Facultad de Veterinaria
Universidad Zaragoza

CIHEAM
IAM ZARAGOZA

Trabajo de Fin de Máster

Effects of using rumen fermentation for the production of microbial protein from agricultural by-products as an alternative to soybean meal in animal nutrition

Autor

Jihed Ben Mabrouk

Director

Gerardo Caja López

Facultad de Veterinaria

2017

Agradecimientos

Quiero dar mis sinceros agradecimientos en primer lugar a mi Director de Tesis, Dr. Gerardo Caja López, por permitirme trabajar bajo su supervisión y haberme confiado este trabajo. Quedo agradecido por haber tenido la oportunidad de beneficiarme de sus conocimientos, orientaciones y su manera de trabajar; y a la Dra. Elena Albanell su ayuda, sus esfuerzos y su visión crítica para que este proyecto sea terminado con éxito.

También, un especial agradecimiento a la Dra. Teresa Gea y al Grup de Compostatge de Residus Sòlids Orgànics del Departament d'Enginyeria Química por la colaboración.

Quisiera agradecer a Dra. María Rodríguez por su inestimable ayuda en la realización de la parte experimental.

Al Instituto Agronómico Mediterráneo de Zaragoza le doy las gracias por permitirme adquirir nuevos conocimientos, en especial al Dr. Armando Occon por la disposición en ayudar en cualquier duda o inconveniente.

Me gustaría hacer una mención especial de agradecimiento a los que han sido el pilar fundamental para la realización con éxito de mi trabajo de tesis, a Monia mi madre, a Lotfi mi padre y a mis hermanas. Gracias por no dejarme en los tiempos difíciles.

Finalmente, y no menos importante, quiero dar las gracias a mis amigos Alaa, Ali, Wellington, Sandra, Nabil y Paola por el apoyo técnico y moral recibido.

Abstract

Use of agro-industrial by-products for the production of microbial protein as an alternative to soybean meal

The aim of this thesis was to evaluate the use of rumen fermentation as a process to increase the protein content of agricultural byproducts, in order to reduce the dependency of the feed industry on soybean meal cake. Three in vitro fermentation experiments (Exp.) were carried out, to test different substrates and ruminal fluid-artificial saliva mixtures, in which gas production (microbial activity) and single cell protein production (protein synthesis) were measured. In Exp 1, gas production and pH were measured at 4 time-points (3 to 24 h) in 3 substrates (barley straw, barley meal, and maize meal) and 8 media concentrations (0.5 to 40 g/100 mL), with or without the addition of urea. Gas production was the greatest in the cereal meals ($P < 0.001$) and the addition of urea according to energy potential, reduced the drop of pH in the case of maize meal ($P < 0.05$), but not in the rest of substrates. Concentrations greater than 10 g/100 mL were considered inadequate for fermentation. In Exp. 2, maize grain, barley straw, corn stover and wood pellets at 7 g/100 mL and with small addition of urea (0.25 g/L) were incubated for 144 h (7 time-points) and digestible protein synthesis measured at 24 and 48 h after HCl-pepsin digestion. The increases of the digestible protein were 82, 225 and 100% for maize meal, barley straw and maize stover at 48 h, respectively. In Exp. 3, the same substrates as Exp. 2 with high urea (2.5 g/L) were used and maize silage included. Incubation lasted for 72 h (5 time-points) and digestible protein synthesis measured at 24 and 72 h. The analysis showed an increase in the digestibility at 72 h compared with 24 h of incubation ($P < 0.05$) for barley straw, maize stover wood pellets and maize silage. Digestible protein increases were 147, 500 and 188% for maize meal, barley straw and maize stover at 72 h, respectively. Evaluation of costs indicated that the process was only cost-effective for prices in the range of 0.03 to 0.06 €/kg for barley straw and 0.3 to 0.5 €/kg for soybean meal. In conclusion, the production of single cell protein from barley straw may be an interesting alternative to imported soybean meal when prices of barley straw are lower than 0.06 €/kg, and soybean meal greater than 0.5 €/kg.

Key words: Rumen fermentation, soybean meal, single cell protein, gas production.

Resumen

Uso de subproductos agroindustriales para la producción de proteína microbiana como alternativa a la harina de soja: El objetivo de esta tesis fue evaluar el uso de la fermentación ruminal como un proceso para aumentar el contenido de proteína de los subproductos agrícolas, con el fin de reducir la dependencia de la torta de harina de soja en la industria de piensos. Para ello se llevaron a cabo 3 experimentos de fermentación *in vitro* (Exp.) Se analizaron diferentes sustratos y mezclas de saliva artificial y líquido ruminal, y se midió la producción de gas (actividad microbiana) y la producción de proteína unicelular (síntesis de proteína). En el Exp. 1, se midió la producción de gas y el pH para 4 tiempos (de 3 a 24 h), 3 sustratos (paja de cebada, harina de cebada y harina de maíz) y 8 concentraciones (0.5-40 g/100 mL), con o sin adición de urea. La producción de gas fue superior en las harinas de cereales ($P < 0,001$), y la adición de urea, dosificada según el potencial energético, redujo la caída del pH en el caso de la harina de maíz ($P < 0,05$), pero no en el resto de los sustratos. Concentraciones superiores a 10 g/100 mL se consideraron inadecuadas para la fermentación. En el Exp. 2, se fermentaron 4 substratos (maíz grano, paja de cebada, cañote de maíz y pellets de madera) a 7 g/100 mL y baja urea (0,25 g/L) durante 144 h (7 medidas), y se evaluó la síntesis de proteína digestible a las 24 y 48 h después de una digestión con HCl-pepsina. Los aumentos de la proteína digestible a las 48 h fueron 82, 225 y 100%, para la harina de maíz, paja de cebada y cañote de maíz, respectivamente. En el Exp. 3, los mismos sustratos que Exp. 2 se fermentaron a mayor dosis de urea (2.5 g / L) y se incluyó ensilado de maíz. La incubación duró 72 h (5 tiempo) y la síntesis de proteína digestible se midió a las 24 y 72 h. El análisis mostró un aumento de la proteína digestible entre las 24 a las 72 h de incubación ($P < 0.05$), siendo el aumento a las 72 h del 147, 500 y 188 %, para la harina de maíz, paja de cebada y cañote de maíz, respectivamente. La evaluación de costes indicó que el proceso solo fue rentable para un precio entre 0,03-0,06 €/kg para la paja de cebada y 0,3-0,5 €/kg para la harina de soja. En conclusión, la producción de proteína unicelular a partir de paja de cebada puede ser una alternativa interesante a la harina de soja importada cuando los precios de la paja de cebada sean inferiores a 0,06 € / kg y los de la harina de soja superiores a 0,5 €/kg.

Palabras clave: Fermentación ruminal, harina de soja, proteína unicelular, producción de gas.

Résumé

Utilisation de sous-produits agro-industriels pour la production de protéines microbiennes comme alternative au tourteau de soja: Le but de cette thèse était d'évaluer l'utilisation de la fermentation ruminale comme un processus visant à augmenter la teneur en protéines des sous-produits agricoles, afin de réduire la dépendance de l'industrie alimentaire à l'égard du tourteau de soja. Trois expériences (Exp.) de fermentation *in vitro* ont été réalisées pour tester différents substrats et mélanges de liquides ruminales et de salive artificielle dans lesquels la production de gaz (activité microbienne) et la production de protéines comme les protéines d'origine unicellulaire (synthèse de protéines) ont été mesurées. Dans l'Exp. 1, la production de gaz et le pH ont été mesurés en 4 points (3 à 24h) sur 3 substrats (paille d'orge, farine d'orge et farine de maïs) et 8 milieux (0,5 à 40 g / 100 mL), avec ou sans l'ajout d'urée. La production de gaz était la plus importante dans les farines de céréales ($P < 0,001$). L'ajout d'urée selon le potentiel énergétique, réduisait la chute de pH dans le cas du maïs ($P < 0,05$), mais pas dans le reste des substrats. Des concentrations supérieures à 10 g/100 mL ont été considérées inadéquates pour la fermentation. Dans l'Exp. 2, le grain de maïs, la paille d'orge, la canne de maïs et les granulés de bois à 7 g / 100 mL et avec une addition d'une petite quantité d'urée (0,25 g / L) ont été incubés pendant 144 h (7 temps). La protéine digestible synthétisée était mesurée à 24h et 48 h après la digestion par le HCl-pepsine. Les augmentations de la protéine digestible étaient respectivement de 82, 225 et 100% pour la farine de maïs, la paille d'orge et la cannes de maïs à 48 h. En Exp. 3, les mêmes substrats que Exp. 2 avec une dose d'urée plus élevée (2,5 g / L) ont été utilisés et de l'ensilage de maïs était inclus. L'incubation a duré 72 h (5 temps) et la synthèse des protéines digestibles a été mesurée à 24 et 72 h. L'analyse a montré une augmentation de la digestibilité à 72 h par rapport à 24 h d'incubation ($P < 0,05$) pour la paille d'orge, les granulés de bois, la farine de maïs et l'ensilage de maïs. Les augmentations des protéines digestibles ont été respectivement de 147, 500 et 188% pour la farine de maïs, la paille d'orge et la canne de maïs à 72 h. L'évaluation des coûts a montré que le procédé n'était rentable que pour des prix compris entre 0,03 et 0,06 € / kg pour la paille d'orge et entre 0,3 et 0,5 € / kg pour le tourteau de soja. En conclusion, la production de protéines unicellulaires à partir de paille d'orge peut constituer une alternative intéressante au tourteau de soja importé lorsque les prix de la paille d'orge sont inférieurs à 0,06 € / kg et que le tourteau de soja dépasse 0,5 € / kg.

Mots-clés: Fermentation ruminale, tourteau de soja, protéines d'origine unicellulaire, production de gaz.

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List of abbreviations

AD	Anaerobic digestion
ADF	Acid detergent fiber
ADL	Acid detergent lignin
CF	Crude fiber
CP	Crude protein (N × 6.25)
DM	Dry matter
EE	Ether extract
GHG	Greenhouse gases
INRA	Institut National de la Recherche Agronomique – French National Institute for Agricultural Research
MBtu	1,000,000 British thermal unit
MP	Microbial protein
NDF	Neutral detergent fiber
Nm^3	Normal cubic meter
OM	Organic matter
PDIE	Protein digested in the small intestine supplied by microbial protein from rumen-fermented OM
PDIN	Protein digested in the small intestine supplied by microbial protein from ruminal degradable protein
VFA	Volatile fatty acids

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1. INTRODUCTION

1. INTRODUCTION

European, Spanish and Catalonian livestock sectors depend markedly on imported soybeans, as a main protein source for animal feeds. This dependency can affect negatively the sustainability of the system of production and makes it very sensitive to changes in the international market prices.

In 2014, total soybean production in Spain was 2,650 t from which only 24 t were produced in Catalonia (Avance Anuario de Estadística, 2016), while in 2011 the Catalonian feed industry used approximately 1.7 Mt of oilseeds (18%) and their respective protein cake meals (82%) (DARP, 2012). From the total oilseeds used, soybean ranks unquestionably first (87%), followed by sunflower (6%) and rapeseed (5%) seeds. Concerning the protein meal used, 68% corresponds to soybean meal cake, followed by the rapeseed meal cake (17%).

On the other hand, agri-food sector in Catalonia produces an important quantity of wastes and by-products, which are in some cases a major environmental problem. Moreover, the treatment of these wastes constitutes a significant cost, because of its volume and its physicochemical characteristics, that is translated to the final users.

The present Master Thesis was done in the framework of the “Altersoy project” which is a research sponsored by the ACVC (Acadèmia de Ciències Veterinàries de Catalunya), within the program of collaboration of the Academies of Catalonia and the Department of Justice of the Generalitat of Catalonia, and was funded by the Banking Foundation "la Caixa". The main objective of the “Altersoy project” is the obtainment of protein supplements from microbial origin destined to the substitution of the soybean protein in livestock diets by means of the fermentation of agricultural and agro-industrial products currently available in Catalonia.

The protein value of feeds and diets for ruminant species is relatively independent from their amino acid profile, depending more on the degradability of the protein used and the contribution of fermentable carbohydrates as the main source of energy for rumen microorganisms. This feature gives to the ruminant digestive system the ability to ferment low quality feeds and to improve their final nutritive value by incorporing microbial protein.

The Altersoy project intends to reproduce this process under *in vitro* conditions, by simulating an industrial process at small scale under laboratory conditions, using different feedstuffs and agricultural by-products from the Catalonian food industry providing both economic and environmental advantages.

Therefore, the main aim of the present study was to investigate the features and possibilities of the bioconversion of agricultural products (used as a substrate) to single cell protein and biogas by using rumen microbiota (as inoculum).

In later stages of the project, not included in this Thesis, the resulting products will be tested as animal feed.

2. LITERATURE REVIEW

2. LITERATURE REVIEW

2.1. History of soybean and current situation of the crop

Soybean (*Glycine max* L.) is a leguminous (family *Fabaceae*) native from Asia (China, Mongolia, Japan, and India) and cultivated for its seed (legume) which has high oil and protein contents. It was domesticated in northern China about 3,000 yr ago, and it is currently cultivated on almost all continents between 53ºN and 53ºS, from the sea level to an altitude of 2,000 m. The optimum soybean growth conditions are template climate (diurnal maximum temperatures of about 30°C), humid (850 mm of annual precipitation, and not less than 500 mm during the growing season), and well drained soils with pH between 5.5 and 7.5 (Heuzé and Tran, 2016).

Europeans begin to better know and give more interest to soybeans after the World Exhibition of Vienna in 1873. The Austro-Hungarian professor Friedrich Haberlandt obtained twenty varieties of soybeans and planted them in 1875 in the botanical garden of the University of Vienna. Since then, various trials have been made to grow soybeans in Europe, but most of them did not have success (Shurtleff and Aoyagi, 2009). Until the third decade of the 20th century, soybean production was limited to the Orient and China, Japan, Indonesia and Korea were the main producers. But starting from the early 1950's, the United States left China and the whole Orient behind and became the first soybean producer (Hymowitz, 1970). At present the US and Brazil contribute over 80% global soybean exports (USDA, 2016)

The China's demand for soybean is currently soaring (**Figure 1**). In 1990, soybean consumption of the US quadruplicated that of China. However, in 2008 the situation changed dramatically. By 2011, China was consuming 70 Mt of soybeans a year, compared to the 50 Mt of the US. In 2015, China soybean imports were around 80 Mt, which is higher than the total soybean imports of the rest of the world. Chinese imports will achieve 112 Mt by 2023 as estimated by the US Division of Agriculture (USDA, 2016).

Soybeans have an important role in human diet, granting protein supply in various forms, sauce, soy milk-like, tofu cheese, oil, flour and bread. Soya complements ideally rice, which is deficient in protein (Shurtleff and Aoyagi, 2009). Soybeans have an important role in human diet, granting protein supply in various forms, sauce, soy milk-like, tofu cheese, oil, flour and bread. Soya complements ideally rice, which is deficient in protein (Shurtleff and Aoyagi, 2009).

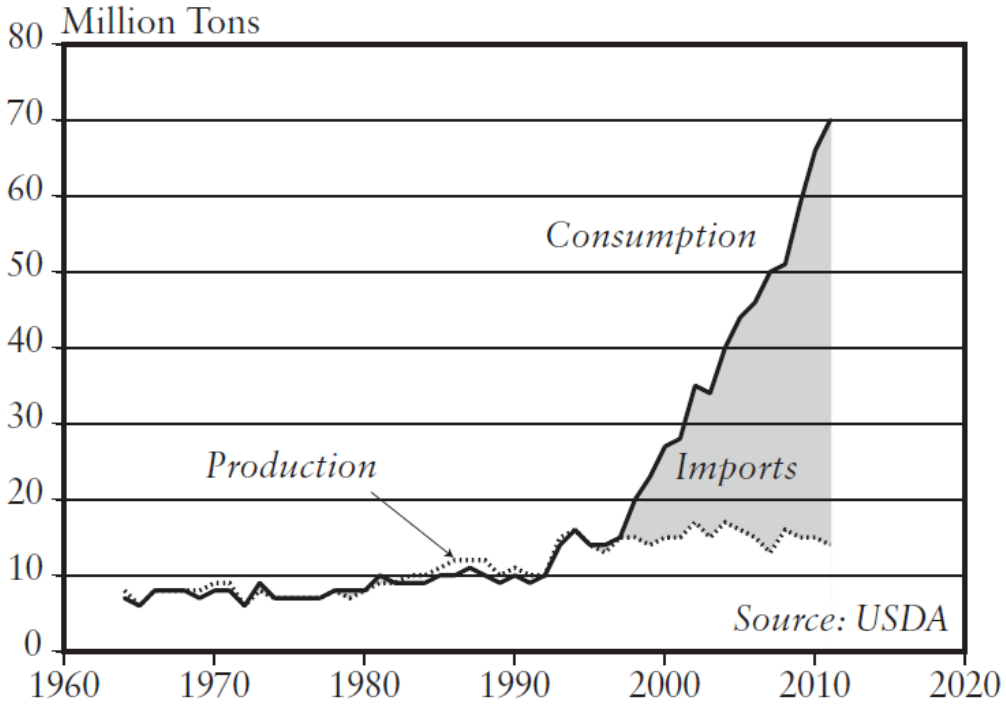


Figure 1. Soybean market in China during 1964–2011 (USDA, 2016).

The high digestibility and biological value of the amino acids of soybean meal in animals, and especially its high lysine content (29.3-35.1 g/kgDM; García-Rebollar et al., 2016) allows the formulation of diets containing less total protein than with other feedstuff sources, which reduces the protein supply in the diet and N excretion to the biosphere (Pettigrew et al., 2008).

2.1.1. Disadvantages of using soybean

As a direct consequence of the increasing incomes in developing countries with large population, China and India for instance, the demand for meat and other high-value food products is rapidly increasing, which means a higher demand for grains and soybean meal for the feed industry, as shown in **Figure 2**.

The main effect of the climbing demand of soybean worldwide is a result of the changes in the agriculture structure in the Americas. In the US, there is currently more land devoted to soybean than to wheat. In Brazil, the surface devoted to soybean surpasses that of all grains.

In Argentina, soybean surface is at present near twofold that of all grains joined, putting the nation perilously near turning into a soybean monoculture (Brown, 2012).

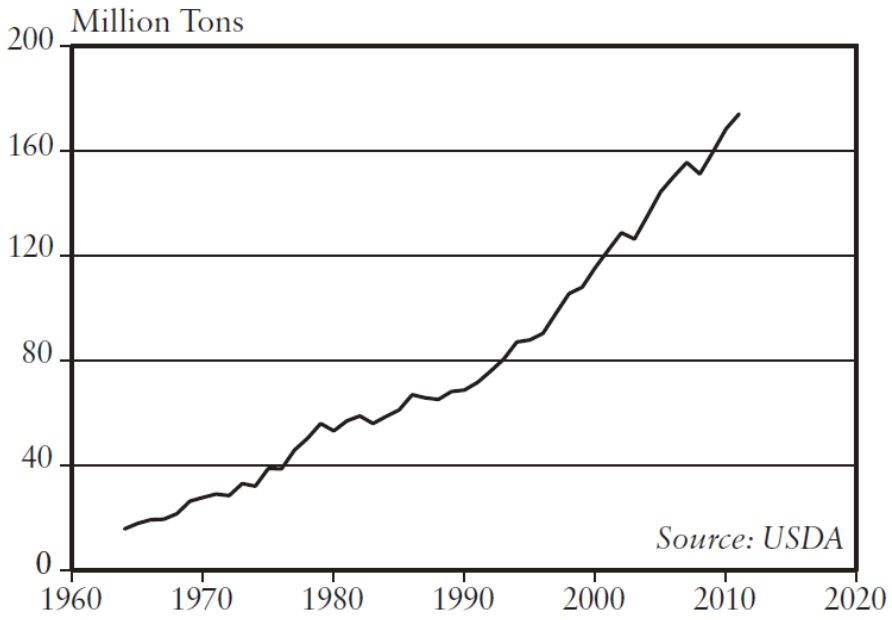


Figure 2. World soybean meal use for feed during 1964–2011 (USDA, 2016).

The global grain yield has almost quadrupled in the second half of the 20th century, basically due to the tripling of the grain yield per unit of land. But the 16-overlap increment in the worldwide soybean yield is due tremendously to the increase in cultivated land. While the soybean surface extended about sevenfold, the crop yield hardly doubled. The global market gets more soybeans principally by planting more soybeans, and that fact may constitute a serious problem for sustainability.

Currently, the US is exploiting all of its available cropland and has no extra land that can be devoted to soybeans. The only alternative is to shift corn or wheat lands to soybean. In Brazil, new soybean lands are made available from the deforestation of the Amazon Basin and the Cerrado, home of a large variety of species and of exclusive fauna and flora, which are essential for the maintenance of the Earth's biodiversity. The state of Mato Grosso, the most important soybean producer in Brazil, soybean production was responsible for 65% deforestation and 14–17% of total Brazilian carbon emissions (Lathuillière et al., 2014). Thus,

new land clearing implies the loss of biodiversity as well as the increase of carbon emissions, aggravating the environmental change for the planet.

The development of innovative feedstuff production beyond the use of agricultural land is hence becoming urgent.

2.2. Agroindustrial by-products

2.2.1. Concept of agricultural wastes

From a strictly productive point of view, it is possible to consider as agricultural residue the fraction not included in the harvest, considering it all the parts usually not utilizable in a strictly commercial sense, and those that do not reach the required quality limits, either by intrinsic or extrinsic reasons (diseases, nutritional deficiencies, etc.). Thus, stems, roots, leaves and unusable fruits, as well as certain aerial parts (e.g., legume and cereal non grain parts, pruning), all of which can be incorporated into the soil (i.e., fertilizer), or destined to the livestock sector (i.e., feedstuff) or used energetically, preferably after being subjected to various treatments of valorization.

The agricultural residues derived from the production or harvesting process are formed by all parts of the crops that are not consumable (e.g., corn stalk, cereal straw, sugar cane bagasse, vegetable stems and leaves, etc.) or marketable (e.g., fruits and grains that do not meet the minimum quality to be marketed, deformed fruits, attacked by pests, that do not reach or exceed the caliper, etc.). They have in common the characteristic of being very heterogeneous and sometimes their elimination being very difficult which poses problems for the environment. Moreover, another common characteristic of agricultural wastes is their dispersion and geographic distribution, their seasonality, the need of removing them quickly from the production area, to avoid the appearance of pests and diseases, their high humidity (in most cases) and the high cost of transport to the treatment or utilization points. In addition, in some cases, agricultural residues contain undesirable or difficult to treat products (e.g., stones, pesticide residues, etc.). The considerable quantity of waste created by the agri-food industries causes genuine ecological issues and also provokes financial losses if not used efficiently.

The carbonaceous nature of most of the vegetal residues makes their reutilization and valorization very diverse, existing throughout the time multiple uses that at present are

testimonials. Currently, new reuse options are being developed that give a new perspective to the consideration as waste. Below are some current and potential uses and applications of these plant materials.

2.2.2. By-products and waste from the agri-food sector

The agri-food industry generates, during the stages of processing in which the raw materials are transformed in final products, large quantities of a wide spectrum of residues. This is a consequence of the large quantities of different raw materials which are processed in a large number of production schemes. These wastes are, in many cases, susceptible of a profitable use, reason why it could be also considered as by-products.

As for wastes, both the nature and volume of production of the by-products depends on the raw material processed and the production scheme used. By-products of the food industry have a wide range of applications among which they stand out their use in agricultural applications, livestock, pharmaceutical and cosmetic industries among others.

The agrifood industry generates different type of residues; some are of animal origin, from meat, fishing, dairy industries, as blood, skins, hooves, and other animal by-products. Others are of vegetal origin.

Concerning the use of the by-products of the agrifood industry, it should be noted that due to the organic nature and physicochemical characteristics of livestock residues, most of them can be valorized by anaerobic digestion or composting, generally using co-substrates which allow the correct development of the process (Red Española de Compostaje, 2014).

According to the Red Española de Compostaje (2014), wastes of vegetable origin may be classified in two large groups:

1. Primary agricultural sector: the waste is generated during the intermediate stages of the production processes, as a result of agronomic practices and tasks such as pruning, defoliation, harvesting, etc.
2. Secondary agricultural sector: the agricultural product is transformed into food product and the residues are mainly produced in the processes of selection of the raw material and during the processes of transformation, mainly through the separation and elimination of parts of the fruits (e.g., seeds, skins, pulp) as well as of the residues resulting from the processes of industrial transformation.

The main industries generating wastes and by-products are fruit and vegetable, oil, sugar, wine and brewing industries.

It is worth noting the large number of research projects and publications carried out in recent years, which attempt to revalue different types of by-products. Diverse research reports have indicated that agrifood industry by-products can conceivably be a wellspring of profitable compounds.

Palumbo et al. (2015) indicated that crop by-products such as barley and wheat straw can be used as raw materials for building thermal insulations. On the other hand, Jahurul et al. (2015) suggested that mango fruit (*Mangifera indica* L.) by-products can be a wellspring of valuable bioactive compounds for pharmaceutical, nutraceutical and cosmetic industries.

Regarding the livestock sector, volatile market and high cost of conventional feed resources such as cereals and legume grains are serious impediments to the industry. In addition, the high competitiveness in demand between humans and livestock regarding these conventional resources require looking for other economical and noncompetitive alternatives which, at the same time, warrant health protection, production yield and product quality.

2.2.3. The use of by-products to produce microbial protein

It has to be taken into consideration that many of the agricultural and agroindustrial residues and by-products contain carbohydrates (i.e., sugars and fiber) and/or proteins, which can be harvested as substrates, either directly or after biological transformation, to obtain compounds of value as animal feed (Bellaver et al., 2005; Molina-Alcaide and Yanez-Ruiz, 2008; Molina-Alcaide et al., 2008; Soto et al., 2015).

The majority of agroindustry by-products and food industry wastes are deficient in proteins and vitamins but rich in low digestible fiber. These characteristics are inadequate for non-ruminant animals, or even for ruminants if the digestibility is very low (Villas-Bôas et al., 2002). The suggested solution to that problem is the use of microorganisms, to bio-convert the agroindustrial wastes into products with better nutritional value, in particular a higher content in protein and vitamin and an improved digestibility (Kuhad et al., 1997; Villas-Bôas et al., 2002).

Fungi, bacteria, yeasts and algae, when cultivated massively (as inoculum) on agro-industrial wastes (as substrate), can become very interesting and advantageous feedstuffs. The resulting

final products, containing both the grown inoculum and the modified substrate, contain high levels of protein (usually with all essential amino acids), and high levels of vitamins and minerals (Villas-Bôas et al., 2002), as well as pre- and probiotics.

In addition, the growth of microbiota on agroindustrial wastes and by-products is able to provide the hydrolytic enzymes frequently added to feed, and to improve the absorption of minerals by the animals. Microbial by-products from the fermentation industries, such as brewing and distilling industries, have been used as protein and energy supplements for ruminant feeding for a long time (Shaver and Batajoo, 1995). Pelczar et al. (1996) reported that using microorganisms to produce nutritionally-enriched feedstuffs have several benefits when compared to conventional feed formulation.

However, microbial products can sometimes hold hazardous and toxic metabolites which represent a risk for both animal and human health. For that reason, the hazard must be evaluated and controlled during the whole bioconversion processes to prevent probable toxicity to animals and humans (Villas-Bôas et al., 2002).

2.2.4. Definitions related to waste and by-products

Directive 2006/12/EC of the European Parliament and of the Council of 5 April 2006 on waste and Directive 2008/98/EC of 19 November 2008 on waste, define:

- **Waste**, is any substance or object from which the holder is detached or has the intention or obligation to discard.
- **Treatment**, is the recovery or disposal operations, including preparation prior to recovery or disposal.
- **Recovery**, means any operation the main result of which is waste serving a useful purpose by replacing other materials which would otherwise have been used to fulfill a particular function or that the waste is prepared to perform that function at the facility or in the economy in general.
- **Elimination**, is any operation other than recovery, even when the operation has as a secondary consequence the use of substances or energy.
- **By-product**, is a substance or object, resulting from a production process, the primary purpose of which is not the production of that substance or object.

  Any substance can be considered as a by-product and not as a waste only, if the following conditions are met:

- It is certain that the substance or object will be used later;
- The substance or article may be used directly without having to undergo further processing other than normal industrial practice;
- The substance or object is produced as an integral part of a production process,
- Subsequent use is legal; meaning that the substance or object meets all relevant requirements for specific application related to products and environmental and health protection, without producing general adverse impacts on the environment or human health.

2.3. Microorganisms as a source of protein

Microorganisms have always played an important role in food processing techniques (e.g., turning fibers to edible food in the case of fermenting dough to bake bread, milk to cheese) permitting the long-term conservation of foods (Caplice and Fitzgerald, 1999). Some microorganisms as yeast and algae are also frequently used as direct food source (Matassa et al., 2016).

By the beginning of the 1960s, the public awareness on world's food shortage has led to the investigation of alternatives to ensure sustainable food for the expanding population (Goldberg, 1987). The efforts made in examination of unconventional food sources highlighted the possibility of using largely available and inexpensive hydrocarbon substrates the microorganisms as high-quality protein additives, called "microbial protein" and "single-cell protein" (Matassa et al., 2016).

The term "Single-cell protein" was proposed by the Massachusetts Institute of Technology, referring to dried cells of microorganisms like bacteria, yeasts, microalgae and fungi, produced in large-scale culture systems to be used as protein sources in human food or animal feed (Matelbs and Tannenbaum, 1968). Those dried cells contain a variety of proteins, lipids, carbohydrates, nucleic acids, inorganic compounds and a mixture of further non-protein nitrogenous substances like vitamins.

With the recent progress made in research and development, and with the tremendous potential of microbes in producing protein, it becomes possible to provide high-quality microbial proteins to animal feed industry and to help to solve the deficit caused by the increasing demand for protein in the world. Microbial protein (MP) can even decrease the use of soybean and cereals in animal feed, hence diminishing the competition between human and

animals of vegetable sources of proteins and improving the human nutrition in underdeveloped nations (Kuhad et al., 1997).

Microorganisms possess numerous advantages over conventional food and feed sources (i.e., vegetal and animal origin). While both microbes and plants have the ability of synthesizing protein form inorganic N, microorganisms can be harvested within hours not seasons like plants. Bacteria are a great source of protein, both quantitatively and qualitatively: They generally contain more than 65% of CP on dry weight (Kuhad et al., 1997). Regarding protein quality, bacteria have a high profile in amino acids, with higher concentration of sulfur-containing amino acids (cysteine and methionine) and lysine (Anupama and Ravindra, 2000).

2.3.1. Environmental advantages of microbial protein

Microorganisms can make use of waste raw materials available in large amount at low cost. The large use of MP products as an alternative for traditional protein feed supplements, such as soybean and fishmeal, can create a chance of diminishing part of the environmental pressure applied by these products on land and water use.

A commercial MP feed produced from the methanotrophic gram-negative bacteria *Methylococcus capsulatus*, marketed under the name of FeedKind® was evaluated by the British Carbon Trust in terms of greenhouse gasses, land and water uses, comparing them with soybean and fishmeal (Cumberlege et al., 2016). As shown in **Figure 3**, the water footprint of the MP was around 20 and 140 times lower than fishmeal and soybean meals, respectively. A similar pattern was estimated for the required land. The higher value in the MP-pelletized form was due to the vegetable oil incorporated during manufacturing, while no cultivable land was needed in the case of the powdered form. In comparison with the 6,655 m^2 land per ton protein needed to produce soybean meal concentrate, the almost zero land foot print shows how significant was the benefit of MP regarding the land footprint.

2.3.2. Microbial protein as a feed

Microbial protein represents a good source of high-quality protein capable to substitute animal protein such as fishmeal in livestock nutrition and aquaculture. Furthermore, MP comply the FAO/WHO requirements regarding the essential amino acid scoring pattern for human nutrition (**Figure 4**); hence humans too could profit considerably from the use of MP as food.

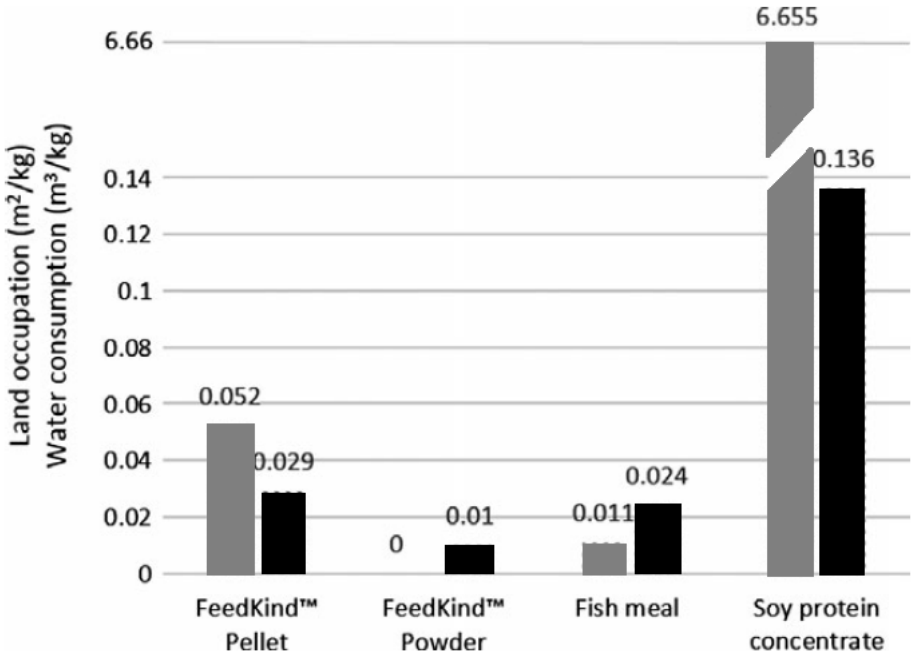


Figure 3. Land (■) and freshwater (■) requirements of microbial protein compared with fishmeal and soy protein concentrate. The values are normalized to the protein content of each product (Matassa et al., 2016).

The main inconvenient in the bacterial protein is its high nucleic acid content, reported to be 15-16% of dry matter (Anupama and Ravindra, 2000). To solve this problem, reducing the nucleic acid content is possible with the use of enzymes such ribonuclease and nuclease enzymes.

2.4. *In vitro* fermentation techniques

Several methods are used to evaluate the nutritive and fermentative potential of feeds, such as *in vivo*, *in situ*, and *in vitro* methods. *In vivo* methods are relatively expensive and difficult to apply to a large scale. For those reasons, alternative methods were developed.

The *in situ* methods are a proxy for the *in vivo*, but they necessitate laborious preparation and continuous maintenance of a significant number of cannulated animals (Mehrez and Orskov, 1977). The *in vitro* methods showed to be more practical in many ways, being less expensive and less time consuming, and permitting a better control and precision of experimental conditions than *in vivo* trials (Raab et al., 1983).

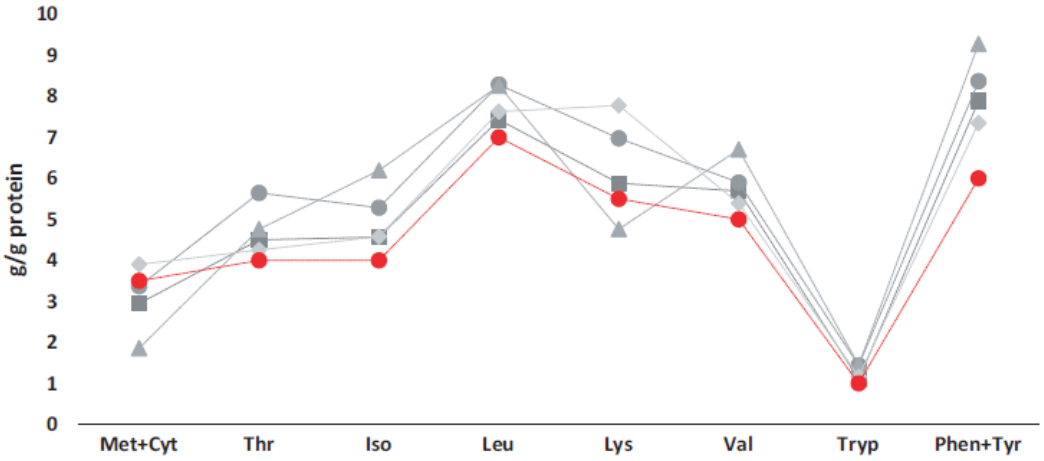


Figure 4. Pattern of essential amino acid scoring of microbial protein from bacteria (*Pseudomonas/Methylophilus* spp.) (■), yeasts (*Candida* spp.) (●), algae (*Spirulina maxima* (▲), compared with a high-quality animal protein from fishmeal (♦) as well as to the FAO/WHO standard (●) for amino acid scoring pattern for human nutrition (Matassa et al., 2016).

Currently many types of biological digestion techniques *in vitro* exists for ruminants, such as measuring the extent of microbial digestion in terms of substrate disappearance, as in the Tilley and Terry (1963), or the indirect estimation of fermentation by measuring gas production (Menke et al., 1979; Theodorou et al., 1994). The use of biological methods instead of simple chemical methods is justified by the fact that microorganisms and enzymes are more responsive to factors influencing the rate and extent of digestion (Van Soest, 1994); hence the results are more accurate.

2.5. Biogas production from agri-food byproducts

The demand for energy is increasing rapidly. According to International Energy Agency, fossil fuels such as oil, coal and natural gas still account for more than 80% of this demand (IEA, 2016). The IEA estimates that the energy demand will multiply by two or three during the 21st century. Simultaneously, there is a rapid rise in the greenhouse gases concentrations (GHG) in the atmosphere, knowing that the main contributor is fossil fuel-derived CO_2 emissions (Weiland, 2010). To decrease the associated global warming and climate change

impacts, GHG emissions must be reduced to less than half of the global emission levels of 1990 (IPCC, 2000).

In these circumstances, biogas produced from agrifood sector residues, and by-products may play an indispensable role in the future.

2.5.1. Biogas utilization

Biogas consists mostly of methane (CH_4) and carbon dioxide (CO_2), although it contains smaller quantities of hydrogen sulfide (SH_2), ammonia (NH_3) and water vapor. Biogas must be desulfurized and desiccated before use to avoid damage of the gas utilization units (Weiland, 2010).

Ruminant CH_4 emissions are produced as a result of the microbial fermentation of carbohydrates in the rumen, the resulting gases containing 66% CO_2 and 27% CH_4 as shown in **Table 1**.

Table 1. Typical composition of rumen gases (Sniffen and Herdt, 1991).

Component	Percentage
Hydrogen	0.2
Oxygen	0.5
Nitrogen	7.0
Methane	26.8
Carbon dioxide	65.5

Biogas is a renewable energy source that can be used for substituting the fossil fuels in power and heat production, and it is possible to be used as gaseous fuel for automobile. Moreover, it can also be purified into bio-methane and used the same way as natural gas as feedstock to manufacture chemicals and materials. It can be fully exploited with maximum energy efficiency to generate heat. Its use is possible as a source of electricity in cogeneration plants or be injected into the natural gas network (EurObserv'er 2014). In Asia, millions of families produce biogas for domestic use by means of their own small-scale digester. Energy produced from biogas in Europe attained 13.4 Mt of oil equivalents (Mtoe) during 2013 with a 10.2% yearly growth (EurObserv'er, 2014).

The conception of an industrial plant to produce biogas is shown in **Figure 5**. The plant consists of an substrate entrance, a digestion tank and a system to separate the solid and liquid phases of the fermented products. The plant can work as a continuous-flow system. The use of an stirring device is recommended for improving the homogenization of microbiota. The fermentation inputs (substrate and inoculum) are introduced into the digestion tank and the outputs separated as solid and liquid phases by filtration. Moreover, the liquid phase is reused to take advantage of the remaining nutrients and ammonia, while the solid phase can be centrifuged and dried to obtain the SCP.

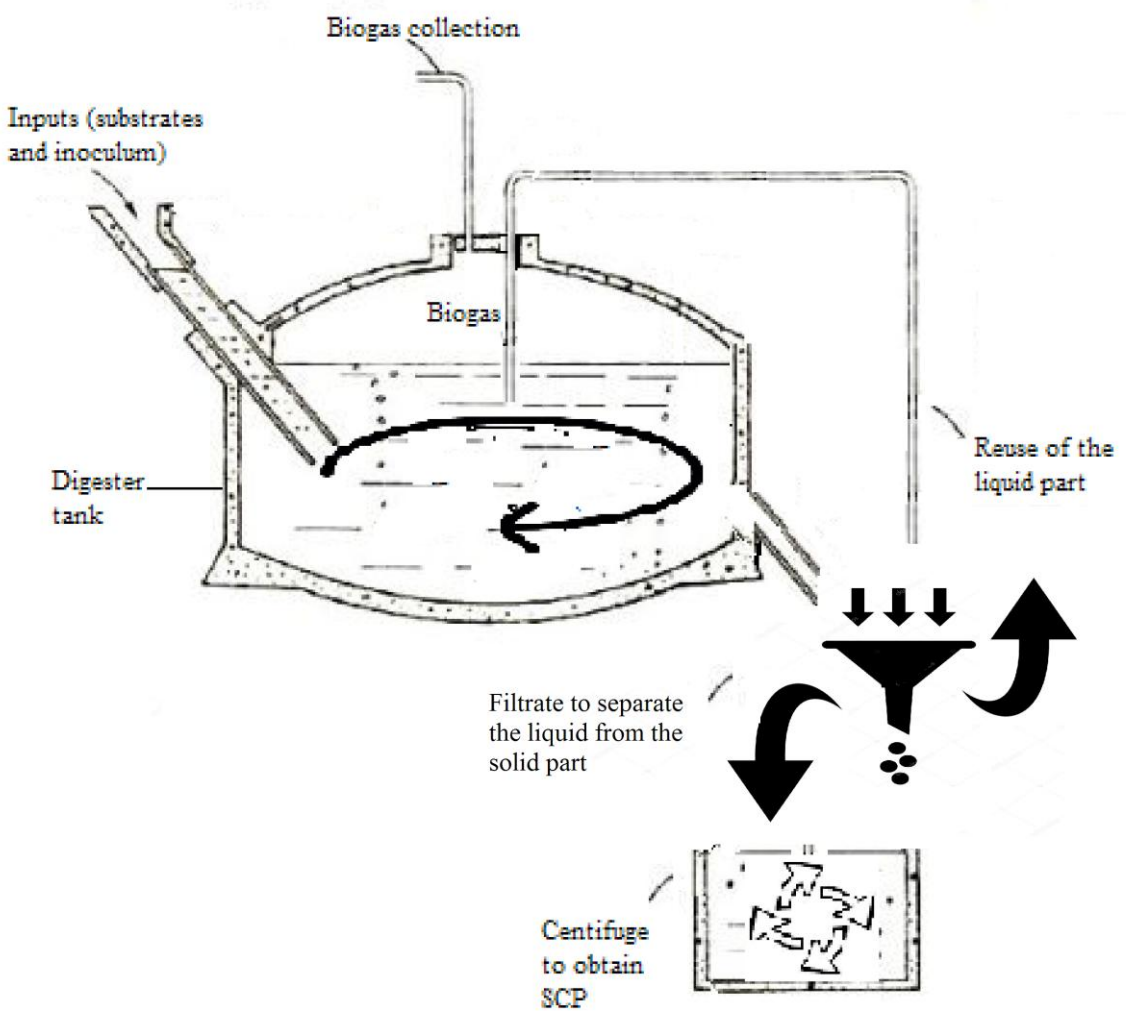


Figure 5. A model of the fermentation system.

The sources to produce biogas are various and abundant, such as agrifood industry residues, vegetable waste and manure. The process is suitable for both small and large scales productions, meaning it can be carried at any place. Biogas production through anaerobic digestion provides important advantages

over other types of bioenergy production. It has been classified between the most energy-efficient and environmentally beneficial technology for bioenergy production (Weiland, 2010). It can considerably decrease GHG emissions compared to fossil fuels by exploitation of locally existing resources.

The residues remaining after the anaerobic digestion of a biodegradable feedstock known as the "digestate" are considered an improved fertilizer to crops in which it can replace mineral fertilizer (Weiland, 2010).

2.5.2. Biochemical process of biogas production

The process of methane fermentation is relatively complex, and it can be segregated into four phases: hydrolysis, acidogenesis, acetogenesis/dehydrogenation and methanation (**Figure 6**).

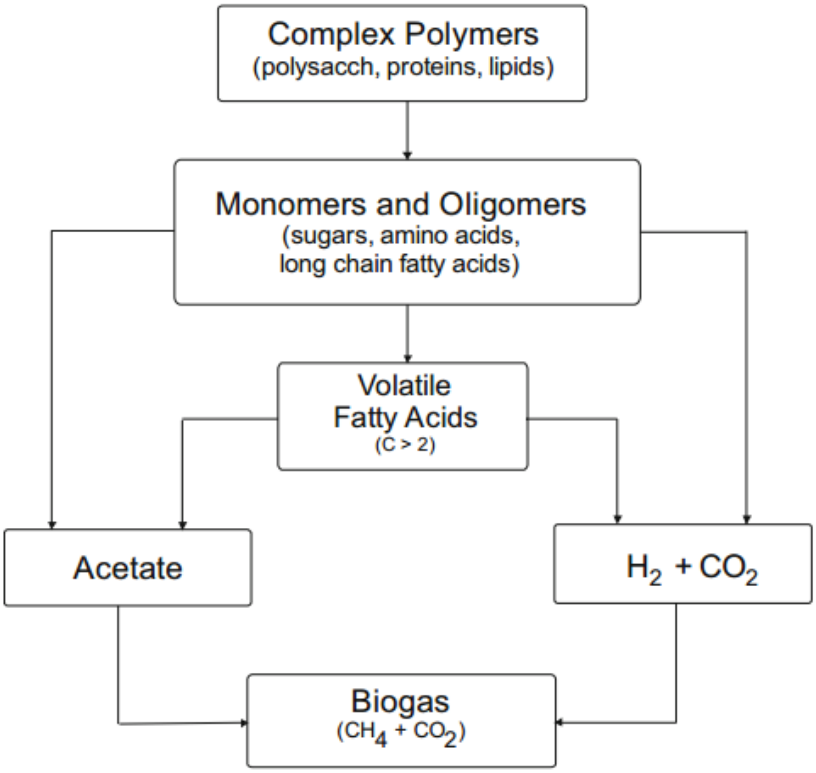


Figure 6. The stages of methane fermentation process (Gujer and Zehnder, 1983).

Hydrolyzing and fermenting microorganisms are responsible for the first attack on both polymers and monomers. This attack produces mostly acetate and hydrogen and different quantities of volatile fatty acids (VFA) like propionate and butyrate.

The excretion of hydrolytic enzymes such as cellulase, cellobiase, xylanase, amylase, lipase, and protease by the hydrolytic microorganisms is essential in the hydrolysis and fermentation of the organic material.

The degradation chain ends with the production of methane by two groups of methanogenic bacteria, using acetate or hydrogen and carbon dioxide. Methanogenic bacteria are strict anaerobes and only few species like *Methanosarcina barkeri*, *Metanonococcus mazei*, and *Methanotrix soehngenii* can degrade acetate into CH_4 and CO_2 (Weiland, 2010).

The digestion process can occur at mesophilic (35-42°C) or thermophilic (45-60°C) temperature conditions. Keeping a constant temperature during the digestion is important to avoid negative effects on biogas production (Weiland, 2010). Nevertheless, mesophilic bacteria resist fluctuations in temperature of 3°C without important reductions in methane production. The growth rate of methanogenic bacteria is higher at thermophilic process temperatures, making the process faster and more efficient. But thermophilic process temperatures assure a higher growth rate of methanogenic bacteria (Weiland, 2010).

The use of biowastes for the dual purpose of producing biomolecules and stabilizing them is more profitable under anaerobic digestion, in comparison to the aerobic process. Anaerobic process leads to the degradation of more than 95% of the organic matter into CH_4 and CO_2. The anaerobic digestion, being a multi-stage process, is produced by various types of microbes (Kalia, 2015). At each step, exist the possibility of taking advantage of the intermediates into a variety of value-added products that can be used as precursors for other biotechnological and medical applications, such as hydrogen, bioactive molecules such as enzymes, VFA, sugars and amino acids (Kalia, 2015).

2.5.3. Feedstock for biogas production

All kinds of biomass are possible substrates for biogas production on the condition that they hold carbohydrate, protein, fat, cellulose, and hemicelluloses as major constituents. The main factors affecting the composition of biogas and the methane production are the feedstock type, the digestion system, and the retention time (Braun, 2007).

The speculative gas production differs with the content of carbohydrates, proteins, and fats (**Table 1**). Strong lignified organic substances such as wood are not suitable in view of the slowly anaerobic decomposition (Weiland, 2010). The genuine methane content in practice is

usually superior to the theoretical values shown in **Table 2** for the reason that a part of CO_2 is dissolved in the digestate.

In the past, anaerobic digestion has been almost always related to the treatment of animal manure and sewage sludge from aerobic wastewater treatment. Currently, the majority of the agricultural biogas plants digest manure from livestock with the addition co-substrates to increase the content of organic matter for attaining a better gas yield. Usually the co-substrates used are harvest residues, such as leaves of sugar beets, agro-industrial wastes, and municipal biowaste.

Table 2. Maximum yield and theoretical methane contents in bio-gas production according to the substrate used (Weiland, 2010).

Substrate	Biogas (Nm^3/t total solids)	CH_4 (%)	CO_2 (%)
Carbohydrate[a]	790–800	50	50
Raw protein	700	70–71	29–30
Raw fat	1,200–1,250	67–68	32–33
Lignin	0	0	0

[a] Only polymers from hexoses, not inulins and single hexoses.

Fat yield the best biogas production, but necessitate an extended retention time because of their deficient bioavailability. Proteins and carbohydrates grant faster conversion rates but lower gas production. All substrates must be pathogen-free; if not, pasteurization at 70°C or sterilization is required (Weiland, 2010).

3. OBJECTIVES

3. OBJECTIVES

The Altersoy project has as specific objectives, to:

- Evaluate the potential agricultural residues and the agri-food industry currently available in Catalonia, as possible substrates for fermentation and transformation into protein of microbial origin destined to animal feed.
- Study the characteristics and variation factors of the fermentative process, including different types of microbial inoculum, to be able to produce profitable and good-quality products. As innovative inoculant, rumen microbioma will be used. This should allow the improvement of the digestibility of the residues and of their nutritional value and, in parallel, the production of biogas, a renewable energy source.
- Study the composition and nutritive value of the obtained products for their use as protein supplements in animal feed.
- Make recommendations for specific actions in order to promote initiatives for the use of best profitable waste for livestock sector in Catalonia.

The first part of the Altersoy project corresponding to evaluating the potential of Catalonia, in agricultural residues and agri-food industry as raw materials that could be used as a substrate for microbial ruminal was already done by Redondo (2016) in a previous Master Thesis. The conclusions and recommendations of that part were the availability of significant quantities of interesting residues such as cereal grains and straw, biomass (forest residue) and corn stover, which could be used as substrate for the fermentation and transformation to bacterial proteins destined to animal feed.

The second part of the Altersoy project, which is developed in the present Thesis, corresponds to the evaluation of ruminal fermentation as a process to increase the protein content of feedstock in order to reduce the dependency from soybean meal cake. The ideal process would consist on a rumen-like fermentor that converts the feedstock to single cell protein (SCP). The biogas produced during the process can be used as an energy source for heating and stirring (**Figure 7**). If this SCP production process were cost-effective, with regard to soybean cake protein, it will be tested at a larger scale.

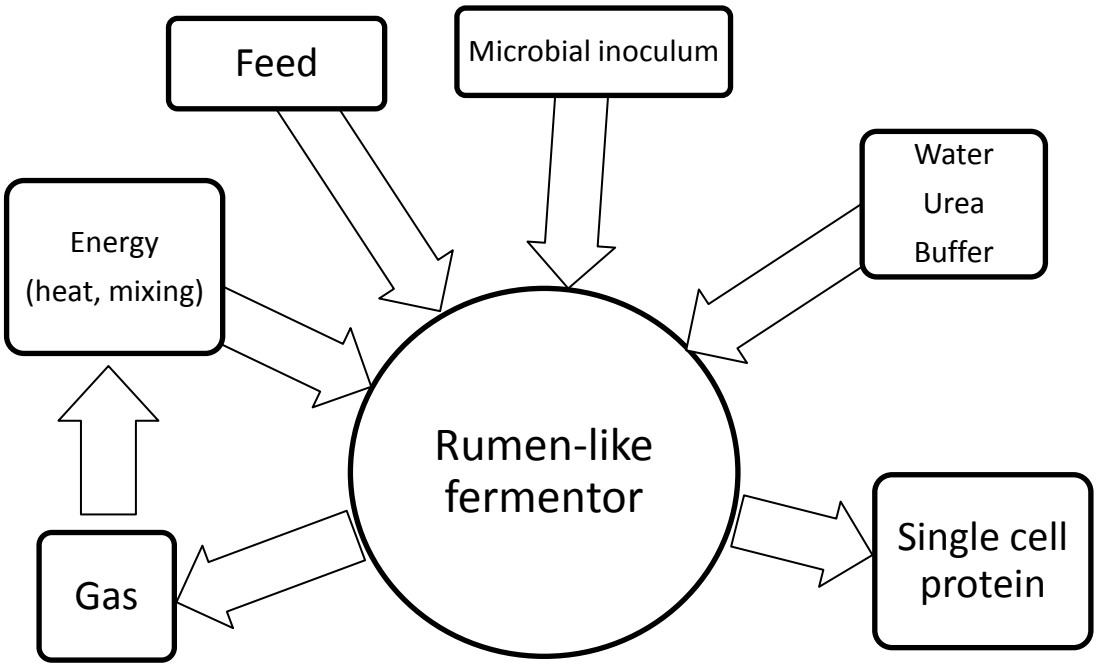


Figure 7. Diagram of the fermentation process showing different inputs and outputs.

4. MATERIAL AND METHODS

4. MATERIAL AND METHODS

4.1. Fermentative products

Based on the conclusions and recommendations of first part of the Altersoy project (Redondo, 2016), the by-products used as substrates for the fermentation and transformation to bacterial proteins were: cereal straw, maize stover and wood. Additionally maize and barley grains were used as reference energy substrates.

4.2. Substrates (Plant matter chemical analysis)

Substrates samples were milled through a 1 mm screen and analyzed for dry matter (**DM**), ash, ether extracts (**EE**) and crude fiber (**CF**) according to the Weende method, using standard procedures as described by the Association of Official Analytical Chemists International (Ref. 934.01, 923.03, 920.39, AOAC International, 2005; and Ref. 962.09,AOAC International, 2010).

Neutral detergent fiber (**NDF**) and acid detergent fiber (**ADF**) were determined sequentially according to the methods of Van Soest et al. (1991) by using the Ankom apparatus (Ankom Tech. Co., Fairport, NY, USA). For the NDF analysis, samples were treated with sodium sulphite and thermostable α-amylase, and corrected for their ash content.

The Dumas method (968.06, AOAC International, 2003) with a Leco analyzer (Leco Corp., St. Joseph, MI, USA) was used for N determinations and crude protein (**CP**) was calculated as percentage of N × 6.25. All chemical analyses were performed in triplicate and expressed on DM basis.

Protein value

The INRA ruminant nutrition system (INRA, 2010) expresses the protein value of feedstuffs as PDI (Digestible Protein in the Intestine). The system determines the PDI value in terms of the amount of amino acids absorbed in the small intestine after the fermentation of the ingested protein. It considers both the rumen non-degraded dietary protein (PDIA) and the potential production of microbial protein (PDIM) under energy- (PDIME) or nitrogen-limiting conditions (PDIMN). Because rumen microbiota is capable of using several sources of non-protein nitrogen (e.g., ammonia, urea, etc.) and the purpose of the current study is the

fermentation of organic substrates, the limiting factor considered for the production of protein was only the energy. Nitrogen deficits would be corrected by the addition of low-quality protein or inorganic nitrogen sources (non-protein nitrogen).

The potential PDI value of the food is thus determined with the sum of the two fractions:

- PDIA: protein digested in the small intestine supplied by the rumen undegradable protein from the feed $PDIA = CP \times (1.11 \times (1\text{-}DT)) \times dr$. With DT being the theoric degradability of the CP of the feed in the rumen and dr being the digestibility of the amino acids from the feed in the intestine.
- PDIM: digestible proteins in the small intestine of microbial origin, synthesized in the rumen, and correspond to the lower of the PDIME or PDIMN values, depending on the limitation in each case.

In order to calculate the value of PDIME, which would be the limiting factor in the situation where the required N was to be supplied; INRA uses a medium efficiency synthesis of microbial nitrogenous materials (MNMIC) of 14.5% on Fermentable Organic Matter (FOM), an MNMIC (amino acids) protein content of 80% and a microbial protein digestibility of 0.8.

The FOM is determined by difference from the Digestible Organic Matter (DOM), subtracting the undegradable components in the rumen, such as the non-soluble and degradable protein and total fat or ether extract (EE), considering that its energy will not be used by the ruminal microbial population, being: $PDIME = FOM \times 0.145 \times 0.8 \times 0.8$.

4.3. Experimentation

In order to evaluate the fermentative potential of the chosen plant material, a gas production experiment in different substrates and conditions was carried out using the method of Theodorou et al. (1994).

4.4. Microbial inoculum

Digesta was taken from a rumen-fistulated non-lactating Holstein dairy cow, fed a diet of 60% Alfalfa hay and 40% cracked corn, housed in the facilities of the SGCE (Servei de Granges i

Camps Experimentals) of the UAB (Universitat Autonoma de Barcelona) at Bellaterra (Barcelona).

The ruminal fluid was extracted before the morning feeding, manually squeezed and filtrated through 2 layers of muslin to get the filtrated rumen-fluid, and post-haste carried to the laboratory in a light proof and isotherm container.

4.5. Culture media

The filtrated rumen-fluid was mixed with McDougall artificial saliva in proportion of 1:4 (**Table 3**) with pH of 6.85, under a steam of CO_2. Fermentation was carried out at 39°C, under anaerobic conditions, in 100 mL serum bottles crimp fastened with butyl rubber stoppers and aluminum crimp seals (**Figure 6**).

Table 3. Composition of artificial ruminant saliva (McDougall, 1948).

Salt	g/l
$NaHCO_3$	9.80
KCl	0.57
$CaCl_2$	0.04
Na_2HPO_4	9.30
NaCl	0.47
$MgSO_4$. $7H_2O$	0.12

4.6. Gas production and pH measurement

Gas production from the in vitro fermentation of substrates with mixed ruminal microbes was measured according to the method of Theodorou et al. (1994) by means of an HD 8804 manometer fitted with a TP804 pressure gauge (Delta Ohm, Caselle di Selvazzano, Italy) attached to hypodermic needles inserted into the 100 mL serum bottles crimp sealed with butyl rubber stoppers.

The gas produced was expelled after each measurement, in order to prevent any pressure effect on microbial activity (Rymer et al., 2005). Blanks bottles containing the inoculum without addition of substrate were used in order to correct data from the residual fermentable

OM in the inoculum. The pH was determined by the mean of a pH meter (Crison micropH 2002, Barcelona, Spain).

Figure 8. Fermentation bottles in the thermostatic water bath.

4.7. Experiment 1

This first experiment was carried out in order to know the fermentation and gas production levels of different substrates with different quantities. Plant materials used in the first experiment as substrates were corn grain, barley grain (Malting variety Peuter, hulled) and barley straw. The quantity of substrate used is shown in **Table 4**, except for the cereal straw; quantities used were fewer than 20 g due to excessive volume of the sample. The repetitions were performed in triplicate.

Table 4. Quantity of substrate and inoculum used.

Substrate, g (as fed)	Inoculum, mL	Maize, g DM	Barley, g DM	Straw, g DM
0.5	100	0.44	0.45	0.46
1	99	0.88	0.90	0.91
2	98	1.76	1.81	1.82
5	95	4.39	4.52	4.56
10	90	8.78	9.03	9.11
20	80	17.55	18.06	-
30	70	26.33	27.09	-
40	60	35.10	36.12	-

Treatments

Two treatments were carried out, one without urea and another with the addition of the required quantity of urea in order to equalize the protein digested in the small intestine supplied by microbial protein from rumen-fermented OM (PDIE) and the protein digested in the small intestine supplied by microbial protein from rumen-fermented protein (PDIN) in every used substrate. Knowing that 1 g of urea is the equivalent of 1.45 g of PDIN (INRA, 2007), the quantity of added urea was as described in the **Table 5**.

Table 5. Quantity of urea added to the substrates.

	PDIE(g/kg)	PDIN(g/kg)	PDIN balance	Urea (mg/g substrate)
Maize grain	97	74	-23	15.86
Barley grain	101	79	-22	15.17
Barley Straw	46	24	-22	15.17

For the urea treatment, only 0, 5, 10 and 40 g of substrates were used. Four blank samples were also included. The total number of bottles used was 94. The gas production was recorded at 3, 6, 10, and 24 h of incubation and corrected for blank bottles incubated in each run. The pH was determined at the end of the incubation (24 h).

4.8. Experiment 2

The purpose was to compare the fermentation pattern of all the substrates and to measure the increment of protein.

The substrates used in the second experiment were maize grain, barley straw, corn stover and wood. The quantity of substrate used was 3.5 grams in 50 mL of inoculum. An amount of 0.25 g/L of urea was added. Same Theodorou et al. (1994) method was used. Gas production were recorded at 6, 10, 24, 48, 72 and 144 h of incubation and corrected for blank bottles. The pH was measured at 6, 10, 24, 48 and 144 h by sacrificing one bottle form each treatment at each sampling time. Since gas was still produced, the experiment was extended until 144 h to observe the kinetics of gas production.

Digestibility

To determine the digestibility of the products obtained two stages were carried on. First, contents of each bottle were transferred to plastic tubes and centrifuged at 6,000 × g for 10

min at 4ºC (Universal 32 R, Hettich, Tuttlingen, Germany). The pellets obtained were dried at 60°C during 48 h and then weighed. The pellets from same treatment were joined and ground to make sure of the homogeneity of the sample.

In second stage, Tilley-Terry method (1963) was used. A quantity of 0.5 grams from every treatment of 24 and 48 h was put in Erlenmeyer flask and digested during 48 h in 38°C in a 50 mL solution containing 0.2 g of pepsin (Pepsin powder from porcine gastric mucosa, ≥ 250 units/mg solid, Sigma-Aldrich) and 2 mL of HCL 6N dissolved in distilled water. The analyses were performed in triplicate.

The products of the enzymatic digestion by pepsin were analyzed for the protein using the Dumas method in order to know the indigested protein

Microbial protein production

The protein content of final products of the fermentation was determined using the Dumas method.

4.9. Experiment 3

From the conclusions of the second experiment, it was decided to include the maize silage, as it is made out of the whole maize plant, rich in both starch and fiber. The second experiment also included the measurement of pH, digestibility and protein production

Urea was added to the artificial saliva by 2.5 g/L to provide a minimum of 10% of CP for all the substrates. Fermentation was carried out for 72 h. The gas production and pH were measured at 3, 6, 10, 24, 48 and 72 h. Same procedures as for experiment 2 to determine digestibility and microbial protein production were carried out in experiment 3.

4.10. Statistical analysis

Comparison between substrate types or concentrations were analyzed using the PROCMIXED procedure of SAS version 9.1.3 (SAS Institute, Cary, NC, USA) for gas production measures, The model contained the fixed effects of the substrate and the time as repeated measure. For all analyzed data, the bottle was used as the experimental unit (n = 3). The GLM procedure of SAS was used for pH, digestibility and protein synthesis measures. Significance was declared at $P < 0.05$ and tendencies at $P < 0.10$. The multiple comparisons

were performed under Tukey method by the MEANS Statement. Results are expressed in terms of means and standard deviation.

5. RESULTS AND DISCUSSION

5. RESULTS AND DISCUSSION

According to the conclusions of the previous research done by Redondo (2016) in the frame of the Altersoy project, cereal straws and grains and wood by-products, are commodities of interest for the production of microbial protein in Catalonia. **Table 6** shows the prices of some of these by-products and the cost of PDI offered under ruminant digestion conditions. For comparison, values of soybean meal are also included. In order to be able to replace Soybean meal, the fermentation process should produce PDI at a cost lower than 1.6 €/kg PDI.

Table 6. Available commodities for the production of microbial protein.

Raw material	Price (€/t)	Cost of PDI (€/kg PDI and %)
Wheat straw	50[2]	1.14 (71%)
Rye straw	50[2]	1.14 (71%)
Barley straw	50[2]	1.14 (71%)
Triticale straw	50[2]	1.14 (71%)
Oat straw	50[2]	1.14 (71%)
Wood, untreated	110[3]	7.33 (451%)
Maize grain	169[4]	1.56 (98%)
Maize stover dried (includes stalks, leaves, husks, and cobs)	70[3]	2.12 (133%)
Barley grain	162[3]	1.64 (97%)
Soybean meal 44 (INRA)	364[1]	1.60 (100%)

[1]Indexmundi, 2016 (Period of reference: August 2015- July 2016). [2]Market price season 2015-2016. [3]AVEBIOM, 2016 (Period of reference: April 2015-April 2016). [4]EUROSTAT, 2016 (Season 2014).

As shown in Table 6, cereal grains and wood by-products had cost values of PDI close to soybean meal (97-98%), while straws had costs around 71%. These results were useful in the selection of the by-products to be used as substrates for the fermentation and transformation into microbial proteins. Additionally maize and barley grains were used as reference energy substrates.

5.1. Chemical composition of substrates used

The chemical composition and the estimated nutritive values of the substrates considered for the fermentation study are summarized in **Table 7**.

Obtained values agreed with small differences those reported in the INRA (1988) feedstuff composition. No data was available for wood pellets, although expected digestibility of OM should be in the range of 5-20% as indicated by Hakkila (1989) for wood residues. All the substrates considered have low protein contents indicating that ammonia addition will be necessary in order to optimize the synthesis of microbial protein.

Table 7. Chemical composition of the substrates used

Item	Maize meal	Barley meal	Barley straw	Maize stover	Maize silage	Wood
DM, % as fed	87.8	90.3	91.1	88.1	35.0	95.3
Component, %DM:						
Ash	1.2	2.5	5.7	12.6	3.9	0.5
OM	98.8	97.5	94.3	87.4	96.2	99.5
EE	3.6	1.9	1.1	0.2	2.9	3.1
CP	7.4	12.5	3.7	4.4	6.6	0.3
Cellulose	2.5	5.3	40.7	40.0	16.9	66.5
NDF	8.8	18.9	97.9	85.2	33.9	84.7
ADF	2.5	6.1	46.5	61.5	17.4	70.3
Nutritive value[1]:						
dMO, %	88.5	83.1	44.0	57.0	73.6	-
UFL/kg DM	1.20	1.08	0.44	0.57	0.96	-
PDIA, g/kg DM	42	37	11	14	15	-
PDIN, g/kg DM	58	85	23	28	42	-
PDIE, g/kg DM	86	103	47	57	67	-
LysDI, %AA	5.96	6.72	7.39	7.33	7.46	-
MetDI, %AA	1.96	1.93	2.06	1.84	1.99	-

[1]According to INRA (1988) by using PrevAlim (v. 3.23).

5.2. Experiment 1.1: Substrate evaluation

5.2.1. In vitro gas production

Total gas production.

Figure 9 shows the *in vitro* gas production according to the different substrate materials and quantity of substrate, when ruminal fluid was used as inoculum. The obtained results showed that the gas production was markedly affected by both variation factors ($P < 0.001$), being the gas production the greatest in the case of maize meal (a), followed by the barley meal (b). The lowest total gas production was observed in barley straw (c). As for the maize and barley meals, the gas curves showed a different pattern when the substrates were lower or greater than 10 g/100 mL.

Theodorou et al. (1994) reported that increasing the amount of substrate generates a linear increase of total gas volume, although without effect on the rate of gas production. This was not observed in our results, the slopes of the curves increasing ($P < 0.05$) as the amount of substrate increased when <10 g/100 mL and the gas production continued for at least for 24 h.

When substrate was >10 g/100 mL, gas production stopped in most cases after 6 h (only maize meal with 10 g/100 mL continued increasing its gas production until h 9), indicating that fermentation reached an early limit for the microbial population. Most likely this was due to the high concentration of substrate used and the accumulation of catabolites in the closed bottles (i.e., bacterial residues and acids), which inhibited their further growth.

Hence, it was decided to continue the next experiments with concentrations of substrates lower than 10 g/100 mL. Moreover, as gas production was still important for barley straw after 24 h, it was also decided to carry out the next experiments for a longer fermentation time.

The greatest gas production was observed between 5 and 10 g/100 mL and maize meal was the substrate which producing the greatest total volume of gas.

Gas production per unit of OM

Figure 10 and **Table 8**, for the values after 24-h incubation, show the *in vitro* gas production per g of OM according to the different substrate materials and the quantity of substrate used. The results clarified those previously reported in Figure 9 and clearly showed that, under

rumen fermentation conditions used, and despite being the maize meal, followed by barley meal, the greatest gas producer, yield of gas per unit of OM decreased as substrate increased.

The lowest values were obtained with barley straw (<400 mL/g OM). In all cases, the maximum gas production (800-1,000 mL/g OM) were achieved with the lowest substrate concentration (0.5 g/100 mL), indicating that an important amount of substrate was not used for microbial fermentation when this concentration was over passed. Additionally, rate of gas production was markedly reduced after h 10 in all substrates and despite the concentration of rumen inoculum used.

The obtained results may also indicate that the measurement of gas production do not reflect the bacterial growth or that other nutrients, different from fermentable OM (i.e., N), may have limited the microbial population growth. Being this an expected limiting factor, we included a parallel treatment with urea correction in this experiment.

5.3. Experiment 1.2: Substrate correction with urea

5.3.1. *In vitro* gas production

A second treatment with the addition of urea was carried out in order to supply the deficient quantity of nitrogen needed for an optimal microbial fermentation. Concentration of urea added intended to offer similar values of protein digested in the small intestine supplied by microbial protein from rumen-fermented OM (PDIE) and the protein digested in the small intestine supplied by microbial protein from rumen-fermented protein (PDIN). Results of fermentation are shown in **Figure 11** (total gas production) and **12** (gas yield per g OM).

According to Sterling et al. (2001) and Yenigün and Demirel (2011), an inhibitory effect of the addition of ammonia on gas production under rumen conditions was expected, but this effect was not observed in all cases, the response to the urea being dependent on the substrate and the concentration of inoculum.

Gas production decreased ($P < 0.05$) for maize meal and barley straw with the addition of urea although the differences were negligible when the concentration of substrate increased, as shown in **Table 8** (gas yield per g OM in 24 h). On the contrary, addition of urea increased gas production in barley straw, the effect being more marled for the low substrate to inoculum concentration (0.5 g/100 mL).

Table 8. Total gas production (mL) at 24 h *in vitro*, according to the type and the quantity of substrate used (mean ± SE).

Substrate	Quantity of substrate (g/100 mL)							
type	0.5	1	2	5	10	20	30	40
Without urea								
Maize meal	546 ± 8^{dx}	763 ± 10^{cx}	985 ± 20^{bx}	$1{,}191 \pm 11^{ax}$	991 ± 2^{bxy}	691 ± 9^{cex}	648 ± 7^{dex}	486 ± 5^{dy}
Barley meal	482 ± 3^{cx}	804 ± 55^{abx}	$1{,}002 \pm 10^{ax}$	$1{,}002 \pm 7^{ay}$	807 ± 25^{abz}	707 ± 12^{bx}	723 ± 8^{bx}	634 ± 8^{cxy}
Barley straw	277 ± 3^{ey}	367 ± 1^{dy}	481 ± 29^{cy}	377 ± 18^{bz}	883 ± 19^{ayz}	-	-	-
With urea								
Maize meal	492 ± 7^{bx}	-	-	-	$1{,}068 \pm 42^{ax}$	-	-	529 ± 5^{bxy}
Barley meal	542 ± 4^{cx}	-	-	-	833 ± 10^{ayz}	-	-	660 ± 18^{bx}
Barley straw	251 ± 26^{by}	-	-	-	732 ± 31^{az}	-	-	-

[a-e]Values within a line by substrate type with different superscripts differ ($P < 0.05$). [x-z]Values within a column with different superscripts differ ($P < 0.05$).

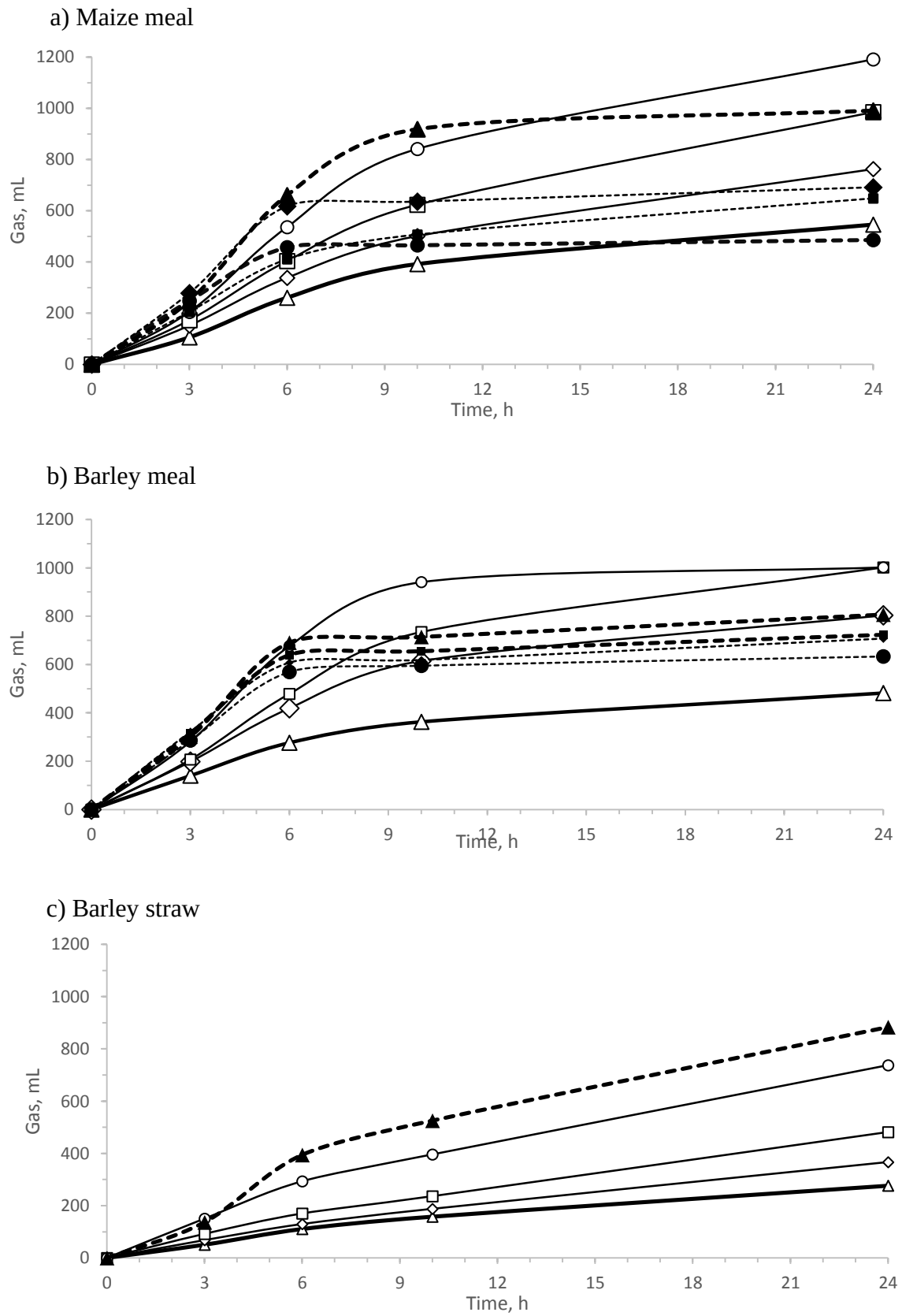


Figure 9. Total volume of gas produced *in vitro* at different incubation times, according to the type (a, maize grain; b, barley grain; c, barley straw) and the quantity of substrate used: <10 g/100 mL (-Δ-, 0.5; -◇-, 1.0; -□-, 2.0; -○-, 5.0) and >10 g/100 mL (--▲--, 10.0; --♦--, 20.0; --■--, 30.0; --●--, 40.0). The 0.5, 10.0 and 40.0 are stressed in bold for comparisons.

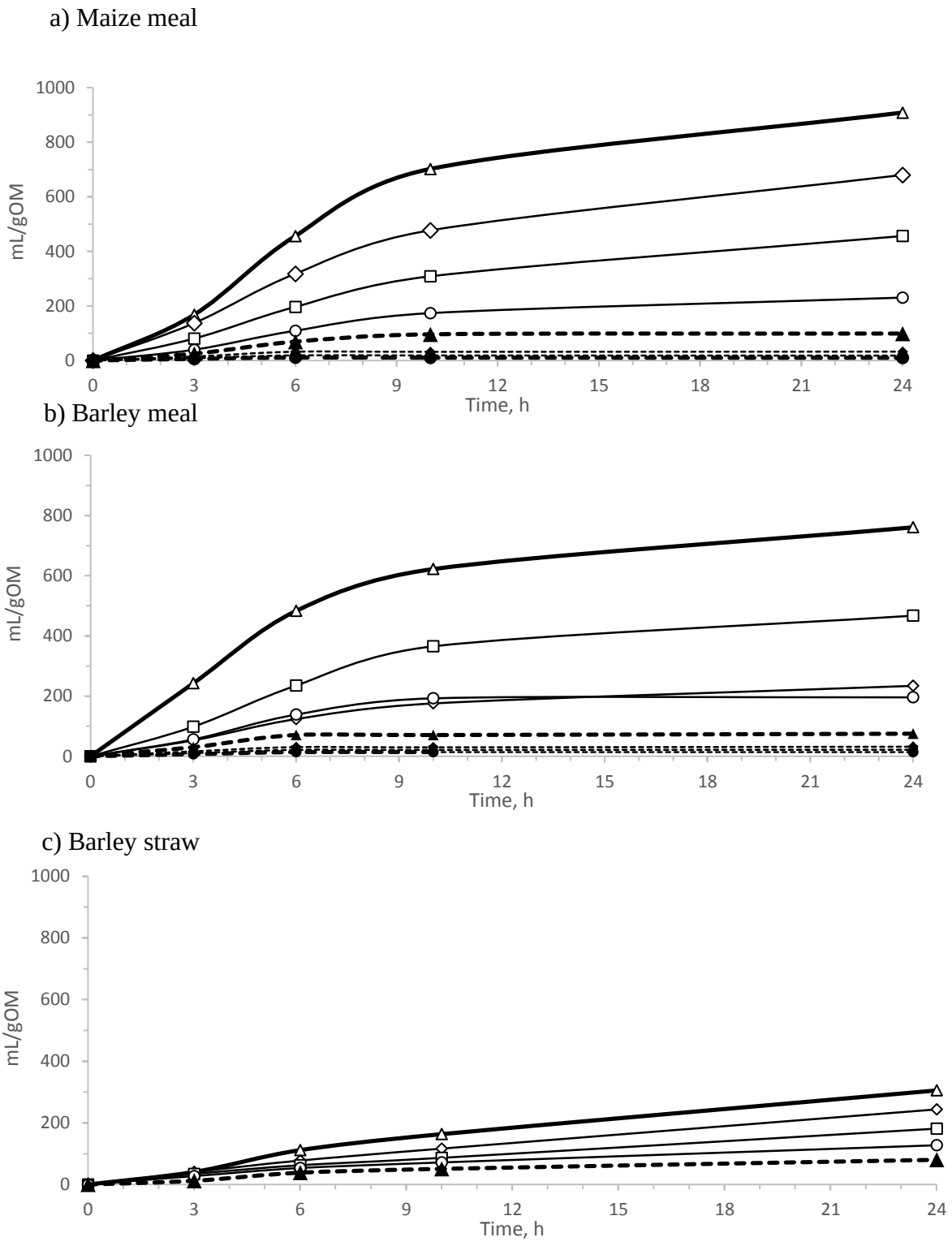


Figure 10. Gas production yield (mL/g OM) *in vitro* at different incubation times, according to the type (a, maize meal; b, barley meal; c, barley straw) and the quantity of substrate used: <10 g/100 mL (-Δ-, 0.5; -◇-, 1.0; -□-, 2.0; -○-, 5.0) and >10 g/100 mL (--▲--, 10.0; --♦--, 20.0; --■--, 30.0; --●--, 40.0). The 0.5, 10.0 and 40.0 are stressed in bold for comparisons.

Nevertheless, this response to the addition of urea may be the result of changes in the microbiota species of the culture that reduced or increased the role of methanogens and increased or decreased, respectively, the single-cell protein production, which was the main objective of this project. This aspect will be studied in the next experiments

Again, the increase of the quantity of substrate used (from 0.5 to 40 g/100 mL), which intended to increase the amount of microbial protein produced by fermentation batch (i.e., assuming that the limiting factor in practice would be the volume of the fermenters), dramatically reduced the gas production yield ($P < 0.001$) per g OM.

Values of Table 8 showed decreases of gas yield of 74-91% when the substrate increased from 0.5 to 10 g/100 mL, and of 98-99% when increased from 0.5 to 40 g/100 mL. These results confirm those of Experiment 1.1 and stated out that the opportunities of producing protein and gas (for co-generation of energy) seem to be very low, and both processes incompatible under ruminal condition conditions.

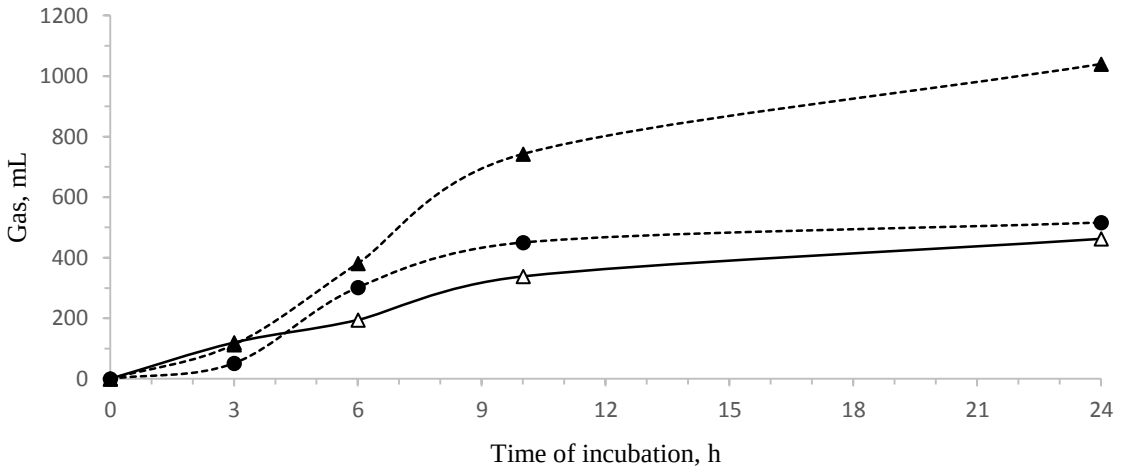
5.3.3. Pattern of incubation pH

At the start of incubation, the inoculum pH was 6.85 (buffer pH), but it dropped rapidly during the incubation in a direct relation with the quantity of substrate added. In the case of the maize and barley meals, the pH of the fermentation media after 24 h dropped below 4.0 when the substrate concentrations were higher or equal to 5 g/100 mL. For 10 g/100 mL of substrate, the decrease of pH of the barley straw was lower, the minimum value being pH = 5.79, while it dropped to pH = 4.07 and pH = 3.95 in maize and barley meals, respectively ($P < 0.01$).

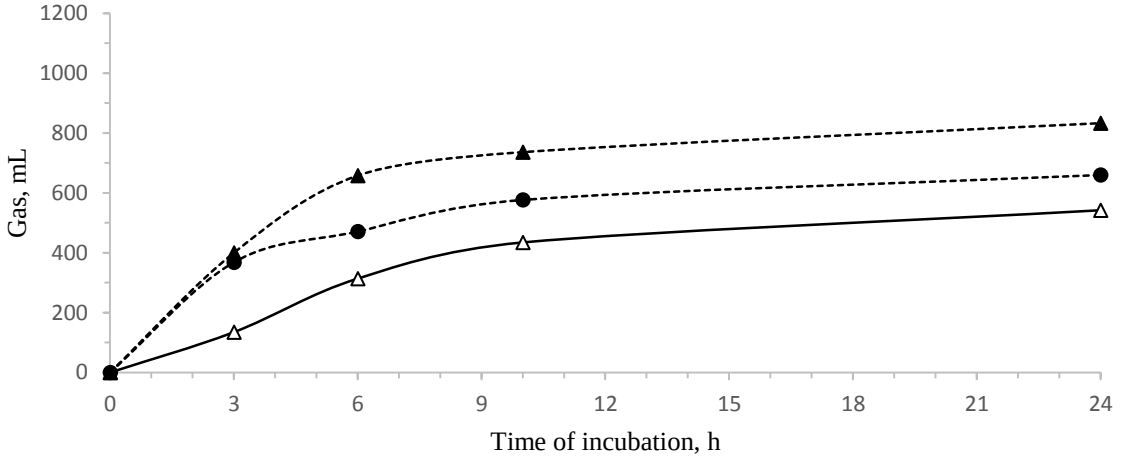
These results evidenced the incapacity of the McDougall saliva to be buffer the rumen media for concentrations of substrate greater than 2 g/100 mL. Moreover it should be taken into account that methanogens is seriously compromised when pH < 6.0 as indicated in ruminant nutrition, resulting in a high accumulation of VFA which will stop the fermentation (i.e., lactic). Van Kessel and Russell (1996) and Hook et al. (2011) reported that methanogenesis is markedly pH-dependent, and it was found that there was no methane production at pH values less than 6.0 and that volatile fatty acids were causing the pH-dependent inhibition, as above indicated.

The addition of urea (alkalinizing material) reduced the drop of pH in the case of maize meal ($P = 0.039$), while it did not have effect on barley meal and barley straw ($P > 0.05$) as shown in **Figures 11** and **12**.

a) Maize meal with urea



b) Barley meal with urea



c) Barley straw with urea

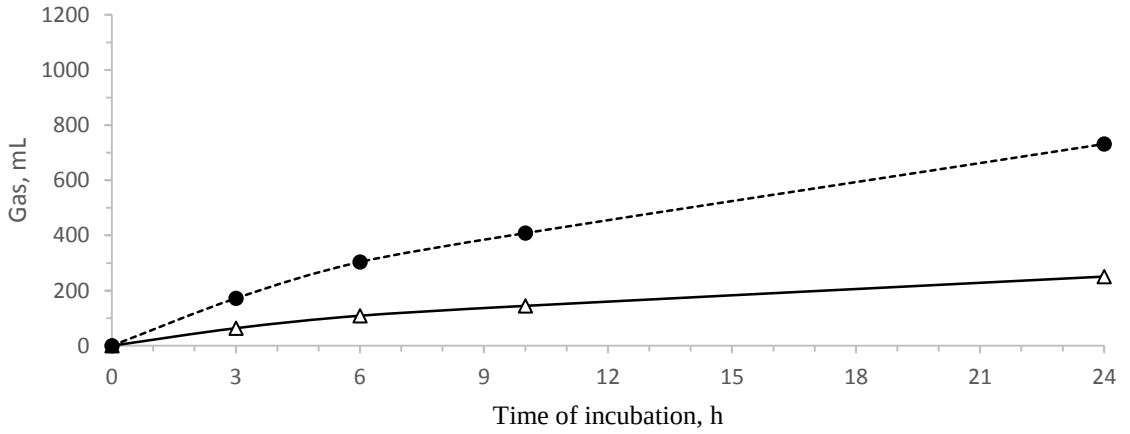
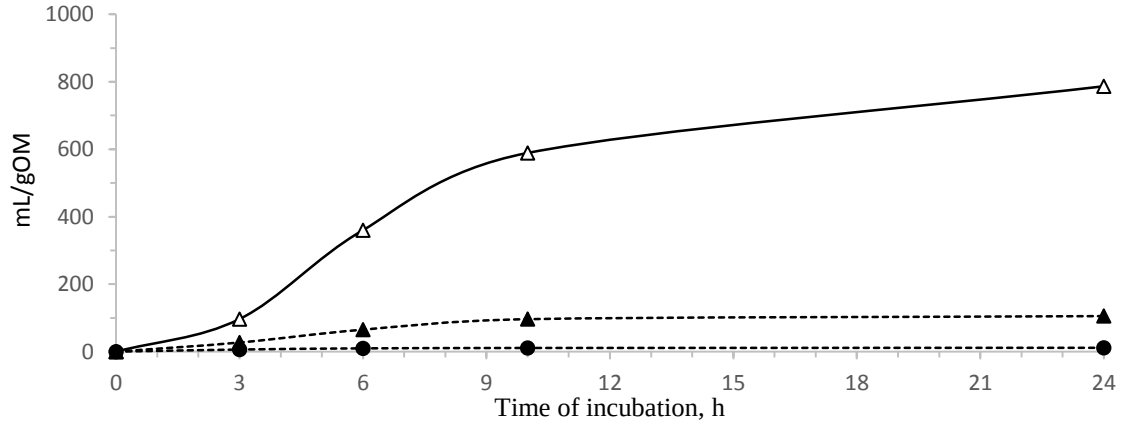
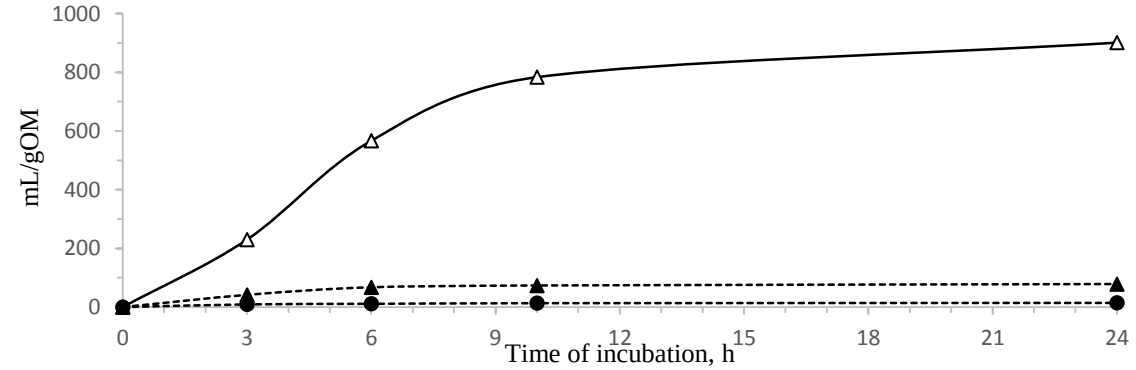


Figure 11. Total volume of gas produced *in vitro* mL at different incubation times with urea, according to the type (a, maize meal; b, barley meal; c, barley straw) and the quantity of substrate used: <10 g/100 mL (-Δ-, 0.5;) and >10 g/100 mL (--▲--, 10.0; --●--, 40.0).

a) Maize meal with urea



a) Barley meal with urea



a) Barley straw with urea

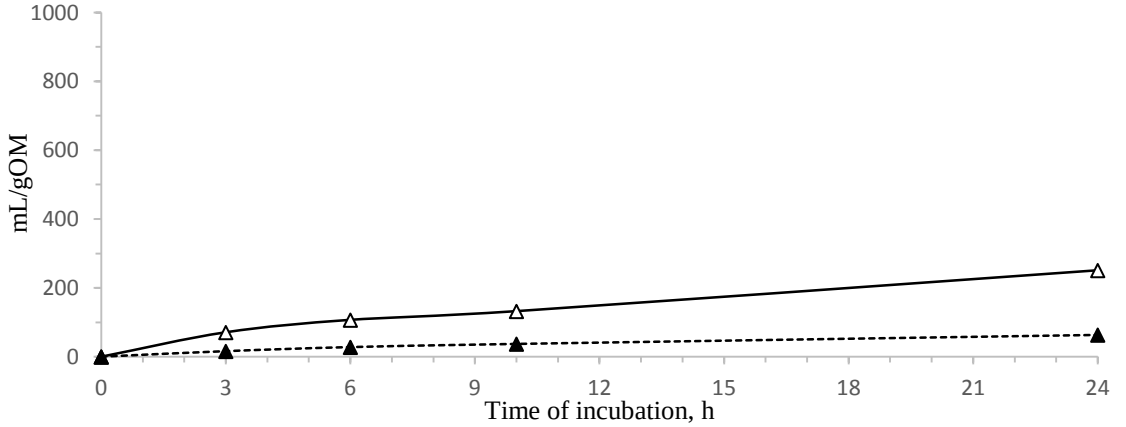


Figure 12. Gas production yield (mL/g OM) *in vitro* at different incubation times with urea, according to the type (a, maize grain; b, barley grain; c, barley straw) and the quantity of substrate used: <10 g/100 mL (-Δ-, 0.5;) and >10 g/100 mL (--▲--, 10.0; --●--, 40.0).

Table 9. Gas production yield (mL/g OM) *in vitro* at 24 h, according to the type and the quantity of substrate used (mean ± SE).

Substrate	Quantity of substrate (g/100 mL)							
type	0.5	1	2	5	10	20	30	40
Without urea								
Maize meal	908 ± 14^{ax}	680 ± 9^{bx}	457 ± 9^{cx}	231 ± 2^{dx}	99 ± 0^{exy}	32 ± 1^{ex}	18 ± 1^{ex}	10 ± 1^{ey}
Barley meal	761 ± 6^{ax}	342 ± 33^{ax}	468 ± 5^{bx}	195 ± 1^{cy}	75 ± 2^{dy}	32 ± 1^{dx}	22 ± 1^{dx}	14 ± 1^{dxy}
Barley straw	306 ± 6^{ay}	244 ± 1^{by}	181 ± 13^{cy}	128 ± 3^{dz}	80 ± 1^{ey}	-	-	-
With urea								
Maize meal	787 ± 14^{ax}	-	-	-	106 ± 4^{bx}	-	-	11 ± 1^{cxy}
Barley meal	901 ± 6^{ax}	-	-	-	78 ± 1^{by}	-	-	15 ± 1^{bx}
Barley straw	251 ± 49^{ay}	-	-	-	64 ± 3^{ay}	-	-	-

$^{a\text{-}e}$Values within a line by substrate type with different superscripts differ ($P < 0.05$). $^{x\text{-}z}$Values within a column with different superscripts differ ($P < 0.05$).

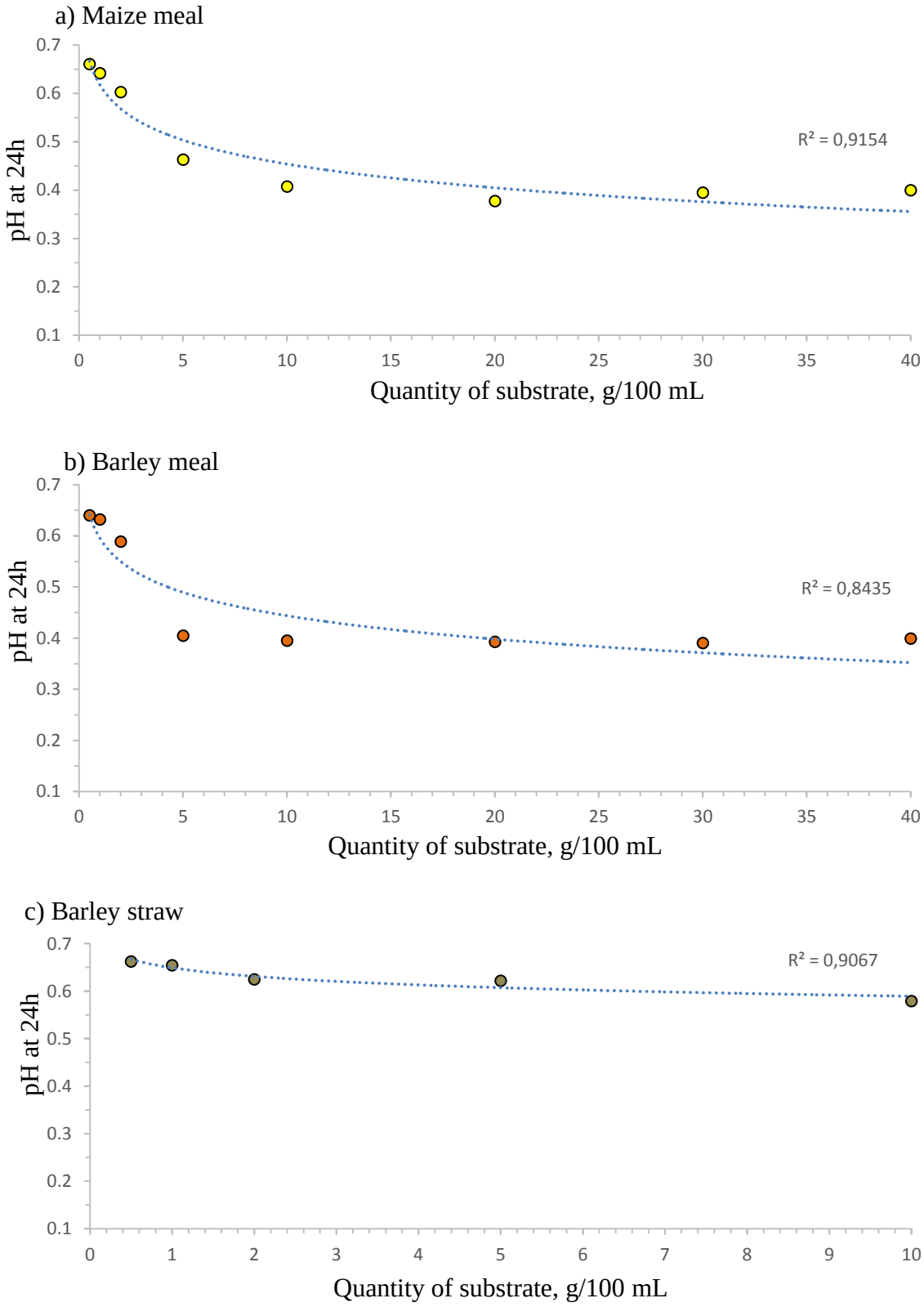


Figure 13. The pH of the media at 24 h of incubation according to quantity of substrate (a, maize meal; b, barley meal; c, barley straw).

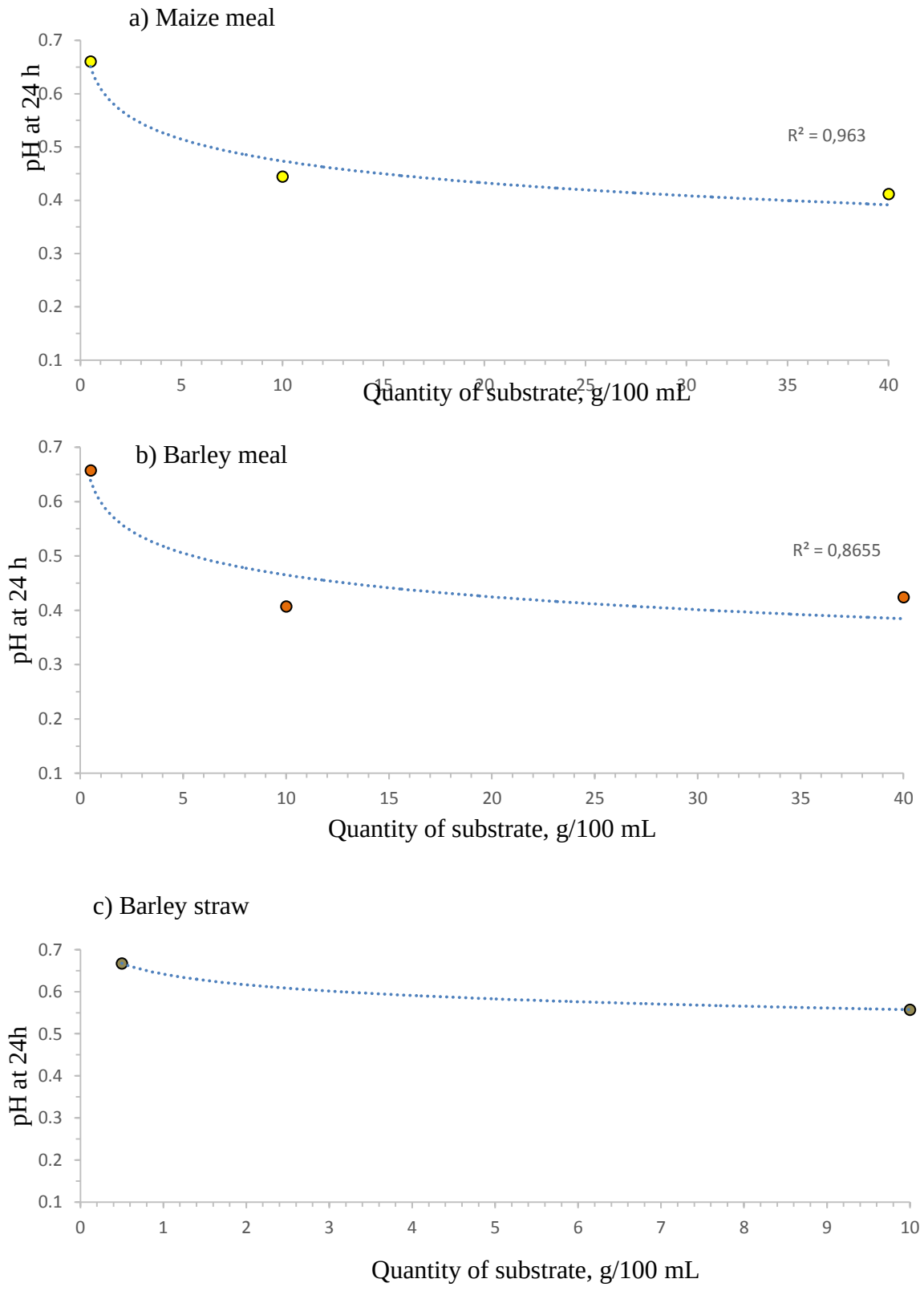


Figure 14. The pH of the media at 24 h of incubation according to quantity of substrate when urea was added (a, maize meal; b, barley meal; c, barley straw).

As shown in **Figures 13** and **14**, the drop of pH depended logarithmically on the concentration of substrate used without (R^2 = 0.84 to 0.92; $P < 0.01$) or with the addition of urea (R^2 = 0.87 to 0.96; $P < 0.01$) and most probably was the limiting factor for both gas and protein production under the rumen fermentation conditions used.

According to the obtained results, the use of a substrate concentration greater than 10 g/100 mL clearly over passed the buffering potential of the media and limited the microbiota growth and consequently was discarded for the following experiments. At the same time, longer incubation times were considered necessary to know the mid-term evolution of the fermentation process.

5.4. Experiment 2: Optimized substrate to inoculum ratio and addition of 0.25 g urea/L

5.4.1. *In vitro* gas production

The chosen substrate concentration of 7 g/100 mL of media produced a similar gas yield to the previous experiment 1 for the maize meal, used as a reference, as shown in **Figure 15**.

Moreover, gas production steadied after 8 h because of the acidification of the media, as shown in **Table 10**. No differences in the gas yield of maize meal were observed after 24 h of fermentation ($P > 0.05$).

Fermentation was slower, but reached a higher gas production in the case of the maize stover and barley straw, as shown in Figure 15. The fibrous substrates took longer (78 h) to reach the maximum yield of gas that were greater than for the maize meal ($P < 0.001$). Ali Shah et al. (2014) reported that cellulose rich substrates need more time to carry out the depolymerization and then the solubilization of the cellulose polymers as the first step in the anaerobic digestion process. On the other hand, wood showed a reduced gas production, which was due to the incapacity of ruminal microflora to ferment its lignocellulosic biomass.

The drop in maize pH was significant ($P < 0.05$; Table 9) since the first hours of incubation. It dropped from 6.85 to 5.42 at 6 h of incubation, while for barley straw and maize stover the pH drop was significant ($P < 0.001$) only after 24 h of incubation.

In the case of maize stover and wood, the pH increased in the first 6 h of incubation, and then dropped. Moreover, the pH in blanks bottles increased in the 6 first hours than it stayed stable, as the changes were not statistically significant ($P > 0.05$) during the rest of the incubation

time. At 144 h of incubation all the incubated substrates had a pH values under 6 except for the wood.

Russell and Dombrowski (1980) studied the effect of pH on rumen microorganisms in continuous culture and they observed a direct relationship between pH and gas yield, due to a change in fermentation products, among other reasons. They indicated also that protein yields were influenced by the uncouplement of microbiota growth at low pH. Energetic uncoupling, or "energy spilling", is basically a distraction of energy from growth to defense mechanisms, and as the extracellular pH becomes acidic, more energy is needed to expel protons.

The different gas pattern curves and pH results observed for concentrate and fibrous materials evidenced their evolution of the inoculum to different microbiota communities according to the substrate used.

Maize meal showed the highest digestibility which was reached at 24 h. Extending the fermentation to 48 h did not increase the digestibility ($P > 0.05$), similarly as previously indicated for gas yield. For the rest of substrates, the digestibility improved at 48 h, compared with this latter at 24 h ($P < 0.05$). However, their values were very low, as expected for the fibrous substrates (**Table 11**).

Table 10. The pH values of fermented substrates during the incubation

Substrate	0 h	6 h	10 h	24 h	48 h	144 h
Maize meal	6.85^{a}	5.42 ± 0.2^{b}	5.14 ± 0.1^{c}	4.63 ± 0.0^{d}	4.38 ± 0.1^{e}	-
Barley straw	6.85^{ab}	6.83 ± 0.0^{b}	6.89 ± 0.0^{a}	6.66 ± 0.1^{c}	6.25 ± 0.0^{d}	5.66 ± 0.0^{e}
Maize stover	6.85^{b}	7.03 ± 0.0^{a}	6.99 ± 0.0^{a}	6.84 ± 0.1^{b}	6.44 ± 0.1^{c}	5.71 ± 0.0^{d}
Wood	6.85^{c}	7.03 ± 0.0^{b}	7.11 ± 0.1^{a}	6.99 ± 0.0^{b}	6.98 ± 0.1^{b}	6.97 ± 0.0^{b}
Blanks bottles	6.85^{b}	7.30 ± 0.1^{a}	7.34 ± 0.1^{a}	7.34 ± 0.1^{a}	7.41 ± 0.0^{a}	7.40 ± 0.0^{a}

$^{a-d}$Values within same substrate type with different superscript differ ($P < 0.05$).

5.4.2 Digestibility of organic matter and protein production

The digestibility of all substrates improved, compared to unfermented substrates, by effect of the rumen fermentation.

This increase may be due to the fact that maize meal and maize silage fermentation led to changes in the endosperm protein fractions and this makes starch more accessible to the digestive enzymes (Khetarpaul and Chauhan, 1990).

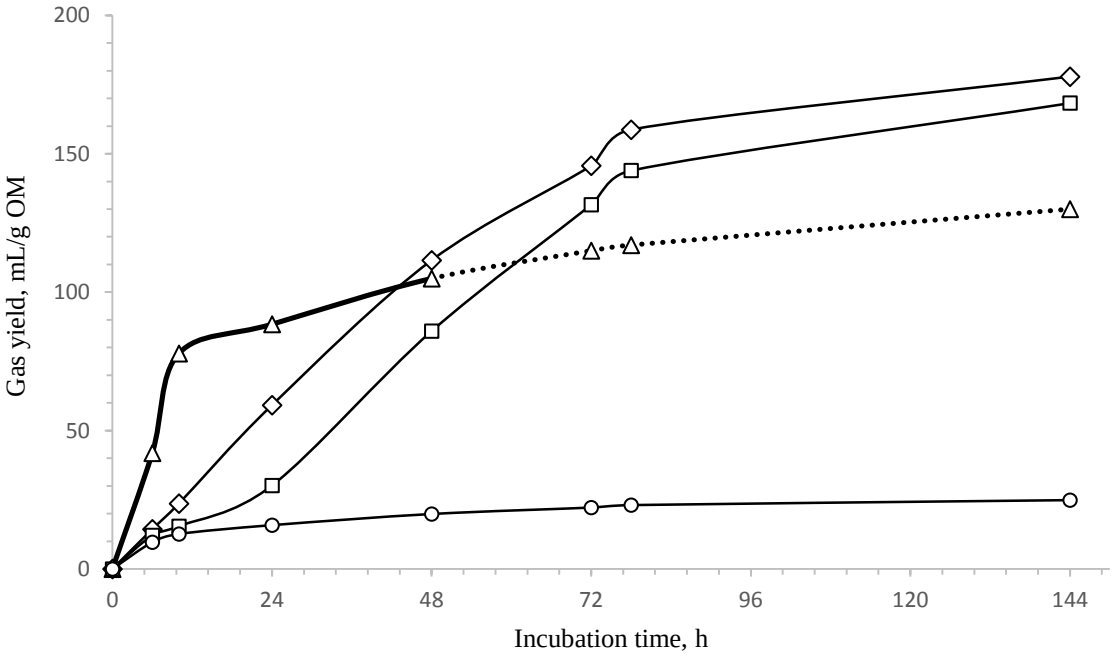


Figure 15. Gas yield (mL/g OM) produced under rumen *in vitro* conditions at different incubation times, according to the substrate: -Δ-: maize meal; -◇-: barley straw; -□-: maize stover; -○-: wood) when 7 g substrate/100 mL and low urea Due to the sacrifice of all 3 maize bottles of fermentation at 48 h, there was no data of maize gas yield from 48 h to 144 h. Hence it was presented in a discontinued line as an estimation of the gas yield based on the results of maize meal gas yield in the experiment 3.

As shown in **Table 12**, the CP content of maize increased by 40% after 24 h of incubation with a digestibility of 88% of the fermented products. While for the maize stover and barley straw the increment was more important at 48 h, which agreed with the results of gas production results.

Despite the 384% of increment in wood CP from 0.3 to 1.2, the digestibility was very low and did not exceed the 25%. Villas-Bôas et al (2002) reported that in case of the bioconversion of lignocellulose into protein-rich animal feed, the fermented product has a lower digestibility than the unfermented lignocellulose.

Table 11. Organic matter digestibility (dOM) of substrates after 24 and 48 h of incubation using the two-stages method of Tilley-Terry modified.

		dOM, %		
Substrate	h	First stage[1]	Second stage[2]	Total digestibility
Maize meal	24	36.4 ± 1.1	38.2 ± 0.2	74.6 ± 0.9[a] (97.5%)
	48	38.7 ± 2.4	37.8 ± 0.3	76.5 ± 2.8[a] (100%)
Barley straw	24	9.1 ± 1.4	8.0 ± 0.2	17.1 ± 1.2[d] (69.8%)
	48	15.5 ± 0.1	9.0 ± 0.2	24.5 ± 0.3[c] (100%)
Maize stover	24	3.6 ± 0.1	8.3 ± 0.3	11.9 ± 0.5[f] (55.9%)
	48	12.1 ± 0.5	9.2 ± 0.3	21.3 ± 0.8[e] (100%)
Wood	24	−0.2 ± 0.1	0.5 ± 0.2	0.3 ± 0.2[g] (14.3%)
	48	0.3 ± 0.3	1.8 ± 0.4	2.1 ± 0.7[g] (100%)

[1]Rumen fermentation; [2]Pepsine-HCl digestion; [a-g]Values within a column and same substrate type with different superscript differ ($P < 0.05$).

Table 12. Crude protein[1] (CP) content and protein digestibility (dCP) changes of substrates after incubation under rumen conditions.

Substrate	h	CP, g/100g DM	Change, %	Digestible CP[2]	Change, %	dCP, %
Maize meal	0	7.4 ± 0.2	-	4.9 ± 0.2	-	66.3[3]
	24	10.4 ± 0.0	40	9.1 ± 0.0	86	87.5
	48	10.1 ± 0.1	37	8.9 ± 0.1	82	88.1
Barley straw	0	3.7 ± 0.1	-	0.8 ± 0.1	-	22.8[3]
	24	4.0 ± 0.1	9	1.5 ± 0.1	88	37.5
	48	5.6 ± 0.4	52	2.6 ± 0.4	225	46.4
Maize stover	0	4.4 ± 0.2	-	0.9 ± 0.1	-	20.0[3]
	24	4.6 ± 0.1	3	0.9 ± 0.1	0	20.0
	48	5.6 ± 0.1	34	1.8 ± 0.1	100	32.1
Wood	0	0.3 ± 0.0	-	-	-	-
	24	1.2 ± 0.0	384	0.3 ± 0.2	-	25.0
	48	1.1 ± 0.0	348	0.2 ± 0.2	-	18.2

[1]CP = N × 6.25; [2]After the second stage digestion of Tilley and Terry; [3] Source: Feedipedia

As a general conclusion from the experiment 2, maize meal showed the higher rate of microbial production but the high drop of pH might be a limiting factor for fermentation and thus for the microbial activity and protein production. For those reasons it was decided to

include the maize silage as substrate in the next experiment as it contain all the components of the maize plant as the rapidly fermentable (i.e., grain) and the fibrous (i.e., footage) carbohydrates which may help to control the pH drop and increase the gas production.

5.5. Experiment 3: Optimized substrate to inoculum ratio corrected with urea

5.5.1. *In vitro* gas production and pH pattern

As shown in **Figure 16**, maize silage behaved similarly to maize meal and shared almost the same slopes and general shape of the gas production curves. While the maize stover and barley straw had slower rate of fermentation due to the need of longer time to achieve the fermentation in the fibrous substrates. Maize stover had lower gas production than barley straw likely due to its higher CP content. Getchew et al. (2004) reported the negative correlation between the CP content of feeds and *in vitro* gas production. The wood had a little gas production as there was no important fermentation of lignocellulosic biomass.

In general, lower gas production was recorded with the addition of 2.5 g/L of urea comparing to the experiment 2; likely due to the increased ammonia accumulation from urea break down in the environment and it inhibited the methanogenic activity.

The pH results shown in **Table 13** were not very different from the previous experiment; maize meal pH dropped rapidly to reach 4.43 at 72 h. Maize silage pH also dropped strongly but less than maize meal to reach 5.30 at 72 h. At 10 h of incubation, the pH values of maize meal and maize silage were already low, 5.24 and 5.85 respectively, meaning that more time of incubation can be unnecessary.

The pH values of maize stover and barley straw augmented at 6 h and then begun to drop, while the pH values of the wood stayed above 7.0 during all the incubation, which means that the low gas production was not due to the pH limiting factors.

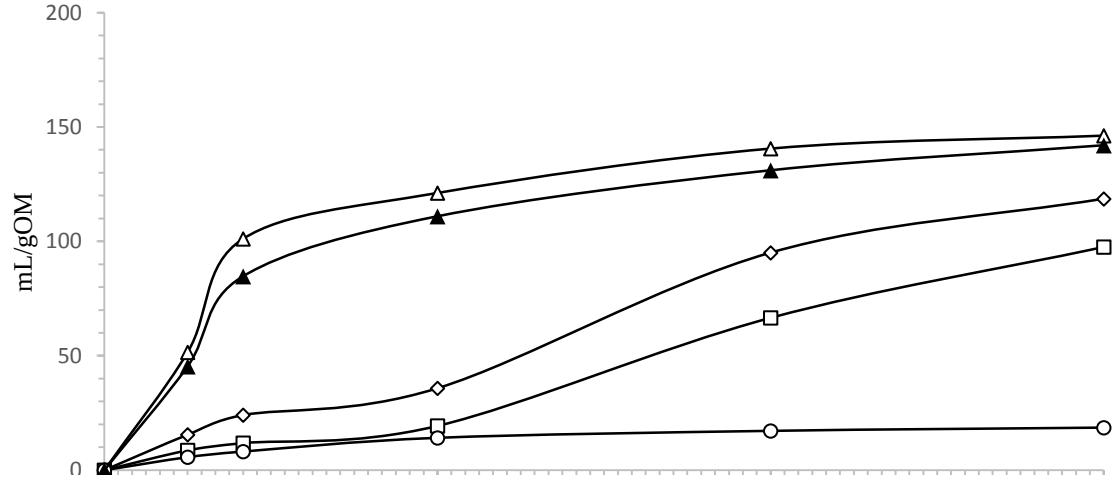


Figure 16. Gas yield (mL/g OM) produced under rumen *in vitro* conditions at different incubation times, according to the substrates: -Δ-: maize meal; -◇-: barley straw; -□-: maize stover; -○-: wood; -▲-: maize silage) when 7 g substrate/100 mL and urea correction were used.

5.5.2 Digestibility of organic matter and protein production

Total maize meal organic matter digestibility was not different between 24 and 72 hours of incubation, while barley straw, maize stover, wood and maize silage digestibility obviously increased ($P < 0.001$) at 72 h. OM digestibility of maize was comparable to the INRA values, 88.5%. The wood showed negative values of digestibility and it improved from -9.4 at 24 to -1.5 at 72 h.

Table 13. The pH pattern of fermented substrates during the incubation

Substrate	0	6	10	24	72
Maize meal	6.85^{a}	6.21 ± 0.2^{b}	5.24 ± 0.1^{c}	4.65 ± 0.1^{d}	4.34 ± 0.0^{e}
Barley straw	6.85^{b}	7.01 ± 0.0^{a}	6.97 ± 0.1^{a}	6.68 ± 0.0^{c}	5.97 ± 0.1^{d}
Maize stover	6.85^{c}	7.17 ± 0.0^{a}	7.11 ± 0.1^{a}	6.99 ± 0.1^{b}	6.57 ± 0.1^{d}
Wood	6.85^{d}	7.24 ± 0.0^{a}	7.16 ± 0.0^{b}	7.16 ± 0.1^{b}	7.07 ± 0.0^{c}
Maize silage	6.85^{a}	6.36 ± 0.0^{b}	5.85 ± 0.1^{c}	5.38 ± 0.0^{d}	5.30 ± 0.0^{e}
Blanks	6.85^{e}	7.61 ± 0.0^{a}	7.58 ± 0.0^{a}	7.52 ± 0.0^{b}	7.44 ± 0.0^{c}

$^{a-e}$Values within same substrate type with different superscript differ ($P < 0.05$).

Those negative values are likely explained by the fact that the produced bacteria can be found stuck between the small pores of wood structures. As McMillan (1994) demonstrated,

pretreatment of lignocellulosic biomass seems to be necessary to improve the bioconversion of wood.

The CP values of incubated substrates are shown in **Table 15**. The results show an increase in CP content compared with the previous experiment which is justified by the supplementation with the urea that boosted the bacterial production (Chamberlain and Thomas, 1980).

An increase in CP content of fermented maize from 7.4%, to 15.7% from which 12.5% are digestible was recorded. Regarding the other fermented substrates, the CP digestibility was low (51.6% for barley straw, 35.1 % for maize stover and 55.1 % for maize silage) but in general it was improved compared to unfermented substrates.

As an implication, the bioconversion of maize into protein-rich animal feed can decrease the percentage of soybean in a 15% CP diet from 21% to 2%.

The improvement of protein content of maize can be an interesting alternative to the high use of soybean in animal feed.

Table 14. Organic matter digestibility (OMD) of substrates after 24 and 72 h of incubation using the two-stage method of Tilley-Terry modified.

		dOM, %		
Substrate	h	First stage[1]	Second stage[2]	Total digestibility
Maize meal	24	48.0 ± 0.2	41.4 ± 0.6	89.4 ± 0.8[a]
	72	48.0 ± 2.0	42.4 ± 0.4	90.5 ± 2.5[a]
Barley straw	24	1.1 ± 1.0	16 ± 0.2	17.1 ± 1.2[d]
	72	13.7 ± 0.4	17.7 ± 1.1	31.4 ± 0.7[c]
Maize stover	24	-6.2 ± 1.2	14.9 ± 0.9	8.6 ± 2.1[f]
	72	2.6 ± 0.2	18.2 ± 0.3	20.8 ± 0.1[e]
Wood	24	-15.1 ± 1.8	5.6 ± 0	-9.4 ± 1.7[h]
	72	-8.9 ± 0.2	7.4 ± 0.2	-1.5 ± 0[g]
Maize silage	24	37.4 ± 0.4	26.3 ± 0.6	63.7 ± 0.2[j]
	72	44.4 ± 0.5	24.7 ± 0.5	69.1 ± 0[i]

[1]Rumen fermentation; [2]Pepsine-HCl digestion; [a-j]Values within a column and same substrate type with different superscript differ ($P < 0.05$).

The costs of CP of the different raw materials and from the fermented products at 24 h and 72 h were calculated and compared with the price of 1 kg of soybeans CP using the current market prices (**Table 16** and **18**). To estimate the final balance of the difference between soybean and SCP from each substrate, the price of gas was also taken into account.

The gas price was estimated using the market price of 2.6 €/MBtu. From the total gas production, only the CH_4 production was considered as valuable, and it was estimated to be 27% of total gas production (Sniffen and Herdt, 1991). To get the MBtu values, the production in cubic meters was converted to cubic feet, by multiplying by 35.3147. Then it was multiplied by 1,037 to covert to Btu. Finally it was divided by 1,000,000 to obtain the unit in MBtu.

The value of gas for each substrate depended on the quantity of substrate needed to produce 1 kg of CP or PDI and on the total gas production of each substrate.

The positive values shown in tables 16, 17, 18 and 19 mean that the process was more cost-effective than soybean. In the case of CP, the substrates that gave positive balance were the barley straw, the maize stover and maize silage. Maize grain showed negative results due to high price of 1 kg of CP of maize when compared to soybean.

The quantity of wood pellets to produce 1 kg of PDI were very high because of the low digestibility of the CP (13 % at 24 h and 20.8 % at 72 h).

In addition, the costs of producing 1 kg of PDI at 24 h and 72 h were calculated and they are shown in Table 16 and Table 18, respectively. Values were in favor of the soybean, except for the the case of barley straw at 72 h where a positive balance of +0.18 € was estimated. The PDI values were considered to be more reliable than CP values.

It appears that with the current prices of the market, cereal straws can be an interesting substrate to produce SCP, starting from 72 h of incubation. As shown in the **Figure 16**, in order to obtain interesting prices for the produced protein, the prices of barley straw should be between 0.03 and 0.06 €/kg, when compared to prices of 0.3 to 0.5 €/kg for soybean meal cake.

Table 15. Crude protein[1] (CP) content and protein digestibility of incubated substrates from fermentation in substrates after 24 and 72 h.

Substrate	h	CP, g/100g DM	Change, %	Digestible CP[2]	Change, %	dCP, %
Maize meal	0	7.4 ± 0.2	-	4.9 ± 0.2	-	66.3[3]
	24	16.9 ± 0.8	129	12.4 ± 0.8	153	73.4
	72	15.7 ± 0.1	112	12.1 ± 0.2	147	77.1
Barley straw	0	3.7 ± 0.2	-	0.8 ± 0.2	-	22.8[3]
	24	7.1 ± 0.1	93	3.3 ± 0.1	312	46.5
	72	9.3 ± 0.2	152	4.8 ± 0.2	500	51.6
Maize stover	0	4.4 ± 0.1	-	0.9 ± 0.1	-	20.0[3]
	24	6.1 ± 0.0	37	1.2 ± 0.0	33	19.6
	72	7.4 ± 0.2	66	2.6 ± 0.2	188	35.1
Wood	0	0.3 ± 0.2	-	-	-	-
	24	2.3 ± 0.2	804	0.3 ± 0.2	-	13.0
	72	2.4 ± 0.0	876	0.5 ± 0.1	-	20.8
Maize silage	0	6.6 ± 0.0	-	4.2 ± 0.0	-	64.0[3]
	24	12.1 ± 0.0	84	6.3 ± 0.1	50	52.1
	72	13.8 ± 0.0	109	7.6 ± 0.0	81	55.1

[1]CP = N × 6.25; [2]After the second stage digestion of Tilley and Terry, [3] Source: Feedipedia

Table 16. Cost of CP produced at 24 h of fermentation of different substrates used compared to soybean.

Raw material	€/kg CP without fermentation	€/kg CP at 24h	Difference with soybean/kg CP	Price of gas	Balance
Maize	2.60	1.91	-0.76	0.04	-0.72
Barley straw	1.48	0.72	0.43	0.02	**0.46**
Maize stover	1.81	1.08	0.07	0.01	**0.08**
Maize silage	3.03	1.00	0.15	0.04	**0.19**
Wood pellets	46.17	4.42	-3.26	0.14	-3.12
Soybean 46	1.16	1.16	0.00	-	-

Table 17. Cost of PDI produced at 24 h of fermentation of different substrates used compared to soybean.

Raw material	€/kg PDI at 24 h	Difference with soybean/kg PDI	Price of gas	Balance
Maize	2.61	-1.23	0.04	-1.19
Barley straw	1.55	-0.17	0.02	-0.15
Maize stover	5.51	-4.13	0.01	-4.12
Maize silage	1.93	-0.55	0.04	-0.51
Wood pellets	33.86	-32.48	0.14	-32.34
Soybean 46	1.38	0	-	-

Table 18. Cost of CP produced at 72 h of fermentation of different substrates used compared to soybean.

Raw material	€/kg CP without fermentation	€/kg CP	Difference with soybean/kg CP	Price of gas	Balance
Maize	2.60	2.17	-1.02	0.05	-0.97
Barley straw	1.48	0.66	0.50	0.07	**0.57**
Maize stover	1.81	1.03	0.12	0.05	**0.17**
Maize silage	3.03	0.99	0.16	0.05	**0.21**
Wood pellets	46.17	4.39	-3.23	0.19	-3.05
Soybean 46	1.16	1.61	0	-	-

Table 19. Cost of PDI produced at 72 h of fermentation of different substrates used compared to soybean.

Raw material	€/kg PDI at 72 h	Difference with Soybean/kg PDI	Price of gas	Balance
Maize	2.82	-1.44	0.05	-1.39
Barley straw	1.27	0.11	0.07	**0.18**
Maize stover	2.94	-1.57	0.05	-1.52
Maize silage	1.81	-0.43	0.05	-0.38
Wood pellets	21.06	-19.68	0.19	-19.49
Soybean 46	1.38	0.00	-	-

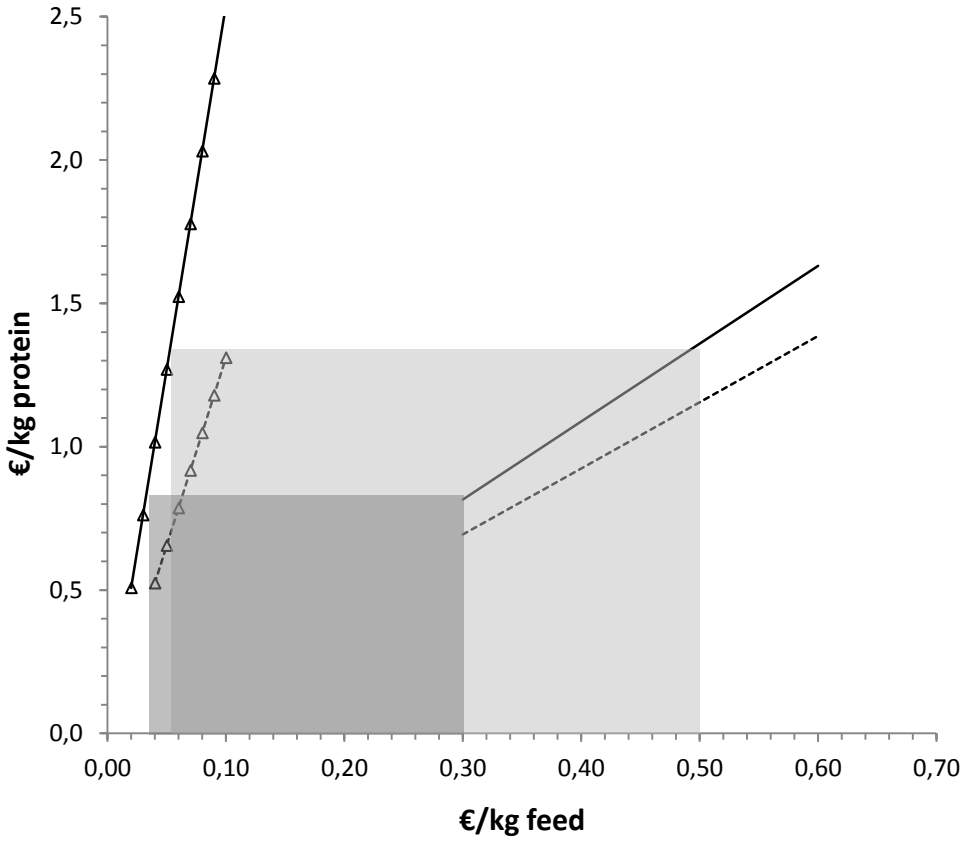


Figure 17. The cost of protein according to the prices of barley straw and soybean meal cake (-Δ-, cost of SCP from barley straw; --Δ--, cost of barley straw's crude protein; -, cost of soybean digestible protein; --, cost of soybean crude protein)

6. CONCLUSIONS

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The results of the present study showed the fermentative potential of some agri-food by-products for SCP production as well as for biogas production. Taking into account the cost-effectiveness of the process, the following conclusions were obtained:

First experiment: the fermentation and gas production levels of different substrates with different quantities:

- The increase of the quantity of substrate used (from 0.5 to 40 g/100 mL), reduced the gas production yield per g OM.
- Addition of urea decreased the gas production
- The greatest gas production was observed between 5 and 10 g/100 mL of substrate per inoculum and maize meal was the substrate which produced the greatest total volume of gas.
- The McDougall saliva was unable to buffer the rumen media for concentrations of substrate greater than 2 g/100 mL.

Second experiment: Optimized substrate to inoculum ratio and addition of urea:

- Maize meal showed the higher rate of microbial production although the high drop of pH recorded may be a limiting factor for fermentation and thus for the microbial activity and protein production. For these reasons it was decided to include the maize silage as substrate in the next experiment as it contain all the components of the maize plant as the rapidly fermentable (i.e., grain) and the fibrous (i.e., forage) carbohydrates which may help to control the pH drop and to increase the gas production.

Experiment 3: Optimized substrate to inoculum ratio corrected with urea:

- The CP content in the final products of fermentation increased, compared with the previous experiment which is justified by the supplementation with urea that boosted bacterial production.
- The process was cost-effective only in the case of barley straw, when the prices were in the range of 0.03 and 0.06 €/kg for barley straw and of 0.3 to 0.5 €/kg to soybean meal.

7. REFERENCE

7. REFERENCES

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