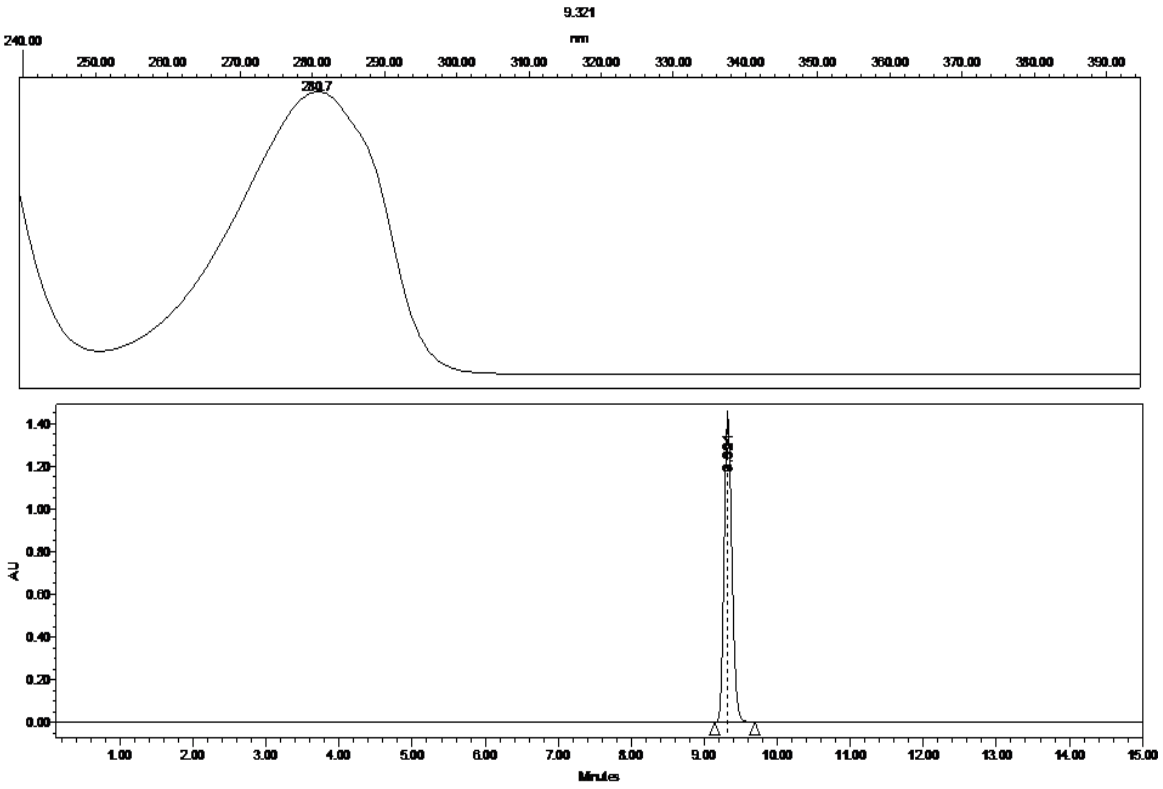

Figure VI.1. Secoisolariciresinol diglucoside (SDG) standard spectrum. Sample concentration 1 mg/ml; Retention time 9.3 minutes; PDA 280.0 nm; Gradient 5-70%MeOH in 15 minutes; Injection Volume 50.00 µL.

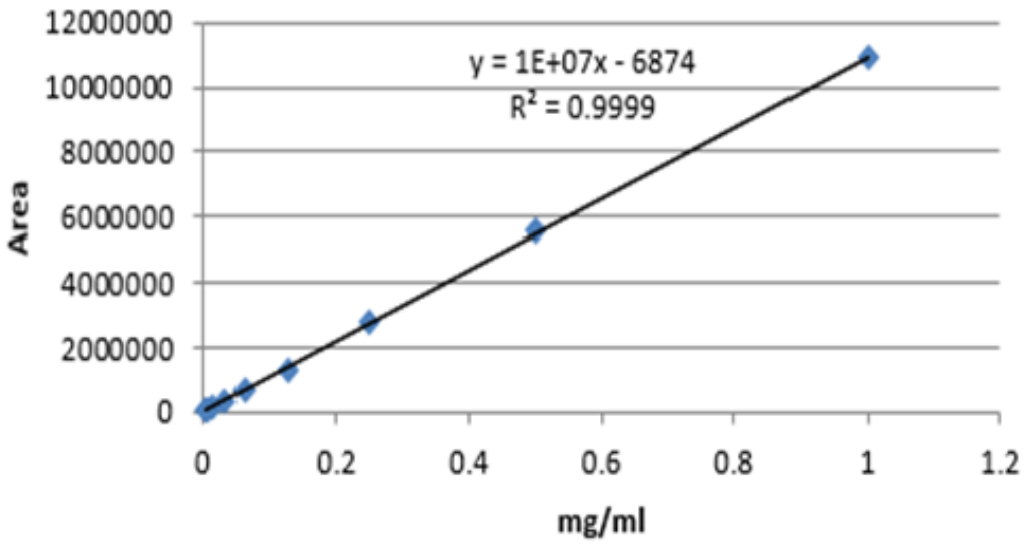

Figure VI.2 Secoisolariciresinol diglucoside (SDG) standard curve for HPLC detection and quantification. Curve concentrations and dilutions (mg/ml): 1 (1:10); 0.5 (1:20); 0.25 (1:40); 0.125 (1:80); 0.0625 (1:160); 0.03125 (1:320); 0.015625 (1:640); 0.007813 (1:1280); and 0.003906 (1:2560).

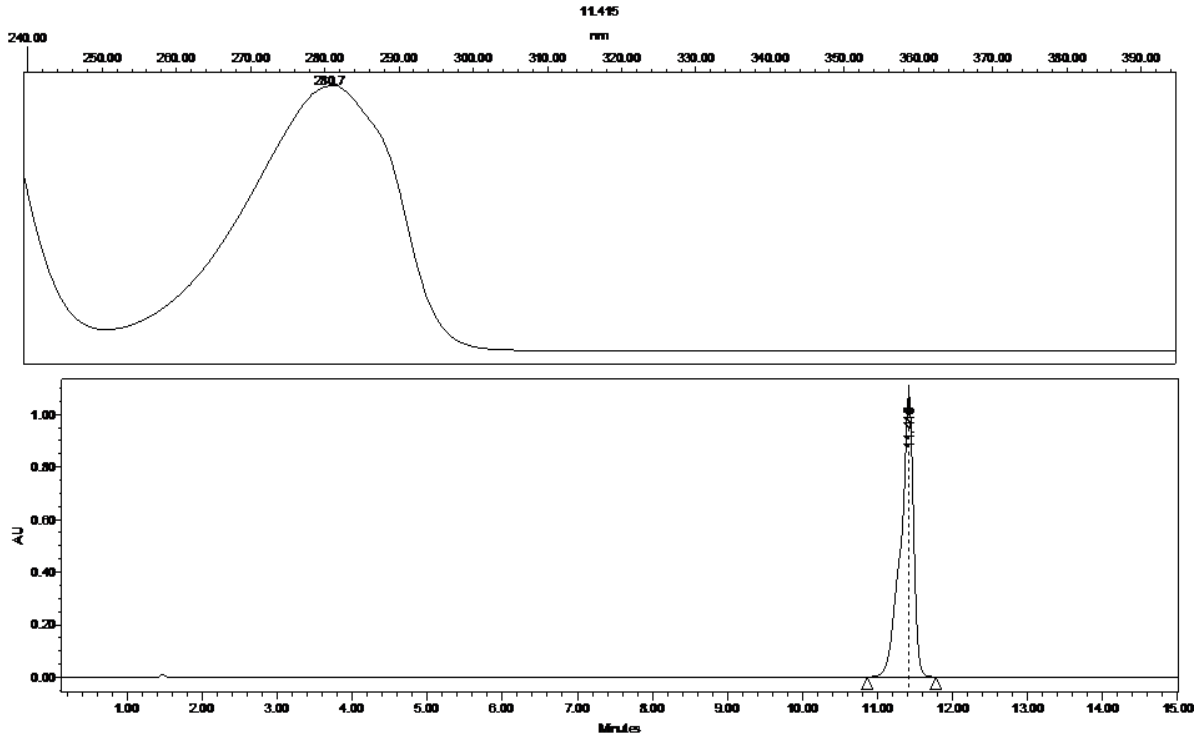

Figure VI.3 Secoisolariciresinol (SECO) standard spectrum. Sample concentration 0.5 mg/ml; Retention time 9.3 minutes; PDA 280.0 nm; Gradient 5-70%MeOH in 15 minutes; Injection Volume 50.00 µL.

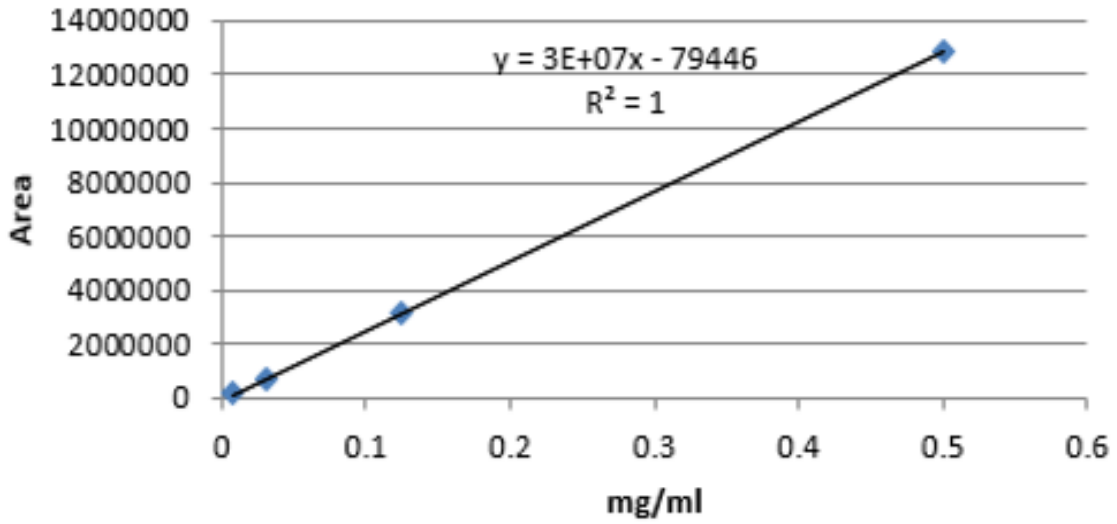

Figure VI.4 Secoisolariciresinol (SECO) standard curve for HPLC detection and quantification. Curve concentrations and dilutions (mg/ml): 0.5 (1:20); 0.125 (1:80); 0.03125 (1:320); and 0.007813 (1:1280).

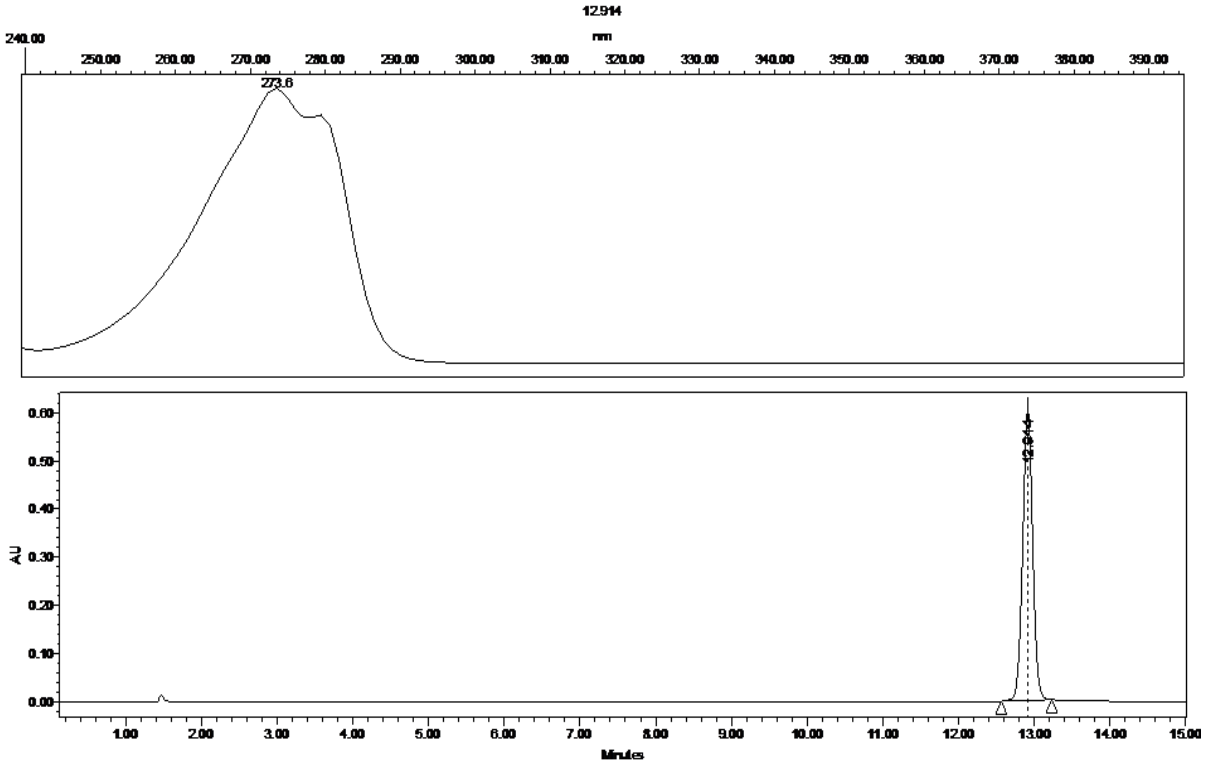

Figure VI.5. Enterodiol (ED) standard spectrum. Sample concentration 0.5 mg/ml; Retention time 12.9 minutes; PDA 280.0 nm; Gradient 5-70%MeOH in 15 minutes; Injection Volume 50.00 µL.

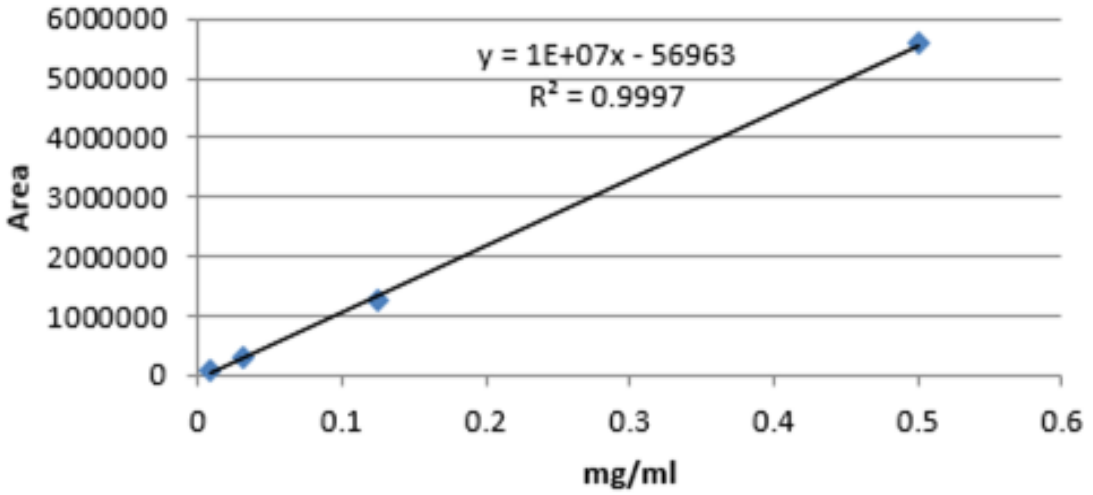

Figure VI.6 Enterodiol (ED) standard curve for HPLC detection and quantification. Curve concentrations and dilutions (mg/ml): 0.5 (1:20); 0.125 (1:80); 0.03125 (1:320); and 0.007813 (1:1280).

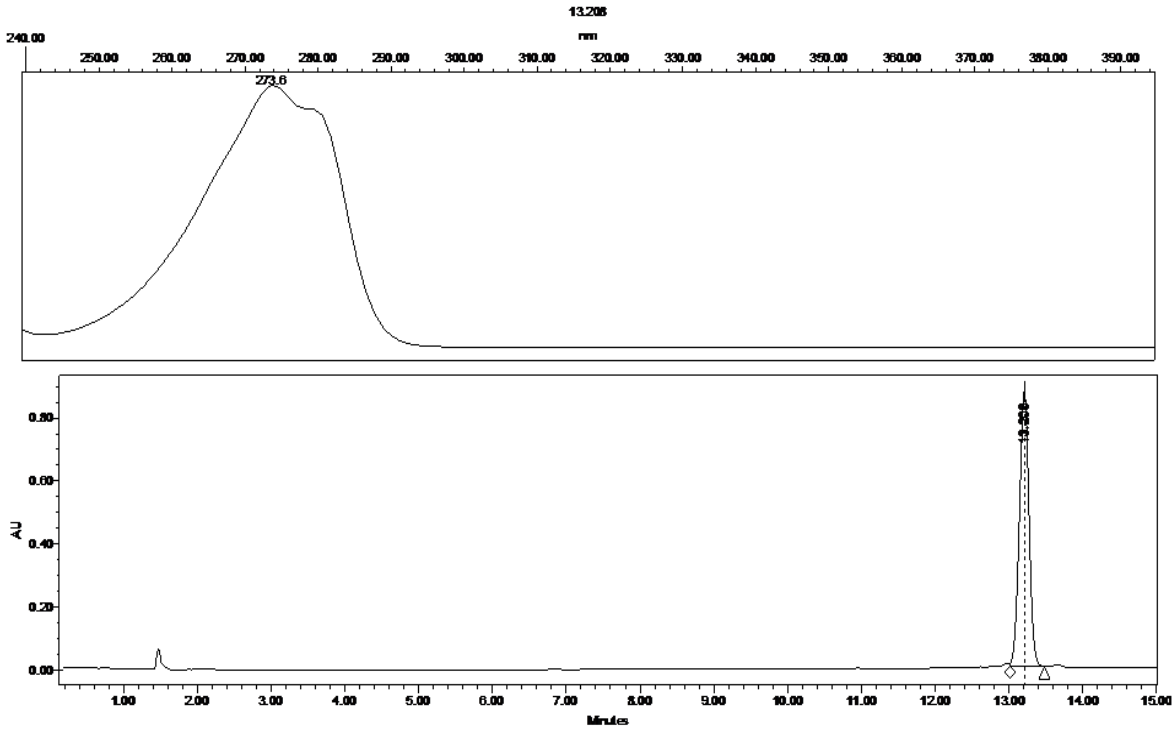

Figure VI.7. Enterolactone (EL) standard spectrum. Sample concentration 0.5 mg/ml; Retention time 13.2 minutes; PDA 280.0 nm; Gradient 5-70%MeOH in 15 minutes; Injection Volume 50.00 µL.

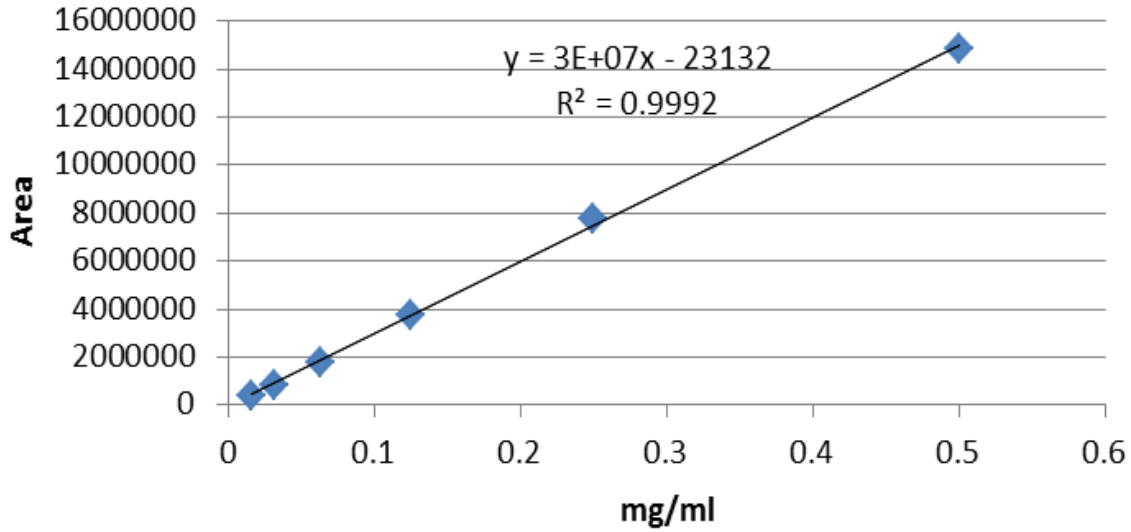

Figure VI.8 Enterolactone (EL) standard curve for HPLC detection and quantification. Curve concentrations and dilutions (mg/ml): 0.5 (1:20); 0.125 (1:80); 0.03125 (1:320); and 0.007813 (1:1280).

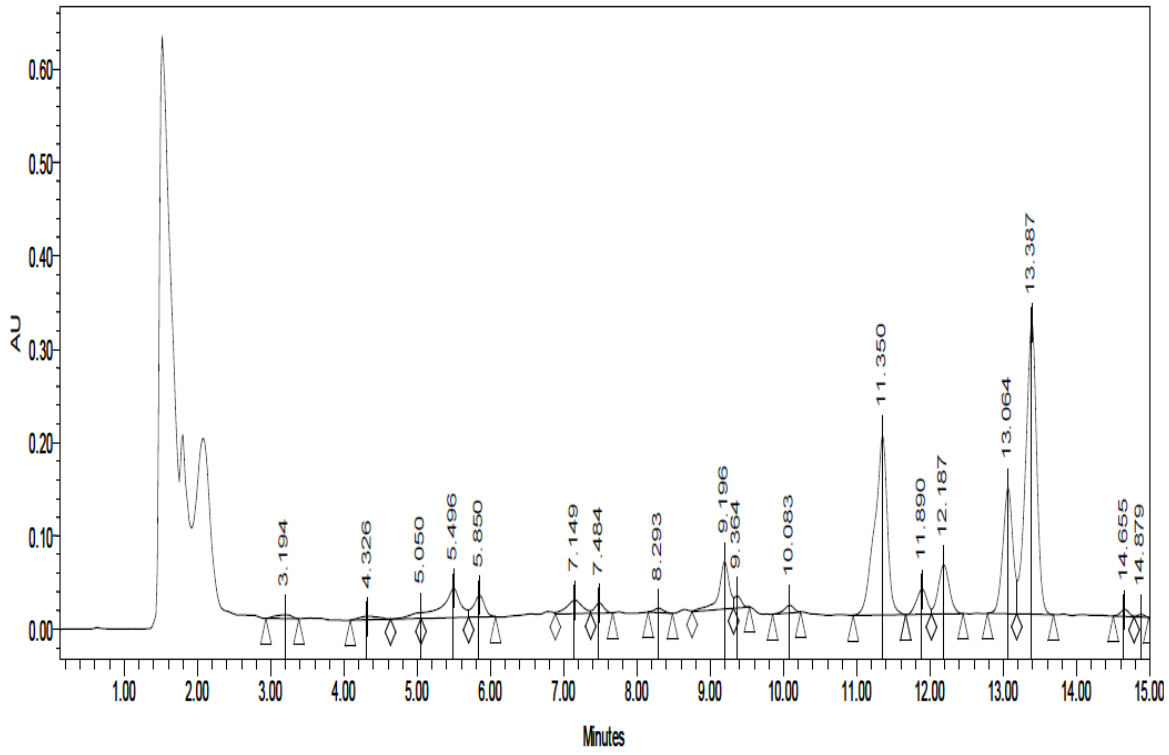

Figure VI.9. Sample of fosmid clone spiked with SECO (retention time 11.3), ED (retention time 13.0) and EL (retention time 13.3). PDA 280.0 nm; Gradient 5-70%MeOH in 15 minutes; Injection Volume 50.00 µL.