Usage of California Agricultural Byproducts to Reduce Enteric Methane in-vitro

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Abstract

The growing demand for high quality food resulting from the increase in world population has incentivized the agriculture industry to increase their food production. However, large amounts of biomass and byproducts from food production are sent to landfills. Many of these wastes, despite no longer being suitable for human consumption, are often still rich in nutrients and represent valuable alternatives to complement animal feed. Livestock, especially ruminants, are an integral part in agriculture as they can convert plant biomass that cannot be digested by humans into high quality animal products such meat, milk and wool. The ability of ruminant animals to degrade recalcitrant plant fibers is due to the symbiotic relationship with the microorganisms that reside in the rumen and that can digest and convert plant biomass into nutrients and metabolic intermediates that can be further utilized by the host animals. Some of these microorganisms produce greenhouse gases, such as methane (CH4), which is released into the atmosphere where it has detrimental effects on the environment. Many byproducts from agriculture may contain bioactive compounds such as phenolics, organosulfur, terpenoids, and fatty acids that can alter rumen fermentation and even inhibit methanogenesis. California is the largest producer of agricultural products in the United States (U.S.), with a large arsenal of byproducts that could hold the key to improve ruminant nutrition and also have the potential to reduce enteric methane production. As of today, there is still only a limited number of studies that have investigated the potential of California byproducts to reduce enteric fermentation. In the work presented here, a panel of byproducts from California agriculture was subjected to chemical profiling and subsequent in vitro rumen fermentation. In addition to providing first insights into the potential effect of the tested local byproducts this work also provides a

framework and the standard operating procedures that should be employed for future analyses to identify potential methane inhibitor and to enable comparisons of results that will be generated over time and from different researchers and institutes. A standardization of the analytical methods to evaluate rumen response will be key to not only identify novel strategies but also provide the framework to study the molecular drivers of methane inhibition and to optimize future methane mitigation strategies.

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Chapter 1. Literature Review (Background)

1. Global Agriculture Production and Greenhouse Gas

The world human population is projected to reach 8.5 and 9.7 billion by 2030 and 2050, respectively (UN, 2022). This will require an increase in food production to meet the growing demand. China and India are countries with the largest human population, with each having over 1.42 billion people (UN, 2022). Given their population size, both countries are also the largest producers of agricultural products (FAO, 2024). However, food waste is still an issue as a lot of biomass is being generated but not utilized as it is estimated that one-third of food produced for consumption, equivalent to 1.3 billion tons, is wasted globally per year (Ishangulyyev et al., 2019).

There are multiple factors that can lead to food waste. At the producer level, farmers can grow more crops than necessary to ensure that there is enough supply to meet demand in the case of unpredictable events such as weather, pests, or a bad harvest season. This leads to an excess supply of crops that are no longer profitable and are not harvested. Other factors that contribute to food waste include mishandling during processing, diseases, and overripe produce that can damage crops and be deemed unfit for human consumption. At the retail level, crops sold are expected to meet certain criteria, especially visual appearance (FAO, 2011). Many retail stores reject fruits and vegetables that have abnormal appearances even if they are perfectly fit for human consumption. In 2022, over 1 billion tons of food were not consumed and wasted with 60% contributed from the consumer level (UNEP, 2024). However, on all three levels of the food supply chain (FSC), food waste can easily be generated from improper storage, causing rapid spoilage. Due to their high population, China and India are also the largest generators of most food waste at 91.6 and 68.7 million tons a year, respectively (UNEP, 2021). Many crops grown,

such as nuts and fruits are also processed into other products such as peanut butter, oils, or jams that create byproducts such as nut meals, hulls, stems, leaves, and pomace. Byproducts and food waste that are sent to landfills or compost generate up to 4.4 gigatons of carbon dioxide equivalent (CO_{2e}), representing 8% of greenhouse gas emitted from all anthropogenic sources yearly (EPA, 2021). Though these byproducts and food waste are not fit for human consumption, they are still nutritive and can be repurposed into animal feed as a method of reducing food waste and greenhouse gas emissions (EPA, 2023).

1.1 Ruminants

Domesticated livestock are an integral part of agriculture as many high-quality foods come from their production such as eggs, milk, and meat. Ruminants, such as cattle, goats, and sheep have a specialized stomach, breaking down into four different chambers with the largest being the rumen. The rumen is an anaerobic environment that can hold anywhere between 113 to 226 liters of feed materials and fluids dependent on the age and size of the animal (Short, 1964) and hosts a variety of microbes such as bacteria, fungi, archaea, and protozoa. Anaerobic fungi possess carbohydrate-active enzymes such as cellulase that can break down structural carbohydrates (cellulose, hemicellulose, and lignin). Bacteria are the most abundant microbes in the rumen and can contain billions within 1 milliliter (mL) of rumen fluid (Matthews et al., 2019). Each having their own niches, they also contain enzymes that can break down carbohydrates ranging from plant cell walls to simpler carbohydrates like starch. Through their metabolism, they produce byproducts that can be utilized by the host in the form of volatile fatty acids (VFA) (Den Besten et al., 2013). Acetate, propionate, and butyrate are the three major VFA that are metabolized and used by the host as their primary energy source (Dijkstra, 1994). The proportion

of VFA is highly dependent on the feed composition with acetate associated with higher fiber degradation and propionate with starch degradation (Wang et al., 2020). Microbes themselves also become a nutrient for the host as they are the source of microbial crude protein (MCP) when they are digested and absorbed in the lower digestive tract (Stern & Hoover, 1979). Through fermentation and digestion, host animals can utilize the VFA and MCP to promote growth, reproduction, and create products such as milk and meat (Seymour et al., 2005). This symbiotic relationship between ruminants and microbes allows them to access a variety of plant-based feed sources without competing with humans.

Aside from VFA and MCP, fermentation also produces other byproducts such as CO_2 and hydrogen (H_2). It is crucial that the H_2 formed from fermentation does not go unregulated as too much will cause the rumen environment pH to decrease (Krause & Oetzel, 2006). Many microbes in the rumen cannot function and may even die off if the rumen becomes too acidic (Slyter, 1976). This can lead to a decrease in fermentation and depression in growth and production, and in some cases detrimental to the health of the animal (Owens et al., 1998). However, there are microbes in the rumen that can utilize hydrogen as substrates for their own metabolism. The main H_2 utilizers are hydrogenotrophic methanogens by reducing CO_2 with H_2 to form methane (CH_4) gas. Other microbes found in the rumen can also utilize hydrogen that can act as competitors to methanogens such as homoacetogens, nitrate-, sulfate-, and fumarate-reducing bacteria. Homoacetogens produce acetate using both H_2 and CO_2, however, methanogens are more efficient at utilizing H_2 when resources are limited (Mackie & Bryant, 1994). Even though sulfate and nitrate are thermodynamically more efficient, substrates for bacteria to use are limited as the concentrations are low in a ruminant's diet (Mackie et al., 2024). Another issue is

the final products of sulfate and nitrate reduction (sulfide and nitrite) are toxic to animals at higher concentrations. Lastly, fumarate-reduction uses hydrogen to produce succinate and eventually into propionate, which can be utilized by the animal compared to CH_4 (Mamuad et al., 2014).

1.2 Greenhouse Gas Production

The greenhouse gases CH_4 and CO_2 along with nitrous oxide (N_2O), water vapor and fluorinated gases are responsible for trapping radiation emitted from the surface of the planet and preventing its dissipation into the atmosphere. The trapping of radiation leads to an increase in the planet's temperature, causing the "greenhouse effect". Carbon dioxide is the most abundant greenhouse gas emitted globally at 65% followed by CH_4 and N_2O at 16 and 11%, respectively (IPCC, 2023). Even though CH_4 constitutes a smaller relative abundance of greenhouse gas and has a shorter life span (12 years), CH_4 has 28 times the global warming potential of CO_2 over a 100-year period. It is estimated that agriculture globally contributed 5.5 gigatons of carbon dioxide equivalent per year ($GtCO_2eq$/year) with enteric fermentation contributing 2.1 $GtCO_2eq$/year in 2010 (IPCC, 2014). This does not include CO_2 emissions as it is considered neutral from carbon fixation. When plants and crops are grown, they take up the CO_2 in the atmosphere, and are released again when they are degraded, thus not creating a net change in CO_2. Enteric fermentation contributes to 32% of global anthropogenic CH_4 while also representing 2-12% loss of gross energy as the animal cannot use it (Johnson & Johnson, 1995). This leads to a decrease in feed efficiency for the animal and becomes costly for producers.

2. Methods of Methane Mitigation from Byproducts

As forementioned, there are other pathways from carbohydrate fermentation that can act as alternative hydrogen sinks such as acetogens and propionate producers. Feed composition plays a big part in ruminal fermentation, with diets containing higher soluble carbohydrates promotes more propionate production whereas diets with higher fiber produce more CH_4 alongside acetate (Yan et al., 2000). Many plant products also contain bioactive compounds such as plant secondary metabolites (PSM) which contain antimicrobial properties that reduce or inhibit CH_4 production. Plant secondary metabolites contribute to their survival via defense against external threats such as pathogens, grazing herbivores, and environmental conditions. These bioactive compounds are also responsible for the unique aroma and taste of different species of plants. Plant secondary metabolites are classified into different families according to their chemical structures: phenols, organosulfur compounds, terpenes, and alkaloids.

2.1 Polyphenolic Compounds

Plant phenolic compounds show promising results for reducing enteric CH_4. Phenols are organic compounds consisting of an aromatic ring binding to at least one hydroxyl group. Polyphenolics are composed of multiple phenols which determine their chemical properties and comprise over 8,000 different polyphenolic compounds. These polyphenolic compounds contain antimicrobial properties that either directly inhibit methanogens or indirectly reduce methanogen activities by reducing fiber degradation from other microbes thus reducing overall fermentation and substrates. Date palm leaves (DPL) are byproducts generated from date production, which are an important food staple in regions with little to no rain fall. Even though they are not fit for human consumption, DPL can be used as a feed for ruminants as they are high in fiber and contain polyphenolic compounds. When replacing berseem hay with DPL in-vitro,

there was a linear decrease in total gas production, CH_4 and CO_2 (Kholif et al., 2022). Date palm leaves had higher fiber content which requires longer time to degrade, resulting in the decrease in fermentation. Thus, DPL was also ensiled for 45 days with fibrolytic bacteria to increase the soluble fiber available for rumen fermentation. When the treated DPL replaced berseem hay at 100%, CH_4 production was decreased to 89.2mL/g degraded DM compared to 111.3mL/g degraded DM, respectively (Kholif et al., 2022). Additionally, in diets containing treated DPL, there was an increase in total VFA concentrations compared to the berseem hay (Kholif et al., 2022). Date kernels (DK) are another byproduct from date processing, containing high nonstructural carbohydrates and can be used as a concentrate in ruminant diets, which supplies energy for the animal (Sabry et al., 2021). They also contain a variety of phenolic compounds that can inhibit ruminal microbe activities. In an in-vitro study that replaced maize with DK from 25 to 100%, up to 39% of CH_4 production was suppressed when DK completely replaced maize (Sabry et al., 2021). Date kernels versus corn had a higher amount of phenol content at 217g/kg DM to 36g/kg DM, respectively. It was found that the most abundant phenolic compound was protocatechuic at 58% followed by p-hydroxybenzoic acid at 16% in date kernels (Sabry et al., 2021).

2.1.1 Hydrolysable Tannins

Tannins are a subgroup of polyphenolic compounds, subdivided into hydrolysable tannins (HT) or condensed tannins (CT). Hydrolysable tannins are characterized by a carbohydrate molecule, most commonly glucose esterified by a phenolic acid. Depending on the phenolic acid, they are grouped into either gallotannins or ellagitannins. Gallic acid is the predominant phenolic acid in gallotannins (also known as tannic acid), whereas ellagitannins contain the

hexahydroxydiphenoyl (HHDP) ester and are modified into ellagic acid, a dimeric form of gallic acid during metabolism (Farha et al., 2020). When HT are broken down by tannin-digesting microbes, gallotannins are transformed into gallic acid and further into pyrogallol. Pyrogallols are toxic to bacteria as they inhibit growth through interactions with proteins or polysaccharides found on their cell walls or directly with the lipid membrane (Kocaçalişkan et al., 2006; Oliveira et al., 2022). However, the exact mechanism in which pyrogallol interacts with rumen microbes is poorly understood. Tannins can be found in pomegranate peels with HT making up 90% of the tannin content (Mo et al., 2022). It is estimated that the pomegranate industry generates over 1.47 tons of byproducts in the form of peels yearly. In an in-vitro study that replaced hay with pomegranate pomace at a rate of 500g/kg of DM, CH_4 production decreased by 28% over a 24 h fermentation period (Giller et al., 2022). Even though CH_4 production significantly decreased, adding pomegranate pomace did not alter VFA production. This shows that pomegranate pomace can be a good candidate for in-vivo studies to potentially reduce CH_4 reduction without compromising the productivity of the host.

2.1.2 Condensed Tannins

Condensed tannins (also known as proanthocyanidins) are polyphenols that are made up of two or more flavan-3-ols compounds and are not water soluble. They bind directly to proteins and polysaccharides, and in doing so reduce the digestibility of feed. With the reduction of fermentation, there are less substrates available such as CO_2 and H_2 for methanogenesis. Mango wet waste such as peels and seeds are also a large part of byproduct waste from mango consumption. Though inedible for humans, they still contain high amounts of carbohydrates, protein, and fats that can be used in animal feed. When replacing yellow corn with mango seed

kernels in-vitro from 5-20%, there was a linear decrease in CH_4 production with no negative effects on VFA production (Shwerab et al., 2023). On the contrary, there was an increase in VFA production in the 10 and 20% treatment, which may be due to seed kernels being more digestible than yellow corn. Mango leaves are also byproducts generated from the mango industry following the pruning process of mango trees. They contain high amounts of tannins that can be used in place of traditional roughages. When replacing wheat bran with deciduous mango leaves in-vitro at 40%, there was a significant decrease in gas production (Mohamed, 2020). Mango leaves contained a higher amount of tannin (2.0mg/100g) compared to wheat bran (0.6mg/ 100g). The lower gas production and fermentation can be attributed from the tannin present but also other compounds such as ether extract (EE) and fiber. Since CT decrease digestibility and fermentation, it should only be given to animals in regulated amounts as too much can be detrimental to the health and production of the host.

2.2 Terpenes and Terpenoids

Terpenes are naturally occurring hydrocarbons built from two or more units of isoprene, a volatile 5-carbon compound, and are responsible for plants' unique aroma, taste, or color. Terpenes can range in size from monoterpenes (2 isoprene units) to polyterpenes of as many as 15,000 isoprene units. Terpenoids, also known as isoprenoids, are modified terpenes which contain oxygen. They also contain functional groups modified by the addition of oxygen atoms. Terpenoids are the largest class of plant secondary metabolite with over 60,000 identified structures. Olive leaves (OL) from the pruning process of olive production contain high amounts of bioactive compounds that can exert antimicrobial effects. When replacing timothy hay with OL by 5% in-vitro, there was a 34% decrease in CH_4 production after 12 h (Lee et al., 2021).

However, the decrease in CH_4 production was only temporary as there was no difference in CH_4 production at 24 h. The temporary decrease may be due to the lower digestibility from the high fat content and terpene-based polyphenols present in OL. Terpene-based compounds exhibit antimicrobial properties by disrupting cellular membranes (Lee et al., 2021). There was also a significant decrease in bacteria, methanogenic archaea, and fungi after 24 h of fermentation in the OL group, though there were no differences in VFA production between the two groups. Further studies are required to understand the long-term effects of OL on rumen microbes and their fermentation in longer in-vitro studies and in-vivo.

2.2.1 Essential Oils

Essential oils (EO) are composed mainly of mono- and sesquiterpenes and are highly concentrated in plants. Essential oils are extremely volatiles and aromatic compounds which plants utilize for a variety of purposes, such as attracting or repelling insects. Essential oils are also used by humans for a variety of purposes, such as ingredients in cosmetics, aromatherapy, or as antimicrobial agents. There are many methods of extractions such as steam distillation, solvent extraction, hydro-distillation, and supercritical CO_2 extraction (Bassolé & Juliani, 2012; Charles & Simon, 1990). Essential oils have been shown to exhibit antimicrobial activity but is highly dependent on the presence of other compounds such as phenols (Bassolé & Juliani, 2012). Orange leaves (OL) generated from pruning of orange trees contain high amounts of essential oils but is highly dependent on their geographical locations (Khalid et al., 2020). They can be used as a feed alternative to traditional forages in ruminant diets. However, when OL replaced alfalfa hay in the ration for lactating goats, there was a decrease in dry matter intake (DMI) but no significant differences in milk yield between the two groups (Fernández et al., 2019). Between

the two groups, the OL had a decrease in CH_4 by 3.8g/kg of milk produced (Fernández et al., 2019). Though they could not identify the compounds directly responsible for the suppression of methanogenesis, it is speculated that the essential oils and tannins inside their leaves may have been responsible. Grapefruit peels also contain citric essential oils (CEO) that have antimicrobial and antioxidant properties that can be effective in inhibiting methanogenesis. When CEO from grapefruit were added in-vitro with rumen fluid from 6 male Hu sheep at 0.8mL/L and 1.6mL/L, there was a negative relationship for CH_4 production as dosage increased (Wu et al., 2018). However, VFA production also declined as CEO do not target a specific group of microbes but rather reduce overall fermentation activity. An in-vivo study was conducted alongside the in-vitro study and found that when CEO was added at a rate of 0.8mL/L of rumen fluid, there were no changes in DMI, body weight (BW), and average daily gain (ADG). Citric essential oils from grapefruit peels were mainly comprised of D-limonene at 80% followed by B-pinene at 5.52%. D-limonene is a monoterpene that has been shown to be an effective antimicrobial compound due to its disruption of cellular membranes (Zhang et al., 2014).

2.3 Organosulfur Compounds

Sulfur is an essential nutrient for the growth and development of plants. Glucosinolates are secondary metabolites that contain glucosides linked with sulfur within their structures. Glucosinolates are mainly found in cruciferous vegetable such as broccoli, cabbages, kale, and other leaf plants (Miękus et al., 2020). There have been around 200 types of glucosinolates identified with differing structures dependent on plant type, environmental factors, and stage of growth (Ishida et al., 2014). Glucosinolates are important in the plant defense system as it is rapidly hydrolyzed by the enzyme myrosinase into mainly isothiocyanates when the plant tissue

is mechanically damaged resulting in a pungent sulfuric odor that acts as a deterrent to herbivores (Connolly et al., 2021). Stalks and leaves from broccoli are usually left unutilized as they are not consumed by humans. Belonging to the *Brassicaceae* family, alongside other crops such as rapeseed, cauliflower, and turnip can have differing effects on rumen fermentation dependent on their vegetive stage. When tested in-vitro using rumen fluid from two sheep, vessels containing only broccoli substrate had a 35% lower CH_4 production compared to treatments containing only rapeseed (Durmic et al., 2016). Though further studies need to be conducted on broccoli when compared to traditional feeds, these results demonstrate that different cultivars under the same family can have differing effects on rumen fermentation.

2.4 Polyunsaturated Fatty Acids

Polyunsaturated fatty acids (PUFAs) are fatty acids containing two or more double bonds as part of their hydrocarbon chain. They are found in some animal sources such as fish but are more commonly found in plant-based sources such as nuts and seeds. The type of fatty acid is dependent on the amount of carbon and double bonds present. Some examples of PUFAs are linolenic acid also known as omega-3 fatty acid (C18:3) and linoleic acid, omega-6 fatty acid (C18:2). PUFAs are high energy dense feed source and are used to supplement high performing animals such as lactating cattle (Castro et al., 2019). Fats provide over two-times more energy than carbohydrates and protein sources. Some PUFAs have been reported to have antimicrobial activities by inhibiting or even cause cell death through cellular membrane interactions (Chanda et al., 2018). Similar to the effects of terpenoids, membranes of microbes are altered, limiting their function or may even cause cell death from electron leakage. Biohydrogenation is seen in the rumen, in which microbes rapidly convert PUFAs to saturated fatty acids.

In addition to their microbial cytotoxicity, PUFAs have been shown to decrease rumen fermentation, though this depends on the types of fatty acids present (Sun et al., 2022). Blueberry seeds may be pressed, and their oils may be extracted to be used as additives, as they contain high PUFAs and polyphenolic compounds (Parry et al., 2005). PUFAs make up over 76% of total fatty acids in blueberry seeds with linoleic acid being the most abundant followed by linolenic acid. Even at a low dosage of 500mg L^{-1} of rumen fluid, addition of blueberry seed oils resulted in a decrease in in-vitro CH_4 production by 16% when compared to a diet without any oils supplemented (Embaby et al., 2019). Embaby et al. concluded that the compounds found in seed oils directly inhibit methanogens rather than altering feed digestibility and overall metabolism, as there were no changes in total VFA production, gas production and DM degradability.

Avocadoes also contain high amounts of unsaturated fatty acids (UFAs), which makes them a high-energy food (Dreher & Davenport, 2013). In an in-vitro study using three adult fistulated goats as donors, 400mg of avocado pulp and peel mixture (81:19 ratio) were added into 48mL of rumen fluid resulted in a significant decrease in potential gas production alongside with CH_4 production when compared to mango peels and pulp mixture (56.5mL vs 153mL and 31.1 mL/g DM vs 61.5mL/g DM, respectively) (Marcos et al., 2020). This is due to the inhibition of bacteria cellulolytic activity and the consequence overall reduction of substrates for downstream metabolic activity, such as methanogenesis (Maczulak et al., 1981). However, in the same study when avocadoes were supplied at a 15% inclusion rate in a multi-nutrient block (which includes other feedstuffs, urea, mineral and vitamin supplements), there were no observed differences in gas production parameters between avocadoes and mango multi-

nutrient blocks. Since inhibiting fiber fermentation can negatively affect the energy available to the host, it is recommended that high producing animals should only be given a maximum of 6% dietary fats in their diets (Bionaz et al., 2020).

3. Conclusion

Many agriculture byproducts have the potential to act as both animal feed and contain bioactive compounds that can reduce greenhouse gas emissions. However, the processing of these byproducts still requires standardization as their composition can heavily vary even between the same byproducts. This is due to the different environmental factors that these crops were grown in, differences in cultivar, and the maturity stage that the crops were harvested at. Storage of these byproducts post-harvest also plays a factor as their composition can rapidly change as some spoil faster than others. Animal hosts also play a large factor in fermentation results as microbiomes are greatly different between animals even if fed the same diet. Furthermore, future studies will require a comparison of byproducts between different ruminant species as well.

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Chapter 2. Identifying California Byproducts to Potentially Reduce Enteric Fermentation

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Abstract

California is the largest producer of crops in the U.S. During crop production large amounts of plant waste is produced that is not suited for human consumption and sent to landfills where it produces GHG emissions, mainly in the form of CO2 and CH4. During the processing of harvested crops additional byproducts are generated and removed from the food chain though they still may contain ample nutrients. California is also the largest dairy producer with over 1.7 million heads of dairy cows which contribute large amounts of CO2 and CH4 through microbial activities in the rumen ecosystem. Many byproducts in agriculture may contain bioactive compounds that can influence fermentation and inhibit methanogenesis. During the work presented here we took advantage of an in vitro rumen system to screen a variety of agricultural byproducts from California for their potential to reduce enteric methane production. We also generated chemical profiles of the tested byproducts to provide a framework that will enable a better understanding of the chemical parameters that dictate the inhibition of rumen methanogenesis. Almond hulls and shells, agave leaves, four different varieties of grape pomace, onion waste, and sunflower meals were tested at inclusion rates from 0.5 to 20%. No significant differences ($p \geq 0.05$) were observed between the different treatments when compared to the basal diet. Due to the runs being only 24 h, further studies with longer exposure to the byproducts may be required to understand the long-term effects on fermentation. In addition to this, further analysis on bioactive compounds such as phenolics, essential oils, and fatty acids will be insightful to understanding the properties of these byproducts.

1. Introduction

The United States generated over $433 billion dollars in animal and crop cash receipts in the year of 2021 with California as the lead contributor of over $51 billion dollars (CDFA, 2022). There were 69,000 operating farms in California, using ~98,000 square kilometer of land, producing 99% or more of many crops, such as almonds, pistachios, walnuts, and grapes (CDFA, 2022). With the production of these crops, there is also a large amount of biomass that is being generated and does not make it through the production process to the consumer. This biomass represents unharvested crops that were grown but left in the field due to overproduction or harvesting is no longer profitable due to declined market prices leading to a burden to the producer and often to the environment. A prominent example are sunflowers with over 294 tons left unharvested on the fields in California in 2021 (CDFA, 2022). Unutilized biomass are crops that were harvested but due to abundance in supply were not incorporated into the food supply chain. It was also reported by CDFA in 2021 that there were over 3,200 tons of onions that were unutilized after harvest.

California is also the largest wine producing state, producing over 2,368 million liters in 2021, making up over 81% of total wine production in the U.S. (Wine Institute, 2024). Grape pomace is a major byproduct that is generated during the wine making process since it cannot be consumed by humans and alternative routes for its utilization are desirable by the wine industry. Many other byproducts, such as hulls, shells, and meals, are generated during the processing of nuts and oils and in most cases these waste products are sent to either compost facilities or landfills that can lead to the production of greenhouse gas (GHG) emissions during anaerobic digestion and fermentation. Recent policies that mandate the reduction of methane

from the livestock industry have led to the growing interest of repurposing plant-based waste into livestock feed that is nutritious but also holds the potential to inhibit methanogenesis in the rumen to reduce environmental impacts.

Livestock, especially ruminants, are an integral part of agriculture as they can consume plant biomass and convert them into high-quality animal products such as meat and milk. On a global scale, 86% of feed consumed by livestock is not edible for humans, making livestock an important portion of human nutrition, especially in places where growing plants for dietary purposes is challenging (Mottet et al., 2017). California is the leading dairy producer with over 1.72 million dairy cows producing over 20 million tons of milk in 2021 (CDFA, 2022) and it is also home to approximately 680,000 heads of beef cattle, which are usually held on pasture where they can graze. Microorganisms that drive feed digestion in the rumen are also producing greenhouse gases such as CO2 and the even more potent CH4, with beef cows emitting between 240 to 396 g CH_4/day and dairy cows producing 269 to 354 g CH_4/day (Broucek, 2014). With the >2 million heads of cows it is therefore not surprising that California's livestock industry also represents a major producers of greenhouse gases.

There has been an increased interest in plant-sourced byproducts as a feed additive to reduce methanogenesis in the rumen with a particular focus on essential oils (Embaby et al., 2019; Wu et al., 2018), phenolic compounds (Hamimed & Kthiri, 2022) or byproducts that has been chemically altered by silaging (Kholif et al., 2022). On the other hand, little work has been performed on the effect on enteric methane production of plant-sourced byproducts from California agriculture and byproducts that have not undergone further resource extensive processing, such as the extraction of essential oils or phenolic compounds. The objective of the

present study was to determine the effects of byproducts on rumen fermentation with a particular focus on methane production and without the need for prior extensive processing of the byproducts. We tested 10 byproducts that were generated from agriculture in California at various inclusion rates to take into consideration that the chemical components of the byproducts we tested might be bioactive at different concentrations. We also generated chemical profiles of byproducts that were tested to provide a foundation to better understand the chemical composition of the different byproducts and to identify and quantify likely compounds, such as organosulfur compounds or tannins, that might have antimicrobial properties and that might drive enteric methane inhibition. We hypothesize that several of the byproducts generated from the production of crops and vegetables have the potential to reduce methanogen activities and consequentially CH_4 production in ruminants.

2. Materials and Methods

2.1 Agricultural Byproducts

Almond hulls, shells, and a mixture of hulls and shells (AH, AS, AHS, respectively), and sunflower meal (SM) samples obtained from a processing facility in Selma, California. Onion waste (OW) (*Allium cepa*) was obtained from a processing facility in Oxnard, CA. Agave Pencas (AP) (*Agave tequilana*) were received from growers in Yolo County, California. Grape pomace from Pinot Noir (*Vitis vinifera*) (GPPN) was obtained from a winery in Modesto, CA and grape pomace of three grape varieties (i.e., *Viognier, Verdelho,* Albariño; GPVI, GPVE, GPA, respectively) were collected locally from the UC Davis Robert Mondavi Institute Teaching and Research Winery immediately after pressing. All samples (Figure 1) were placed in 3.79-liter bags and stored in a -20°C freezer until further analysis.

2.2 Animals and Diet

Rumen contents for in-vitro rumen fermentation was obtained from rumen-fistulated non-lactating dairy cows housed at the University of California, Davis (UCD) Dairy Teaching and Research Facility. The donor animals were fed a dry cow total mixed ration (TMR) consisting of 50% wheat, 25% alfalfa, 21.43% almond hulls, and 3.57% dry cow pellet on a dry-matter (DM) basis. Animals were fed four times a day at 5:00 am, 11:00 am, 6:00 pm, and 11:00 pm and had free access to water.

2.3 Rumen Fluid Collection

Rumen contents were collected from three fistulated cows one hour after feeding and just prior to the start of the in-vitro rumen fermentation. Rumen fluid was collected in accordance with the Institution of Animal Care and Use Committee (IACUC) at UCD under protocol number 22753. Approximately 1,500 mL of rumen fluid were collected from each cow via a perforated PVC pipe, 500 mL syringe and Tygon tubing (Saint-Gobain North America, Malvern, PA, USA), passed through a strainer and into individually pre-warmed insulated thermos (Coleman Company, Chicago, IL, USA). Rumen solid was collected by hand from each cow directly after rumen fluid was collected and solids were placed in a sterile container apart from the rumen fluid. Temperature and pH of the rumen fluid were immediately measured once rumen fluid had been collected from each cow and transported back to the laboratory within 30 minutes.

2.4 Experimental Design

Byproducts were grounded up using an Oster 14-Speed blender (Sunbeam, Boca Raton, FL, USA) and dried in a Fisher brand Gravity Oven (Thermo Fisher Scientific, Pittsburg, PA, USA) at 55°C for 72 h. Total mixed ration was collected from the UCD Dairy Facility with all components

mixed to represent the animals' typical diet. A total of 1g of TMR were weighed into 100mL serum bottles with 0.5%, 1%, 5%, 10% and 20% (DM-basis) of the TMR replaced with the byproduct under evaluation. Vessels with TMR only were used as negative controls. As positive control, 2% of the TMR were replaced with *Asparagopsis taxiformis*. Rumen fluid collected from at least two cows was combined in equal amounts to account for biological variation and the rumen fluid mixture was further mixed with artificial saliva buffer (1:3 v/v). Artificial saliva buffer was according to Oeztuerk et al. (2005). All treatment groups had 5 replicates (n = 5), totaling 35 serum bottles per experiment. Every vessel was flushed with high-purity nitrogen for 15 seconds and sealed with a rubber cork attached to a Tygon tube to connect to 1L gas bags (Restek, Bellefonte, PA, USA). Vessels were placed into a shaking water bath (Sheldon Manufacturing, Cornerlius, OR, USA) at 39°C.

2.5 In-vitro Measurements

Temperature and pH were measured from individual rumen fluid samples immediately after rumen fluid was collected, after individual rumen fluid samples were combined, after the artificial saliva buffer was added to the rumen fluid mixture, and after 24 h of fermentation. After fermentation contents of serum bottles representing a particular treatment were pooled before the pH was measured. Gas samples were collected after 24 h of fermentation from each serum bottle and gas analysis was performed within 24 h using gas chromatography.

2.6 Greenhouse Gas Analysis

Gas analysis was performed using an SRI Gas Chromatograph 8610C (SRI, Torrance, CA, USA) equipped with a Haysep D column (3' x 1/8" stainless steel) and a flame ionization detector (FID) held at 300°C. High-purity hydrogen was used as the carrier gas with a flow rate of 30

mL/min with the oven temperature held at 50°C for 2 minutes. A 7-point calibration curve was developed using a CH_4 and CO_2 gas standard provided by Mesa (MESA Specialty Gases & Equipment, Santa Ana, CA, USA). Measurement of gas production from each treatment was prepared by extracting 1 mL of sample from the gas bags and diluted with 29 mL of N_2 gas and injected directly into the column. Total gas production (TGP) was determined by extracting gas using a 60 mL syringe and a double female luer lock until no gas remained in the gas bags.

2.7 Statistical Analysis

One-way ANOVA followed by Dunnett's multiple comparison test was performed using GraphPad Prism 10 version 10.0.3 for Windows (GraphPad Software, Boston, MA, USA). All parameters measured including CH_4, CO_2, TGP, and pH were tested for statistical differences and compared to the negative control in the follow up multiple comparison. A nonlinear regression test was also conducted on CH_4 production from all treatment groups to determine second order polynomial (quadratic) effects using GraphPad Prism. A statistical difference was considered when p-values < 0.05.

2.8 Chemical Analysis

Wet chemistry analysis was conducted for each byproduct to determine DM, energy value, fiber content, ash content, non-fiber carbohydrate (NFC), and fat concentrations. Samples were placed into individual 1-liter sized plastic bags and mailed to Forage Labs (Cumberland Valley Analytical Services, Waynesboro, PA, USA) for analysis following their standard procedures listed on their services website (https://www.foragelab.com/Resources/Lab-Procedures). Dry matter followed a two-step process with the samples first partially dried adapted from Goering & Van Soest (1970), then followed by 3 h of drying at 105°C. Neutral detergent fiber (NDF) used

the method published from Van Soest et al. (1991) and acid detergent fiber (ADF) following method 973.18 from AOAC (2000). Crude fat was measured using the Gravimetric Method (954.02) from AOAC (2000). Ash content was analyzed using method 942.05 from AOAC (2000).

3. Results

3.1 Gas Production Parameters

3.1.1 Methane Production

During enteric fermentation methane is produced under anaerobic conditions. To determine the potential of the various byproducts, in vitro methane production in the presence and absence of the byproducts was determined (Figure 2). As previously shown, inclusion of *A. taxiformis* resulted an almost complete shutdown of methanogenesis when compared to the negative control ($p \leq 0.01$). This significant reduction was observed for all in-vitro runs that were performed as part of this study. No significant change of CH4 production was observed between any of the other byproducts with onion waste being the only exception with a trend ($p = 0.08$) to produce less methane compared to the negative control.

3.1.2 Carbon Dioxide Production

Carbon dioxide production, commonly also used as an indicator for microbial growth, was also monitored and results suggested that none of the treatments had strong antimicrobial properties, which would result significant decreased CO2 production. Carbon dioxide production remained consistent across all treatment groups ($p > 0.05$), with agave at 10% inclusion rate showing a trend ($p = 0.07$) of lower CO2 production being an exception and with A. taxiformis stimulating microbial fermentation (Figure 3).

3.1.3 Total Gas Production

Total gas production was measured after 24 h of in vitro rumen incubation and results did not reveal any significant differences in the amount that was produced between the different treatment groups and the negative control ($p > 0.05$). There was also no difference in the amount of gas that was produced by negative and the positive control, the latter containing 2% *A. taxiformis* (Figure 4).

3.2 Physical Parameters

The pH remained largely unaffected by the treatment and ranged between 5.4 and 6.0 after 24 h of in vitro fermentation (data not shown).

3.3 Chemical Analysis

The chemical composition of the byproducts tested in this work is summarized in Table 1.

4. Discussion

4.1 Agave

Agave has been of particular interest as a feed additive since it grows under very arid conditions and provides farmers in areas where rainfall is limited the opportunity to provide extra fiber at low or no cost to the animals. The leaves of the agave utilized in this study was notably fibrous but also contained relatively high levels of moisture (84%) which is similar to what was reported previously by Iñiguez-Covarrubias et al. (2001) who investigated the effect of agave bagasse which are the residual fibers after sugar extraction as a feed replacement on 36 male sheep. Detailed chemical analyses (Table 1) revealed that total fiber content of NDF (consisting of cellulose, hemicellulose, and lignin) only accounted for 28.9% of the leaves' total DM. Most of the fiber content is from cellulose and lignin as ADF makes up 26.1% of the leave's total DM.

Lignin content of leaves used here was lower compared to Corbin et al. (2015), but this may be due to the differences in the maturity level of the leaves, whereas ash content had similar levels between the two studies. Crude protein and crude fat were low in agave (i.e., 7.8% and 1.99%, respectively). This is not surprising and low crude protein and fat concentrations also provide the foundation for why agave is categorized as lower quality forage. However, total digestible nutrients (TDN) values can be used to determine the energy provided by feedstuff which accounts for carbohydrates, lipids, and protein content (Jayanegara et al., 2019). Agave leaves had a TDN index of 58.8 and ME at 0.94 Mcal/lb, which can potentially be considered as a forage replacement especially in areas that require higher water requirements.

The availability of studies on using agave leaf as animal feed is scarce, especially when looking at specifically those of the *Agave tequilana* cultivar. However, in a study by Dias et al., (2023) used the more common *Agave americana,* which happens to be reported as invasive in certain countries, to repurpose them as animal feed and observe rumen fermentation. However, they reported that adding *A. americana* did not affect in vitro rumen fermentation, which agrees with our current work as total gas production did not differ between any of the treatment groups with our negative control. However, it is noteworthy that there was a weak trend towards CO_2 production when 10% of agave replaced traditional feed. Since CO_2 acts as a major H_2 sink in CH_4 production, if available CO_2 is reduced, it could potentially lead to lower CH_4 production from methanogenesis. However, longer studies including microbiome profiling may be needed to better understand the long-term effects of agave leaves on fermentation patterns.

4.2 Almonds

Almond hulls have gained popularity as cheap feed alternative for high producing cattle as almond hulls are low in fiber and energy rich. This is reflected in our chemical analysis with almond hulls having 12.4% crude fiber and a TDN of 65.8%. Diets containing higher soluble sugar and starches with lower fiber contents produce less methane during fermentation compared to high fiber diets (Wallace et al., 2014) making almond hulls a promising candidate for reducing methane. Even though the control diet, a diet commonly used in the dairy industry, contained small amounts of almond hulls (21.4%), we wanted to investigate if increasing the non-structural carbohydrate concentrations influenced enteric methane production. Consistent with other studies that substituted dairy cattle diets with almond hulls (Swanson et al., 2021; Williams et al., 2018) no changes in TGP, CH_4, or CO_2, were observed, suggesting that raw almond hulls have no methane mitigation effect. However, it needs to be noted that the purity of almond hulls and subsequentially the chemical composition and nutritional value of almond hulls can vary significantly between charges, processing procedures and from year to year (DePeters et al., 2020). The impact of additional components in feed additives, intentional or unintentional, was highlighted by the observation that the mixture of almond hulls and almond shells, a combination that is natural intermediate in the almond processing process, possessing a lower ME and higher fiber content than pure almond hulls. We also tested almond shells which are considered low quality feed due to their high fiber content and a low lower energy index (Li et al., 2018; Duncan et al., 2024). When mixed with almond hulls, the TDN of the mixture decreased by 7.5% compared to pure hulls. However, since almond shells also contain bioactive compounds such as anthraquinones, flavonoids, and phenolic compounds that may contain antimicrobial properties (Li et al., 2018) we hypothesized that although the addition of shells to the cattle diet might

reduce the nutritional value of the supplemented diet, a potential reduction of enteric methane caused by these bioactives might compensate for this. We expected to see a decrease in fermentation rate when shells were added into the diet alongside hulls since NDF contributed to almost 50% of the mixed diet and providing less ME. Interestingly, no significant differences in the gas parameters between the treatment groups containing the different almond byproducts was observed. This may be due to the limited duration of the in-vitro rumen fermentation potentially not allowing differences in microbial responses to manifest themselves sufficiently.

4.3 Onions

Onion waste was comprised of different components of the plant, including outer skin, peels, bulbs, and roots. Chemical analysis revealed that the mixture contained high levels of fiber (NDF = 41.2%) but still had high total nutrient digestibility (69.2). Onion peels have also shown to contain bioactive compounds, mainly in the form of quercetin accounting for up to 80% of their flavonoid content (Stoica et al., 2023) which have been shown to have antimicrobial activity by inhibiting the formation of biofilms in bacteria (Nguyen & Bhattacharya, 2022). In a study by Lubberding et al. (1988) onion waste tested in fermenters inoculated with rumen fluid and found that ~60% of onion biomass was degraded within 60 h. Quercetin availability in onion waste can vary from 14.7 to 21.8 mg/g dependent on type of onion (yellow or red) and the type of extraction (Osojnik Črnivec et al., 2021). The onion waste tested in-vitro had minimal processing as it was only grinded down to mimic chewing from the cow to represent the quality of onion waste when received from producers, which could have possibly resulted in a lower concentration of quercetin exposed to microbes in the tested rumen fluid compared to other studies where onion peels are extracted using different methods to increase the available amount (Jin et al., 2011).

We did not observe any significant differences in the gas production parameters when onion waste was added, which corresponds with results from Alabi et al. (2024) looking at the effects of onion waste on rumen fermentation. There was a tendency of lower methane production when 1% of onion waste was added, however, longer exposure to onion waste is required to understand its effect on fermentation.

4.4 Sunflower

Nitrogen that is not utilized by rumen microbes for ammino acid synthesis is converted to ammonia and eventually secreted by the ruminant animal as urea (Savari et al., 2018). The reduction of available nitrogen through microbial protein can no longer be absorbed in the lower digestive tract resulting in an decrease of CH4 production (Haro et al., 2019). Due to their high protein content, sunflower meal has been widely used as a protein replacement for both growing animals and lactating cows (Richardson et al., 1981; Schingoethe et al., 1977) and some researchers suggested that sunflower meal has the potential to reduce enteric CH_4 production. Although Haro and colleagues were able to confirm this with processed sunflower meal in 2018 and 2020 in two independent studies, no changes in CH4 production was observed in this study. Since our sunflower meal was added without any being processed, which usually changes the chemical composition, to the feed, it is feasible that the differences in chemical composition between processed and unprocessed sunflower meal might cause the difference in methane production. When analyzing for second order of effects, sunflower meal appeared to have a critical inclusion rate between 10 and 20% ($p = 0.015$), that may have potential to inhibit methanogenesis at 24 h of exposure. However, further studies with processed and unprocessed sunflower meal at higher inclusion rates and for longer fermentation times will be required.

4.5 Grape

Grape pomace contains bioactive compounds, such as phenolics, that can have antimicrobial activities also affecting the rumen microbiome (Hassan et al., 2019). Since grape pomace is usually high in moisture, which makes it prone to spoilage, prolonged storage is a challenge and, in most cases, not economical. To avoid contamination, spoilage and subsequently changes in their phenolic profile (Yu & Ahmedna, 2013), freezing grape pomace was shown to trigger no differences in rumen fermentation (Russo et al., 2017), hence grape pomace used in this study was immediately transferred into -20C after it was collected. Besides phenolics, lignin is also a significant component of grape pomace which results in decreased digestibility when added at increased inclusion rates to the animal's diet. From the samples we evaluated grape pomace from Viognier had the lowest concentration and grape pomace from Pinot Noir grapes had the highest concentration of lignin (13.73% of DM and 38.04% of DM, respectively), suggesting that grape pomace from Pinot Noir would reduce digestibility and CH4 production due to its increased lignin content per se if added in sufficient amount. However, no differences in gas production or methane production were observed for all varieties of grape pomace. This may have been caused by an insufficiently low inclusion rate or rumen fermentation time. Evidence from Moate et al., (2020) who fed different varieties of grape pomace (red and white) to lactating dairy cows over 28 days at an inclusion rate of 33% and found a reduction in methane by 15% when compared to a basal ryegrass diet, suggest that the latter is the case and prolonged in vitro rumen fermentation will be needed before differences in gas and methane production manifest itself and can be detected. In the same study, it was found that the red grape pomace had considerably less condensed tannins than white grape pomace, however, there were no

differences in fermentation between the two groups. Many factors can affect the fermentability of grape pomace such as the composition and different ratios of skin, seed and stem. This would affect the lignin and available metabolizable energy for microbial activity.

5. Conclusion

California plays in important role in providing food at the national and global level, generating a diverse repertoire of plant-based products. In addition to this California is also a leader in dairy and beef production, providing a significant portion of animal protein to the US market. Rerouting plant-based byproducts away from the landfill and utilizing them as animal feed is a promising strategy to decrease waste from agriculture, while also making animal production more economical. If these byproducts have the additional benefit of reducing the emission of greenhouse gases such as CH4 from enteric fermentation, the rerouting and up-grading of byproducts also provides an attractive strategy to reduce to environmental footprint of the livestock industry.

Over recent years there has been a great interest in identifying feed additives that might upgrade conventional cattle feed and that could provide additional avenues for methane mitigation. However, in many cases results from these studies have been inconsistent at best often even contradicting each other. This is not surprising, since these studies varied greatly in the experimental design and utilized compounds without generating detailed chemical profiles that would enable to identification of potential bioactives that drive rumen methanogenesis or its inhibition. The work presented here provides detailed chemical analyses of some common agricultural byproducts generated in California and therefore a valuable foundation for

subsequent more refined in vitro rumen fermentation studies. Results obtained from the work presented here also suggest that a prolonged in-vitro rumen fermentation time might be essential to detect any methane mitigation effect from compounds that contain bioactives with lower potency than bromoform or the red seaweed Asparagopsis taxiformis that trigger methane mitigation to the extent that it can be reliably detected after 24 h of rumen incubation. Byproducts from plant-based agriculture remain a promising option as feed supplement and replacement with the potential to reduce enteric methane emission, but more refined in-vitro screening protocols and detailed biochemical information of the byproducts will be necessary before they can employ reliably and at large scale.

Tables

Table 1. Chemical composition of byproducts on a dry matter (DM) basis.

Items	Dry Matter %	Crude Protein %	Crude Fat %	Ash %	Calcium %	Phosphorous %	ADF_1 %	$aNDF_2$ %	Lignin	TDN_3 % DM	ME_4 (Mcal/lb)
Almond Hulls/Shell Mix	88.6	5	1.98	7.18	0.27	0.1	32.6	49.9	17.03	48.6	0.78
Almond Hulls	87.2	4.9	2.15	6.63	0.23	0.13	12.4	24.3	9.79	65.8	1.12
Almond Shell	89	5.2	2.19	6.01	0.25	0.09	23.2	43.4	11.99	56.1	0.93
Agave	15.6	7.8	1.99	17.05	3.83	0.42	26.1	28.9	6.51	56.8	0.94
Onion Waste	90.1	8.3	3.17	6.19	1.18	0.33	21.7	41.2	3.33	69.2	1.19
Grape Pomace (Viognier)	34.6	7.5	5.30	3.73	0.18	0.18	13.6	21.5	13.73	72.3	1.26
Grape Pomace (Verdelho)	35.8	7.2	5.14	4.45	0.2	0.2	16.1	25	17.21	67.7	1.16
Grape Pomace (Albarino)	46.3	11.5	10.7	4.39	0.39	0.3	22.1	33.8	25.24	66.2	1.13
Grape Pomace (Pinot Noir)	45.7	13.3	9.12	4.7	0.56	0.27	40.9	50.6	38.04	48.6	0.78
Sun Meal	92.5	32.8	2.75	6.35	0.43	0.95	30.8	41.7	10.41	59.1	0.99

1. Acid detergent fiber
2. Neutral detergent fiber
3. Total digestible energy
4. Metabolizable energy

Figures

Figure 1. Byproducts tested for their effects on gas production during in-vitro rumen fermentation.

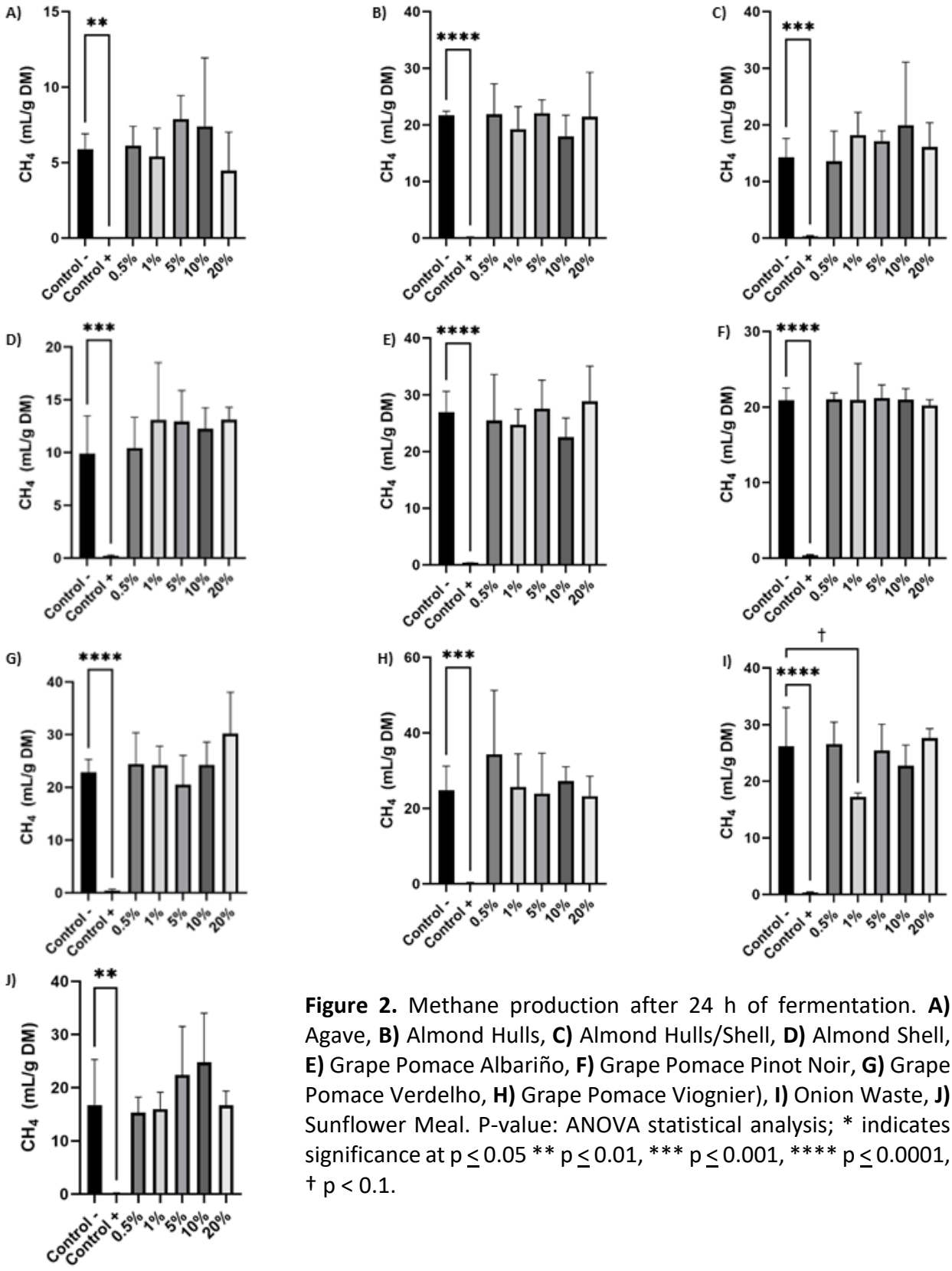

Figure 2. Methane production after 24 h of fermentation. **A)** Agave, **B)** Almond Hulls, **C)** Almond Hulls/Shell, **D)** Almond Shell, **E)** Grape Pomace Albariño, **F)** Grape Pomace Pinot Noir, **G)** Grape Pomace Verdelho, **H)** Grape Pomace Viognier), **I)** Onion Waste, **J)** Sunflower Meal. P-value: ANOVA statistical analysis; * indicates significance at $p \leq 0.05$ ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, † $p < 0.1$.

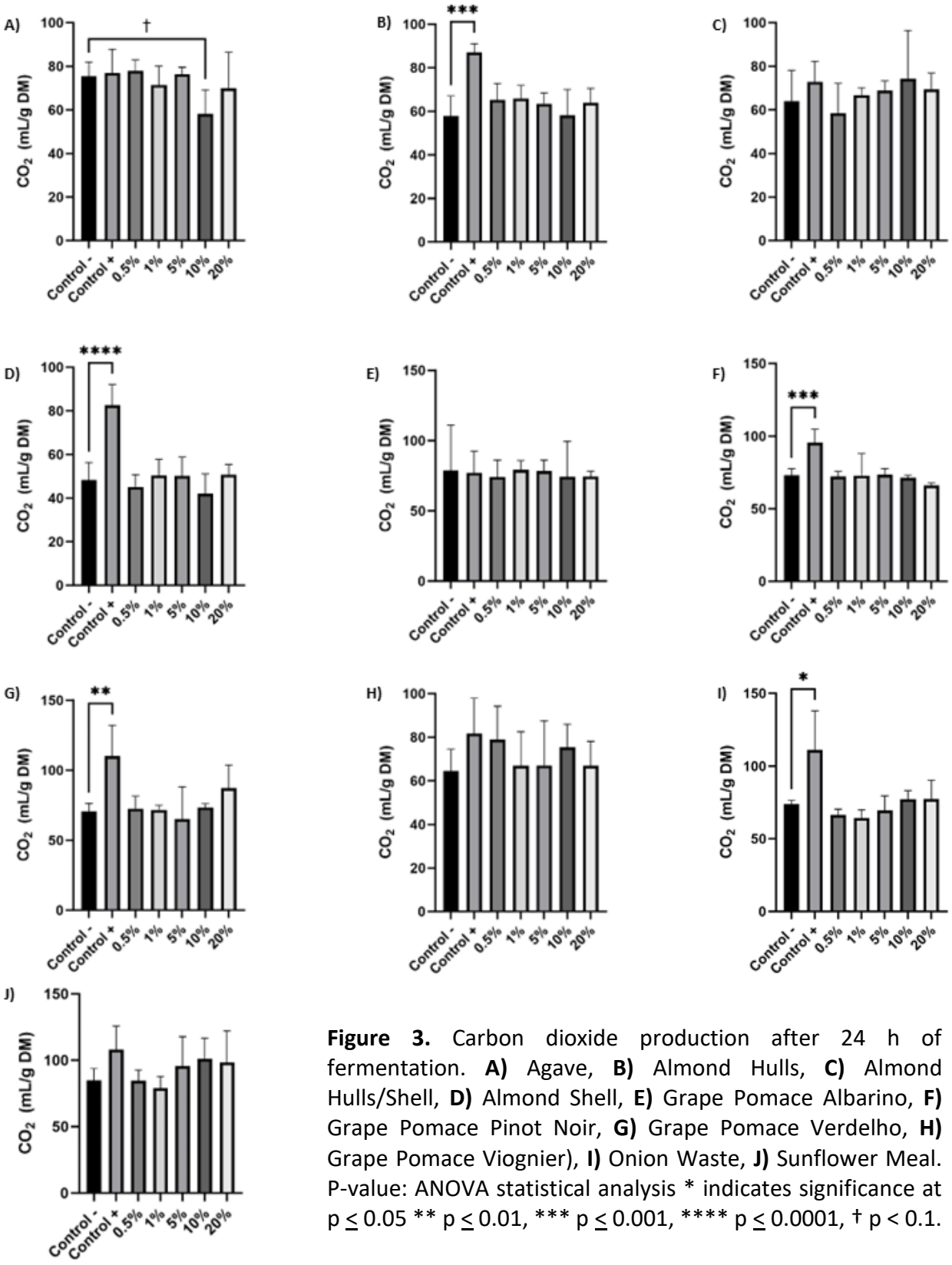

Figure 3. Carbon dioxide production after 24 h of fermentation. **A)** Agave, **B)** Almond Hulls, **C)** Almond Hulls/Shell, **D)** Almond Shell, **E)** Grape Pomace Albarino, **F)** Grape Pomace Pinot Noir, **G)** Grape Pomace Verdelho, **H)** Grape Pomace Viognier), **I)** Onion Waste, **J)** Sunflower Meal. P-value: ANOVA statistical analysis * indicates significance at $p \leq 0.05$ ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, † $p < 0.1$.

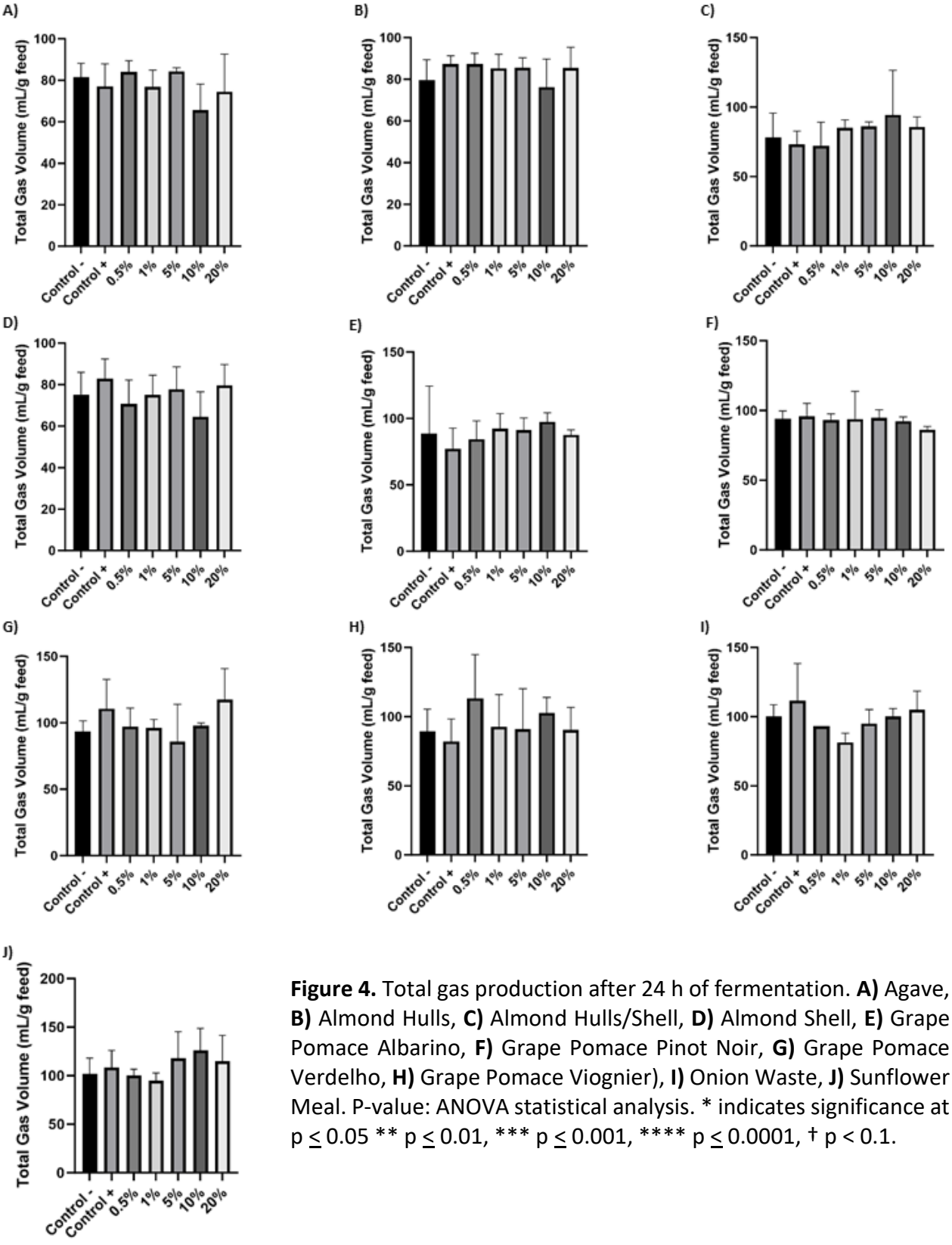

Figure 4. Total gas production after 24 h of fermentation. **A)** Agave, **B)** Almond Hulls, **C)** Almond Hulls/Shell, **D)** Almond Shell, **E)** Grape Pomace Albarino, **F)** Grape Pomace Pinot Noir, **G)** Grape Pomace Verdelho, **H)** Grape Pomace Viognier), **I)** Onion Waste, **J)** Sunflower Meal. P-value: ANOVA statistical analysis. * indicates significance at $p \leq 0.05$ ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, † $p < 0.1$.

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