Chemical composition and *in vitro* ruminal fermentation of red grape pomace spent mushroom substrates

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Dissertation accepted in fulfilment of the requirements for the degree *Master of Science in Animal Science* at the North-West University

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Graduation: May 2021

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DECLARATION

I, Godfrey Mhlongo, declare that this thesis entitled "Chemical composition and *in vitro* ruminal fermentation of red grape pomace spent mushroom substrates" is my work. All the resources, which are used or quoted have been acknowledged accordingly in the form of a complete reference list per chapter. This thesis has not to be submitted by me or anyone else before to any other university than the North-West University.

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GENERAL ABSTRACT

Red grape pomace (GP) is a by-product of grape wine and juice making industries. The pomace is produced in large quantities and thus tends to have detrimental effects on the environment when it is disposed into the landfills or when it is incinerated. The use of GP in ruminant diets can mitigate the negative effects it has on the environment whilst at the same time, improving food and nutrition security in South Africa. However, the alternative use of GP as a feed source or ingredient in ruminant rations may be limited by high levels of structural carbohydrates and condensed tannins, which reduce nutrient digestibility and utilization. It is, therefore, important to evaluate innovative strategies to reduce the negative nutrient effect of these anti-nutrients such that high levels of GP can be included in ruminant diets for economic and environmental sustainability. One such previously unexplored strategy is the use of mushrooms to reduce fibre content and modify condensed tannins in GP. The aim of the current study was, therefore, to examine the influence of oyster mushroom (*Pleurotus ostreatus*) spawning rates and duration on the chemical composition, *in vitro* ruminal gas production and *in vitro* ruminal dry matter degradability (DMD) of red grape pomace (*Vitis vinifera* L. var. Shiraz). The red GP substrates were inoculated with oyster mushroom spawns at 0, 200, 300, 400, and 500 g/kg. The first chapter of the thesis provides a background on the grapes and ruminant industries, the problem statement, the justification and objectives of the study. The second chapter is a literature review on ruminants, the nutritional composition of grape pomace and the utilization of oyster mushrooms to improve the nutritive value of fibrous feeds for ruminants. The third chapter investigated the chemical composition of red GP treated with incremental spawn levels. The results from chapter three showed that the oyster mushroom (OM) spawning rates and incubation duration influenced the chemical composition (fibre and crude protein) of spent GP mushroom substrate (SMS). Both linear and quadratic trends (P <0.05) were observed for dry matter (DM), organic matter (OM), neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL) and crude protein (CP) in weeks 3 and 4.

Higher OM spawning levels (20 – 50%) and longer incubation periods (3 – 4 weeks) improved the NDF, ADF, ADL and CP content for the treated substrates relative to the untreated substrates. The fourth chapter investigated the *in vitro* ruminal gas production and the *in vitro* ruminal dry matter degradability parameters of the SMS, which was only evaluated using week 4 based on the maximum effectiveness of the oyster mushroom on the chemical composition of the red GP in week 4. The spawning rates linearly increased the rate of gas production at 12, 24 and 48 h of *in vitro* incubation. Quadratic trends were observed for the rate of gas production across all weeks. The treated substrates had a higher rate of gas production relative to the untreated substrates in week 2, 3 and 4. There were significant linear trends for the immediate fermentable fraction (*a*), the slowly fermentable fraction (*b*), the fermentation rate of fraction (*c*), the potential gas production (*Pgas*) and the effective gas production (*Egas*) in weeks 2 and 4. Quadratic responses were also observed in weeks 1, 2, 3 and 4 for the *in vitro* ruminal gas production kinetics. Inoculation with spawn (20 - 50%) had an effect (P <0.05) on the *in vitro* ruminal gas production kinetics substrates. Linear and quadratic trends were observed for the immediately degradable fraction (*a*), DMD48, the effective degradability (*EDeg*), the *in vitro* organic matter degradability (*iv*OMD) at 36 and 48 h, and the partitioning factor (PF) at 48 h, respectively. The oyster mushroom spawn treatment had an effect (P <0.05) on the fraction *a*, the slowly degradable fraction (*b*), potential degradability (*PDeg*), *EDeg* and PF (at 12, 24, 36 and 48 h). Substrate GP40 had a higher (P <0.05) fraction *b* (277.8 g/kg DM) and *PDeg* (147.5 g/kg DM) than the control post-incubation. It was, therefore, concluded that inoculating GP with graded levels of oyster mushroom spawn and incubating it for a sufficient amount of time (28 days) has the potential to reduce the fibre content of GP and improve the CP content. Furthermore, it has the potential to improve the *in vitro* ruminal dry matter degradability and fermentation efficiency of the red GP waste. However, it was not possible to deduce the optimum spawning rate to improve the chemical composition and *in vitro* fermentation of red grape pomace spent oyster mushroom substrate.

Keywords: Chemical composition; *In vitro* ruminal fermentation; Oyster mushroom; Red grape pomace; spent mushroom substrates

ACKNOWLEDGEMENTS

The financial assistance granted by the National Research Foundation (Freestanding Scholarship) and NWU Postgraduate Masters Bursary towards this research is hereby acknowledged.

A special appreciation to my supervisors, Prof. C.M. Mnisi and Prof. V. Mlambo, who have been guiding me throughout this research project. Thank you, Prof. Mnisi and Prof. Mlambo, for imparting your research and writing skills. Your guidance throughout the entire research project is greatly appreciated.

I am also grateful to Dr L.E. Motsei and Dr C. Kumanda for assisting me with laboratory analysis during my research.

Another special appreciation to my colleagues, Zuziwe Nkosi, Dante Lentsoe, Thabiso Masenya, Mveleli Marareni and Steve Mahlake, for their help and support, which resulted in this research project becoming a success against all odds.

All praises to GOD and my family, for the love, encouragement, and support they gave to me throughout my life.

DEDICATION

I dedicate this dissertation to my family and friends, more especially to my mother, Suzan Nompumelelo Ngomane, who has been my pillar of strength throughout my entire life.

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Mhlongo G, Mnisi, C.M. Mlambo, V. 2020. Cultivating oyster mushrooms on red grape pomace waste enhances potential nutritional value of the spent substrate for ruminants. PLoS One. (**Accepted**).

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LIST OF ABBREVIATIONS

AOAC	:	Association of Official Analytical Chemists
CP	:	Crude protein
DMD	:	Dry matter degradability
GLM	:	General linear model
GP	:	Grape pomace
*iv*OMD	:	*In vitro* organic matter degradability
SAS	:	Statistical analysis software
SMS	:	Spent mushroom substrate
SOP	:	Standard operating procedure
SSF	:	Solid-state fermentation
VFAs	:	Volatile fatty acids

1 CHAPTER ONE - GENERAL INTRODUCTION

1.1 Background

In a study by the South African Table Grape Industry (SATGI), it has been reported that South Africa is the most reliable and oldest supplier of table grapes (freshly harvested grapes) to the Northern hemispheres. According to DAFF (2016), the Western Cape Province accounts for more than 80% of grape production in South Africa. Other production areas include the Eastern and Northern Cape, Free State, Limpopo and the Mpumalanga provinces. In South Africa, grapes are grown for direct consumption, for drying into raisins and pressing for juice and winemaking. Pressing and/or fermentation processes that take place during winemaking and grape juice production generates solid organic waste called grape pomace (Cuccia, 2015). Large quantities (9 million metric tons) of grape pomace (leaves, stems, seeds, skins, and pulp) are generated per annum worldwide (Wadhwa *et al*., 2015). Arvanitoyannis & Varzakas (2008) reported that GP represents about 20% of the weight of processed grapes. Jianmei & Mohammed (2013) reported that grape pomace consists of a wide range of phenolic compounds and other bioactive compounds with potential beneficial applications in the nutraceutical industry. Teixeira *et al*. (2014) stated that wastes (GP) from vineyards production have been reported to be rich sources of natural cellulose, minerals, polyunsaturated fatty acids, and phytochemicals, which have nutritional, antioxidant, antimicrobial and anthelmintic properties that can be useful for ruminant production. In addition, Dwyer *et al*. (2014) reported that approximately 30 g/kg of GP is reused as animal feed, which also has potential phenolic and antioxidant fibres in their skin that can be beneficial to ruminants. However, their nutritional and chemical composition varies among the grape cultivars.

1.2 Problem statement

The world population growth causes an increased demand and pressure for food on agricultural production systems (Klapa, 2015). Ruminants are an important source of food for the growing population but may also compete with humans for food if reared on maize grain and soybeans. To ensure that ruminant production complements other food production systems, they must be reared on alternative feed resources that have no direct food value to humans. This can be achieved through identifying and evaluating alternative non-conventional and low-cost feedstuffs such as red grape pomace. According to Dwyer *et al*. (2014), a large quantity of GP is produced in a short period of harvesting time, which in return causes detrimental effects on the environment during incineration and deposition on landfills. The significant amounts of antimicrobial phenolic compounds in GP reduces pomace pH thus limiting the process of biodegradation by microorganisms. Other environmental problems caused by the dumping of GP include plant growth interference, contamination of underground water resulting in the death of sensitive marine organisms due to a build-up of chemicals (Kalli *et al*., 2018). Fontana (2013) stated that some animals are intolerant to certain components in GP such as polymeric polyphenols and condensed tannins, which negatively affect digestibility through inhibiting the cellulolytic and proteolytic enzymes, and the growth of rumen microbes.

Ruminants have a diverse ruminal microbial population, which primarily consist of the protozoa, bacteria and fungi which synthesizes and secretes the β-1,4-cellulase enzyme complex, which hydrolyses the plant cell walls. However, the actual conversion of fibrous feed by ruminants to produce meat and milk is not very efficient with only 10 – 35% of the energy intake being captured as net energy, while the rest is not utilized (Varga & Kolver, 1997). Grape pomace contains high amounts of non-digestible fibre and phytochemicals that can be a problem for ruminant digestion processes. Given that a large percentage of fibre is not digested by ruminants, there is a need to identify and evaluate lignin degradation methods such as treatments with alkaline, urea, exogenous

enzymes as well as the use of fungi like white rot and mushrooms. These strategies can be used to breakdown the fibrous matrix of GP before they are used in ruminant diets.

1.3 Justification

According to Tomovic *et al*. (2017), GP can be adopted as an ingredient in ruminant diets to minimize feed shortages and costs for low and middle-income countries, while at the same time improving animal nutrition, health, welfare, production, fatty acid profile, and shelf life. Furthermore, the use of GP as dietary supplements can produce meat with beneficial human health-promoting properties, while helping to mitigate environmental challenges that would result from the disposal of winery wastes using traditional methods. However, the use of GP as a ruminant feed by Alipour & Rouzbehan (2007) is limited by high levels of the complex cell wall fraction and tannin content, which can adversely affect nutrient utilization when fed at high levels. Machado & Ferraz (2016) reported that the alternative use of biological methods such as the solid-state fermentation with white-rot fungi is economically and environmentally sustainable. Van Kuijk *et al*. (2015) stated that most white-rot fungi often require a long fermentation time for their growth, which will eventually lead to the significant reduction of hemicellulose and cellulose constituents of their substrates. Furthermore, Saratale *et al*. (2008) reported that the use of white-rot fungi has a lot of advantages such as being environmentally friendly, cost-effective and requires low energy for the simultaneous degradation of cellulose and the lignin content of plant cell walls. The use of fungi such as white rot and mushrooms to breakdown the fibre matrix of potential feedstuffs is an ingenious, low cost and relatively safer approach to valorise GP. Zadrazil (1997) stated that the use of white-rot fungi could be an innovative approach to convert low-quality agricultural by-products such as wheat straw into a higher quality of ruminant feed. In a study by Moyson & Verachtert (1991), an increase in crude protein (CP) and ash was recorded when the straw was treated with white-rot fungi. Moreover, Fazaeli *et al*. (2004) reported an increase in both the *in vitro* dry matter digestibility and *in vitro* organic matter digestibility when the wheat straw

was treated with five species of *Pleurotus* fungi compared to the untreated. However, Tripathi & Yadav (1991) reported that such improvements depend on the fungal strain and culture conditions. In addition, mushrooms can also be a direct source of food for the growing human population thus their use in enhancing ruminant feeds is easier to sell to small scale farmers. Tuyen *et al*. (2013), stated that oyster mushroom (*Pleurotus ostreatus*), a white-rot fungus, can selectively degrade lignin leaving out cellulose. This, in turn, will facilitate access of rumen microbial enzymes to the polysaccharide cellulose, which is a major source of energy for the ruminant. Consequently, the selective removal of lignin from fibrous material increases the digestibility of the substrate in ruminants (Kuijk, 2016). However, it appears that no studies have investigated the use of oyster mushrooms to enhance the nutritive value of red GP, a potential feed resource for ruminants.

1.4 Aim and specific objectives

The study is designed to investigate the use of oyster mushroom (*Pleurotus ostreatus*) to enhance the nutritive value of red grape pomace (*Vitis vinifera* L. var. Shiraz) for ruminants. The following specific objectives guided the study:

a) To determine the chemical composition and *in vitro* ruminal fermentation parameters of red grape pomace spent oyster mushroom substrates.
b) To determine the quantity of the oyster mushroom spawn required to maximize the nutritive value of red grape pomace.
c) To determine the optimum amount of time required for the oyster mushroom to maximize the nutritive value of red grape pomace.

1.5 Hypothesis

a) Using red grape pomace as an oyster mushroom substrate will improve its chemical constituents.

b) Red grape pomace spent oyster mushroom substrates have better ruminal fermentation parameters compared to untreated substrates.

1.6 References

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2 CHAPTER TWO - LITERATURE REVIEW

2.1 Introduction

Ruminant production plays an important role in ensuring food and nutrition security all over the world through the production of meat, milk and milk by-products (Mlambo & Mnisi, 2019). According to Pulina *et al*. (2017), the ruminant industry plays a pivotal role as an important risk aversion strategy that supports over 600 million smallholder livestock farmers in the resource-poor households of the low-to-middle-income countries. Indeed, Upton (2004) stated that ruminants in developing countries far outnumber most of the non-ruminant animals as a source of employment, capital accumulation, income, fibre, power and manure. The importance of ruminant production and their products (meat, and dairy products) is increasing globally due to the increasing consumer demand in the low-to-middle-income countries. This increasing demand is exacerbated by the rapidly growing global human population and rising incomes in the low-to-middle-income countries (Wadhwa *et al*., 2015). To meet this demand, ruminant products should be at least 60 - 70% to feed the human population, which is predicted to grow from 7.3 - 9.6 billion between the year 2015 and 2050, in which most of this increase will be from low-to-middle-income countries (Thornton, 2010). For these reasons, there is currently a global need to increase ruminant production whilst minimizing the negative impact that livestock production has on the environment.

Arowolo & He (2018) reported that ruminant livestock production in tropical countries suffers greater setbacks owing to the available feed resources. The growing demand for animal products in these countries imposes huge demand on the available feed resources. In response to this demand, large amounts of feed resources are currently needed, thus compromising the sustainability of the current feeding systems (Wadhwa, 2015). However, the availability of grazing and browsing land for ruminants has become scarce, owing to the growing human population,

industrialization, urbanization and rising demand for utilization of agricultural land for crop production (Thornton, 2010; Pulina *et al*., 2017). As such, there is a need to identify and utilize non-conventional waste products such as grape pomace (GP) in ruminant production. However, to the best of our knowledge, there is limited data on the valorisation of GP waste product for ruminants.

2.2 Ruminants

The ruminant digestive system is uniquely suited for efficient utilisation of high-fibre forages and roughages. According to Koeing *et al*. (2003), the feed consumed by ruminants is subjected to rumen fermentation, where it is degraded into short-term intermediates of simple carbohydrates and protein such as sugar and amino acids by rumen microbes. Ruminal degradation produces metabolic end-products such as carbon dioxide, methane, ammonia, and volatile fatty acid (VFA). Bergman (1990) noted that ruminants absorb VFAs through the rumen wall covered by numerous small finger-like projections called papillae, whose function is to increase the surface absorption area for the digestion of proteins, lipids, microbial carbohydrate components and feed waste entering the small intestine to provide maintenance requirements for milk and meat production. Ruminants derive approximately 70% of their metabolic energy from the microbial fermentation of feed particles and microbial protein, which accounts for about 90% of the amino acids that reach the small intestine (Bergman, 1990).

2.3 Dietary importance and role of fibre in ruminants

According to Bargo *et al*. (2001), the main components of fibre in forages are cellulose and hemicellulose. Forages constitute about 40 - 100% of a ruminant ration and are important for animal production and health maintenance. Beauchemin *et al*. (2003) reported that high fibre diets play an important role in supplying the fibrolytic microbes with beneficial rumen flora substrates that helps in the saliva production, which in return increases rumination time during the digestive

process. Adesogan *et al*. (2018), however, stated that the linkage between the lignin and hydroxycinnamic acids (such as ferulic acid) in the cell wall of forages is the greatest inhibitor to an efficient digestion and use of nutrients from forages for energy by ruminants.

2.4 Grape pomace as an animal feed resource

Grapes are one of the most extensively cultivated crops in the world with approximately 63 million tons produced globally, with a vast majority of the total grape production (75%) used to produce wine (FAOSTAT, 2013). According to Charalampia & Koutelidakis (2016), grape pomace (GP) is considered a biodegradable solid by-product of the winemaking process that is formed after the pressing of grapes, which mainly consists of the peels, seeds, and some part of stems, depending on the technological process used during pressing and the targeted product. Ahmad & Ali Siahsar (2011) reported that GP is composed of proteins, carbohydrates, water, minerals, vitamins, lipids and bioactive compounds with important biological properties such as phenolic compounds (phenolic acids, tannins, anthocyanins and resveratrol), vitamin C and some portion of fibre, depending on the type of cultivar, waste, climatic, and cultivation conditions.

According to Karakaya *et al*. (2001), GP contains phenolic compounds as well as crude cellulose (25 - 35%), crude protein (8 - 14%), crude fat (4 - 10%), hemicellulose (4 - 8%), pectin (5 - 10%), and non-nitrogen substances (30 - 45%). Meyer *et al*. (1997) reported that GP can be utilised as a feed additive with nutritional benefits because it represents a potentially valuable source of phenolic antioxidants. As a result, GP has attracted the interest of the food, pharmaceutical, and cosmetic industry as a profitable source of natural antioxidants (Bucic-Kojic *et al*., 2013). Spanghero *et al*. (2009) stated that there is currently limited literature regarding the use of the grape by-products as animal feed, probably because of the high fibre and tannin content that is known to reduce digestibility and energy value. However, Waghorn & McNabb (2003) reported that the presence of moderate concentrations of dietary polyphenols was able to increase

productive performances in ruminants which were achieved by increasing utilization efficiency of nitrogen, reducing gas emission and improving the health and quality of derived products. So for these reasons, the use of GP in ruminant feeding has been deemed to be an attractive research field.

2.5 Chemical composition of grape pomace

The chemical compositions (Table 2.1) of GP vary depending on factors such as growth environment, grape maturity, and viticultural practices (Deng *et al*., 2011). According to Robredo *et al*. (1991), the composition of grapes may vary depending on both the intrinsic (maturity, variety, and sanitary conditions) and extrinsic factors (edaphoclimatic conditions and viticultural practices). The composition of GP is mostly influenced by both the type of process and wine-making conditions that were employed (Perez-Magarino & Gonzalez-SanJose, 2000). The variation between grape varieties and the different wine-making process explains why there are different reported composition variations in the literature of GP and its main components (skins, stems and seeds).

Table 2.1. Chemical composition (g/kg) of red grape pomace.

Components	Quantity
Protein	112 – 138
Fat	56 – 117
Ash	24 – 58
Fibre	325 – 563
Neutral detergent fibre	542 – 708
Acid detergent fibre	480 – 704
Acid detergent lignin	307 – 475
Calcium	5 – 7
Phosphorus	2 – 3
Iron	640 – 1850
Copper	650 – 1240
Magnesium	130 – 170

Source: Brenes *et al*. (2016)

2.6 Oyster mushrooms

According to Adejoye *et al*. (2006), mushrooms are saprophytic microorganisms that usually grow on the organic matter of vegetative origin and can utilize almost all forest and agricultural residues as substrates. Khan *et al*. (2012) stated that mushrooms can be cultivated in developing countries because of their ability to grow or be grown on agricultural wastes such as sawdust, straw, rice hull, etc. Growing mushrooms promote environmental conversation by recycling wastes that can be acquired at low prices or even for free, which can be used for human consumption or to produce feed for ruminants (spent mushroom substrates). Cohen *et al*. (2002) stated that oyster mushroom (OYM) can grow on different kind of substrates than any other mushrooms. Kues & Lui (2000) reported that among all the available species of mushrooms, OYMs are the second most cultivated mushrooms in the whole world after the white button mushroom (*Agaricus bisporus*). Oyster mushroom belongs to the genus *Pleurotus* and the family *Pleurotaceae*, and is an active lignin degrader in the forest, appearing as a cluster on dead trees, mostly in the winter and spring. The *Pleurotus* species is said to be distributed all over the world especially in subtropical and temperate forest zones (Pokhrel, 2016). Van Peer *et al*. (2009) reported that the environment plays an important role in the production of OYMs with various mushrooms known to be sensitive to certain major climatic conditions such as temperature, humidity and fresh air in mushroom production (AMGA, 2004). Furthermore, OYMs require a small piece of land to be produced with shorter production cycles and low production inputs. *Pleurotus* sp. grows in a wide range of temperature (15-30°C) and humidity (60-90%), which also vary from species to species. The mycelial development of OYM is said to be optimum at a temperature ranging between 18°C and 28°C and a relative humidity range of 70 – 90% (Islam *et al*., 2007). For the primordial formation of OYM, Viziteu (2000) reported that the temperature should range from 18°C to 25°C.

2.6.1 Utilization of oyster mushroom as human food

The consumption of mushrooms is an ancient practice by human beings. However, in recent years, the consumption of mushrooms has become an integral part of every continental dish because of its good nutritional value, taste, and flavour. Mushrooms contain nutrients such as proteins, fibre, vitamins, and minerals (Pokhrel *et al*., 2006). Dunkwal & Singh (2007) reported that the consumption of mushrooms is a growing fast commodity due to its balanced nutritional composition and health improvement benefits for humans. Mushrooms are said to be low in calories, sodium, fat, and cholesterol but they are rich in fibre, vitamins, and minerals. In addition, OYMs can be a viable alternative to alleviate issues of protein and energy malnutrition in underdeveloped and developing countries. Indeed, Eswaran & Ramabadran (2000) reported that mushrooms have been identified as an excellent food source to alleviate malnutrition in developing countries because of their nutritional value, flavour, texture, high productivity per unit area and as a potentially good income-generating enterprise for the landless poor people. Bioactive compounds in OYM such as anti-tumour substances have stirred a growing interest in the industry, the media, and the scientific community (Pokhrel *et al*., 2006). Hossain *et al*. (2003) reported that *Pleurotus ostreatus* can decrease cholesterol levels in experimental animals. Adams & Wermuth (1999) reported that *P. sajor-caju* contains antioxidants that can be exploited as protective agents that can help reduce oxidative damage of the human body without any interference.

2.6.2 Utilization of oyster mushrooms to improve the nutritive value of fibrous feeds

In recent years, the natural delignification of agricultural waste products by solid-state fermentation (SSF) has been considered because of its ability to breakdown lignin (Moyson & Verachtert, 1991). According to Zadrazil *et al*. (1996), fungal treatment could be a way to improve crop by-products for ruminant nutrition. Kundu (1994) stated that during SSF by fungi, the neutral detergent fibre (NDF), acid detergent fibre (ADL) and organic matter content (OM) of the substrate were reduced whereas the lignin content was selectively removed from the lignocellulosic

complex. It is, however, worth noting that such modifications depend on the type of fungi and cultural conditions (Tripathi & Yadav, 1991). Zadrazil *et al*. (1996) stated that the *Pleurotus* species is more efficient among the edible white-rot fungi in terms of reducing the lignin content of straw. Singh *et al*. (1990) reported that *P. ostreatus* and *P. eryngii* have the capacity to reduce indigestible cell wall components of wheat straw while increasing dry matter digestibility. According to Zadrazil (1997), few strains of the *P. ostreatus* increased the *in vitro* digestibility of wheat straw up to 25.5% while others lowered the digestibility by 13.8% due to its poor enzyme efficacy on the substrate. Jalc *et al*. (1998) reported that the *in vitro* dry matter digestibility of the *Pleurotus* fungi treated wheat straw was increased up to 10% when it was treated for a 30-day fermentation period. Furthermore, Karunanandaa & Varga (1996) reported in a 30 days SSF of the rice straw with *Cyathus stercoreus* that there was an increase in the apparent digestion of DM (44 vs. 35.1%) and OM (50.6 vs. 41.5%).

2.7 The biodegradation process

Agricultural by-products are composed of three major structural polymers, which namely are cellulose, hemicellulose and lignin (Bargo *et al*., 2001). These three major structural components can be broken down by lignocellulolytic enzymes. According to Adebayo *et al*. (2012), *Pleurotus* species are regarded as the most efficient lignin-degrading organisms among white-rot fungi because they have the ability to produce crucial biodegradation extracellular lignin modifying enzymes such as laccases, lignin peroxidase (LiP) and manganese peroxidase (MnP) (Figure 2.1). Bilal & Asgher (2016) reported that the lignin peroxide enzyme is able to oxidise various aromatic compounds, while the manganese peroxidase almost exclusively oxidises Mn^{+2} to Mn^{+3}, which in return turn results in the degradation phenolic compounds. Laccases, blue copper-containing oxidases, are said to be able to reduce molecular oxygen to water and in turn oxidise phenolic compounds. In addition, Sanchez (2010) reported that the laccases or ligninolytic peroxidases (LiP and MnP) oxidizes the lignin polymer, thereby generating aromatic radicals. These in return evolve

into different non-enzymatic reactions that include the C-4-ether breakdown, aromatic ring cleavage, carbon alpha and beta (Cα-Cβ) breakdown and lastly the demethoxylation of the substrate. Valadares *et al*. (2016) stated that the white-rot fungi biodegradation process would proceed until the cellulose and the hemicellulose matrix are degraded by cellulases, by the process of hydrolysis of beta-1, 4-glycosidic linkage bonds. The hemicellulose is then degraded into monosaccharides and acetic acid by hemicellulases through the formation of oligosaccharides from xylan (Balan *et al*., 2008), resulting in the process of biodegradation by white-rot fungi.

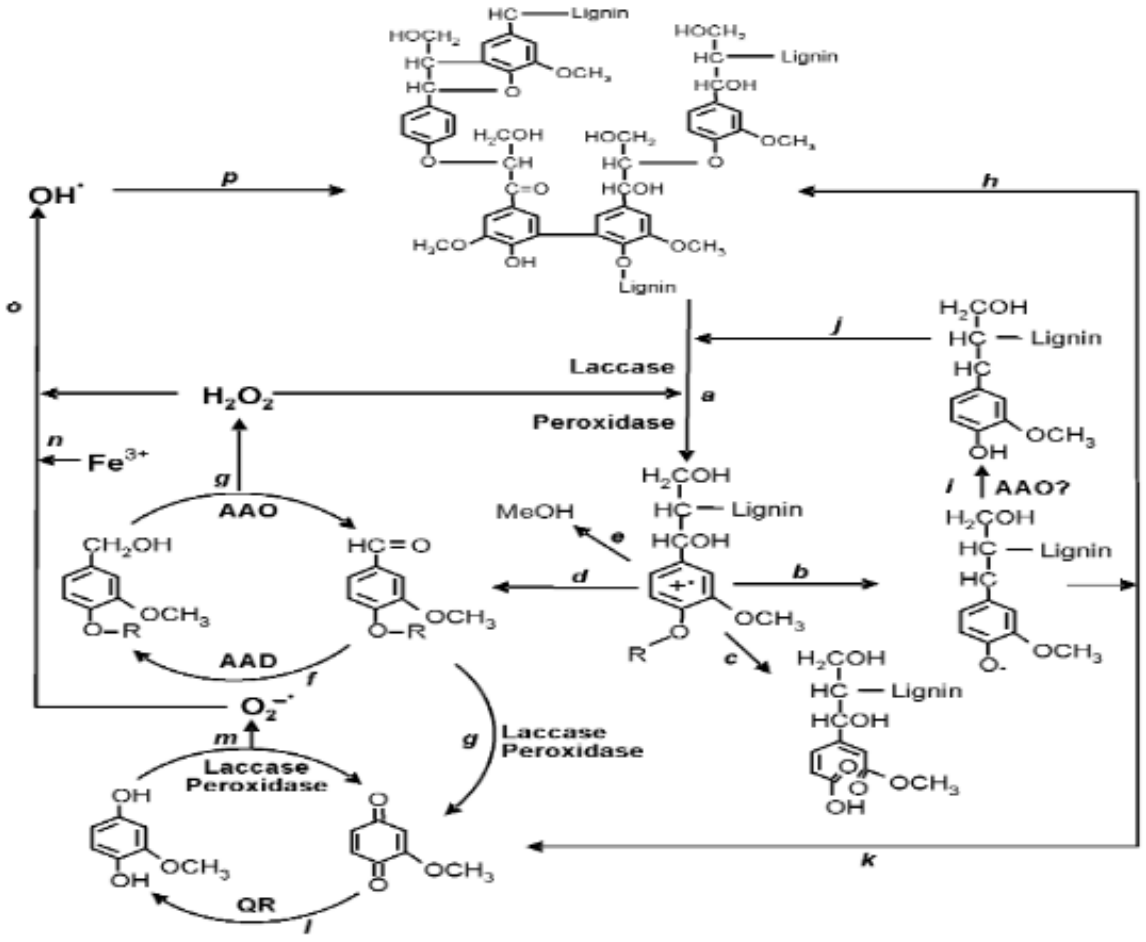

Figure 2.1. A schematic diagram of lignin biodegradation by white-rot enzymes (Source: Martinez *et al*., 2005).

2.8 Strategies to improve the feed value of fibrous feed resources for ruminants

2.8.1 Sodium hydroxide treatment

Various chemical treatments have been used to breakdown the resistant cell wall of fibrous crop residues such as lignin to increase the accessibility and susceptibility of lignocellulose substrate for degradation by rumen microbes (Egan, 1990). Arieli (1997) reported that several sodium hydroxide (NaOH) treatment methods have been developed to improve crop residues for ruminant feeding. Vadiveloo (2000), reported that the main advantages of the various NaOH treatment methods include increased palatability and degradability of treated rice straw, compared to the control. Fahey *et al*. (1993) reported that the *in vitro* ruminal OM digestibility of barley straw increased from 52 to 76% and the digestibility of DM increased by 22% in 29 other crop residues that were treated with NaOH. Furthermore, Miron *et al*. (1997) reported that dairy cows fed a diet containing sorghum grains treated with 4% NaOH had a greater digestibility of NDF when compared to those fed untreated sorghum grain. However, Chandra *et al*. (2012), reported that the NaOH becomes corrosive when more than 4% is used in the pre-treatment of crop residues like rice straw. The NaOH completely damages the cell wall structure (causes excessive fibre damage) of the rice straw, which is required by methanogenic bacteria during the rumen fermentation. Furthermore, NRC (1984) the application NaOH can be a nuisance to the environment by polluting water bodies (oceans, sea and rivers) resulting in increased pH levels that affect or destroy aquatic life. Additionally, NaOH is also expensive and may not be easily accessed by small-scale farmers.

2.8.2 Ammonia and urea treatment

According to Fadel-Elseed *et al*. (2003), the use of aqueous ammonia, anhydrous ammonia, urea and other ammonia-releasing compounds in treating of cereal straw has been widely investigated to improve degradability. Abou-el-Enin *et al*. (1999) reported that the treatment of rice straw with ammonia (NH_3) not only increases the degradability of fibre but also improves the nitrogen

content, while at the same time, preserving the straw by inhibiting mould growth (Calzado & Rolz, 1990). In a study by Oji *et al*. (2007), improvements in the digestibility of nitrogen, dry matter (DM), NDF, ADF, and OM values were reported when a diet containing a mixture of corn cobs, corn husks, and corn stalk treated with 3% of aqueous ammonia was fed to sheep. Besides the improvement in degradability of structural carbohydrates, the treatment of fibre-rich feeds with ammonia is an effective way of reducing large amounts of supplemental nitrogen, cutting the costs of purchasing protein-rich feedstuffs, and enhancing acceptability and voluntary feed intake of the treated substrates by ruminants. Liu *et al*. (2002) stated that treating fibrous material with ammonia is less efficient in improving the energy value of straw than using NaOH. However, using NH_3 is deemed to be more profitable for farmers because the added ammonia serves as a source of nitrogen. Aruwayo (2018) reported that treating crop residues like straws with ammonia should be kept at a dosage of 3% by weight of the straw DM.

Schiere & Ibrahim (1989) reported that the use of urea by farmers is more practical and safer than using anhydrous or aqueous ammonia and improve the nitrogen content of the straw and other crop residues. According to Sundstol & Coxworth (1984), urea is a solid chemical that is easy to handle, transport and can easily be obtained by farmers in many developing countries. Vadiveloo (2003) reported that different type of rice with a low degradability responded better to urea treatments than higher-quality straw, which resulted in increased *in vitro* dry matter degradability from 45% to 62%. Urea treatment may, therefore, be most suitable for farmers to improve the quality of straws, particularly in varieties showing poor digestibility. For this reason, the use of urea is regarded as the most practical and available method in livestock production in developing countries, because it is relatively cheap, adds nitrogen to the ration and is relatively safe to use. Urea dosage needed to treat straw may vary a lot. Chenost & Kayouli (1997) reported that to avoid urea poisoning for ruminants, it thus recommended that the urea inclusion level should be kept

between 4-6 % on DM basis, taking into consideration the effect of ammoniation and the of cost urea.

2.8.3 Biological treatment

Eriksson *et al*. (1990) stated that white-rot fungi are wood-decaying microorganisms belonging to *Basidiomycetes*. White-rot fungi are lignocellulolytic microorganisms that can decompose and metabolize cellulose, hemicellulose and lignin. Various kinds of white-rot fungi species that are effective lignin degraders have been utilized to assess their ability to improve the nutritive value of fodder for ruminants (Howard *et al*., 2003). Lechner & Papinutti (2006) reported that they consist of lignin-peroxidase (LiP), manganese-dependent peroxidase (MnP), laccase (phenoloxidase) and hydrogen peroxide-producing oxidase (aryl-alcohol oxidase; AAO and glyoxal oxidase) as their extracellular lignin-modifying enzymes. Jung *et al*. (1992) reported that white-rot fungi significantly increase *in vitro* dry matter digestibility of cereal straws through delignification. The delignification processes increase the accessibility of cellulose and hemicellulose by the microbial enzymes thereby increasing the digestibility of fibre in ruminants. Karunanandaa *et al*. (1995) reported that the *in vitro* dry matter digestibility was enhanced when rice straws (leaves and stems) were incubated for 30 days with white-rot fungi (*Pleurotus sajor-caju*). Furthermore, the entire rice straw (both the leaves and stem) treated with *C. stercoreus* had the highest digestibility when compared to other fungi treated species. However, Rouches *et al*. (2016) stated that the main challenges of using the biological treatment methods is that they require proper optimization of the fungal strain, inoculum concentration, temperature, moisture content, substrate pH, aeration and incubation time for the enzymes to effectively reduce the carbohydrates.

2.9 Summary

Incorporating red GP waste in ruminant diets can be one of the new sustainable alternatives to the GP disposal methods. The addition of GP in ruminant diets can be used as a source of nutrients

and bioactive compounds to supplement the available forages and enhance ruminant productivity as well as reducing environmental pollution caused by the disposal of this by-product into landfills. This approach may help improve food and nutritional security while reducing waste from wine production. Various studies have reported different results in terms of the nutritional value of GP, which could be due to factors such as the grape cultivar, climatic conditions, growth environment, and winemaking processes. There is currently limited information on the use of this non-conventional feedstuff in ruminant diets due to its high fibre content. So innovative strategies such as the use of OYM need to be evaluated and employed because it is deemed as one of the environmentally friendly methods to improve the nutritive value of various agricultural by-products by other researchers. However, there is currently no available research on the use of OYM to break down GP fibre content so that more nutrients can be available for digestion by the ruminants.

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3 CHAPTER THREE - CHEMICAL COMPOSITIONAL CHANGES IN RED GRAPE POMACE USED AS A SUBSTRATE FOR OYSTER MUSHROOMS

Abstract

The study was designed to investigate the effect of oyster mushroom (*Pleurotus ostreatus*) spawning rates treatment and harvesting time on the chemical composition of red grape pomace (*Vitis vinifera* L. var. Shiraz). The grape pomace (GP) substrate was moistened through the sprinkling of distilled water in a closed container to increase the moisture content, without allowing the nutrients to leach. After 12 h, the GP substrate was sterilized using an autoclave (121°C) for 1 hour at 100 kPa and then allowed to cool for 16 h. A total of 50 pots (experimental units), each with 500 g of GP substrates, were inoculated with the spawn at the following rates: 0, 20, 30, 40, and 50% (w/w). After inoculation, the pots were covered with black refuse bags and kept at a room temperature of between 20 - 25°C for 28 days and were occasionally irrigated to keep them humid. About 125 g was sampled on a weekly basis (7, 14, 21, and 28 days post-inoculation) to determine the effect of the oyster mushroom spawn on the chemical composition of GP substrate (spent mushroom substrate). The spent mushroom substrates (SMS) were then oven-dried (60°C) until constant weight and milled for chemical analyses. In week 1, there were no linear effects (P >0.05) with the increasing spawning rate on dry matter (DM), organic matter (OM), and crude protein (CP) content, whereas linear effects were observed for neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) content. Significant quadratic trends were observed for NDF and ADF. There were significant linear trends for DM and OM, but no quadratic trends were observed for all chemical components in week 2. In week 3, there were linear effects (P <0.05) for DM, NDF and ADL, and a quadratic trend (P <0.05) for DM. In week 4, there were significant linear effects for OM, NDF, ADF, ADL and CP, and significant quadratic trends for

NDF, ADF and CP in response to the different spawning rates. In all the weeks, the SMS had higher (P <0.05) DM, OM, and CP contents than the untreated substrates. The fibre constituents (NDF, ADF and ADL) improved with an increase in the incubation period and spawning levels (week 3 and 4) for the treated substrates. There was a significant linear trend for sulphur and a quadratic trend (P <0.05) for phosphorus in week 1. In week 2, there were significant linear effects for sulphur and calcium, but no quadratic effects (P >0.05) for all the macro-minerals. Phosphorus linearly decreased (P <0.05) whereas sulphur linearly increased (P <0.05) with spawning rates in week 3. In week 4, there was a linear effect (P <0.05) on sodium and quadratic trends (P <0.05) for magnesium, phosphorus, and calcium in response to increasing spawning rates. In week 1 and 2, there were significant linear trends for iron and selenium, but there were no quadratic effects (P >0.05) in both weeks. There were significant linear effects observed on manganese and zinc and a significant quadratic effect for cobalt in week 3. In week 4, there were linear effects (P <0.05) on manganese, cobalt, copper and selenium, a quadratic effect (P <0.05) on selenium in response to spawning rates. It can be concluded that treatment with oyster mushroom spawn improves the crude protein content and the fibre content of red GP with the increase in spawning rates and the incubation duration.

Keywords: Chemical composition; Oyster mushroom; Red grape pomace; Substrate.

3.1 Introduction

The cost of animal feeds has increased in certain parts of the world due to the dry climatic conditions and water shortages (Alipour & Rouzbehan, 2006). It is, therefore, imperative to identify non-conventional feed ingredients that have no direct food value for humans to use in ruminant diets. According to Gonzalez-Centeno *et al*. (2013), by-products such as GP need to be better exploited because such waste products are valuable raw materials, which have attracted research interests due to high levels of natural bioactive compounds. Sousa (2014) reported that

GP is composed of proteins, carbohydrates, minerals, lipids, vitamins and compounds with beneficial biological properties such as phenolic compounds (i.e. phenolic acids, tannins, anthocyanins and resveratrol). However, these components depend on the type of grape by-product, the cultivar and climatic conditions. Yu & Ahmedna (2013) stated that GP is a rich source of dietary fibre, ethanol, citric acid, malates, hydrocolloids and tartrate. In addition, Corbin *et al*. (2015) stated that red GP is a potential source of water-soluble monosaccharides (i.e. glucose and fructose), structurally complex water-insoluble polysaccharides (i.e. xyloglucan, pectin and cellulose), and beneficial bioactive compounds. The valorisation of GP does not only help to overcome environmental issues but also addresses feed shortages for ruminants across the world (Baumgartel *et al*., 2007). However, the use of GP as a ruminant feed may be limited by the presence of non-starch polysaccharides, high level of lignified cell wall fractions and high tannin content (Alipour & Rouzbehan, 2006).

Grape pomace consists of three major structural polymers namely cellulose, hemicellulose and lignin, which are not easily digestible (Adebayo *et al*., 2012). As such, the use of *P*. *ostreatus* can help reduce the high lignin content of GP and at the same time improve the nutritional value of GP for ruminants. Mikiashvili *et al*. (2006) reported that *Pleurotus* species present high adaptability to produce basidiomata within a wide range of agro-industrial lignocellulosic residues due to their production of ligninolytic and hydrolytic enzymes such as cellulase, xylanase and pectinase. A study by van Kuijk *et al*. (2016), reported that there was a general decrease in fibre and lignin contents with an increase in incubation time (0, 2, 4, 8 and 12 weeks respectively) when straw substrates were treated with *Lentinula edodes*. Fungal treated straw had higher CP, ether extract and ash contents whereas lower neutral detergent fibre, acid detergent fibre, acid detergent lignin, organic matter, cellulose and hemicellulose contents were recorded for the untreated straw (El-Rahman *et al*., 2014). Furthermore, Jonathan *et al*. (2012) reported an increase in CP content on *P*. *pulmonarius* fungi treatment of rice straws after 40 days of fermentation. However, due to

limited literature on the use of *P. ostreatus* to improve the nutritive value of GP for ruminants, there is a need for more research to be conducted. Thus, the objective of the current study was to determine the optimum quantity of the oyster mushroom spawn required to improve chemical composition of red grape pomace and the amount of time required for the oyster mushroom to reduce the fibre content of red GP. It was hypothesized that using red grape pomace as oyster mushroom substrates would reduce its fibre content.

3.2 Materials and methods

3.2.1 Study site and ingredient sources

The study was carried out at Molelwane Research Farm (25°86′00″ S, 25°64′52″ E) of the North-West University (North West Province, South Africa). The farm is located in the semi-arid region of the province, with temperatures ranging from 4°C to 27°C during winter. The average rainfall in the area ranges from 300 to 600 mm per annum. Fresh red grape pomace (*Vitis vinifera* L. var. Shiraz) (GP) was acquired from Blaauwklipan Wine Estate (33.969° S; 18.844° E) located in Stellenbosch (Western Cape, South Africa). The area experiences cold wet winter and dry hot summer seasons, with an average daily temperature of 16.4°C and an average annual rainfall of 802 mm. The estate soil type ranges from dark alluvial to clay. The oyster mushroom spawn was supplied by Eco-Agro Enterprise (PTY) LTD (Nelspruit, South Africa).

3.2.2 Development of the oyster mushroom spawn

According to the company, the oyster mushroom was split into half and a small piece of mycelial tissue was extracted from the top part of the stem. It was then placed aseptically, using a needle, on sterile potato dextrose agar (PDA) and incubated at 25°C for 7-10 days. The mycelium was allowed to develop from the spores until it fully invaded PDA. For the spawn preparation, 100 g sorghum (*Sorghum bicolor* L.) grains were soaked using tap water and kept overnight in 500 ml

flask. The grains were then partially cooked for 1 hour, excess water was drained off and they were allowed to cool at room temperature. It was then spread evenly on a sterilized plastic sheet (70% ethanol) to attain moisture content of about 50 - 54%. The grains were thoroughly mixed with calcium carbonate as a nutrient supplement, and then packed in plastic bags and sterilized in an autoclave for 1 h at 121ºC. After sterilization, the mycelia culture was sub-cultured inside a laminar airflow chamber into plastic bags. The inoculated plastic bags were then incubated at 24 ± 3°C for 10 - 15 days.

3.2.3 Substrate preparation

The GP substrate was moistened by sprinkling distilled water on top of the substrate to increase the moisture content overnight, without allowing the nutrients from leaching (Shah *et al*., 2004). The GP substrate was then sterilized in an autoclave set at a temperature of 121°C for 1 hour at 100 kPa (Tuyen *et al*., 2012). After sterilization, the GP substrate was cooled at room temperature.

3.2.4 Inoculation and experimental design

A total of 50 pots (experimental units), each with 500 g of GP substrates, were inoculated with the oyster mushroom spawn at 0, 200, 300, 400 and 500 g/kg of red GP. An electronic balance was used to weigh the spawn before inoculation. The GP substrate was placed in plant pots (223 cm^3) prior to inoculation with the oyster mushroom spawn. The pots were then inoculated with the spawn and kept at room temperature, covered with black refuse bags from the start of the incubation period until the end. The inoculated GP substrates were irrigated occasionally to keep the surface moist and humid (Chang & Wasser, 2017). In a completely randomized design (CRD), the experiment was carried out with 50 pots (experimental replicates) in which 5 treatment levels per spawning rate were replicated 10 times per level. About 125 g of the substrate was sampled from each replicate pot on a weekly basis (7, 14, 21, and 28 day's period) for chemical analysis, which would indicate the fibrolytic enzymatic activity of OMYs.

3.2.5 Temperature and humidity

The pots were kept at room temperature (between 20-25°C) and relative humidity (ranged between 70 – 80%) of the room was recorded on a two days interval from the first day of inoculation until the last sampling day using a multi-meter (HTC-1, Xuzhou Sanhe Automatic Control Equipment Co., Ltd, China) in order to monitor changes during the mycelial development.

3.2.6 Preparation of the spent mushroom substrates (SMS)

The remaining mycelial development was removed by hand from the spent mushroom substrates (SMS) before oven-drying at a temperature of 60°C until constant weight. The oven-dried SMS were milled (1 mm sieve; Polymix PX-MFC 90 D, Switzerland) and stored in their respective labelled sample bottles, which was based on spawning rate and time of harvest, pending chemical analysis.

3.2.7 Chemical analyses

3.2.7.1 Proximate analyses

Spent mushroom substrates were analysed in the Animal Science laboratory of the North-West University. For DM determination (AOAC, 2005: method no. 930.15), approximately 1 g of SMS and the control substrates were placed in pre-weighed crucibles that were then inserted in an oven set at 105ºC for a duration of 12 h. The DM was calculated as the difference between the initial sample and the moisture weights, while the moisture content was measured as the loss in weight. For organic matter (OM) content determination (AOAC, 2005: method no. 942.05), samples used to determine DM were incinerated in a muffle furnace set at a temperature of 600°C for 6 h. The loss in weight was measured as OM content and the remaining residues as ash. The ANKOM^{2000} fibre analyser (ANKOM Technology, Macedon New York) was used to determine the neutral detergent fibre (NDF) and acid detergent fibre (ADF) by refluxing about 0.45 – 0.5 g samples with

neutral detergent and acid detergent solutions for 1 h and 1 h 15 min, respectively, according to van Soest *et al*. (1991). A heat-stable α-amylase was used for NDF determination. The lignin content was determined by treating the ADF bags with 72% sulfuric acid to dissolve the cellulose after drying for 24 h (105°C). The standard macro-Kjeldahl method (AOAC, 2005: method no. 984.13) was used to determine the total N content, which was then converted to CP by multiplying percentage N by the factor 6.25.

3.2.7.2 Mineral analysis

Minerals were analysed in the Animal Health laboratory following the guidelines provided by the Agri-Laboratory Association of Southern Africa (AgriLASA, 1998). The ash content of SMS and the control substrates samples were digested with 1 mL of 55% nitric acid and 10 mL of 32% hydrochloric acid using the wet digestion method. Samples were digested for 45 min, cooled, and transferred into respective volumetric flasks (100 mL). The volumetric flasks were topped-up with distilled water and left standing for 24 h to allow the sediment to settle down. After 24 h, the digested samples were slowly transferred to McCartney bottles (28 mL) without disturbing the sediment. The ICP Mass Spectrometer (Perkin-Elmer, 1982, NexION 300Q) was used to determine the concentrations of Na, Mg, P, S, Ca, Mg, Mn, Fe, Co, Cu, Zn and Se. The macro-minerals were presented as g/kg DM whereas and the micro-minerals were presented as mg/kg DM.

3.2.8 Statistical analysis

Linear and quadratic effects were evaluated using polynomial contrasts for the chemical composition data. Response surface regression analysis (Proc RSREG; SAS, 2010) was employed to estimate the optimum spawning rate of the oyster mushroom for each GP substrate using the following quadratic equation:

$$y = c + bx + ax^2$$

Where: y = response variable; a and b are the coefficients of the quadratic equation; c is the intercept; x is spawning rate, and $-b/2a$ is the x value for optimal response.

Repeated measures analysis was used to analyse weekly measured chemical composition data using SAS (2010) according to the following model:

$$Y_{ijk} = \mu + S_i + T_j + (S\times T)_{ij} + E_{ijk},$$

Where, Y_{ijk} = the observation of the dependent variable, μ = the fixed effect of the population mean for the variable, S_i = the effect of spawning rate, T_j = time of incubation in weeks, $(S\times T)_{ij}$ = the interaction effect between the spawning rate and incubation time, and E_{ijk} = the random error effect associated with the observation ijk, in which was deduced to be normally and independently distributed.

Chemical composition data were analysed using the GLM procedure of SAS (2010) in a completely randomised design (CRD) using the following statistical linear model:

$$Y_{ij} = \mu + S_i + E_{ij}$$

Where, Y_{ij} = the observation of the dependent variable, μ = the fixed effect of the population mean for the variable, S_i = the effect of spawning rate, and E_{ij} = the random error effect associated with the observation ij, which was deduced to be normally and independently distributed. For all the statistical tests, the significance was declared at $P < 0.05$. The least-squares means were compared using the probability of difference option in SAS.

3.3 Results

Repeated measures analysis showed a significant week × treatment interaction effect for all chemical components. Table 3.1 shows the chemical composition of red grape pomace spent oyster mushroom substrate. In week 1, there were no linear effects (P >0.05) for DM, OM and CP, with the exception of ADL, which linearly increased [y = 1.79 (±0.875)x + 331.10 (±10.110); R^2 = 0.282; P = 0.0001] with spawning rates. There were significant quadratic effects observed for NDF [y = 442.880 (±10.685) + 4.44 (±0.925)x - 0.067 (±0.0180)x^2; R^2 = 0.3953; P = 0.0005] and ADF [y = 364.4 (±9.616) + 4.03 (±0.832)x - 0.058 (±0.012)x^2; R^2 = 0.426; P = 0.0008]. There were significant linear trends for DM [R^2 = 0.116; P = 0.023] and OM [R^2 = 0.133; P = 0.034] but there were no quadratic trends for all the chemical components in week 2 in response to the different oyster mushroom spawning rates.

In week 3, there were no linear effects (P >0.05) for OM, ADF and CP, with the exception of DM [R^2 = 0.439; P = 0.003], NDF [R^2 = 0.2542; P = 0.005] and ADL [R^2 = 0.1521; P = 0.036]. A significant quadratic trend observed for DM [y = 932.3 (±1.88) + 0.495 (±0.154)x - 0.013 (±0.003)x^2; R^2 = 0.439; P = 0.0001]. In week 4, there were significant linear effects for OM [R^2 = 0.174; P = 0.006], NDF [R^2 = 0.525; P = 0.0001], ADF [R^2 = 0.653; P = 0.0001], ADL [R^2 = 0.200; P = 0.019] and CP [R^2 = 0.515; P = 0.0001]. There were significant quadratic trends for NDF [y = 0.058 (±0.012)x^2 - 3.862 (±0.650)x + 688.2 (±8.253); R^2 = 0.525; P = 0.0001], ADF [y= 0.049 (±0.011)x^2 - 3.851 (±0.590)x + 627.0 (±7.493); R^2 = 0.653 ; P = 0.0001] and CP [y = 108.1 (±2.2077) + 1.037 (±0.19105)x - 0.014 (±0.0037)x^2; R^2 = 0.515; P = 0.0004] in response to the different oyster mushroom spawning rates, for which an optimum spawning rate was calculated to be 30%.

Table 3.1. Chemical composition (g/kg DM, unless stated otherwise) of red grape pomace spent oyster mushroom substrate.

	[1]Substrates						*P* value	
[2]Parameters	GP0	GP20	GP30	GP40	GP50	[3]SEM	Linear	Quadratic
Week 1								
DM (g/kg)	933.0	936.4	935.0	930.8	931.1	2.29	0.403	0.222
OM	864.8	865.7	866.7	859.7	861.5	3.61	0.352	0.603
NDF	438.4^{a}	521.7^{b}	510.4^{b}	496.1^{b}	508.0^{b}	10.35	0.000	0.001
ADF	359.9^{a}	438.6^{b}	428.3^{b}	415.1^{b}	432.1^{b}	9.12	0.000	0.001
ADL	328.8^{a}	377.8^{b}	359.4^{ab}	380.3^{b}	391.6^{b}	9.98	0.000	0.430
CP	105.6	107.2	107.0	107.4	108.4	3.92	0.628	0.989
Week 2								
DM (g/kg)	946.4^{bc}	948.5^{c}	934.8^{a}	942.4^{abc}	940.2^{ab}	2.02	0.023	0.540
OM	875.8^{b}	877.2^{b}	858.5^{a}	867.9^{ab}	870.2^{b}	2.69	0.034	0.152
NDF	612.1^{c}	571.2^{a}	576.4^{ab}	607.1^{bc}	578.3^{ab}	9.12	0.170	0.091
ADF	486.2	499.4	503.4	527.1	500.1	9.91	0.060	0.296
ADL	397.5	414.1	392.9	427.9	459.7	19.75	0.097	0.809
CP	102.9	105.1	103.9	101.1	108.4	2.16	0.283	0.472
Week 3								
DM (g/kg)	933.6^{b}	936.2^{b}	932.6^{b}	937.1^{b}	921.9^{a}	1.31	0.003	0.000
OM	861.4^{ab}	862.5^{ab}	858.1^{ab}	867.8^{b}	850.9^{a}	3.42	0.390	0.187
NDF	660.7^{b}	616.0^{a}	602.4^{a}	619.9^{a}	605.6^{a}	10.09	0.005	0.067
ADF	571.4	546.1	536.1	546.3	543.7	8.30	0.060	0.099
ADL	507.9	545.8	525.6	512.7	569.6	23.48	0.036	0.237
CP	107.8^{a}	120.0^{b}	107.7^{a}	109.0^{ab}	113.0^{ab}	2.82	0.791	0.351
Week 4								
DM (g/kg)	936.5	937.2	941.2	938.1	938.1	1.23	0.283	0.121
OM	859.7^{ab}	857.7^{a}	863.5^{ab}	861.6^{ab}	864.4^{b}	1.50	0.006	0.328
NDF	692.0^{c}	615.4^{a}	629.5^{ab}	638.5^{b}	633.7^{ab}	5.74	0.000	0.000
ADF	632.9^{b}	551.1^{a}	559.9^{a}	564.5^{a}	552.0^{a}	5.15	0.000	0.000
ADL	595.0^{b}	522.2^{ab}	487.7^{a}	501.8^{a}	508.8^{a}	21.13	0.019	0.059
CP	107.3^{a}	126.9^{b}	124.1^{b}	125.4^{b}	126.4^{b}	2.16	0.000	0.0004

[1]Substrates: GP0 = GP with no spawn; GP20 = GP inoculated with 200 g/kg spawn; GP inoculated with 300 g/kg spawn; GP40 = GP inoculated with 400 g/kg spawn; GP50 = GP inoculated with 500 g/kg spawn.
[2]Parameters: DM = dry matter; OM = organic matter; NDF = neutral detergent fibre; ADF = acid detergent fibre; ADL = acid detergent fibre; CP = crude protein.
[3]SEM = standard error of the mean.
[a, b, c] In a row, different superscripts denote significant differences (P <0.05) between spawning rates.

In week 1, there were no significant (P >0.05) differences observed for DM, OM and CP content in all the treatments. Substrate GP20 had a higher NDF content (521.7 g/kg DM) than substrate GP0 (438.4 g/kg DM). Substrate GP20 had the same NDF content as substrates GP30, GP40 and GP50 produced at 12 h post-inoculation. Substrate GP20 had a higher ADF content (438.63 g/kg DM) than substrate GP0 (359.9 g/kg DM). Substrates GP20, GP30, GP40 and GP50 had the same ADF content post-inoculation. Substrate GP50 had a higher ADL content (391.6 g/kg DM) compared to substrate GP0 (328.8 g/kg). Substrate GP0 had similar (P >0.05) ADL content as substrate GP30. Substrates GP20, GP30, GP40 and GP50 had the same ADL content post-inoculation.

In week 2, substrate GP20 had a higher DM content (948.5 g/kg) than GP0 and GP30 (934.8 g/kg DM). There no were significant differences among substrates GP30, GP40 and GP50 in terms of the DM content. Substrate GP0 had a similar DM content as substrates GP20, GP40 and GP50 post-inoculation. Substrate GP20 had a higher OM content (877.2 g/kg DM) than substrate GP30 (858.5 g/kg DM). There were no significant differences among substrates GP30 and GP40 in terms of the OM. Substrate GP0 had a similar OM content as substrates GP20, GP40 and GP50 post-inoculation. Substrate GP0 had a higher NDF content (612.1 g/kg DM) compared to substrate GP20 (571.2 g/kg DM). There were no significant differences among substrates GP20, GP20 and GP50 in terms of the NDF content. Substrate GP0 and GP40 had a similar NDF content post-inoculation. There were no significant differences observed for ADF, ADL and CP in all the treatments.

In week 3, substrate GP40 had a higher DM content (937.1 g/kg) than substrate GP50 (921.9 g/kg DM). Substrate GP0 had a similar DM content as substrates GP20, GP30 and GP50 post-inoculation. Substrate GP40 had a higher (P <0.05) OM content (867.8 g/kg DM) than substrates GP50 (850.9 g/kg DM). Substrate GP0 had a similar OM concentration as GP20, GP30 and GP40 substrates post-inoculation. Substrate GP0 had a higher (P <0.05) NDF content (660.7 g/kg DM)

than substrates GP20 (616.0 g/kg DM), GP30 (602.4 g/kg DM), GP40 (619.9 g/kg DM) and GP50 (605.6 g/kg DM). There were no significant differences (P >0.05) among substrates GP20, GP30, GP40 and GP50 in terms of the NDF content. There were no significant differences observed for ADF and ADL in all the treatments Substrate GP20 had a higher CP content (120.0 g/kg DM) than substrates GP0 (107.8 g/kg DM) and GP30 (107.7 g/kg DM). There were no significant (P >0.05) differences between substrate GP20, GP40 and GP50. Substrate GP0 had a similar CP concentration as GP30, GP40 and GP50 substrates post-inoculation.

In week 4, there was no significant (P >0.05) difference observed for DM in all the treatments. Substrate GP50 had a higher (P <0.05) OM content (864.4 g/kg DM) than substrate GP20 (857.7 g/kg DM). Substrate GP0 had a similar OM concentration as substrates GP20, GP30 GP40 and GP50 post-inoculation. Substrate GP0 had a higher NDF content (692.0 g/kg DM) than substrate GP20 (615.4 g/kg DM). There were no significant (P >0.05) differences between substrates GP30, GP40 and GP50 in terms of the NDF content. Substrate GP0 had a higher ADF content (632.9 g/kg DM) than substrates GP20 (551.1 g/kg DM), GP30 (559.9 g/kg DM), GP40 (564.5 g/kg DM) and GP50 (552.0 g/kg DM), which were the same (P <0.05). Substrate GP0 had a higher (P <0.05) ADL content (595.0 g/kg DM) than substrate GP30 (487.7 g/kg DM), GP40 (501.8 g/kg DM) and GP50 (508.8 g/kg DM), which did not differ (P <0.05). There were no significant (P >0.05) differences between GP20, GP30, GP40 and GP50 in terms of the ADL content. Substrate GP0 had the ADL content as substrate GP20 post-inoculation. Substrate GP20 had a higher (P <0.05) CP content (126.9 g/kg DM) compared to substrates GP0 (107.3 g/kg DM). There were no differences (P >0.05) among substrates GP20, GP30, GP40 and GP50 post-inoculation.

Table 3.2 shows the macro-minerals content of red grape pomace spent oyster mushroom substrate. In week 1, there was a significant linear trend for sulphur [R^2 = 0.315; P = 0.015] and a quadratic trend (P <0.05) for phosphorus [R^2 = 0.261; P = 0.031]. In week 2, there were significant linear effect for sulphur [R^2 = 0.527; P = 0.0006] and calcium [R^2 = 0.395; P = 0.012], but no quadratic

effects (P >0.05) were observed for all the macro-minerals in response to spawning rates of oyster mushroom.

In week 3, no significant quadratic trends were observed for all macro-minerals except for phosphorus and sulphur. Phosphorus [R^2 = 0.604; P = 0.004] linearly decreased whereas sulphur [R^2 = 0.801; P = 0.0001] linearly increased with spawning rates. In week 4, there was a linear effect (P <0.05) for sodium [R^2 = 0.434; P = 0.018] and quadratic trends for magnesium [R^2 = 0.445; P = 0.014], phosphorus [R^2 = 0.381; P = 0.027] and calcium [R^2 = 0.417; P = 0.037] in response to increasing spawning rates of oyster mushroom.

In week 1, there were no significant differences (P >0.05) observed for Na, Mg, P, S and Ca content in all the treatments. In week 2, substrate GP30 had a higher Na content (3.70 g/kg DM) than substrates GP0 (2.47 g/kg DM) and GP20 (2.24 g/kg DM), which did not differ (P <0.05). There were no differences (P <0.05) among substrates GP30, GP40 and GP50. Substrate GP0 had a similar Na content as substrates GP20, GP40 and GP50 post-inoculation. There were no significant differences observed for Mg and P in all the treatments. Substrate GP0 had a higher (P <0.05) S content (4.78 g/kg DM) than substrates GP40 (1.99 g/kg DM). There were no significant (P >0.05) differences between GP30, GP40 and GP50 in terms of S content. Substrate GP0 had a similar S content as substrates GP20, GP30 and GP50 post-inoculation. There was no significant (P >0.05) difference for the Ca content in all the treatments.

Table 3.2. Macro-minerals (g/kg DM) content in red grape pomace spent oyster mushroom substrate.

	[1]Substrates						*P* value	
	GP0	GP20	GP30	GP40	GP50	[2]SEM	Linear	Quadratic
Week 1								
Sodium	4.55	5.16	4.12	3.85	3.98	0.36	0.068	0.325
Magnesium	2.22	2.54	2.41	2.22	2.25	0.23	0.518	0.130
Phosphorus	1.77	2.37	2.21	1.94	2.02	0.19	0.751	0.031
Sulphur	4.56	3.84	3.36	3.31	2.84	0.47	0.015	0.887
Calcium	32.5	35.7	33.1	29.5	31.0	3.97	0.505	0.494
Week 2								
Sodium	2.47^{a}	2.24^{a}	3.70^{b}	2.61^{ab}	2.79^{ab}	0.27	0.336	0.409
Magnesium	1.17	1.42	1.64	1.18	1.31	0.17	0.411	0.063
Phosphorus	1.13	1.27	1.17	0.96	1.20	0.10	0.719	0.388
Sulphur	4.78^{b}	4.32^{b}	2.98^{ab}	1.99^{a}	2.36^{ab}	0.58	0.000	0.999
Calcium	23.5	27.8	30.9	26.2	29.6	3.11	0.012	0.145
Week 3								
Sodium	3.39	3.09	3.19	2.59	2.70	0.34	0.096	0.885
Magnesium	1.72	1.80	1.80	1.25	1.44	0.36	0.431	0819
Phosphorus	1.57	1.30	1.35	1.29	1.25	0.09	0.004	0.387
Sulphur	1.2^{a}		3.96^{b}	4.12^{b}	4.32^{b}	0.41	0.000	0.074
Calcium	26.1	21.7	26.5	27.4	23.7	1.79	0.230	0.554
Week 4								
Sodium	4.24^{bc}	4.38^{c}	3.12^{ab}	3.79^{abc}	2.98^{a}	0.29	0.018	0.388
Magnesium	2.31^{bc}	2.81^{c}	1.99^{b}	2.32^{bc}	1.72^{a}	0.20	0.644	0.014
Phosphorus	1.83^{ab}	2.21^{b}	1.54^{a}	1.83^{ab}	1.54^{a}	0.11	0.610	0.027
Sulphur	1.98	2.49	1.93	2.05	1.50	1.25	0.788	0.487
Calcium	30.9^{a}	39.7^{b}	30.2^{a}	35.7^{ab}	31.3^{a}	2.01	0.161	0.037

[1]Substrates: GP0 = GP with no spawn; GP20 = GP inoculated with 20% spawn; GP inoculated with 30% spawn; GP40 = GP inoculated with 40% spawn; GP50 = GP inoculated with 50% spawn.
[2]SEM = standard error of the mean.
[a, b, c] In a row, different superscripts denote significant differences (P <0.05) between spawning rates.

In week 3, there were no significant differences observed for Na, Mg and P in all the treatments. Substrate GP50 had a higher (P <0.05) S content (4.324 g/kg DM) than substrate GP0 (1.2 g/kg DM). There were no differences (P >0.05) among substrates GP30, GP40 and GP50 in terms of S content. In week 4, substrate GP20 had a higher (P <0.05) Na content (4.38 g/kg DM) than substrate GP50 (2.98 g/kg DM). There were no differences (P <0.05) among substrates GP30, GP40 and GP50 in terms of the Na content. Substrate GP0 had the same Na content as substrates GP20, GP30 and GP40 post-inoculation. Substrate GP20 had a higher (P <0.05) Mg concentration

(2.81 g/kg DM) than substrate GP50 (1.72 g/kg DM). Substrate GP0 had the same Mg content as substrates GP20, GP30 and GP40. Substrate GP20 had a higher (P <0.05) P content (2.21 g/kg DM) than substrates GP30 (1.54 g/kg DM) and GP50 (1.54 g/kg DM). There were no significant (P >0.05) differences between GP30 and GP50 in terms of the P content. There was no significant difference observed for S in all the substrates. Substrate GP20 had the highest (P <0.05) Ca content (39.7 g/kg DM) compared to treatments GP0, GP30 and GP50, which were the same (P >0.05). There were no differences (P >0.05) between GP20 and GP40 in terms of the Ca content.

Table 3.3 shows the micro-minerals content in red grape pomace spent oyster mushroom substrate. There was a significant linear trend for iron [y = 1.26 (±0.265) - 0.007 (±0.023x); R^2 = 0.276; P = 0.041] but there were no quadratic effects (P >0.05) in week 1. In week 2, there was a significant linear effect for selenium [R^2 = 0.367; P = 0.008] but there were no quadratic effects (P >0.05) for all the micro-minerals in response to the different spawning rates. In week 3, there were significant linear effects observed for manganese [R^2 = 0.505; P = 0.001], zinc [R^2 = 0.589; P = 0.006] and a significant quadratic effect for cobalt [R^2 = 0.5226; P = 0.008]. In week 4, there were significant linear effects for manganese [R^2 = 0.488; P = 0.008], cobalt [R^2 = 0.461; P = 0.018], copper [R^2 = 0.546; P = 0.004] and selenium [R^2 = 0.753; P = 0.046], there was a significant quadratic effect for selenium [R^2 = 0.753; P = 0.046] in response to spawning rates of oyster mushroom.

Table 3.3. Micro-minerals (mg/kg DM) content in red grape pomace spent oyster mushroom substrate.

	[1]Substrates						*P* value	
	GP0	GP20	GP30	GP40	GP50	[2]SEM	Linear	Quadratic
Week 1								
Manganese	20	20	20	20	20	0.002	0.442	0.620
Iron	1210	1600	1070	1940	1960	0.25	0.041	0.306
Cobalt	0.2	0.3	0.2	0.4	0.2	0.70	0.796	0.793
Copper	20	10	10	20	10	5.00	0.224	0.686
Zinc	30	30	30	30	40	0.004	0.308	0.616
Selenium	0.5	0.5	0.5	0.4	0.4	0.000	0.069	0.095
Week 2								
Manganese	20	18	18	14	22	0.002	0.644	0.121
Iron	1680	1005	1120	1080	940	0.280	0.109	0.426
Cobalt	0.2	0.2	0.2	0.2	0.3	0.000	0.933	0.137
Copper	12	10	9	11	11	0.001	0.646	0.141
Zinc	20	21	17	16	18	0.002	0.087	0.703
Selenium	0.5	0.6	0.6	0.5	0.7	0.000	0.008	0.813
Week 3								
Manganese	25	16	20	19	16	0.002	0.001	0.859
Iron	1680	1220	1640	1580	780	0.260	0.158	0.174
Cobalt	0.2	0.3	0.3	0.3	0.2	0.000	0.719	0.008
Copper	16	9	10	16	12	0.003	0.376	0.584
Zinc	58	65	18	20	17	0.015	0.006	0.168
Selenium	0.5	0.6	0.4	0.4	0.4	0.000	0.233	0.931
Week 4								
Manganese	30^{b}	20^{a}	20^{a}	19^{a}	19^{a}	0.001	0.008	0.946
Iron	2001	1850	950	940	1690	0.340	0.051	0.952
Cobalt	0.4	0.2	0.2	0.1	0.2	0.000	0.018	0.227
Copper	13^{c}	12^{bc}	8^{ab}	8^{ab}	7^{a}	0.001	0.004	0.872
Zinc	30	20	20	20	20	0.002	0.261	0.759
Selenium	0.5^{c}	0.5^{c}	0.4^{bc}	0.3^{ab}	0.2^{a}	0.000	0.000	0.046

[1]Substrates: GP0 = GP with no spawn; GP20 = GP inoculated with 20% spawn; GP inoculated with 30% spawn; GP40 = GP inoculated with 40% spawn; GP50 = GP inoculated with 50% spawn.
[2]SEM = standard error of the mean
[a, b, c] In a row, different superscripts denote significant differences (P <0.05) between spawning rates.

In weeks 1, 2 and 3, there were no spawning rate differences (P >0.05) for Mn, Fe, Co, Cu, Zn and Se. In week 4, substrate GP0 had a higher (P <0.05) Mn content (30 mg/kg DM) compared to substrate GP20 (20 mg/kg DM), GP30 (20 mg/kg DM), GP40 (19 mg/kg DM) and GP50 (19 mg/kg DM), which were the same (P >0.05). There were significant differences between Fe and

Co content in all the treatments. Substrate GP0 had a higher (P <0.05) Cu content (13 mg/kg DM) than substrate GP50 (7 mg/kg DM). There were no significant (P >0.05) differences between GP30, GP40 and GP50 in terms of the Cu content. Substrate GP0 had a similar Cu content as substrates GP20. There was no significant difference observed Zn content in all the treatments. Substrate GP0 and GP20 had a higher (P <0.05) Se content (0.5 mg/kg DM) than substrate GP50 (0.2 mg/kg DM). There were no significant (P >0.05) differences between GP40 and GP50 substrate in terms of the Se content. Substrate GP0 had a similar Se content as substrates GP20 and GP30 post-inoculation.

3.4 Discussion

3.4.1 Proximate composition

According to Barnes *et al*. (2007), it is paramount to understand the chemical constituents of the feed and their interactions to improve its utilisation by ruminants. This study aimed to improve the nutritive value of GP for ruminants using oyster mushroom spawn. The use of fungal treatment has been reported to be effective in reducing fibre constituents (Lynch *et al*., 2014; Shrivastava *et al*., 2014). It was not the case in the 1st and 2nd week of the current study because as more of the oyster mushroom spawn was added into the substrate it linearly increased the ADL content and showed quadratic response towards the NDF and ADF content. However, in week 3 and 4, the oyster mushroom spawn had an effect on the fibre content of GP as it was reduced with the increase in spawning rate. The increase in spawning rate linearly decreased the DM and OM content of the treated substrates compared to the untreated substrates in this study. This was in line with the results that were reported by Nasehi *et al*. (2017), whereby the DM and OM content of pea straw was lowered by solid-state fermentation compared to the untreated. A quadratic response in CP with an increase in spawning level and the incubation duration was observed in the present study for the treated GP substrates relative to untreated GP substrates. Previous studies (Fazaeli *et al*.,

2004; Rodrigues *et al*., 2008) also recorded an increase in protein when agricultural by-products are treated with different strains of white-rot fungi.

The oyster mushroom (*Pleurotus ostreatus)* spent substrates in weeks 1, 2 and 3 had low DM and OM contents in some of the treated substrates compared to the untreated in response to increasing spawning rates (30 - 40%). These findings were in agreement with those of Akinfemi *et al*. (2010) and Rahman *et al*. (2011), who reported slight losses in both the DM and OM content of fungal-treated agro by-products. According to Mukherjee & Nandi (2004), the low DM and OM content in fungal-treated agricultural by-products could be due to the degree of degradation of the substrate carbohydrates in the cell wall or the extent of fungal growth on the agro by-product substrates. In week 4, the increase in the spawning rate did not influence the DM and OM content. The use of incremental spawning rate on the GP substrate increased the CP content in all the weeks compared to untreated substrates. This was in line with the finding by other researchers (Bhuvnesh *et al*., 2011). Presumably, Akinfemi *et al*. (2010) reported that these could be due to the hydrolysis of carbohydrates and the subsequent use of carbon to synthesize fungal biomass that is rich in protein. Furthermore, Sallam *et al*. (2007) reported that the increase in CP content might be triggered by the excess capturing of nitrogen through the release of extracellular protein enzyme by the mycelia into the substrate during the fermentation process.

In this study, it was expected that the use of oyster mushroom spawn as an innovative strategy would significantly reduce the high amount of cellulose and lignin content in the GP, which hinders its effective utilization by rumen microbes. However, there was a gradual increase in the amount of fibre content (NDF, ADF and ADL) of the treated substrates relative to the untreated GP substrate in week 1 and 2 response to increasing spawning rates and the incubation period was recorded. These findings agreed with the results of Tuyen *et al*. (2012, 2013), whereby there was an increase in ADF and ADL contents for the autoclaved and *Pleurotus ostreatus*- treated sugarcane bagasse compared to the untreated substrates. Niu *et al*. (2018) reported an increase in

amylase-treated neutral detergent fibre, ADF and lignin content of wheat straw treated with *Irpex lacteus* and *P. ostreatus* compared to the untreated substrate after 7 days of incubation. Furthermore, van Kuijk *et al*. (2015) reported an increase in the lignin content during the early period of incubation of wheat straw, silver grass, wood chips and rice straw, which might be due to the fact that the white-rot fungi were able to consume a relatively high proportion of the easily digestible nutrients rather than lignin and cellulose for growth purposes (van Kuijk *et al*., 2015; Niu *et al*., 2018). The increase in the fibre content of the SMS in the present study might be presumably caused by external factors such as contamination, the fungi strain and the culturing of the spawn. Adamovic *et al*. (1998) reported that the chemical composition of the substrate might be affected by many factors such as the mixing ratios of ingredients, substrate ingredients and cultivation methods. However, when the GP substrate was incubated for 3 to 4 weeks, OYM tended to reduce the fibre content (NDF, ADF and ADL) of the treated substrates relative to the untreated GP substrates.

3.4.2 Mineral composition

There increase in the spawning rate and incubation duration linearly increased the calcium and sulphur content post-inoculation. This shows that the oyster mushroom spawn influenced the GP substrates. However, there was a slight decrease when more of the oyster mushroom spawn was added into the GP substrates. Sales-Campus *et al*. (2009) also reported a slight decrease in the P, Na and K content, which might be due to the basidioma selectively removing nutrients for the process of fruiting body formation. The incremental spawning rates and incubation time also had a quadratic effect for phosphorus magnesium, phosphorus, and calcium content in response to increasing spawning rates and in incubation time of oyster mushroom. This suggests that the increasing spawning rate had a positive effect on the substrate by increasing the macro-mineral content. An increase in mineral content of the spent substrates from the untreated was also reported

by Sales-Campos *et al*. (2009), with the exception of potassium which had lower values than the untreated, whose values were lower in all spent substrates than the untreated. The macro-minerals (Na, Mg, P, S and Ca) of the SMS did not have a great variation from the untreated substrates in weeks 1, 2, 3 and 4 because they were both within the required dietary range for ruminants as recommended by NRC (2005). Even though, CSIRO (2007) reported that the concentration of dietary mineral that is deemed to be adequate for ruminant requirements cannot be closely defined and estimated reliably from the feed analysis.

A linear decrease in micro-minerals (iron, manganese, zinc, cobalt and copper) and a linear increase in selenium content together with a quadratic trend for selenium and cobalt were observed with an increase in incubation time and spawning rates post-inoculation. This was expected in the current study because Lee (2009), also reported an increase in the micro-mineral (Al, Fe, Ni and Pb) values of the *H. marmoreus*, *F. velutipes*, and *P. eryngii* spent substrates than the untreated. However, Lee *et al*. (2009), reported that in all the weeks, an extremely deficient amount of micro-minerals (Mn, Co, Cu, Zn, and Se) was also observed in both the SMS and the untreated substrates, which were both similar in terms of the micro-mineral content. The micro-minerals were below the required range for ruminants as reported by NRC (2005), except for Cu for small ruminants and Fe for both small and large ruminants. The *P. ostreatus* is deemed to be an effective lignin degrader, however, Jung *et al*. (1992) reported that it is not a potent fungus that can be used to improve the nutritive value of fibrous by-products fed to ruminants. This implies that ruminants are likely to suffer from micro-mineral deficiencies when fed GP and SMS, thus trace minerals supplementation either in a form of lick blocks or in-feed would be necessary.

3.5 Conclusion

The use of oyster mushroom spawn reduced the fibre content when it was incubated for 3 to 4 weeks relative to the untreated red grape pomace. This shows that *P. ostreatus* spawn has the

potential to degrade the lignin content of grape pomace. The use of *P. ostreatus* spawn improved the crude protein content of the substrates. This implies that the spent mushroom substrate can be used by farmers as a possible source of protein for ruminants that are feed on low protein forages particularly during the dry seasons. In addition, ruminants fed grape pomace and grape pomace spent mushroom substrates would require trace mineral supplementation because the micro-minerals were below the required range for ruminants. It was, therefore, not possible to deduce the optimum spawning rate, suggesting a need to explore more research on the use of various *Pleurotus* species in diets containing grape pomace.

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4 CHAPTER FOUR – *IN VITRO* RUMINAL FERMENTATION PARAMETERS OF RED GRAPE POMACE SPENT OYSTER MUSHROOM SUBSTRATES

Abstract

The objective of this study was to investigate the *in vitro* ruminal fermentation parameters of red grape pomace spent oyster mushroom substrates. The grape pomace spent mushroom substrate produced in Chapter 3 was fermented using the Reading Pressure Technique for *in vitro* ruminal gas production and ANKOM Daisy^{II} incubator for the *in vitro* ruminal dry matter degradability (DMD). The *in vitro* ruminal dry matter degradability was only evaluated using 4-week red grape pomace spent mushroom substrates due to the maximum chemical modification of GP that was observed in week 4 (Chapter 3). In week 2, a significant (P <0.05) linear effect was observed for cumulative gas production at 48 h post-incubation, whereas significant (P <0.05) quadratic trends were observed for cumulative gas production at 12, 24, 36 and 48 in weeks 1 and 2. Both linear and quadratic trends were observed for the cumulative gas produced at 12, 24, 36 and 48 h post-inoculation in weeks 3 and 4, respectively. In all the weeks, a higher cumulative gas production was observed on the treated substrates (20 - 50 %) relative to the untreated substrates. The rate of gas produced at 24, 36 and 48 h post-incubation linearly increased with spawning rates in weeks 2 and 3. In all the weeks, there were significant (P <0.05) quadratic trends for the rate of gas production (between 12 - 48 h) post-incubation. The substrates inoculated with the spawn had a lower rate of gas production than the untreated substrates in week 1, whereas the treated substrates had a higher rate of gas production than the untreated substrates in weeks 2, 3 and 4. Both linear and quadratic trends were observed for the fraction (*a*), the slowly fermentable fraction (*b*), the fermentation rate of fraction (*c*), potential gas production (*Pgas*) and the *Egas* in almost all of the weeks. In all the weeks, the increase in the amount of spawn (20 - 50 %) had an effect (P <0.05)

on the *in vitro* ruminal gas productions kinetics except for the fraction *b* of the control substrate in week 2, the fraction *b* and *Pgas* of the control substrate in week 4, which produced higher *in vitro* gas than the treated substrates.

A quadratic trend was observed for DMD48. At 48 h, substrate GP30 had the highest (P <0.05) DMD (176.7 g/kg DM) while the lowest DMD (124.9 g/kg DM) was recorded for substrate GP0. The increase in spawning levels linearly increased the immediately degradable fraction (*a*). In response to the increasing spawning levels, effective degradability (*EDeg*) was found to be quadratic. The oyster mushroom spawn treatment had an effect (P <0.05) on fraction *a*, *b*, *PDeg* and *EDeg*. Quadratic responses were observed for *in vitro* organic matter degradability (*iv*OMD) at 36 and 48 h, and the partitioning factor (PF) at 48 h. The PF values at 12, 24, 36 and 48 h were significantly affected (P <0.05) by the spawning levels, with substrate GP20 having a higher (P < 0.05) PF value at 12 and 24 h post-incubation than the control substrate. It was, therefore, concluded that inoculating GP with graded levels of oyster mushroom spawn has the potential to improve the rate of gas production, the *in vitro* ruminal gas production kinetics, the degradability parameters, and fermentation efficiency of red GP.

Keywords: *In vitro* ruminal gas production; *In vitro* ruminal dry matter degradability; Red grape pomace; Ruminants; Spent oyster mushroom substrates.

4.1 Introduction

During the winemaking process, waste by-products such as red grape pomace (GP) are produced in large quantities posing several environmental challenges. The disposal of GP might result in plant growth interference and water body contamination through the build-up of chemicals (eutrophication). According to Bustamante *et al*. (2008), red GP is a rich source of biodegradable organic matter that causes environmental hazards and pollution if not discarded properly. Thus, it is important to find alternative uses of red GP that can improve its value while reducing the

negative impact it has on the environment. Corbin *et al*. (2015) reported that GP has the potential to be incorporated into ruminant rations because of the presence of the beneficial bioactive compounds such as phenolic and flavonoids, monosaccharides such as fructose and glucose, and the complex polysaccharides such as cellulose, xyloglucan and pectin. Grape pomace is a rich source of sugars which are in a form of a complex lignocellulosic matrix that constitutes around 34 - 50% cellulose, 19 - 34% hemicellulose and 11 - 30% lignin (Rogalinski *et al*., 2008). In addition, GP contains a high dietary fibre content that represents about 80% of DM, and the insoluble fibre accounts for a major part of the dietary fibre followed by protein. According to Baumgartel *et al*. (2007), the presence of a high level of lignified cell wall matrix is, however, deemed to be a major limitation in using GP in ruminant diets. Moreover, Moate *et al*. (2014) stated that the extractable and bound condensed tannins quantity in dried GP was 0.3 and 20.1 g/kg DM, respectively. These naturally occurring phenolic compounds tend to have the ability to bind and precipitate proteins. They additionally reduce feed intake in ruminants through their astringent taste. The high dietary fibre causes poor feed utilization, low degradability, and increased emission of greenhouse gases (GHGs) through enteric fermentation, which can cause air pollution (Kholif *et al*., 2017)

However, in recent years, there has been extensive interest among researchers (Zhang *et al*., 2007; Yu *et al*., 2009) in the use of biological treatments to improve the accessibility of the cell wall complex fractions of crop residues, which can improve their digestibility and feeding value. Zadrazil *et al*. (1996) reported that the use of fungal treatment could be a possible approach to improve crop residues for ruminant nutrition. Attempts to identify such fungal species that can grow and improve agricultural by-products have been made (Yamakawa *et al*., 1992). Among these fungal species is the edible white-rot fungi, the *Pleurotus* species, which has been identified as an efficient fungal species for this purpose (Zadrazil *et al*., 1996). Oyster mushroom is a preeminent wood decomposer that grows on a wide range of forest and agricultural wastes. They

can thrive on almost all the agricultural by-products such as sawdust, cereal straws, and other crop residues (Adebayo & Oloke 2017). A report by Mahmood *et al*. (2019) indicated that when the lignolytic fungus (*P*. *ostreatus*) was used in wheat straw, there was a reduction in lignin content, an increase in crude protein and improved digestibility. Calzada *et al*. (1987) reported a significant decrease in the lignin content and an increase *in vitro* dry matter digestibility (DMD) from 14.3 to 29.5% during solid-state fermentation of wheat straw by *P*. *ostreatus*. In an *in vitro* study conducted by Singh *et al*. (1990), *P*. *eryngii* and *P*. *ostreatus* were shown to reduce the high fibre fraction and increased DMD of wheat straw. In addition, an increase in DMD from 4.4% to 8.9% was observed when *P*. *ostreatus* was cultured and harvested on wheat straws (Zadrazil, 1997).

Although there are a lot of studies in the use of oyster mushroom to improve the digestion of lignin in a variety of crop residues, none of these investigations focuses on GP. Accordingly, the optimum spawning rates and the duration of incubation for this ubiquitous substrate is unknown. There is, therefore, a need to determine *in vitro* ruminal fermentation parameters of oyster mushroom spent substrates. Thus, the objective of the current study was to determine the *in vitro* ruminal gas production and the *in vitro* ruminal dry matter degradability parameters of red grape pomace spent oyster mushroom substrates. It was, therefore, hypothesized that inoculation of red GP with oyster mushroom for four weeks would significantly improve the ruminal fermentation parameters of the spent substrate in a dose and incubation time-dependent manner.

4.2 Materials and methods

4.2.1 Study site and ingredient sources

The study site and ingredient sources were as described in Chapter 3, Section 3.2.1.

4.2.2 Substrate preparation

The GP substrate preparation was done as it was described in Chapter 3, Section 3.2.2

4.2.3 Inoculation and experimental design

The inoculation and incubation of the 50 pots (experimental units), the experimental design, subsampling and processing of spent mushroom substrates for *in vitro* ruminal fermentation was carried out as described in Chapter 3, Sections 3.2.3 and 3.2.4.

4.2.4 Temperature and humidity

The pots were kept at a room temperature (between 20 - 25°C) and the relative humidity (ranged between 70 – 80%) was recorded using a multi-meter as mentioned in Chapter 3, Section 3.2.5. The spent mushroom substrates preparation was carried out as mentioned in Section 3.2.6 prior to analyses.

4.2.5 Preparation of spent mushroom substrates (SMS)

The preparation of the spent mushroom substrates was carried out as described in Chapter 3, Section 3.2.6.

4.2.6 In vitro ruminal gas production

The *in vitro* ruminal gas production parameters were determined using the Reading Pressure Technique (RPT) developed by Mauricio *et al*. (1999). The rumen inoculum was collected in the morning prior to feeding the fistulated Bonsmara cow (600 kg body weight) in the squeeze chute. The cow was receiving a mixture of blue buffalo grass (*Cenchrus ciliaris*) and Lucerne hay *ad libitum* before collecting the rumen inoculum. Rumen fluid was collected into pre-warmed thermal flasks and quickly taken to the laboratory, where it was blended and filtered through a two-layer of muslin cloth. The filtered rumen fluid was readily purged with carbon dioxide gas (CO_2) to mimic the anaerobic environment of the rumen (39°C). Milled SMS and untreated samples (1 ± 0.001 g) were weighed and placed into 125 mL glass serum bottles. An ANKOM buffer solution

was prepared according to ANKOM technology method no 3, whereby two buffer solutions, A and B (Appendix 3) were prepared beforehand at a ratio of 1:5. Ninety (90) mL of the ANKOM buffer solution was added in each of the bottles followed by the inoculation with 10 mL of the rumen fluid before sealing the serum bottles with air-tight rubber stoppers. The correction of gas was produced from the rumen liquor by allowing the inclusion of two serum bottles without samples. The glass serum bottles were then incubated at 39°C for a period of 48 h. The headspace gas pressure was measured at different intervals starting from 12, 24, 36 and 48 h post-inoculation by inserting a 23-gauge needle attached to a pressure transducer (model PX4200-015GI, Omega Engineering, Inc., Laval, QC, Canada) into the blue rubber stoppers of the serum bottles. The needles were left on the serum bottles after the insertion to allow all the available gas to move out of the glass serum bottle. The readings from the pressure transducer were recorded using a phone by taking a video to narrow the time and ensure that the correct peak reading per sample was recorded as they display. The gas pressure readings (psi) were then converted to the gas volume (mL) using the following site-specific equation:

$$y = 0.034x^2 + 6.2325x + 1.8143,$$

Where y = gas volume (mL) and x = measured gas pressure (psi). The rate of gas produced per period was calculated by dividing the amount of gas volume produced for each period by the number of hours within that period.

Cumulative gas production parameters were estimated by fitting data into the Ørskov & McDonald (1979) non-linear model:

$$y = a + b(1 - e^{-c(t)})$$

Where, Y = gas produced at time "t"; a = the gas production from the immediately fermentable fraction (mL); b = the gas production from the slowly fermentable fraction (mL); c = the gas production rate constant for the insoluble fraction, b; t = incubation time (h). Potential gas

production (*Pgas*) was calculated as the summation of fractions *a* and *b*, whereas the effective gas production (*Egas)* was calculated using the following formula: *Egas* = $a + \frac{bc}{k+c}$, where *k* is the rumen outflow rate assumed to be 2% per hour, and *a*, *b*, and c are the Ørskov-McDonald parameters already described.

4.2.7 In vitro ruminal dry matter degradability

The *in vitro* ruminal dry matter degradability of SMS and untreated substrates were determined using the Daisy^{II} incubator according to the ANKOM Technology Method no. 3 for the *in vitro* true digestibility (ANKOM Technology, 2001). Ground samples were weighed (0.45 – 0.5 g) into the ANKOM F57 filter bags and sealed using an impulse heat sealer and thereafter placed in the Daisy^{II} incubator jars. Accordingly, two buffer solutions A and B were prepared in advance and combined at a ratio of 1:5 with a final pH of 6.8. A total of 1600 mL of the mixed buffer solution was added into each of the Daisy^{II} incubator jars and pre-warmed at 39°C overnight. The next morning, rumen inoculum was collected from the same fistulated Bonsmara cow and processed as mentioned on the *in vitro* ruminal gas production. Each of the jars was then inoculated with 400 mL of rumen inoculum and purged with CO_2 before placing them in the Daisy^{II} incubation chamber. The ANKOM filter bags were withdrawn until 72 h post-inoculation. The withdrawn bags were then washed with tap water for about 20 min before being dried at a temperature of 105°C for 12 h. The *in vitro* ruminal DMD was determined using the following formula:

$$\%IVTD(DM\ basis) = \frac{100 - (W3 - (W1 \times C1))}{W2\ \times DM} \times 100$$

Where, W1 = bag weight, W2 = sample weight, W3 = bag weight plus residue after *in vitro* treatment, and C1 = correction factor (final oven-dried bag weight/original blank bag weight). The dry matter degradability parameters: *a*, *b*, and *c* were estimated using the Ørskov & McDonald (1979) non-linear procedure as described above. The potential degradability (*PDeg*) was

determined using the following equation: *PDeg* = *a* + *b*, whereas the effective degradability (*EDeg*) was calculated using the following equation: $EDeg = a + \frac{bc}{k+c}$, where *a* = the immediately degradable fraction, *b* = the slowly degradable fraction, *c* = rate of degradation of the slowly degradable fraction '*b*' (%/hour), and *k* = the rumen outflow rate assumed to be 2% per hour.

4.2.8 Estimation of the degradable substrate

The *in vitro* ruminal organic matter degradability (*iv*OMD) was determined through the incineration of the ANKOM F57 filter bags with residues placed on pre-weighed crucibles using a muffle furnace set at 600°C for 12 h. Partitioning factors (mL/g OM), a measure of fermentation efficiency, was calculated as a ratio of the Cumgas and the *iv*OMD at 12, 24, 36 and 48 h post-incubation.

4.2.9 Statistical analysis

The cumulative gas production, the rate of gas produced per period, the *in vitro* ruminal gas production kinetics (*a*, *b*, *c*, *Pgas* and *Egas*), the dry matter degradability, *in vitro* ruminal dry matter degradability kinetics (*a*, *b*, *c*, *PDeg* and *EDeg*), *in vitro* organic matter degradability and the partitioning factors data were evaluated for linear and quadratic effects using polynomial contrasts. Response surface regression analysis (Proc RSREG; SAS, 2010) was employed to estimate the optimum spawning rate of the oyster mushroom for each GP substrate using the quadratic equation described in Chapter 3, Section 3.4.

Repeated measures analysis was used to analyse weekly measured *in vitro* ruminal gas production data using SAS (2010) as described in Chapter 3. In a CRD, cumulative gas production, the rate of gas produced per period, the *in vitro* ruminal gas production, *in vitro* ruminal dry matter degradation and partitioning factors were analysed using the GLM procedure of SAS (2010) using the one-way ANOVA model as described in Chapter 3. For all the statistical tests, the significance

was declared at P <0.05. The least-squares means were compared using the probability of difference option in SAS.

4.3 Results

4.3.1 In vitro ruminal gas production

Table 4.1 shows the cumulative gas production of red grape pomace spent oyster mushroom substrate. In week 1, there were no linear trends (P >0.05) for cumulative gas production at 12, 24, 36 and 48 h post-inoculation. However, there were significant (P <0.05) quadratic trends for cumulative gas production at 12, 24, 36 and 48 h post-inoculation in response to the different spawning rates of oyster mushroom. In week 2, there were no linear effects (P >0.05) for cumulative gas production at 12, 24 and 36 h except for cumulative gas production at 48 h post-inoculation which differed (P <0.05). There were significant quadratic effects for cumulative gas production at 12, 24, 36 and 48 h. In week 3, there were significant linear and quadratic effects for cumulative gas production at 24, 36 and 48 h, except for cumulative gas production at 12 h post-inoculation which did not differ (P <0.05) among the treatments. In week 4, there were both significant (P <0.05) linear and quadratic trends for the cumulative gas produced at 12, 24, 36 and 48 h post-inoculation in response to the different spawning rates of oyster mushroom.

Table 4.1. Cumulative gas production (mL/g OM) of red grape pomace spent oyster mushroom substrate.

	[1]Substrates						Significance	
[2]Parameters	GP0	GP20	GP30	GP40	GP50	[3]SEM	Linear	Quadratic
Week 1								
Cumgas12	16.76^{ab}	12.59^{a}	14.60^{ab}	13.50^{ab}	18.54^{b}	1.214	0.543	0.006
Cumgas24	21.59^{ab}	15.10^{a}	19.14^{ab}	19.00^{ab}	23.20^{b}	1.824	0.569	0.014
Cumgas36	25.70	17.91	22.53	22.81	26.62	2.158	0.741	0.018
Cumgas48	29.36	20.24	25.25	26.26	29.23	2.348	0.920	0.017
Week 2								
Cumgas12	11.56^{ab}	14.72^{b}	13.44^{ab}	11.49^{a}	12.28^{ab}	0.661	0.672	0.003
Cumgas24	13.95^{a}	17.86^{b}	17.88^{b}	13.43^{a}	14.12^{a}	0.922	0.394	0.000
Cumgas36	17.36^{ab}	21.05^{b}	21.43^{b}	15.81^{a}	15.93^{a}	1.104	0.295	0.000
Cumgas48	20.62^{ab}	24.02^{b}	24.74^{b}	17.98^{a}	17.62^{a}	1.288	0.023	0.000
Week 3								
Cumgas12	6.98	9.14	8.65	8.23	8.87	0.723	0.118	0.420
Cumgas24	7.78^{a}	11.33^{ab}	12.24^{b}	12.07^{b}	9.97^{ab}	1.000	0.005	0.022
Cumgas36	9.44^{a}	13.92^{ab}	14.89^{b}	15.56^{b}	12.45^{ab}	1.237	0.002	0.028
Cumgas48	11.57^{a}	16.04^{ab}	17.65^{b}	18.53^{b}	14.41^{ab}	1.404	0.003	0.027
Week 4								
Cumgas12	8.02^{a}	11.22^{b}	11.38^{b}	10.32^{ab}	10.25^{ab}	0.648	0.002	0.009
Cumgas24	9.10^{a}	12.41^{b}	12.15^{b}	12.11^{b}	12.05^{b}	0.517	0.000	0.007
Cumgas36	11.15^{a}	14.50^{b}	14.05^{b}	14.09^{b}	13.93^{b}	0.559	0.000	0.011
Cumgas48	13.07^{a}	16.29^{b}	16.01^{b}	15.46^{ab}	15.55^{ab}	0.628	0.006	0.017

[a,b] Means in a row with different superscripts denote significant differences (P <0.05) between spawning rates.

[1]Substrates: GP0 = red grape pomace with no spawn; GP20 = red grape pomace inoculated with 20% of oyster mushroom spawn; red grape pomace inoculated with 30% of oyster mushroom spawn; GP40 = red grape pomace inoculated with 40% of oyster mushroom spawn; GP50 = red grape pomace inoculated with 50% of oyster mushroom spawn. [2]Parameters: cumgas12 = cumulative gas production at 12 h post-inoculation; cumgas24 = cumulative gas at 24 h post-inoculation; cumgas36 = cumulative gas at 36 h post-inoculation; cumgas48 = cumulative gas at 48 h post-inoculation.

[3]SEM = standard error of the mean.

In week 1, substrate GP50 had higher (P <0.05) cumulative gas production at 12 h (18.54 mL/g OM) than substrate GP20 (12.59 mL/g OM). Substrate GP0 had similar cumulative gas production

at 12 h post-inoculation as substrates GP20, GP30, GP40 and GP50. Substrate GP50 had higher (P <0.05) cumulative gas production at 24 h (23.20 mL/g OM) than substrate GP20 (15.10 mL/g OM). Substrates GP0, GP20, GP30, GP40 and GP50 had the same cumulative gas production at 24 h post-inoculation.

In week 2, substrate GP20 had higher (P <0.05) cumulative gas production at 12 h (14.72 mL/g OM) than substrate GP40 (11.49 mL/g OM). The untreated substrates GP0 had similar (P >0.05) cumulative gas production at 12 h as substrates GP20, GP30, GP40 and GP50. Substrate GP30 had higher (P <0.05) cumulative gas production at 24 h (17.88 mL/g OM) than substrate GP0, GP40 and GP50, which did not differ (P >0.05). Substrate GP30 had a higher (P <0.05) cumulative gas production at 36 h (21.43 mL/g OM) than substrate GP40 and GP50, which did not differ (P >0.05). There were no significant differences between GP10, GP20 and GP50 in terms of cumulative gas production at 36 h post-inoculation. Substrate GP30 had higher (P <0.05) cumulative gas production at 48 h (24.74 mL/g OM) than substrates GP40 and GP20, which did not differ (P >0.05). Substrate GP0 had the same cumulative gas production at 48 h as substrate GP20, GP30, GP40 and GP50.

In week 3, there was no significant difference in cumulative gas production at 12 h in all the treatments. Substrate GP30 had higher (P <0.05) cumulative gas production at 24 h (12.24 mL/g OM) compared to substrate GP0 (7.78 mL/g OM). Substrates GP0, GP20 and GP50 had a similar (P <0.05) cumulative gas production at 24 h post-inoculation. Substrates GP40 had a higher (P <0.05) cumulative gas production at 36 h (15.56 mL/g OM) than substrate GP0 (15.56 mL/g OM). Substrate GP0 had the same (P <0.05) cumulative gas production at 36 h post-inoculation as GP20 and GP50 substrates. Substrate GP40 had higher (P <0.05) cumulative gas production at 48 h (18.53 mL/g OM) than substrate GP0 (11.57 mL/g OM). Substrates GP0, GP20 and GP50 had similar (P <0.05) cumulative gas production at 48 h post-inoculation.

In week 4, substrate GP30 had higher (P <0.05) cumulative gas production at 12 h (11.38 mL/g OM) than substrate GP0 (8.02 mL/g OM). There were no significant differences among substrates GP20, GP30, GP40 and GP50 in terms of the cumulative gas produced at the 12 h. Substrate GP0 had the same (P >0.05) cumulative gas production at 12 h post-inoculation as substrates GP40 and GP50. Substrate GP20 had higher (P <0.05) cumulative gas production at 24 h (12.41 mL/g OM) than substrate GP0 (9.10 mL/g OM). Substrate GP20 had the highest (P <0.05) cumulative gas production at 36 h (14.50 mL/g OM) than substrates GP0 (11.15 mL/g OM). Substrate GP20 had higher (P <0.05) cumulative gas production at 48 h (16.29 mL/g OM) than substrate GP0 (13.07 mL/g OM). Substrates GP0, GP40 and GP50 had the same cumulative gas production at 48 h post-inoculation.

Table 4.2. Rate of gas produced (mL/h OM) of red grape pomace spent oyster mushroom substrate.

	[1]Substrates						Significance	
[2]Parameters	GP0	GP20	GP30	GP40	GP50	[3]SEM	Linear	Quadratic
Week 1								
Rtgas12	1.52^{b}	1.05^{a}	1.24^{ab}	1.17^{ab}	1.45^{ab}	0.120	0.567	0.009
Rtgas24	0.69	0.41	0.63	0.72	0.70	0.087	0.601	0.066
Rtgas36	0.68	0.47	0.57	0.63	0.64	0.062	0.859	0.045
Rtgas48	0.69^{b}	0.46^{a}	0.55^{ab}	0.64^{ab}	0.61^{ab}	0.052	0.586	0.019
Week 2								
Rtgas12	0.88^{a}	1.13^{b}	1.14^{b}	1.00^{ab}	1.03^{ab}	0.062	0.182	0.005
Rtgas24	0.36^{ab}	0.47^{a}	0.62^{b}	0.33^{a}	0.33^{a}	0.041	0.388	0.000
Rtgas36	0.49^{bc}	0.51^{bc}	0.59^{c}	0.40^{ab}	0.35^{a}	0.032	0.005	0.000
Rtgas48	0.52^{bc}	0.53^{bc}	0.62^{c}	0.41^{ab}	0.36^{a}	0.033	0.001	0.000
Week 3								
Rtgas12	0.73	0.77	0.83	0.84	0.73	0.063	0.278	0.489
Rtgas24	0.19^{a}	0.33^{ab}	0.47^{b}	0.47^{b}	0.32^{ab}	0.058	0.005	0.014
Rtgas36	0.27^{a}	0.40^{ab}	0.43^{ab}	0.49^{b}	0.39^{ab}	0.040	0.001	0.113
Rtgas48	0.33^{a}	0.39^{ab}	0.47^{ab}	0.49^{b}	0.38^{ab}	0.036	0.008	0.073
Week 4								
Rtgas12	0.68^{ab}	0.80^{b}	0.77^{ab}	0.67^{ab}	0.53^{a}	0.066	0.089	0.007
Rtgas24	0.21	0.27	0.22	0.31	0.31	0.038	0.064	0.838
Rtgas36	0.32	0.38	0.34	0.35	0.34	0.017	0.590	0.079
Rtgas48	0.34	0.38	0.38	0.32	0.34	0.019	0.574	0.080

[a,b] In a row, different superscripts denote significant differences (P <0.05) between spawning rates.

[1]Substrates: GP0 = red grape pomace with no spawn; GP20 = red grape pomace inoculated with 20% of oyster mushroom spawn; red grape pomace inoculated with 30% of oyster mushroom spawn; GP40 = red grape pomace inoculated with 40% of oyster mushroom spawn; GP50 = red grape pomace inoculated with 50% of oyster mushroom spawn.

[2]Parameters: Rtgas12 = rate of gas produced at 12 h post-inoculation; Rtgas24 = rate of gas produced at 24 h post-inoculation; Rtgas36 = rate of gas produced at 36 h post-inoculation; Rtgas48 = rate of gas produced at 48 h post-inoculation.

[3]SEM = standard error of the mean.

Table 4.2 shows that there were no linear trends (P >0.05) for the rate of gas produced at 12, 24, 36 and 48 h post-inoculation, but there were quadratic trends (P <0.05) for the rate of gas produced at 12, 36 and 48 h post-inoculation in response to spawning levels in week 1. In week 2, there were

no linear effects (P >0.05) for the rate of gas produced at 12 and 24 h, except for the rate of gas produced at 36 and 48 h post-inoculation. Quadratic trends (P <0.05) were observed for the rate of gas produced at 12, 24, 36 and 48 h post-inoculation. In week 3, there were significant linear effects for the rate of gas produced at 24, 36 and 48 h post-inoculation, whereas a quadratic effect (P <0.05) was observed for the rate of gas produced at 24 h post-inoculation. In week 4, there were no linear trends (P >0.05) for the rate of gas produced at 12, 24, 36 and 48 post-inoculation, however, a quadratic trend (P <0.05) was observed for the rate of gas produced at 12 h after incubation in response to the different spawning rates of oyster mushroom.

In week 1, substrate GP0 had a higher (P <0.05) rate of gas produced at 12 h (1.52 mL/h OM) than substrate GP20 (1.05 mL/h OM). Substrates GP20, GP30, GP40 and GP50 had the same rate of gas produced at 12 h post-inoculation. There were no significant (P >0.05) differences in the rate of gas produced at 24 and 36 h in all the treatments. Substrate GP0 had a higher (P <0.05) rate of gas produced at 48 h (0.69 mL/h OM) than substrate GP20 (0.46 mL/h OM).

In week 2, substrate GP30 had a higher (P <0.05) rate of gas produced at 12 h (1.14 mL/h OM) than substrate GP0 (0.88 mL/h OM). Substrate GP0 had a similar (P >0.05) rate of gas produced at 12 h as substrates GP40 and GP50 post-inoculation. Substrate GP30 had a higher (P <0.05) rate of gas produced at 24 h (0.62 mL/h OM) than substrates GP20, GP40 and GP50, which did not differ (P >0.05). Substrate GP0 had the same (P >0.05) rate of gas produced at 24 h as GP20, GP20, GP30, GP40 and GP50. Substrate GP30 had a higher (P <0.05) rate of gas produced at 36 h (0.59 mL/h OM) than substrates GP40 and GP50, which did not differ (P >0.05). Substrate GP0 had the same rate of gas produced at 36 h post-inoculation as substrates GP20 and GP30. Substrate GP30 had a higher rate of gas produced at 48 h (0.62 mL/h OM) than substrates GP40 and GP50, which were the same (P <0.05). Substrate GP0 had a similar rate of gas produced at 48 h post-inoculation as substrates GP20 and GP30

In week 3, there were no significant (P >0.05) differences in the rate of gas produced at 12 h in all the treatments. Substrate GP30 and GP40 had a higher (P <0.05) rate of gas produced at 24 h post-inoculation (0.47 mL/h OM) than the untreated substrate GP0 (0.19 mL/h OM). Substrate GP20, GP30, GP40 and GP50 had a similar rate of gas produced at 24 h post-inoculation. Substrate GP40 had a higher (P <0.05) rate of gas produced at 36 h (0.49 mL/h OM) than substrate GP0 (0.27 mL/h OM). Substrates GP20, GP30 and GP50 had the same (P >0.05) rate of gas produced at 36 h post-inoculation. Substrate GP40 had a higher (P <0.05) rate of gas produced at 48 h (0.49 mL/h OM) than substrate GP0 (0.33 mL/h OM). Substrate GP20, GP30, GP40 and GP50 had the same rate of gas produced at 48 h post-inoculation.

In week 4, substrate GP20 had a higher (P <0.05) rate of gas produced at 12 h post-inoculation (0.80 mL/h OM) than substrate GP50 (0.53 mL/h OM). Substrates GP0, GP20, GP30, GP40 and GP50 had a similar (P >0.05) rate of gas produced at 12 h post-inoculation. There were no significant differences observed for the rate of gas produced at 24, 36 and 48 h for all the treatments.

Table 4.3. *In vitro* ruminal gas production kinetics (mL/g OM, unless stated otherwise) of red grape pomace spent oyster mushroom substrate.

	[1]Substrates						Significance	
[2]Parameters	GP0	GP20	GP30	GP40	GP50	[3]SEM	Linear	Quadratic
Week 1								
a	5.31^{a}	3.86^{a}	5.80^{ab}	5.99^{ab}	7.56^{b}	0.533	0.004	0.022
b	30.58^{ab}	20.58^{a}	27.17^{ab}	33.37^{b}	26.62^{ab}	2.569	0.975	0.139
c (%/h)	0.03^{ab}	0.04^{b}	0.03^{ab}	0.02^{a}	0.04^{b}	0.003	0.819	0.322
Pgas	35.89^{b}	24.44^{a}	32.97^{ab}	39.35^{b}	34.17^{ab}	2.836	0.636	0.086
Egas	24.72^{ab}	17.17^{a}	21.97^{ab}	23.26^{ab}	25.26^{b}	1.951	0.653	0.020
Week 2								
a	4.78	5.39	5.78	4.62	4.55	0.451	0.490	0.035
b	28.48^{b}	27.06^{b}	28.63^{b}	18.51^{a}	16.02^{a}	1.802	0.001	0.003
c (%/h)	0.02^{a}	0.03^{b}	0.03^{b}	0.03^{b}	0.04^{c}	0.002	0.000	0.008
Pgas	33.27^{b}	32.45^{b}	34.42^{b}	23.12^{a}	20.57^{a}	1.977	0.000	0.000
Egas	18.24^{ab}	20.89^{b}	21.47^{b}	15.66^{a}	15.41^{a}	1.105	0.014	0.000
Week 3								
a	2.73	3.76	4.29	3.76	3.26	0.459	0.554	0.012
b	17.78^{a}	19.06^{ab}	26.18^{bc}	27.87^{c}	16.16^{a}	2.085	0.114	0.029
c (%/h)	0.02^{a}	0.02^{a}	0.02^{a}	0.02^{a}	0.05^{b}	0.007	0.129	0.097
Pgas	20.08^{a}	22.81^{ab}	30.01^{b}	31.20^{b}	18.19^{a}	2.377	0.108	0.014
Egas	10.23^{a}	14.00^{ab}	15.95^{b}	16.42^{b}	12.06^{ab}	1.262	0.006	0.013
Week 4								
a	4.27^{a}	6.02^{b}	5.44^{ab}	6.43^{bc}	7.80^{c}	0.425	0.0001	0.468
b	18.11^{b}	15.56^{ab}	14.54^{ab}	13.18^{a}	12.26^{a}	1.035	0.0001	0.922
c (%/h)	0.016^{a}	0.024^{ab}	0.029^{b}	0.028^{ab}	0.026^{ab}	0.003	0.018	0.036
Pgas	22.38	21.58	19.98	19.62	19.03	1.103	0.037	0.848
Egas	11.85^{a}	14.43^{b}	13.91^{ab}	13.71^{ab}	13.64^{ab}	0.562	0.009	0.477

[a,b,c] In a row, different superscripts denote significant differences (P <0.05) between spawning rates.

[1]Substrates: GP0 = red grape pomace with no spawn; GP20 = red grape pomace inoculated with 20% of oyster mushroom spawn; red grape pomace inoculated with 30% of oyster mushroom spawn; GP40 = red grape pomace inoculated with 40% of oyster mushroom spawn; GP50 = red grape pomace inoculated with 50% of oyster mushroom spawn.

[2]Parameters: *a*: the immediate fermentable fraction; *b*: the slowly fermentable fraction; *c*: fermentation rate of fraction *b*; *Pgas* = potential gas production; *Egas* = effective gas production.

[3]SEM = standard error of the mean.

Table 4.3 shows that there were no linear effects (P >0.05) for the slowly fermentable fraction (*b*), the fermentation rate of fraction *b* (*c*), the potential gas production (*Pgas*) and the effective gas production (*Egas*), with the exception of the immediate fermentable fraction (*a*) in response to spawning levels in week 1. No quadratic effects (P >0.05) were observed for fractions *b*, *c* and *Pgas*, with the exception of the fraction *a* and *Egas*. In week 2, there were quadratic trends (P <0.05) for all gas production parameters in response to different spawning rates of oyster mushroom. Linear responses (P <0.05) were observed for fraction *b*, *c*, *Egas* and *Pgas* in response to spawning levels. In week 3, there were no linear effects (P >0.05) for fraction a, b, c and *Pgas* except for *Egas*. Significant quadratic trends (P <0.05) were observed for fraction *a*, *b*, *Pgas* and *Egas* except for fraction *c*. In week 4, there were linear effects for fraction *a*, *b*, *c*, *Pgas* and *Egas*, whereas a significant quadratic trend was observed for the fraction *c* in response to the different spawning rates of oyster mushroom.

In week 1, substrate GP50 had higher (P <0.05) fraction *a* (7.56 mL/g OM) than substrates GP0 and GP20, which did not differ (P >0.05). Substrate GP40 had higher (P <0.05) fraction *b* (33.37 mL/g OM) than substrate GP20 (20.58 mL/g OM). Substrate GP0 had a similar (P >0.05) fraction *b* as substrates GP20, GP30, GP40 and GP50. Substrate GP20 had higher (P <0.05) fraction *c* (0.04 mL/g OM) than substrate GP40 (0.02 mL/g OM). Substrate GP0 had a similar (P >0.05) gas produced from the fraction *c* as substrates GP20, GP30, GP40 and GP50. Substrate GP40 had higher (P <0.05) *Pgas* (39.35 mL/g OM) than substrate GP20 (24.44 mL/g OM). There were no significant differences among GP0, GP30, GP40 and GP50 substrates in terms of *Pgas*. Substrate GP50 had higher (P <0.05) *Egas* (25.26 mL/g OM) than substrate GP20 (17.17 mL/g OM). Substrate GP0 had a similar (P >0.05) *Egas* as substrates GP20, GP30, GP40 and GP50.

In week 2, there were no significant (P >0.05) differences for the fraction *a* in all the treatments. Substrate GP30 had a higher (P <0.05) gas produced for the fraction *b* (28.63 mL/g OM) than substrates GP50 (16.02 mL/g OM). There were no significant differences between GP40 and GP50

in terms of the fraction *b*. Substrates GP0, GP20 and GP30 had the same gas produced from the fraction *b* post-inoculation. Substrate GP50 had a higher (P <0.05) gas produced from the fraction *c* (0.04 mL/g OM) than substrate GP0 (0.02 mL/g OM). There were no significant differences between GP20, GP30 and GP40 in terms of fraction *c*. Substrate GP30 had a higher (P <0.05) *Pgas* produced (34.42 mL/g OM) than substrates GP40 (23.12 mL/g OM) and GP50 (20.57 mL/g OM), which did not differ. Substrates GP0, GP20 and GP30 had the same *Pgas* produced post-inoculation. Substrate GP30 had a higher (P <0.05) *Egas* produced than substrates GP40 (16.66 mL/g OM) and GP50 (15.41 mL/g OM), which were the same. Substrate GP0 had a similar (P >0.05) *Egas* produced as substrates GP20, GP30, GP40 and GP50.

In week 3, there was no significant (P >0.05) differences observed for fraction *a* in all the treatments. Substrate GP40 had a higher (P < 0.05) gas produced from fraction *b* (27.87 mL/g OM) than substrates GP0 (17.78 mL/g OM) and GP50 (16.16 mL/g OM), which did not differ. There were differences (P < 0.05) among substrates GP20, GP30 and GP40 in terms of fraction *b*. Substrates GP0, GP20 and GP50 had the same post-inoculation fraction *b*. Substrate GP50 had a higher (P < 0.05) gas produced from fraction *c* (0.05 mL/g OM) than substrates GP0 (0.02 mL/g OM), GP20 (0.02 mL/g OM), GP30 (0.02 mL/g OM) and GP40 (0.02 mL/g OM), which did differ (P <0.05). Substrate GP40 had a higher (P <0.05) *Pgas* produced (31.20 mL/g OM) than substrate GP50 (18.19 mL/g OM). There were no differences (P >0.05) among substrates GP20, GP30 and GP40 in terms of the *Pgas* produced. Substrates GP0, GP20 and GP50 had the same *Pgas* produced post-inoculation. Substrate GP40 had a higher (P <0.05) *Egas* produced (16.42 mL/g OM) than substrate GP0 (10.23 mL/g OM). There were significant differences among substrates GP20, GP30 GP40 and GP50 in terms of the *Egas* produced. Substrate GP0 had a similar (P >0.05) *Egas* produced as G20 and GP50 substrates.

In week 4, Substrate GP50 had a higher (P <0.05) gas produced from the fraction *a* (7.80 mL/g OM) than substrate GP0 (4.27 mL/g OM). There were differences (P <0.05) among substrates

GP20, GP30 and GP40 in terms of the fraction *a*. Substrates GP0 and GP30 had the same gas produced from the fraction *a*. Substrate GP0 had a higher (P <0.05) gas produced from fraction *b* (18.11 mL/g OM) than substrates GP40 (13.18 mL/g OM) and GP50 (mL/g OM), which were the same. There were no significant differences among substrates GP20, GP30, GP40 and GP50 in terms of the gas produced from fraction *b*. Substrates GP0, GP20 and GP30 had the same gas produced from fraction *b*. Substrate GP30 had a higher (P <0.05) gas produced from fraction *c* (0.029 mL/g OM) than substrates GP0 (0.016 mL/g OM). Substrates GP0, GP20, GP40 and GP50 had the same gas produced from fraction *c*. There were no significant differences for the *Pgas* produced in all the treatments. Substrate GP20 had a higher (P <0.05) *Egas* produced (14.43 mL/g OM) than substrates GP0 (11.85 mL/g OM). Substrate GP0 had a similar (P >0.05) *Egas* produced as substrate GP30, GP40 and GP50. There were no significant (P >0.05) differences among substrates GP20, GP30, GP40 and GP50 in terms of the *Egas* produced. Substrate GP0 had a similar *Egas* produced as substrates GP30, GP40 and GP50.

4.3.2 In vitro ruminal dry matter degradability parameters

Table 4.4 shows that there were no significant (P >0.05) linear and quadratic trends for DMD12, DMD24 and DMD36, except for DMD48 [y = 3.244 (±0.592)x - 0.006 (±0.010)x^2 + 131.8 (±8.121); R^2 = 0.838; P = 0.002] which had a quadratic (P <0.05) response to the spawning levels. There were no significant differences on DMD12 among the substrates. Substrate GP20 had higher (P <0.05) DMD24 (155.4 g/kg DM) than the control substrate GP0 (80.10 g/kg DM). Nonetheless, substrates GP20, GP30, GP40 and GP50 had the same (P >0.05) DMD24. Substrate GP50 had higher (P <0.05) DMD36 (131.2 g/kg DM) than the control substrate GP0 (59.70 g/kg DM). However, substrate GP0 had a similar DMD36 (P >0.05) with substrates GP20, GP30 and GP40. Substrate GP30 had higher (P <0.05) DMD48 (176.7 g/kg DM) than substrate GP0 (124.9 g/kg

DM). Substrates GP20, GP30, GP40 and GP50 had the same dry matter degradability 48 h post-inoculation.

Table 4.4. *In vitro* ruminal dry matter degradability (g/kg DM) of red grape pomace spent oyster mushroom substrate harvested at 4 weeks post-inoculation.

	[1]Substrates						*P*-value	
[2]Parameters	GP0	GP20	GP30	GP40	GP50	[3]SEM	Linear	Quadratic
DMD12	75.97	329.6	126.1	117.5	120.7	0.303	0.505	0.197
DMD24	80.10[a]	155.4[b]	143.2[b]	122.9[b]	148.0[b]	0.390	0.856	0.099
DMD36	59.70[a]	104.6[ab]	127.7[b]	87.42[ab]	131.2[c]	0.263	0.460	0.264
DMD48	124.9[a]	164.8[b]	176.7[b]	164.5[b]	158.9[b]	0.838	0.492	0.002

[a,b,c] In a row, different superscripts denote significant differences (P <0.05) between spawning rates.
[1]Substrates: GP0 = red grape pomace with no spawn; GP20 = red grape pomace inoculated with 20% of oyster mushroom spawn; red grape pomace inoculated with 30% of oyster mushroom spawn; GP40 = red grape pomace inoculated with 40% of oyster mushroom spawn; GP50 = red grape pomace inoculated with 50% of oyster mushroom spawn.
[2]Parameters: DMD12 = dry matter degradability at 12 h after inoculation; DMD24 = dry matter degradability at 24 h after inoculation; DMD36 = dry matter degradability at 36 h after inoculation; DMD48 = dry matter degradability at 48 h after inoculation.
[3]SEM = standard error of the mean.

Table 4.5 shows that the increase in spawning levels linearly increased the fraction *a* [y = 3.590 (±1.686)x + 13.90 (±23.13); R^2 = 0.587; P = 0.042] and showed a quadratic response for *EDeg* [y = 3.197 (±1.051)x - 0.046 (±0.018)x^2 + 87.35 (±14.42); R^2 = 0.648; P = 0.048] with the increase in oyster mushroom spawning levels. Neither linear nor quadratic trends (P >0.05) were observed from fractions *b* and *c*, as well as *PDeg* with oyster mushroom spawn levels. Substrate GP50 had higher (P <0.05) fraction *a* (101.32 g/kg DM) than substrate GP0 (23.98 g/kg DM). Whereas, substrate GP0 had similar (P >0.05) fraction *a* as substrate GP20. Substrate GP40 had higher (P <0.05) fraction *b* (277.8 g/kg DM) than substrates GP0, GP20, GP30 and GP50, which did not differ (P >0.05). Substrate GP0 had a higher (P <0.05) fraction *c* (0.980 %/h) than substrates GP20, GP30 and GP50, which did not differ (P >0.05). Substrate GP40 had higher (P <0.05) *PDeg*

produced (364.7 g/kg DM) than substrates GP0, GP20, GP30 and GP50, which did not differ (*P* >0.05). Substrate GP0 (92.38 g/kg DM) had lower (*P* <0.05) *EDeg* (147.5 g/kg DM) than the GP20, GP30, GP40 and GP50 substrates, which were similar (*P* >0.05).

Table 4.5. Kinetics of *in vitro* ruminal dry matter degradability (g/kg DM, unless otherwise stated) of red grape pomace spent oyster mushroom substrate harvested at 4 weeks post-inoculation.

	[1]Substrates						*P* value	
[2]Parameters	GP0	GP20	GP30	GP40	GP50	[3]SEM	Linear	Quadratic
a	23.98[a]	49.89[ab]	90.58[bc]	92.47[bc]	101.32[c]	0.587	0.042	0.214
b	71.19[a]	85.76[a]	87.49[a]	277.8[b]	71.06[a]	0.129	0.422	0.717
c (%/h)	0.980[b]	0.536[b]	0.029[b]	0.004[a]	0.022[b]	0.413	0.093	0.650
PDeg	86.01[a]	135.65[a]	178.1[a]	364.7[b]	172.4[a]	0.514	0.065	0.301
EDeg	92.38[a]	125.58[b]	147.5[b]	133.5[b]	138.8[b]	0.648	0.068	0.048

[a,b,c] In a row, different superscripts denote significant differences (*P* <0.05) between spawning rates.

[1]Substrates: GP0 = red grape pomace with no spawn; GP20 = red grape pomace inoculated with 20% of oyster mushroom spawn; red grape pomace inoculated with 30% of oyster mushroom spawn; GP40 = red grape pomace inoculated with 40% of oyster mushroom spawn; GP50 = red grape pomace inoculated with 50% of oyster mushroom spawn.

[2]Parameters: *a*: the immediately degradable fraction; *b*: the slowly degradable fraction; *c*: degradation rate of fraction *b*; *PDeg* = potential degradability; *EDeg* = effective degradability.

[3]SEM = standard error of the mean.

4.3.3 In vitro ruminal fermentation efficiency

Table 4.6 shows the *in vitro* ruminal organic matter degradability and partitioning factors of the spent oyster mushroom substrate. Both the linear and quadratic trend were observed for *iv*OMD36 [$y = 0.095\ (\pm0.036)x^2 - 6.800\ (\pm2.070)x + 878.54\ (\pm28.388)$; $R^2 = 0.689$; $P = 0.040$] in response to oyster mushroom spawning levels. Significant quadratic trends (*P* <0.05) were observed for the *iv*OMD48 [$y = 0.046\ (\pm0.011)x^2 + 2.800\ (\pm0.621)x + 771.9\ (\pm8.522)$; $R^2 = 0.773$; $P = 0.006$] and

PF48 [$y = 0.046 (\pm 0.011)x^2 + 2.800 (\pm 0.621)x + 771.9 (\pm 8.522)$; $R^2 = 0.773$; $P = 0.006$] in response to incremental levels of oyster mushroom spawn.

Table 4.6. *In vitro* ruminal organic matter degradability (g/kg OM) and partitioning factors (mL/mg OM) of red grape pomace spent oyster mushroom substrate harvested at four weeks post-inoculation.

	[1]Substrates						*P* value	
[2]Parameters	GP0	GP20	GP30	GP40	GP50	[3]SEM	Linear	Quadratic
*iv*OMD12	804.9[a]	766.5[a]	775.0[a]	764.9[a]	773.9[a]	0.149	0.531	0.466
*iv*OMD24	804.5	751.1	761.6	772.3	515.7	0.377	0.194	0.268
*iv*OMD36	859.8[b]	777.4[a]	772.7[a]	793.2[a]	763.4[a]	0.689	0.044	0.040
*iv*OMD48	775.1	773.8	729.4	745.3	727.2	0.773	0.133	0.006
PF12	0.094[a]	0.167[b]	0.163[ab]	0.123[ab]	0.156[ab]	0.261	0.573	0.232
PF24	0.100[a]	0.208[b]	0.189[ab]	0.162[ab]	0.170[ab]	0.383	0.505	0.123
PF36	0.059[a]	0.136[ab]	0.168[b]	0.111[ab]	0.169[b]	0.278	0.450	0.245
PF48	0.161[a]	0.215[b]	0.242[b]	0.221[b]	0.218[b]	0.824	0.452	0.002

[a,b,c] In a row, different superscripts denote significant differences ($P < 0.05$) between spawning rates.
[1]Substrates: GP0 = red grape pomace with no spawn; GP20 = red grape pomace inoculated with 20% of oyster mushroom spawn; red grape pomace inoculated with 30% of oyster mushroom spawn; GP40 = red grape pomace inoculated with 40% of oyster mushroom spawn; GP50 = red grape pomace inoculated with 50% of oyster mushroom spawn.
[2]Parameters: *iv*OMD12 = *in vitro* organic matter degradability at 12 h after inoculation; *iv*OMD24 = *in vitro* organic matter degradability at 24 h after inoculation; *iv*OMD36 = *in vitro* organic matter degradability at 36 h after inoculation; *iv*OMD48 = *in vitro* organic matter degradability at 48 h after inoculation; PF12 = partitioning factors at 12 h post-incubation; PF24 = partitioning factors at 24 h post-incubation; PF36 = partitioning factors at 36 h post-incubation; PF48 = partitioning factors at 48 h post-incubation.
[3]SEM = standard error of the mean.

There were no significant ($P > 0.05$) differences on *iv*OMD24 and *iv*OMD48 among the substrates. There were no significant differences amongst substrates GP0, GP20, GP30, GP40 and GP50 in terms of the *iv*OMD12 post-incubation. Substrate GP0 had higher ($P < 0.05$) *iv*OMD36 (859.8 g/kg OM) than substrates GP20, GP30, GP40 and GP50, which did not differ ($P > 0.05$). Substrate GP20

had higher (P <0.05) PF12 (0.167 g/kg OM) and PF24 (0.208 g/kg OM) than the control substrate GP0. However, substrate GP0 had a similar (P >0.05) PF12 and PF24 as substrates GP30, GP40 and GP50. Substrate GP30 and GP50 had higher (P <0.05) PF36 than the control substrate GP0, which was similar to substrates GP20 and GP40. The control substrate GP0 (0.161 g/kg DM) had lower PF48 as the oyster mushroom spawn inoculated substrates, which did not differ (P >0.05).

4.4 Discussion

4.4.1 Cumulative gas production

The increase in incubation time and spawning rate of the oyster mushroom spawn linearly increased, decreased and showed a quadratic response to the cumulative gas produced at 12, 24, 36 and 48 h in all the weeks post-incubation. This could be caused by the fact that there was an increase in NDF, ADF and ADL of the treated substrates than the untreated in the present study, resulting in poor microbial fermentation and energy loss in the rumen. Akinfemi (2010) reported a higher cumulative gas volume at 24 and 48 h post-incubation in the *P. ostreatus* and *P. pulmonarius* treated maize cob compared to the untreated maize cob. Furthermore, Rahman *et al*. (2011), also reported higher cumulative gas production for chopped oil palm fronds treated with *C. subvermispora* for three weeks. Tuyen *et al*. (2012) suggested that the increased cumulative gas production of the treated substrates could be caused by the fungi excreting some compounds that have inhibitory effects on the fermentation process of the rumen microorganisms. Substrate GP50 had the highest cumulative gas produced (18.54 and 23.20, respectively) at 12 and 24 h in week 1 than the untreated substrates and the other substrates inoculated with the spawn. Menke & Steingass (1988) suggested that there is a relationship between the gas volumes at 24 h post-incubation with metabolisable energy in feedstuffs. There was an increase in cumulative gas production at 12, 24, 36, and mostly at 48 h post-incubation when 20-50% of the spawn was inoculated to the substrates compared to the untreated. This could be caused by the high fibre

constituents (NDF, ADF and ADL) in the inoculated substrates, resulting in poor fermentation by the microorganisms and energy loss because the microorganisms cannot access the complex carbohydrates. Okano *et al*. (2006) reported a similar gas production trend that showed a large increase on *in vitro* ruminal gas production at 48 h of incubation when sugar bagasse was treated with *C. subvermispora* and *L. edodes* for 8 - 16 weeks.

4.4.2 Rate of gas production

The increase in spawning rate and incubation time linearly increased the rate of gas produced at 24, 36 and 48 h in week 2 and 3 post-incubation. A quadratic response was also observed in all the weeks for the rate of gas produced at 12, 36 and 48 mostly. This could be due to the fact that the treated substrates in the current study had a lower NDF and ADF content than the untreated in week 3 and a lower NDF, ADF and ADL than the untreated in week 4, thus resulting in a higher rate of gas production because of the improved availability of fermentable nutrients for the rumen microbes. Consequently, Akinfemi (2010) reported that the high rates of gas production in the *P. ostreatus* treated maize cob and *P. pulmonarius* treated maize cob could be associated with the low content of CF, NDF, ADF and ADL and high CP content. The treated substrates (GP20 to GP50) had no effect on the rate of gas production in week 1, except for the rate of gas produced at the 12 and 48 h whereby the untreated substrates had a higher rate of gas production than the treated substrates. This suggests that the inoculated substrates (GP20 to GP50) had higher fibre constituents which were not accessible for fermentation by the rumen microbes. This, in that case, resulted in prolonged rates of gas production in treated substrates than the untreated resulting in poor fermentation. Tuyen *et al*. (2013) stated that this could be caused by the poor delignification of substrates by the *P. ostreatus* and *P. eryngii* fungi. However, the treated substrates had higher rates of gas production than the untreated substrates with the increased incubation time (12, 24, 36 and 48 h respectively) and spawning level (20 - 40%) in week 2, 3 and 4. This was expected in the

present study because the oyster mushroom spawn is reported to influence the carbohydrate fractions by certain authors (Valizadeh & Sobhanirad, 2009; Nasehi *et al*., 2017), making the feed to be more accessible for rumen microbial fermentation.

4.4.3 In vitro ruminal gas production kinetics

The *in vitro* ruminal gas production kinetics is primarily dependent on the relative proportions of soluble, insoluble but degradable, and non-degradable components of the feed, and the gas production curve consists of two to three phases (Zicarelli *et al*., 2011). There increase in spawning level and incubation duration linearly decreased the fraction (*a*) in week 1. This implies that the presence of the high fibre content on the treated substrates than the untreated had a negative correlation towards the fraction *a*. Both linear and quadratic responses were observed for fermentation of fraction *b* in response to the increasing spawning rates and an incubation time of the oyster mushroom in week 2, 3 and 4 showed a positive trend for fraction *b*. This suggests that that the fibre fraction was improved by the spawn especially in week 3 and 4 in the present study. Akinfemi (2010) also reported a higher fraction *b* for the fungal treated substrates than the untreated, which might be associated with the improved fibre constituents making it possible for the microbial population to attack the readily available carbohydrates for the treated substrates. Cone *et al*. (1996) reported that in the first phase, the soluble components have the highest contribution towards the gas production and the insoluble but degradable components mainly contribute to the gas production of the second phase, and there is no clear separate sequence of degradation of these components in the rumen.

Substrate GP40 in week 1 and substrates GP20, GP30 and GP40 in week 3 had higher fraction *b* than the untreated substrate. This is in line with the study by Chumpawadee *et al*. (2005) and Akinfeni (2010), whereby the fungal treated maize husk had a higher fraction *b* compared to

untreated. This might be influenced by the carbohydrate fraction readily available to the microbial population. In the present study a higher *Pgas* was observed for substrate GP40 in week 1, substrate GP30 in week 2 and substrate GP40 in week 3 than the untreated. These findings were in line with those of Rahman *et al*. (2011) whereby the treated maize husk had a higher *Pgas* than the untreated substrates at 3 and 9 weeks respectively. In the increase in spawning rate and incubation did not influence the fermentation rate of fraction *b* (*c*) compared to the untreated because they had the same values in week 1, 2 and 3. However, in week 4, treated substrates GP20, GP30, GP40 and GP50 had a higher fermentation rate of fraction *b* (c) than the untreated with GP30 (0.029 mL/g OM) having the highest fraction *b* (*c*). Akinfemi *et al*. (2009) reported that this could be due to the fact that that the *P. tuber-regium* treated substrates carbohydrate fraction were readily available and highly accessible to the rumen microbes than the untreated maize husk substrates, thus resulting in a higher rate of fermentation than the untreated.

4.4.4 In vitro ruminal dry matter parameters

In recent years, the use of several fibre treatments (physical and chemical) has been proposed to improve the degradability of certain agricultural by-products and subsequent feed intake by ruminants. However, safety concerns, cost, and the negative impact they might have on the environment limit their application (Raghuwanshi *et al*., 2014). This allowed more attention to be given to the use of white-rot fungi that have been reported to improve the nutritive value and *in vitro* dry matter degradability of certain agricultural by-products (Fazaeli *et al*., 2004; Samsudin *et al*., 2013). The increase in the spawning rate had a linear effect on the immediately degradable fraction (*a*), a quadratic response was observed for the DMD48, *EDeg* and the *iv*OMD36. This was due to the reduction of the fibre fraction (NDF, ADF and ADL) on the treated GP substrates relative to the untreated substrate by the oyster mushroom spawn in week 4 (Chapter 3), which implies that the rumen microbes had easy access to the treated substrates for increased

degradability. This is supported by a study by Khattab *et al*. (2013), where fungal treatment of rice straws reduced the fibre content thereby increasing nutrient bioavailability for rumen microbes. This in turn increased and sustained the longevity of the ruminal microflora and improved rumen DMD.

The treated GP substrates had a higher DMD at 24, 36 and 48 h post-incubation than the untreated substrates in response to increasing spawning rates. This was due to the fact the oyster mushroom spawn degraded the fibre constituents (NDF, ADF and ADL) of the GP treated substrates relative to the untreated substrates in week 4 (Chapter 3). This confirms that the oyster mushroom treated substrates were more accessible for the degradation of nutrients by the rumen microbes. These results were in line with the findings of Karunanandaa *et al*. (1995), who reported that the *P*. *sajor-caju* and *C*. *stercoreus* improved the *in vitro* DMD of rice straw leaves and stems after 30 days incubation period, with the *C*. *stercoreus* treatment having the highest *in vitro* DMD than the other fungi. Akinfemi & Ogunwole (2012) stated that the improved digestibility of treated straw than the control was a result of the significant increase in crude protein, the reduction of crude fibre and the fibre components (NDF, ADF and ADL) when rice straw was treated with *P*. *ostreatus, P*. *pulmonarius* and *P*. *tuber-regium* spawns and acid detergent lignin.

Higher degradable fractions for fraction *a* and *b* were observed on the treated GP substrates than the untreated substrates, with GP50 (101.32 g/kg DM) and GP40 (277.8 g/kg DM) being the highest amongst all the spawning levels, respectively. Furthermore, higher *PDeg* and *EDeg* on the treated substrates than the control was recorded in response to increasing spawning rates. These could be attributed to the lower NDF, ADF and ADL contents of the treated substrates relative to the control in week 4 (Chapter 3). The current results are supported by Valmaseda *et al*. (1991) and Gutierrez *et al*. (1996) who both noted a decreased cell wall contents and increased soluble fractions of carbohydrates when the wheat straws were fermented with the *Pleurotus* fungi, which could have been due to enzymatic degradation. Addition, Feazeli *et al*. (2004) reported higher

fraction *a*, *b* and *c*, *PDeg* and *EDeg* for the fungal treated straws, which suggested the *P. florida* (P-41) treated straws were highly degraded compared to the *P. eringi* (P-21), *P. eringi* × *P. local* (P-30), *Pleurotus* spp. (P-60) and the control substrate.

4.4.5 In vitro ruminal fermentation efficiency

The increasing spawn rates had a linear effect on the *iv*OMD at 36 h post-incubation and a quadratic response on *iv*OMD at 36 and 48 hours, as well as PF48. These results suggest that the oyster mushroom spawn was able to degrade the structural carbohydrates for higher and faster ruminal fermentation efficiency, as observed by Colombatto *et al*. (2003). In terms of *iv*OMD post-incubation, *iv*OMD decreased with increasing spawning rates. This might be due to the lack of effects on the treated GP substrates by the oyster mushroom, which might have extensively degraded the material which could have been degraded in the rumen by the microbes. According to a study by Baba *et al*. (2002), a higher PF value translates to higher fermentation efficiency in the rumen. Thus, the increase in PF values at 36 and 48 h post-incubation of the treated substrates suggest that there was an increase in the utilisation of the substrates by rumen microbes for efficient fermentation.

4.5 Conclusion

Increasing spawning rates improved the *in vitro* ruminal fermentation of red grape pomace. It was, therefore, concluded that oyster mushroom spawn improves nutritive value and fermentation efficiency of red grape pomace for ruminants, with the ideal treatment duration being 28 days.

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5 CHAPTER FIVE – GENERAL DISCUSSION, CONCLUSIONS AND FUTURE RESEARCH

5.1 General discussion and conclusions

The utilization of GP as a ruminant feedstuff is limited by the presence of structural carbohydrates and low molecular polyphenolic acids. Kalli *et al*. (2018) reported that in a short period of grape harvesting, a large amount of GP is produced, which increases the concentration of this waste by-product per disposal area. This, in turn, becomes detrimental to the environment. The disposal of GP thus leads to serious pollution of surface and groundwater and foul odours, which attract diseases vectors such as flies and pests. Chand *et al*. (2009) and Bekhit *et al*. (2016) stated that the presence of tannins and other compounds in GP has the potential to increase oxygen depletion and nitrogen leaching when the GP is used as a fertilizer or compost. In addition, Baumgartel *et al*. (2007) stated that phytopathological issues may arise from the discarding of GP back into the vineyards as a fertilizer or compost. Therefore, it is increasingly necessary to find environmentally friendly alternative uses of GP such as livestock feeding. This will help reduce the negative impact that both the disposal of GP and the feeding of ruminants have on the environment. In addition, Salami *et al*. (2019) stated that the utilisation of plant-based by-products can be of sustainable value because they tend to be of low cost and they help alleviate the environmental impact of the ruminant industry, as they reduce enteric greenhouse gas emissions. However, GP has high fibre content, which compromises its feed value for ruminants.

This study was, therefore, designed to investigate an innovative strategy of using the fibrolytic capacity of oyster mushrooms to improve the nutritive value of red GP for ruminants. Graded levels (0, 200, 300, 400, and 500 g/kg) of the oyster mushroom spawn were used to inoculate 500 g of red GP and sampled at 7, 14, 21 and 28 days. The proximate components, minerals, *in vitro* ruminal gas production, *in vitro* ruminal dry matter degradability and fermentation efficiency of

the raw and oyster mushroom-treated GP substrates, were analysed. The increase in spawning rates and the incubation duration influenced the neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL) and the crude protein (CP) content. In week 3 and 4, the treated red GP substrates had a lower NDF, ADF and ADL contents relative to the control. In addition, improved CP content was observed on the GP treated substrates than the control. In terms of the mineral content, there was not much variation between untreated and treated GP substrates with the increase in spawning rates. The macro-mineral content was within the required range for ruminant feeding (NRC, 2005).

Higher cumulative gas production was observed for the treated GP substrates compared to the control. This could be attributed to the high fibre fraction (NDF, ADF and ADL) of the treated GP substrates relative to then untreated substrates in week 1 and 2. However, the increase in spawning rates and incubation duration resulted in higher rates of gas production and improved *in vitro* ruminal gas production kinetics. This could be attributed to the improved NDF, ADF and ADL content of the treated GP substrates in week 3 and 4 (Chapter 3), which allowed for the efficient fermentation by the rumen microbes. The increasing spawning rates improved the dry matter degradability (DMD) at 24 and 48 for all the spawning levels relative to the control. Furthermore, the substrate GP50 had a higher immediately degradable fraction (*a*) than the control and treated. Substrate GP40 had a higher slowly degradable fraction (*b*) and the potential degradability (*PDeg*) compared to the control. This was because the fibre content of the treated GP substrates was degraded by the oyster mushroom in week 4 (Chapter 3), which resulted in the efficient fermentation by the rumen microbes. It was also found that the utilization of oyster mushroom improved the partitioning factors (PF) of the treated GP substrates compared to control substrate week 4, this revealed the red GP fermentation efficiency was increased by the use of the oyster mushroom spawn. The current results can be supported by Baba *et al*. (2002) whereby it was

reported that the increased PF values postulate to a higher fermentation efficiency by the microbes in the rumen.

It was, therefore, concluded that the use of oyster mushroom spawn is beneficial towards the reduction of red GP fibre fractions. An increase in CP content was also achieved, although it was a secondary benefit. Furthermore, the treating of red GP with incremental levels of the spawn had a positive effect on the *in vitro* ruminal gas production kinetics, the *in vitro* dry matter degradability parameters, and the partitioning factors when it was incubated for sufficient amount of time (28 days ideally). However, it was not possible to deduce the optimum spawn to improve the chemical composition and *in vitro* ruminal fermentation of red grape pomace spent oyster mushroom substrate.

5.2 Recommendations and future research

The current study served as the base for future research into the use of white-rot fungi (*Pleurotus ostreatus*) spawning rates to improve the nutritive value of red grape pomace. It is thus recommended that additional research is conducted in the future to evaluate the *in vivo* utilization of spent GP substrates-containing diets in ruminants to improve the accuracy of the current findings. Future studies can also be designed to explore the growth of oyster mushrooms using different substrates, which can be included in ruminant diets as spent mushroom substrates or as a combination of the SMS and the mushrooms to improve ruminant performance and ensure sustainable intensification.

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6 LIST OF APPENDICES

6.1 Appendix 1. Ethics Certificate of the Study

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Research Ethics Regulatory Committee
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ETHICS APPROVAL LETTER OF STUDY

Based on approval by the **North-West University Animal Production Sciences Research Ethics Committee (NWU-AnimProdREC)** on 01/11/2019, the NWU Animal Production Sciences Research Ethics Committee hereby **approves** your study as indicated below. This implies that the North-West University Senate Committee for Research Ethics (NWU-SCRE) grants its permission that, provided the special conditions specified below are met and pending any other authorisation that may be necessary, the study may be initiated, using the ethics number below.

Study title: Chemical composition and in vitro ruminal fermentation of red grape pomace spent mushroom substrates.

Study Leader/Supervisor (Principal Investigator)/Researcher: Dr K Mnisi, Prof V Mlambo

Student: Mhlongo G

Ethics number:

N	W	U	-	0	1	8	8	7	-	1	9	-	S	5
Institution				Study Number						Year			Status	

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

Application Type: Single Study
Commencement date: 2019/11/06 **Risk Category:** 2
Expiry date: 2020/10/05
Approval of the study is initially provided for a year, after which continuation of the study is dependent on receipt and review of the annual (or as otherwise stipulated) monitoring report and the concomitant issuing of a letter of continuation.

Special in process conditions of the research for approval (if applicable):

- Any research at governmental or private institutions, permission must still be obtained from relevant authorities and provided to the NWU-AnimProdREC. Ethics approval is required BEFORE approval can be obtained from these authorities.

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, the following general terms and conditions will apply:

- *The study leader/supervisor (principle investigator)/researcher must report in the prescribed format to the NWU-AnimProdREC:*
  - *annually (or as otherwise requested) on the monitoring of the study, whereby a letter of continuation will be provided, and upon completion of the study; and*
  - *without any delay in case of any adverse event or incident (or any matter that interrupts sound ethical principles) during the course of the study.*
- *The approval applies strictly to the proposal as stipulated in the application form. Should any amendments to the proposal be deemed necessary during the course of the study, the study leader/researcher must apply for approval of these amendments at the NWU-AnimProdREC, prior to implementation. Should there be any deviations from the study proposal without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited.*
- *Annually a number of studies may be randomly selected for an external audit.*
- *The date of approval indicates the first date that the study may be started.*
- *In the interest of ethical responsibility, the NWU-SCRE and NWU-AnimProdREC reserves the right to:*

- *request access to any information or data at any time during the course or after completion of the study;*
- *to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process;*
- *withdraw or postpone approval if:*
  - *any unethical principles or practices of the study are revealed or suspected;*
  - *it becomes apparent that any relevant information was withheld from the NWU-AnimProdREC or that information has been false or misrepresented;*
  - *submission of the annual (or otherwise stipulated) monitoring report, the required amendments, or reporting of adverse events or incidents was not done in a timely manner and accurately; and / or*
  - *new institutional rules, national legislation or international conventions deem it necessary.*

- *NWU-AnimProdREC can be contacted for further information or any report templates via upenyu.marume@nwu.ac.za or 018 389 2725.*

The NWU-AnimProdREC would like to remain at your service as scientist and researcher, and wishes you well with your study. Please do not hesitate to contact the NWU-AnimProdREC or the NWU-SCRE for any further enquiries or requests for assistance.

Yours sincerely

Prof Upenyu Marume
Chairperson NWU Animal Production Sciences Research Ethics Committee

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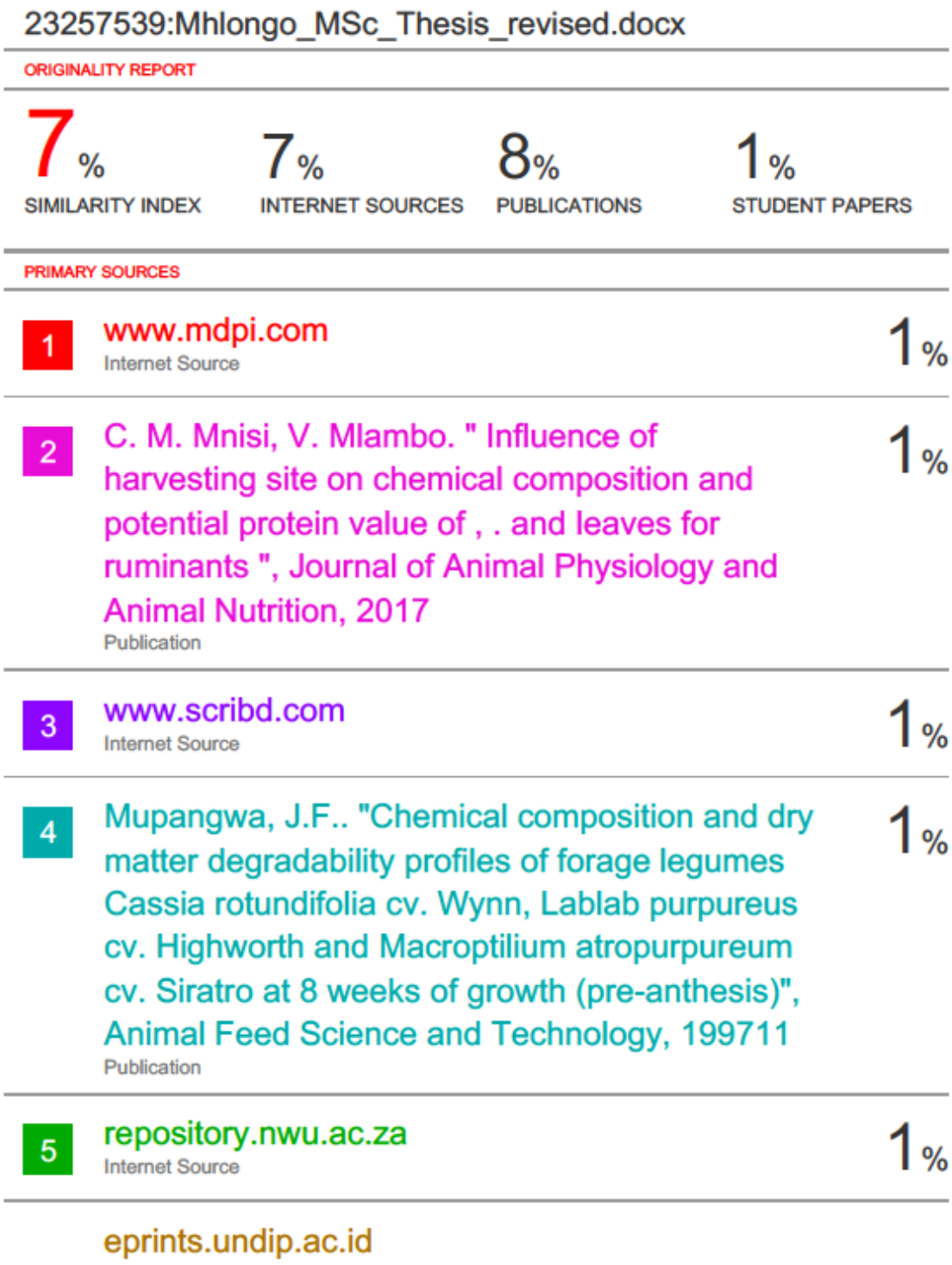
6.2 Appendix 2. Turnitin Report



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6.3 Appendix 3. Language Editing Certificate

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Date: 26th October, 2020

To Whom It May Concern,

REF: Language Editing and Proof-reading of Dissertations/Theses

Dear Sir or Madam,

This serves to confirm that I have proof-read and edited the MSc dissertation of G. **Mhlongo** (26735970; **orcid.org/0000-0002-9806-914X**) entitled: **Chemical composition and *in vitro* ruminal fermentation of red grape pomace spent mushroom substrates.** The candidate then later corrected all the identified language and technical errors to my and the supervisor's utmost satisfaction. Thus the document presented here is of sufficient and acceptable academic standards.

Editor

Prof. O Ruzvidzo

Supervisor

Dr C.M. Mnisi

6.4 Appendix 4. Recipe for the *in vitro* ruminal gas production buffer solution

Reagents

Solution A Buffer:	g/litre
KH_2PO_4	: 10.0
$MgSO_4.7H_2O$	: 0.5
NaCl	: 0.5
$CaCl_2.2H_2O$	: 0.1
Solution B Buffer:	
Na_2CO_3	: 15.0
$Na_2S.9H_2O$	: 1.0

Buffer A and Buffer B were combined using a ratio of 1:5 to adjust pH to obtain a final pH of 6.8.