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THE UNIVERSITY OF ALBERTA

THE EFFECT OF NITRITE ON THE CARBOHYDRATE AND AMINO ACID METABOLISM OF *MEGASPHAERA ELSDENII*

BY

ALASTAIR FURTADO

A THESIS

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DEDICATION

This thesis is dedicated to my parents who have always encouraged and supported me in all my endeavors.

THE EFFECT OF NITRITE ON THE CARBOHYDRATE AND AMINO ACID METABOLISM OF MEGASPHAERA ELSDENII

Megasphaera elsdenii, an anaerobic rumen bacterium, plays an important role in the rumen in lactate metabolism, amino acid catabolism and subsequent branched-chain fatty acid production. M. elsdenii is capable of reducing NO_2^-, a toxin at sufficiently high concentrations, to NH_4^+ and possibly use NO_2^- as an alternate electron acceptor. Pure culture studies were conducted to determine the effect of NO_2^- on carbohydrate and amino acid catabolism.

M. elsdenii, in the presence of 3.57 mM NO_2^--N, was able to reduce all NO_2^- to NH_4^+. No increase in oxidation products was observed although H_2 production decreased with lactate as the substrate. Amino acid catabolism was increased in the presence of NO_2^-, suggesting either an inhibition of pyruvate:ferrodoxin oxidoreductase by NO_2^- and a shift in the major source of energy from carbohydrate catabolism to amino acid catabolism or greater oxidative deamination and subsequent decarboxylation when NO_2^- was available as an alternate electron acceptor.

The production of an uncommon amino acid identified as 2-aminobutyric acid was observed. Its production paralleled that of alanine in response to the various treatments. 2-Aminobutyric acid was derived from threonine but not serine, methionine or aspartate; all of which are potential sources for 2-ketobutyric acid from which 2-aminobutyric acid is formed. Treatments resulting in increased 2-aminobutyric acid production resulted in decreased alanine production, suggesting a competition for NH_4^+, electrons or enzyme systems. The presence of L-cycloserine, an inhibitor of alanine transaminases,

decreased 2-aminobutyric acid production suggesting that either 2-aminobutyric acid synthesis is under the same metabolic controls as alanine synthesis or that 2-ketobutyric acid can act as a substrate for the alanine transaminases resulting in 2-aminobutyric acid production. It was suggested that 2-ketobutyric acid acts as an electron and NH_4^+ acceptor in transamination, possibly it is involved specifically in the degradation of the branched-chain amino acids since their concentrations were inversely related to those of alanine and 2-aminobutyric acid.

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I. INTRODUCTION

I-1. Electron Disposal in the Rumen

In aerobic environments, electrons are transferred to O_2 resulting in the formation of water. Oxygen is the terminal electron acceptor of choice since the greatest amount of energy can be obtained using it due to a greater difference between the oxidation-reduction (redox) potential of the substrate and molecular oxygen relative to other biologically significant electron acceptors (Lehninger, 1979). However, O_2 is not always present in all ecological niches. The reticulo-rumen is essentially an anaerobic environment in which O_2 is virtually absent and resident microbes must use alternate terminal electron acceptors. These can be derived from the animal's feed directly or from metabolism. Electron acceptors can be organic compounds such as pyruvate, fumarate, amino acids, CO_2 and unsaturated fatty acids or inorganic ions such as H^+, NO_3^-, NO_2^-, SO_4^{-2}, SO_3^{-2} or SSO_3^{-2} ions (Prins, 1977; Gottschalk, 1979; Erfle et al. 1986).

Knowledge of the disposal of electrons is essential to an understanding of anaerobic metabolism. Electrons derived from the oxidation of substrates are transferred to electron carriers and finally to the specific terminal electron acceptors used by the organism. Re-oxidation of the electron carriers is essential since they are present in catalytic rather than stoichiometric amounts with respect to the substrate. If this were not to occur, further oxidation would be

blocked and the organism would be unable to continue substrate metabolism.

I-2. Fermentation and Organic Electron Acceptors

Substrate fermentation can be seen as an oxidation and reduction of intermediates derived from a common substrate and can be expressed as:

$$(1) \quad C_{int} \text{ ---> } C_{ox} + ne^-$$

$$(2) \quad C_{int} + ne^- \text{ ---> } C_{red}$$

where C_{int}, C_{ox}, C_{red} and ne^- represent organic intermediates, oxidized organic compounds, reduced organic compounds and transferred electrons, respectively. The above equations can be summarized as:

$$(3) \quad C_{int} \text{ ---> } C_{ox} + C_{red}$$

The above reactions result in a portion of the substrate being oxidized to CO_2 or formate and the other portion being reduced to products which include lactate, succinate and the reduced volatile fatty acids (which include propionate, butyrate, valerate, and others) (Gottschalk, 1979); that is, there is dismutation of the substrate. Amino acids can also serve as both electron donors and acceptors. While some amino acids can be fermented singly, others can be fermented in pairs in a Stickland-type reaction in which a reduced amino acid (relative to other amino

acids) acts as an electron donor and is oxidized while an oxidized amino acid acts as the electron acceptor (Stanier et al., 1976). The Stickland-type reaction has been studied extensively in Clostridia spp. but its significance in the rumen has not yet been clearly demonstrated (Broderick and Balthrop, 1979; Hino and Russell, 1985).

Utilization of alternate electron acceptors not derived from substrates allows more of the substrate to be metabolized in the energy-yielding oxidation pathways because electrons derived from reaction (1) can reduce the alternate electron acceptor therefore less substrate is needed in reaction (2). Unsaturated free fatty acids found in the rumen can act as such electron acceptors. Hydrogenation of acids such as linoleic and linolenic acid occurs rapidly in the rumen and this process is not confined to lipolytic bacteria (Dawson and Kemp, 1970; Kemp et al., 1975; Hazlewood et al., 1976). This suggests opportunistic utilization of these acids as electron acceptors, as opposed to a functional requirement for lipid metabolism.

Carbon dioxide is used as an electron acceptor by highly specialized methanogenic bacteria. These bacteria are able to reduce CO_2 to CH_4 using H_2, formate, or methylamine as electron donors (Balch et al., 1979). Acetate can also be decarboxylated to CO_2 and CH_4 (Jones et al., 1987) by some genera. These bacteria play an important role in maintaining the low partial pressure of H_2 found in the rumen. This is beneficial since formation of H_2 from reduced pyridine nucleotides is reversible and highly sensitive to the accumulation of H_2 as shown in the following reaction catalyzed by hydrogenase:

$$(4)\quad NADH + H^+ + 2e^- \longleftrightarrow NAD^+ + H_2$$

Partial pressures of H_2 greater than approximately 2.85 kPa (0.001 atm) cause a shift in the equilibrium resulting in the formation of reduced pyridine nucleotides thereby stopping regeneration of the oxidized form in those bacteria using NADH-dependent hydrogenases (Wolin, 1979). Selenomonas ruminantium and Ruminococcus flavefaciens produce H_2 using such a system and will produce only small amounts of H_2 before metabolism becomes inhibited unless they are co-cultured with a H_2-utilizer, such as a methanogen, that can maintain a low H_2 partial pressure (Latham and Wolin, 1977; Chen and Wolin, 1977). The concentration of H_2 in the rumen is maintained at approximately 0.77 kPa (0.0003 atm) (Hungate, 1967) which is below the theoretical equilibrium point.

I-3. Inorganic Electron Acceptors

The electrons for reduction of H^+ to H_2 can also be obtained from reduced ferredoxin (Fd) (Wolin, 1979; Gottschalk, 1979) as an alternative to NADH. Since the redox potential for the oxidation of Fd is much lower than that of NAD, this reduction is less susceptible to a mass action inhibition by H_2 accumulation and proceeds even when H_2 is abundant.

Sulfate, sulfite and thiosulfite ions can act as inorganic electron acceptors. The reduction of these oxyanions is performed by several bacterial genera (Devereux et al., 1990), two of which have been found to inhabit the rumen. Desulfovibrio desulfuricans (Howard and Hungate, 1976) is believed to be the major sulfate reducer in the rumen with

Desulfotomaculum ruminis (Coleman, 1960) playing a lesser role. Nitrates and nitrites also act as electron acceptors although problems arise due to the toxicity of NO_2^-.

The ability to reduce NO_3^- or NO_2^- is widespread among rumen bacterial species. In the rumen, NO_3^- is rapidly reduced to NO_2^- which is subsequently reduced, but at a much slower rate, to NH_4^+ (Pfander et al., 1957; Wang et al., 1961).

$$(5) \quad NO_3^- + 2e^- + 2H^+ \longrightarrow NO_2^- + H_2O$$

$$(6) \quad NO_2^- + 6e^- + 8H^+ \longrightarrow NH_4^+ + 2H_2O$$

Ammonia is the terminal product since denitrification (reduction to N_2 gas) does not appear to be a significant occurrence in the rumen (Kaspar and Tiedje, 1981). When NO_3^- is present in sufficient quantities such that the rate of its reduction exceeds the capacity for NO_2^- reduction, accumulated NO_2^- will diffuse into the bloodstream. NO_2^- oxidizes hemoglobin to methemoglobin (which cannot transport O_2) and also acts as a vasoconstrictor (Deeb and Sloan, 1975). It is expected that the presence of NO_3^- and NO_2^- would influence the pattern of rumen fermentation by acting as electron acceptors and facilitate production of oxidized products (Allison and MacFarlane, 1988; Bryant, 1965; Farra and Satter, 1971; Yamamoto et al., 1982; Alaboudi and Jones, 1985) and by selecting against NO_2^--sensitive microbes (Marais et al., 1988; Cheng et al., 1988). The mechanism by which NO_2^- inhibits bacterial growth is still unclear. Chemical modification of membrane sulfhydryl groups or

interaction with media components resulting in formation of inhibitory compounds have been suggested (Moran et al., 1975; Buchman and Hansen, 1987). NO_2^- has also been shown to inhibit the phosphoroclastic reaction of Clostridium sporogenes by binding and inactivating the pyruvate:ferredoxin oxidoreductase (Woods et al., 1981). NO_2^- reduction has also been correlated with the ability of rumen bacteria to degrade the nitrotoxins 3-nitropropionic acid and 3-nitropropanol (Majak and Cheng, 1981) and has been suggested as a step in their intermediary metabolism.

The reduction of NO_3^- and NO_2^- in the rumen is mainly an enzymatic process carried out by its resident microorganisms. Limited chemical reduction does occur due to the reduced state of the rumen fluid and its solutes but such reduction is is generally very slow. Dissimilatory reduction of NO_3^- to NO_2^- is catalyzed by the molybdenum-containing NO_3^- reductase enzyme which is usually associated with an energy-yielding electron transport system (Inderlied and Delwiche, 1973; de Vries et al., 1974). NO_2^- reductase then catalyzes the six electron reduction to NH_4^+ (Blackmore and Brittain, 1986; Yordy and Delwiche, 1979). This process generally does not result in energy conservation though de Vries et al., (1982) reported transmembrane proton gradient formation linked to NO_2^- reduction and Bokranz et al. (1983) demonstrated electron transport phosphorylation coupled to NO_2^- reduction.

Not all microorganisms are able to reduce both NO_3^- and NO_2^-. On the basis of NO_3^- and NO_2^- reduction, rumen microorganism can be divided into four categories; those that contain both NO_3^- and NO_2^- reductases, those that contain a NO_3^-, those that contain a NO_2^- reductase and those

that contain neither enzyme system. The ability to reduce NO_3^- is used routinely in bacterial identification hence many NO_3^- reducers have been identified. Studies on NO_2^- reduction have generally been done on bacteria that possess both NO_3^- and NO_2^- reductases. Thus, until recently, bacteria that reduce only NO_2^- had not been reported. Cheng et al. (1988) screened 51 strains of anaerobic rumen bacteria for NO_2^- reduction ability and determined that many of the 25 strains that were able to reduce NO_2^- have been shown previously to be unable to reduce NO_3^- (Hungate, 1966; Holdeman et al., 1977). This included five strains of the rumen bacterium Megasphaera elsdenii.

I-4. Role of Megasphaera elsdenii

Megasphaera elsdenii (formerly Peptostreptococcus elsdenii [Gutierrez et al., 1959]) is an anaerobic Gram-negative cocci present in the rumen of sheep and cattle (Elsden et al., 1951; Giesecke et al., 1970). It has also been isolated from the cecum and feces of pigs and from the human intestine and feces (Sugihara et al., 1974). It also plays an important role in amino acid catabolism and branched-chain volatile fatty acid production (Scheifinger et al., 1976; Allison, 1978; Wallace, 1986). It can grow on glucose but is abundant mainly when lactate is being produced by starch-utilizing bacteria (e.g., Streptococcus bovis) on a high concentrate diet (Baldwin et al., 1963; Wallnofer et al., 1966; Hungate, 1966). Counette et al., (1981) estimated that M. elsdenii is responsible for approximately 74% of the lactate metabolized in the rumen. Lactate is fermented via the acrylate

pathway to propionate and acetate, from which valerate and caproate can also be formed (Ladd, 1959; Stadtman et al., 1949). Butyric acid is produced from the reductive condensation of two acetate units and is the major product of glucose metabolism (Holdeman et al., 1977; Marounek et al., 1989). H_2 is also produced in significant amounts by M. elsdenii through catalysis by a hydrogenase that can accept electrons from either ferredoxin or flavodoxin (van Dijk et al., 1979; 1980) which are in turn reduced either by lactic dehydrogenase (Brockman and Wood, 1975) or by a phosphoroclastic-type pyruvate oxidoreductase (Peel, 1960).

M. elsdenii is an important contributor to amino acid catabolism in the rumen. Serine and threonine are the most rapidly degraded. They are acted upon by the same enzyme, threonine deaminase (4.2.1.16), and the initial products are pyruvate and 2-ketobutyrate which can yield acetate and propionate, respectively (Lewis and Elsden, 1955; Walker, 1958; Umbarger, 1973). Recently, Wallace (1986) demonstrated that 2-aminobutyric acid is formed from threonine (but not serine) presumably via the amination or transamination of 2-ketobutyric acid. 2-aminobutyric acid is also produced by various other microbes (Holden, 1962; Loefer, 1962) including the Group I type Clostridia (Mead, 1971) as well as in plant and animal tissues (Soupart, 1962; Meister, 1965; Chen, 1962; Steward and Pollard, 1962) but is not found to be a constituent of any protein or peptide. The branched-chain amino acids, leucine, isoleucine and valine are also degraded by M. elsdenii, forming 3-methylbutyrate, 2-methylbutyrate and isobutyrate, respectively, (Hungate, 1966; Wallace, 1986) which are essential growth factors for other rumen microbes.

M. elsdenii also utilizes other amino acids to different extents and may actually cause an increase in some of them in the culture supernatant depending on the strain of M. elsdenii and culture conditions. Results differ among experiments using amino acids versus peptides as amino acid source as well as among those using batch versus continuous culture incubation (Scheifinger et al., 1976; Cotta and Russell, 1982; Wallace, 1986).

I-5. Objectives of the Present Study

The utilization of inorganic electron acceptors generally results in a greater energy yield due to more of the substrate being oxidized rather than being used as an electron sink. The reduction of NO_2^- not only provides the benefits of an inorganic electron acceptor but also detoxifies a potential toxin and provides NH_4^+ for the rumen microbiota. M. elsdenii was chosen as the experimental organism because of its ability to reduce NO_2^- at relatively rapid rates (Cheng et al., 1988) and because of its importance in the rumen ecosystem in amino acid catabolism, branched-chain VFA production and lactate metabolism. It's role in amino acid catabolism is also significant as this is potentially a major process of oxidation and reduction in M. elsdenii yet its significance in electron metabolism is not yet clear. The purpose of this investigation was to determine the effects of NO_2^- reduction on the metabolism of five strains of M. elsdenii in order to gain further insights, not only into NO_2^- reduction in the rumen, but also into

aspects of electron metabo[illegible] relation to amino acid catabolism in this species.

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II. The Effect of Nitrite on Carbohydrate and Amino Acid Fermentation by Megasphaera elsdenii

II-1. Introduction

In the rumen, ingested NO_3^- is rapidly reduced to NO_2^- by the rumen microbiota. Nitrite is subsequently slowly reduced to NH_4^+ (Pfander et al., 1957; Wang et al., 1961). The discrepancy in these rates results in an accumulation of NO_2^- which may be absorbed into the animal's bloodstream where it oxidizes hemoglobin to methemoglobin and impairs O_2 transport (Deeb and Sloan, 1975). At high levels of NO_3^- intake, this condition can be fatal to the animal.

Detoxification of NO_2^- by reduction to NH_4^+ can be accomplished by numerous rumen species (Cheng et al., 1988), one of which is Megasphaera elsdenii (formerly Peptostreptococcus elsdenii [Gutierrez et al., 1959]). This bacterium is a lactate-utilizing, amino acid-fermenting Gram-negative coccus present in the rumen of sheep and cattle (Elsden et al., 1951; Scheifinger et al., 1976). This species is most abundant under feeding conditions that favour lactate production, such as the intake of a high proportion of concentrate (Baldwin et al., 1962; 1963; Wallnofer et al., 1966; Hungate, 1966). It ferments lactate by the acrylate pathway (Ladd, 1959) and also ferments many of the amino acids (Scheifinger et al., 1976; Wallace, 1986) resulting in the production of acetate, propionate, isobutyrate, butyrate, 2- and 3-methylbutyrate, valerate and caproate plus CO_2 and H_2 (Elsden and

Lewis 1953). This species is believed to be of quantitative importance in ruminal amino acid catabolism in producing branched-chain volatile fatty acids that are essential for other rumen species (Hungate, 1966). Four of the five strains of M. elsdenii screened by Cheng et al. (1988) for NO_2^- reduction were able to reduce almost all of the NO_2^- in the medium (approximately 10 µg NO_2^--N/mL) within 24 hours, while one strain required several days.

In an anaerobic environment, reduction of oxidized compounds such as NO_3^- or NO_2^- accomplishes electron disposal and offsets some of the need to use carbon-containing substrate for this component of dismutation. If inorganic ions such as NO_3^- or NO_2^- act as electron acceptors, a greater growth yield may be realized because a greater proportion of the carbon substrate is oxidized rather than being used as an electron sink, thereby permitting greater energy conservation via substrate level phosphorylation. Nitrite can also alter rumen fermentation through inhibition of specific classes of rumen bacteria. Marais et al., (1988) have shown that NO_2^- can inhibit cellulolytic and methanogenic bacteria as well as other species which conserve energy by electron transport processes.

The following experiments were undertaken to determine the effect of NO_2^- supplementation on the carbohydrate and amino acid metabolism of five strains of M. elsdenii. Supplementation with NH_4^+ was also investigated to determine any effects on metabolism and NO_2^- reduction since NH_4^+ is the product of both NO_2^- reduction and the deamination of amino acids . The nature of the NO_2^- reductase system in M. elsdenii is

not known. Generally, NH_4^+ only inhibits or represses assimilatory NO_3^- and NO_2^- reduction systems (Kemp and Atkinson, 1966; Prakash and Sadana, 1972; Gottschalk, 1979). However, Yordy and Delwiche (1979) demonstrated that NH_4^+ salts were repressive to NO_2^- reduction in the rumen microbe Veillonella alcalescens, in which the NO_3^- and NO_2^- reductases have characteristics of both assimilatory and dissimilatory reduction systems (Inderlied and Delwiche, 1973; Yordy and Delwiche, 1979).

II-2. Materials and Methods

II-2-1. Organisms. Five strains of M. elsdenii from the culture collection of the Agriculture Canada Research Station at Lethbridge, Alberta were used. Strains J1 and LC1 (Elsden et al., 1951) were originally obtained from S. O. Mann of the Rowett Research Institute, Aberdeen. Strains AW106, T81 and B159 (Bryant et al., 1958) were from Dr. M. P. Bryant of the University of Illinois, Urbana. These five cultures were maintained on media containing (per litre) 0.45 g K_2HPO_4, 0.45 g KH_2PO_4, 0.9 g $(NH_4)_2SO_4$, 0.9 g NaCl, 0.09 g $MgSO_4$, 0.09 g $CaCl_2$, 0.03 g $FeSO_4$, 10 g Bacto-casitone or Bacto-casamino acids (Difco Laboratories, Detroit, Mich.), 2.5 g yeast extract (Difco), 0.001 g resazurin, and 6 g of either sodium lactate or glucose. Where specified, $(NH_4)_2SO_4$ and Bacto-casitone were omitted or substituted. Following adjustment of the pH of the above mixture to 6.5, it was dispensed in 300 mL aliquots to 500 mL round-bottomed flasks, and 15 mL

of 8% Na_2CO_3 (w/v) were added to each. All subsequent reduction procedures were performed anaerobically under O_2-free CO_2 (obtained by passing Coleman or Instrument grade CO_2 through a copper furnace heated to approximately 350°C). The medium was brought to boiling, 0.3 g crystalline cysteine-HCl was added and the solution was brought to boiling again and then allowed to cool for five min. The solution was then bubbled for 10 min with CO_2, distributed, sealed and autoclaved for 20 min at 120°C. Anaerobic culture techniques were essentially those of Hungate (1950) as modified by Bryant and Burkey (1953).

II-2-2. Experimental protocol. In the first experiment, the effect of NO_2^- on carbohydrate fermentation and gas production was investigated. Triplicate 20 mL cultures of each bacterial strain, plus triplicate uninoculated controls, were incubated at the same time (24 h, 39°C) in media containing either glucose or lactate as substrate, and to which either filter-sterilized $NaNO_2$ (final concentration - 3.57 mM NO_2^--N) or an equal volume of sterile water had been added. Cultures were incubated in 30 mL serum bottles (Wheaton) sealed with injectable sleeve-style rubber stoppers.

In the second experiment, the effect of NO_2^- and NH_4^+ on amino acid catabolism was investigated. Five mL volumes of each strain were cultured in lactate media either alone, with NH_4^+ (13.63 mM NH_4^+-N, with NO_2^- (3.57 mM NO_2^--N) or with both NH_4^+ and NO_2^-. This level of $(NH_4)_2SO_4$ (0.09%) was chosen as it was the level usually used in the media and thus allowed comparison with the regular media. For these studies, the Bacto-casitone or Bacto-casamino acids was replaced with a mixture of

pure L-amino acids (Asp[1], Glu, Ser, His, Gly, Thr, Arg, Ala, Tyr, Trp, Met, Val, Phe, Ile, Leu and Lys) each at 1.5 mM final concentration. Inoculated cultures plus uninoculated controls were incubated (24 h, 39°C) in 16 x 125 mm Pyrex test tubes sealed with butyl rubber stoppers. At the end of the incubation period, H_2 and total gas production were estimated immediately and cultures were centrifuged at approximately 12,000xg (10,000 rpm) for 15 min. Cell-free supernatants were stored at -40°C until used for amino acid analyses. One mL aliquots of the supernatant for volatile fatty acid (VFA) analysis were mixed in a 5:1:1 ratio of supernatant:25% phosphoric acid:internal standard and stored at 4°C.

Each of the above two experiments was run once.

II-2-3. Methods of analysis. VFA analyses were performed using a temperature- programmed Varian 3700 Gas Chromatograph (GC) (Varian Canada, Inc, Georgetown, Ont) equipped with a flame ionization detector. Columns used were: OV 351 (Supelco Canada Ltd., Oakville, Ont) (30 m x 0.25 mm I.D. with a 0.25 µm film thickness) with N_2 as a carrier gas and a Hewlett-Packard 3350 data system (Hewlett-Packard (Canada) Ltd., Mississauga,Ont) or 10% SP1000/1% H_3PO_4 on 100/120 Chromosorb W (Supelco) AW (6' x 0.125", glass) with He as carrier gas and a Shimadzu

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[1]Abbreviations used: aspartate, Asp; asparagine, Asn; glutamate, Glu; glutamine, Gln; serine, Ser; threonine, Thr; glycine, Gly; histidine, His; arginine, Arg; tyrosine, Tyr; phenylalanine, Phe; tryptophan, Trp; alanine, Ala; valine, Val; leucine, Leu; isoleucine, Ile; methionine, Met; lysine; Lys; cysteine, Cys; proline, Pro.

Chromatopac C-R1B (Shimadzu Corporation, Kyoto, Japan) data system. Neither system allowed separation of 2-methylbutyric from 3-methylbutyric (isovaleric) acid. The internal standard was 2-ethylhexanoic acid. Hydrogen determination was also performed on the Varian 3700, equipped with a thermal conductivity detector. Porapak Q 80/100 columns (6' x 1/8", SS) packed with 6% FFAP (Supelco) were used, and N_2 was the carrier gas. Sample injection size was 50 µL and quantitation was performed using a calibration curve based on peak areas. Total gas production was determined by injecting with a syringe connected to a simple U-tube water manometer through the serum vial stopper and observing the volume displaced.

Prior to analysis, *o*-phthaldialdehyde (OPA) derivatives of amino acids were prepared according to the method of Jones and Gilligan (1983). Analyses were done on a Varian 5000 or a Varian 5560 high performance liquid chromatograph (HPLC) using a Varian 2070 Spectrofluorometer or a Fluorichrom detector at an excitation wavelength of 340 nm and emission wavelength of 450 nm. Data were processed by a Hewlett-Packard 3350 data system. The column used was a Supelcosil 3 µm LC-18 reverse phase (4.6 x 150 mm; Supelco) equipped with a guard column (4.6 x 50 mm) packed with LC-18 reverse phase packing (20-40 µm; Supelco). Ethanolamine was the internal standard. A two-solvent gradient system based on the method of Jones and Gilligan (1983) was used for elution. Proline and Cys were not measurable by this method. The standard used was a commercially available preparation (AA-S-18; Sigma) to which Asn, Trp and Gln were added. Amino acids were quantitated by determination of peak areas, except for 2-aminobutyric

acid, which was calculated later using a calibration curve applied to initial peak areas.

Aliquots for NO_3^-, NO_2^-, and NH_4^+ assay were stored at 4 °C with 15% (saturated) lead acetate as a preservative (Alaboudi and Jones, 1985) added in a 2:1 ratio (supernatant:lead acetate). Before these analyses, the Pb^{2+} was precipitated by the addition of 10% (w/v) sodium sulfate (volume equal to that of lead acetate). Other preservatives were compared (tungstate/sulfuric acid and trichloracetate) but substantial NO_2^- loss occurred. NO_3^- was estimated by the method of Cataldo et al. (1975), NO_2^- by the method of Schneider and Yeary (1973) and ammonia by the method of Weatherburn (1967).

T-tests and Least Square Differences multiple range test were used for comparison of means between treatments using the GLM procedure of the SAS System (SAS Institute Inc., 1985).

II-3. Results

The inability to reduce NO_3^- was confirmed initially in all five strains of M. elsdenii. NO_2^- reduction was then determined at 10, 50 and 100 µg NO_2^--N/mL (equivalent to 0.71, 3.57 and 7.14 mM NO_2^--N) in glucose-containing medium. Nitrate reduction and growth occurred at the two lower concentrations with strains T81, AW106, J1 and LC1 but the growth rate of B159 was slightly inhibited by 0.71 mM NO_2^--N and completely inhibited by 3.57 mM NO_2^--N. All strains were completely inhibited at 7.14 mM NO_2^--N. Nitrite at 3.57 mM NO_2^--N was selected as the experimental concentration and strain B159 was retained as a

negative control.

Tables II-1 and II-2 (summarized from Appendix 1 and 2, respectively) show the effects of 3.57 mM NO_2^--N on H_2 production and medium VFA concentrations by the five strains of M. elsdenii grown on glucose and lactate, respectively. Generally, the major VFA products from glucose were acetate, butyrate and isovalerate. Final concentrations of caproate and isobutyrate in the medium varied among strains. Strain J1 showed relatively high levels of all the VFAs and included propionate which was not expected with glucose as a substrate (Marounek et al., 1989; Forsberg, 1978). In the presence of NO_2^-, final butyrate concentration was increased ($P < 0.05$) by the four uninhibited strains. Significant decreases in production of isovalerate, caproate and H_2 by strain T81, isovalerate and caproate by strain AW106 and propionate and valerate by strain J1 were also observed.

The major products from lactate were propionate, acetate, valerate with lesser amounts of butyrate, isovalerate and isobutyrate. Strains T81 and B159 were inhibited by the presence of NO_2^-. Negative values for CO_2 production were observed for both inhibited strains suggesting that whatever metabolism occurred may have involved the uptake of CO_2. For the other three strains, there was a decrease in H_2 and CO_2 production. An increase in acetate final concentration and decreases in butyrate and valerate final concentrations were observed for strain LC1. Decreases in isobutyrate and isovalerate concentrations in the medium occurred with strain AW106 whereas there was a decrease in propionate concentration and an increase in butyrate and caproate concentrations by strain J1. In a preliminary experiment in which gas production was not

determined (results not shown), similar inconsistent patterns of VFA production were observed among the strains but strain T81 was not inhibited by NO_2^-.

The effects of NO_2^- and NH_4^+ on amino acid catabolism are presented in Table II-3 (summarized from Appendix 4). The amino acids were added to achieve a final concentration of approximately 1.5 mM. Analysis of the uninoculated media showed that concentrations of Asn, His, Tyr, Trp and Met were approximately 1.6-1.8 mM, with the remainder of the amino acids being approximately 2.0-2.3 mM except for Ala which was approximately 3.0 mM. Gln was almost completely lost after reduction and autoclaving of the media and this chemical degradation of Gln may have contributed to the extra Ala present. Small amounts of amino acids may have been introduced by the addition of the Yeast Extract (Difco).

The percentage disappearance of the amino acids (where [AA] is amino acid concentration) was determined by the following formula:

$$\% \text{ disappearance} = \{([AA]_{initial} - [AA]_{final})/[AA]_{initial}\} \times 100$$

All values were corrected for non-bacterial degradation by subtraction of percentage disappearance values of an uninoculated control. Disappearance of Thr in the presence of NH_4^+ alone was greater than 100% in all strains except B159. The value for the control for $[Thr]_{final}$ in the presence of NH_4^+ was 26.0% greater than that of $[Thr]_{initial}$ resulting in a negative percentage disappearance which subsequently raised the corrected bacterial values above 100%. Values of greater than 100% disappearance were also observed for Thr in the presence of both NH_4^+

and NO_2^- with strains LC1 and J1. Once again, the $[Thr]_{final}$ value was 13.4% greater than the $[Thr]_{initial}$ value for this treatment also resulting in raised corrected values. It is not known what caused this increased Thr value or if this also occurred in the control when bacteria were not present. Ammonia, the common factor between the two treatment, is derivatized by OPA but elutes several minutes after Thr and therefore was not believed to be the cause.

Consistently, Ser and Thr h[illegible] the greatest percentage disappearance followed by Val, Leu and Ile with values varying among treatments and strains (strain LC1 had much higher values of Leu percentage disappearance relative to the other strains which corresponded to higher medium concentrations of isovalerate). This pattern is similar to those obtained by Wallace (1986) and Scheifinger et al. (1976). Ala was also consistently produced by all strains with all treatments except for strain J1 in the presence of NO_2^- alone.

In the media without $(NH_4)_2SO_4$, NO_2^- generally increased amino acid percentage disappearance for strains T81, AW106 and J1 but had little effect on strain LC1. Ammonia also caused a general increase in amino acid percentage disappearance with strains T81, AW106 and LC1 but had little or no effect with strains J1 and B159. Together, NO_2^- and NH_4^+ caused an increase with strain T81 but the increase was not greater than that caused by the NO_2^- or NH_4^+ alone. With strain LC1, NO_2^- and NH_4^+ together tended to decrease disappearance of amino acids while with strain AW106, any gain caused by the individual compounds was negated when they were present together. With strain J1, the increases caused by NO_2^- and NH_4^+ together were greater than those caused by the presence

of NH_4^+ but not as great as those caused by NO_2^- alone.

With strain T81, the presence of NO_2^- in medium supplemented with $(NH_4)_2SO_4$ caused little effect on percentage disappearance of amino acids but with strains LC1 and AW106, there was a general decrease in percentage disappearance whereas an increase was seen with strain J1. The presence of NH_4^+ in medium supplemented with NO_2^- also had little effect on strain T81 but decreases were observed with strains LC1, AW106 and J1.

The major VFA products from M. elsdenii (results in Appendix 5) were propionate, acetate, butyrate and valerate and varying amounts of isovaleric acid. There was little similarity between these results and those of the previous incubation (Appendix 1 and 2) possibly due to the difference in amino acid form (peptide and amino acid mixture vs free amino acids). No patterns between strains were seen from the VFA results in response to NO_2^- or NH_4^+.

An unknown amino acid was also produced in substantial quantities by all strains and was suspected to be 2-aminobutyric acid as previously reported by Wallace (1986). This was confirmed by comparison of its retention time to published values (Jones and Gilligan, 1983) and by co-elution with pure 2-aminobutyric acid. The production of 2-aminobutyric acid varied among strains; B159 having the lowest final concentration at 1.31 mM with the other four strains having concentrations ranging from 3.4 to 4.6 mM when no additional NO_2^- or NH_4^+ was added (Table 1-4). The presence of NO_2^- and NH_4^+ alone or together caused either no change or decreases in 2-aminobutyric acid production.

Strain B159 also showed a decrease in 2-aminobutyric acid production in the presence of NH_4^+ without NO_2^-.

II-4. Discussion

Strain J1 unexpectedly produced large amounts of propionate when grown with glucose as a substrate. Marounek et al. (1989) detected no propionate from this strain and there is no reason to suspect this is simply a reaction to the presence of NO_2^- since no such effect was seen with the other strains Therefore it is suspected that the innoculum of this strain in this trial was contaminated. As the cultures were checked regularly for contamination by microscopic examination, it is believed the contamination was introduced at the time of inoculation of the serum vials and was not present in the inocula source.

The results shown in Tables II-1 and II-2 did not follow the expected pattern of increased oxidized and decreased reduced VFAs with the addition of an alternate electron acceptor. Other investigators have studied the effect of NO_3^- using pure cultures of rumen bacteria (de Vries et al., 1974), rumen liquor (Bryant, 1965) and with in vivo experiments (Farra and Satter, 1971; Alaboudi and Jones, 1985) and have demonstrated increases in acetate and decreases in reduced products (ie. propionate and butyrate). Similar results have also been obtained in non-rumen anaerobic systems (Keith et al., 1982; Yamamoto et al., 1982; Allison and MacFarlane, 1988). With M. elsdenii using glucose as a substrate, increased butyrate production was the only consistent result among the four uninhibited strains T81, LC1, AW106 and J1. Glucose is

metabolized via the Embden-Meyerhof glycolysis pathway to pyruvate. Acetyl-CoA is produced from pyruvate in a reaction catalyzed by pyruvate:ferrodoxin oxidoreductase. Ferredoxin (Fd) is the electron acceptor and the reduced form can act as an electron donor for the hydrogenase of M. elsdenii (Peel, 1960; van Dijk et al., 1979). If the reduction of NO_2^- is Fd-coupled, as it is in Veillonella alcalescens (Yordy and Delwiche, 1979), reduction of additional NO_2^- may have allowed further substrate to be metabolized via the pyruvate:ferrodoxin oxidoreductase by re-oxidizing reduced Fd. This could lead to increased butyrate production without a corresponding increase in H_2 production which would imply that the cells were limited for oxidized Fd. Greater CO_2 production would also be expected due to the increased pyruvate being converted to acetate. Strain LC1 growing with glucose (Table II-1) had increased CO_2 production following the proposed scheme but no change occurred with strain AW106. The decrease in CO_2 and H_2 production with strain T81, without a decrease in the VFAs would be indicative of a shift in metabolism prior to the pyruvate oxidoreductase step in the metabolic pathway. However no VFAs are seen to increase sufficiently to dispose of the electrons not being utilized in H_2 production. This suggests the possibility of unmeasured routes of electron disposal.

Production of VFAs was much lower with glucose as the substrate than with lactate (Table II-2). This was probably due to the greater energy conservation from glucose. Theoretical ATP yields for the conversion of glucose to acetate or butyrate are four and three ATP/glucose, respectively, while the acrylate pathway yields one

ATP/three lactate (Gottschalk, 1979). Valerate production also differed greatly between substrates. The greater production of valerate from lactate can be attributed to greater production of acetate and propionate, from which valerate is formed by the acrylate pathway (Prins, 1977). Valerate production by strain LC1 decreased with NO_2^- addition; this corresponded to the increase in acetate production. This may be due to a decreased need to dispose of electrons via valerate production.

Utilizing lactate, the three uninhibited strains, LC1, AW106 and J1 all displayed a decrease in H_2 production in the presence of NO_2^- (Table II-2). Unlike growth on glucose, this supports the likelihood of either the involvement of Fd in NO_2^- reduction (assuming the decrease was due to competition between the hydrogenase and NO_2^- reductase systems for the reduced Fd) or the ability of H_2 formed by the hydrogenase system to act as an electron donor for the NO_2^- reductase system. The hydrogenase of M. elsdenii has been shown to accept electrons from Fd, flavodoxin and methyl viologen (van Dijk et al., 1979). A decrease in H_2 production is also found in mixed cultures using human faecal slurries in response to KNO_3 addition (Allison and MacFarlane, 1988) and H_2 and formate have been shown to be good electron donors for both mixed or pure cultures (Allison and MacFarlane, 1988; Jones, 1972; Yordy and Delwiche, 1979). The observed decrease in H_2 alone was insufficient to account for the amount of NO_2^- reduced. The reduction in measured H_2 is further significant because in the rumen, this would have the potential to reduce CH_4 production.

In order to perform the six electron reduction of the added NO_2^-,

approximately 428 µmoles of electrons or 214 µmoles of electron pairs would have been required. The changes in VFAs in terms of electron pairs, in response to the presence of NO_2^-, were calculated (see Appendix 3) with respect to their contribution to the electron pool assuming production of two µmoles electron pairs per µmole acetate, isobutyrate and isovalerate (the latter two via amino acid deamination), three µmoles electron pairs per µmole butyrate and four µmoles electron pairs per µmole caproate as well as the utilization of one µmole electron pair per propionate and H_2 produced and none per µmole valerate. These were determined assuming the Embden-Meyerhoff pathway for growth on glucose and the acrylate pathway for growth on lactate (Gottschalk, 1979). Using these values a deficit of approximately 145 electron pairs was determined for strain T81 and a surplus of approximately 229 and 163 electron pairs for strains LC1 and AW106 were determined for growth on glucose respectively. With lactate as a substrate, surpluses of approximately 164, 290 and 320 electron pairs were calculated for strains LC1, AW106, and J1, respectively. A balance was not achieved considering VFAs alone but these calculations do not take into account the role of amino acid metabolism in electron disposal.

Nitrite, generally, stimulated amino acid catabolism but the extent of stimulation differed between strains with T81 and J1 showing the greatest increases. Ammonia also was stimulatory especially with strains T81 and LC1. Low concentrations of NH_4^+ have been shown to increase rates of protein degradation. Both Erfle *et al.* (1977), in an *in vitro* experiment using NH_4^+ ranging in concentration from 0.5 to 3

mM, and Wallace (1979), in an *in vivo* experiment using NH_4^+ ranging from 6.1 to 13.4 mM, demonstrated increases in amino acid and protein degradation corresponding to increasing NH_4^+ concentration. The amount of $(NH_4)_2SO_4$ (0.09%) used in the media was equivalent to 13.63 mM NH_4^+-N or 191 µg NH_4^+-N/mL. Since NO_2^- is reduced to NH_4^+, the same mechanism might be expected to mediate NO_2^- and NH_4^+ stimulation of amino acid degradation. However, the presence of added NH_4^+ generally negated the effects of NO_2^- so the effect of NO_2^- on amino acid degradation appears to be different in mechanism from that of NH_4^+.

Nitrite may have dual effects as both an electron acceptor and an inhibitor of metabolism. Nitrite is able to inhibit the phosphoroclastic pyruvate:ferrodoxin oxidoreductase in *Clostridium sporogenes* (Woods *et al.*, 1981) and may affect *M. elsdenii* if the oxidoreductases are similar. If the enzyme is inhibited, other pathways such as amino acid degradation may become of greater importance for energy conservation. As well, electrons are diverted for NO_2^- detoxification. The inhibition of the pyruvate:ferrodoxin oxidoreductase would account for the reduction in CO_2 and H_2 production which was more evident when the strains were grown on lactate. The increase in CO_2 shown by strain LC1 when grown on glucose indicates no such inhibition of the oxidoreductase with this strain and possibly a lower susceptibility to this mechanism of NO_2^- inhibition.

The production of Ala from pyruvate is a reductive amination or transamination therefore the decrease in production may be due to

pyruvate role as an electron acceptor in the following reaction:

$$\underset{\text{pyruvate}}{CH_3\overset{\overset{O}{\|}}{C}COO^-} + NH_4^+ + 2e^- + 2H^+ \longrightarrow \underset{\text{alanine}}{CH_3\overset{\overset{NH_3^+}{|}}{C}HCOO^-} + H_2O$$

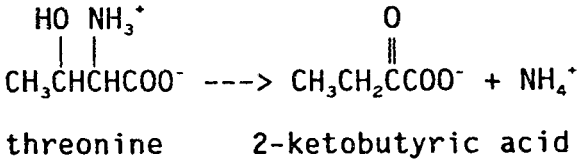
The presence of NO_2^-, as an alternate electron acceptor, would be expected to decrease Ala production if can compete with pyruvate for electrons. However, the above reaction does not account for the decrease in Ala production in the presence of NH_4^+. If Ala is also an NH_4^+ sink, Ala production should increase in the presence of NH_4^+ but the opposite was observed. This decrease in Ala production may be due to Ala also being subject to the general increased amino acid catabolism caused by the presence of NH_4^+.

Hino and Russell (1985) observed that methylene blue, an agent capable of oxidizing NADH, increased the rate of deamination of the relatively reduced amino acids (Ala, Leu, Ile and Val) but not of the neutral (Ser, Thr, Phe and His) or relatively oxidized (Gly, Pro, Arg and Trp) amino acids. NO_2^- may act similarily by facilitating the re-oxidation of reduced electron carriers (produced in deamination and decarboxylation) either chemically or via enzyme-mediated processes. This re-oxidation mechanism could account for differences in effects of NO_2^- and NH_4^+ on amino acid catabolism. However, in the present study, no differentiation was observed between the oxidized, neutral or reduced amino acids.

The decrease in Ala concentration in the presence of NH_4^+ was inconsistent with the experiments of Erfle *et al.* (1977) and Wallace

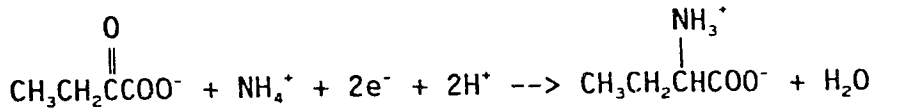
(1979) who observed an increase in Ala concentration with rumen fluid at high NH_4^+ concentrations. Erfle et al. (1977) suggested a role for Ala as a NH_4^+ storage depot or as a mechanism to transport pyruvate (converted to Ala by amination or transamination) into the cell. The former does not appear to be a role for Ala production since neither added NH_4^+ nor NO_2^-, which increased amino acid catabolism and would have increased NH_4^+ concentration concurrently (in the second experiment), caused increased Ala levels. It is possible, however, that any increase in Ala production may have been masked by the general increase in amino acid catabolism caused by either the presence of NO_2^- or NH_4^+.

The unusual amino acid 2-aminobutyric acid was produced by all strains (Table II-4). Changes in 2-aminobutyric acid concentration were found to parallel those of Ala (Table II-5) suggesting either similar controls or mechanism of production. Wallace (1986) demonstrated that 2-aminobutyric acid is produced by M. elsdenii from Thr but not from Ser but production from other amino acids was not checked. Catabolism of Thr by M. elsdenii is performed by threonine deaminase which forms 2-ketobutyric acid (Walker 1958).



Group I type Clostridia also produce 2-aminobutyric acid from Thr. Some Group I type Clostridia also produce 2-aminobutyric acid from Met.

These two amino acids are catabolized to 2-ketobutyric acid (Mead, 1971) which can subsequently be aminated or transaminated to form 2-aminobutyric acid similar to Ala formation from pyruvate.



2-ketobutyric acid 2-aminobutyric acid

The similarity in response of 2-aminobutyric acid and Ala to NO_2^- and NH_4^+ implies that the treatments may have affected each synthetic pathway similarily or that 2-ketobutyric acid, a structural analogue of pyruvate, may also serve as a substrate for the system(s) producing Ala from pyruvate. The reduction of 2-ketobutyric acid could also be performed without the need of amination or transamination, resulting in the formation of 2-hydroxybutyrate and still fulfill the need for electron disposal. Analysis for 2-hydroxybutyrate was attempted but levels in culture supernatant were too low to determine quantitatively. However, 2-hydroxybutyrate can also act as a substrate for lactate dehydrogenase (Brockman and Wood, 1975) and thus it would not be expected to be detected in the culture supernatant. This suggests a role as an electron sink for 2-aminobutyric acid and 2-ketobutyric acid using the same enzyme system(s) responsible for the production of Ala from pyruvate. Both Ala and 2-aminobutyric acid have been shown to participate in transamination reactions in cell-free extracts from Neurospora crassa (Fincham and Boulter, 1956) and Escherichia coli (Rudman and Meister, 1953). These amino acids may also play a role in electron and NH_4^+ disposal in a transamination reaction linked to the catabolism of other amino acids.

The amount of 2-aminobutyric acid in the final medium was much greater than the amount of Thr present initially in the media suggesting either another source of 2-ketobutyric acid (assuming it is the precursor of 2-aminobutyric acid) or alternate routes of 2-aminobutyric acid production. Two other possible sources of 2-ketobutyric acid are cleavage of cystathionine to Cys and 2-ketobutyric acid (cystathionine having been formed by the condensation of Ser and homocysteine which can be formed from Met) or by the action of homoserine dehydratase on homoserine formed from Asp (Davis and Metzler, 1970). However, the loss from the media of the possible 2-ketobutyric acid sources does not yield a balance between amino acid loss and 2-aminobutyric acid production. Another possibility is that the method of quantitation may have given inaccurate values, although if this occurred, the 2-aminobutyric acid values relative to each other would still be valid. This is possible since the quantitation was done by the external standard method after the initial analysis in which the HPLC running conditions were reproduced as closely as possible. The samples could not be rerun by the internal standard method due to lack of sample.

Some investigators have shown that NO_2^- is more readily reduced when the host is consuming a high proportion of concentrates in its diet (Nakamura et al., 1979; Takahashi, 1980) and when lactate levels are higher in the diet (Nakamura et al., 1981). This is consistent with M. elsdenii's ecological niche, suggesting that the organism may play an important role in NO_2^- reduction in the rumen along with other lactate-utilizing NO_2^- reducers such as V. alcalescens (Yordy and Delwiche, 1979).

The NO_2^- reductase from M. elsdenii is unique from the dissimilatory NO_2^- reductases previously studied from other species including V. alcalescens (Yordy and Delwiche, 1979), Clostridium butyricum (Keith et al., 1982) Clostridium perfringens (Hasan and Hall, 1975), Wolinella succinogenes [formerly Vibrio succinogenes] (Blackmore et al., 1986) since it is not associated with a NO_3^- reductase. The role of NO_2^- reductase in these other systems appears to be detoxification of the NO_2^- produced by the energy-yielding NO_3^- reductase. Nitrite reduction is generally not an energy-yielding process (Hasan and Hall, 1975; Cole and Brown, 1980) although de Vries et al. (1982) had evidence that it may have been in Campylobacter sputorum and Bokranz et al. (1983) have demonstrated electron transport phosphorylation coupled to reduction of NO_2^- to NH_4^+ in W. succinogenes). It is not known if the reduction of NO_2^- via the reductase enzyme of M. elsdenii is an energy-yielding process or not. If NO_2^- reduction is not an energy-yielding process, its purpose in M. elsdenii may strictly be one of opportunistic electron disposal and coincidentally detoxifying the NO_2^- present in its environment to the benefit of itself and neighboring microbes. It could also have a role in supplying NH_4^+ when NH_4^+ or amino acids are insufficient and NO_2^- is present. However the existence of a NO_2^- reductase enzyme system in M. elsdenii has not been prover M. elsdenii has the ability to reduce NO_2^- but the mechanism is not known and could be a catalytic activity of another enzyme system or chemical reduction by a compound produced by the bacterium. In relation to this last point, NO_2^- is a strong oxidant and, as such, is very reactive. One can speculate that M. elsdenii could produce compounds

such as sulphides from the catabolism of the sulphur amino acids which may be able to react with NO_2^- reducing it to NH_4^+ and thereby detoxifying it.

A surprising result of this investigation is the lack of metabolic consistency between strains of M. elsdenii. Aspects such as VFA production were very similar but response to NO_2^- and NH_4^+, with respect to amino acid metabolism and H_2 production, although qualitatively similar were quantitatively different. Further classification on the basis of patterns of amino acid utilization may be applicable to this species in a manner similar to that used to classify Clostridia species (Mead, 1971) with which M. elsdenii shares many biochemical characteristics. Both genera are amino acid fermentors, although amino acids are not a major source of energy (Russell and Baldwin, 1979, Wallace 1986) for M. elsdenii as they are for Clostridia. The phosphoroclastic-type reaction to cleave pyruvate, the acrylate pathway for propionate production, and production of 2-aminobutyric acid are also common to both genera. The similarity between the metabolism of M. elsdenii and C. kluyveri in the production of n-VFAs higher than n-butyrate was noted by Elsden and Lewis (1953).

The presence of NO_2^- did not give the expected changes in the VFA metabolism of M. elsdenii although amino acid metabolism was more significantly affected. Amino acid catabolism generally increased in the presence of NO_2^-. This is likely due to the ability of NO_2^- to act as an electron acceptor, possibly for the reduced electron carriers produced during the deamination and decarboxylation of amino acids.

The production of 2-aminobutyric acid was found to parallel that of

Ala and both amino acids likely play a role in electron disposal and possibly in NH_4^+ metabolism as well. Wallace (1986) observed the production of 2-aminobutyric acid by M. elsdenii from Thr but little other research has been done on 2-aminobutyric acid in bacterial systems. 2-Aminobutyric acid is found to occur normally in human serum and urine (Soupart, 1962) as well as in body fluids and tissues from other mammals (Gordon, 1949; Meister, 1965). It is also found in plant tissues (Steward and Pollard, 1962), some insect species (Chen, 1962), and various microbes including the Group I type Clostridia (Holden, 1962; Loefer, 1962; Mead, 1971). This amino acid is not normally detected in rumen fluid and is likely metabolized via the general pattern for amino acid degradation entailing deamination and subsequent decarboxylation. This route would yield propionate. 2-Ketobutyric acid would not be expected to be detected in rumen fluid since the production of 2-aminobutyric acid appears to be an intracellular process. The source and role of 2-aminobutyric acid production and its significance in the rumen is not well understood and the discrepancy between Thr present and 2-aminobutyric acid detected in the present study points to a need for a more thorough investigation of 2-aminobutyric acid metabolism.

Table II-1. Effect of NO_2^- (3.57 mM NO_2^--N) on volatile fatty acid production by M. elsdenii grown on glucose.

Strain	Increased	Decreased
T81	Butyrate	Isovalerate, caproate, H_2
AW106	Butyrate	Isovalerate, caproate
LC1	Butyrate	Propionate, valerate

Table II-2. Effect of NO_2^- (3.57 mM NO_2^--N) on volatile fatty acid production by M. elsdenii grown on lactate.

Strain	Increased	Decreased
AW106		Isobutyrate, isovalerate, H_2, CO_2
LCI	Acetate	Butyrate, valerate, H_2, CO_2
J1	Butyrate, Caproate	Propionate, H_2, CO_2

Table II-3. Effect of NO_2^- (3.57 mM NO_2^--N) and/or NH_4^+ (13.63 mM NH_4^+-N) on amino acid percentage disappearance by M. elsdenii grown on lactate.

Strain	NO_2^-	NH_4^+	NO_2^- + NH_4^+
T81	↑	↑	↑
B159	N/A	-	N/A
LCI	-	↑	↓
AW106	↑	↑	-
J1	↑	-	↑

Table II-4. Effect of NO_2^- (3.57 mM NO_2^-N) and/or NH_4^+ (13.63 mM NH_4^+-N) on 2-aminobutyrate production.*

	T81	B159	LC1	AW106	J1
None	4.13	1.31	4.41	4.59	3.42
NO_2^-	3.40	0.28	4.49	3.90	2.66
NH_4^+	3.70	1.15	3.40	4.67	3.58
NO_2^- + NH_4^+	2.08	0.14	4.18	4.28	2.82
S.E.	0.12	0.01	0.08	0.09	0.12

* n = 3 for all means.

Table II-5. Effect of NO_2^- (3.57 mM NO_2^--N) and/or NH_4^+ (13.63 mM NH_4^+-N) on alanine and 2-aminobutyric acid production by M. elsdenii.

Strain	Compound	NO_2^-	NH_4^+	NO_2^- + NH_4^+
T81	Alanine	↓	↓	↓
	2-aminobutyric acid	↓	↓	↓
B159	Alanine	N/A	↓	N/A
	2-aminobutyric acid	N/A	-	N/A
LC1	Alanine	-	↓	-
	2-aminobutyric acid	-	↓	-
AW106	Alanine	-	↓	↓
	2-aminobutyric acid	↓	-	-
J1	Alanine	↓	-	↓
	2-aminobutyric acid	↓	-	↓

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III. Production of 2-Aminobutyric Acid by Megasphaera elsdenii

III-1. Introduction.

Megasphaera elsdenii (formerly called Peptostreptococcus elsdenii [Gutierrez et al., 1959]) is an anaerobic gram-negative cocci present in the rumen of sheep and cattle (Elsden et al., 1951; Scheifinger et al., 1976). This species is most abundant when cattle are fed diets containing high levels of concentrates that favour the production of lactate (Baldwin et al., 1962; Baldwin et al., 1963; Wallnofer et al., 1966; Hungate, 1966) which it ferments via the acrylate pathway (Ladd, 1959). M. elsdenii also plays an important role in amino acid catabolism (Scheifinger et al., 1976, Wallace, 1986) resulting in the production of branched-chain volatile fatty acids (VFAs) and NH_4^+ essential for the growth of some rumen species (Hungate, 1966). The major end products from lactate and amino acid fermentation are acetate, propionate, butyrate, valerate, CO_2, and H_2 with lesser amounts of isobutyrate, 2- and 3-methylbutyrate (isovalerate) and caproate.

In the previous experiments, 2-aminobutyric acid was observed as an end product produced by five strains of M. elsdenii (T81, B159, LC1, AW106 and J1). 2-Aminobutyric acid is found to occur normally in human serum and urine (Soupart, 1962) as well as in body fluids and various tissues of other mammals (Gordon, 1949; Meister, 1965). It is also found in plant tissues (Steward and Pollard, 1962), some insect species (Chen, 1962) and various microbes including the Group I type Clostridia (Holden, 1962; Loefer, 1962; Mead, 1971). 2-Aminobutyric acid has not

been found to be a constituent of any protein although the L isomer is a component of the tripeptide opthalmic acid whereas the D isomer is a component of staphylomycin S (Meister, 1965). This amino acid is not normally detected in rumen fluid. If present and if it is metabolized via the general pattern for microbial amino acid degradation consisting of deamination and subsequent decarboxylation, propionate would be produced from 2-aminobutyric acid.

The source and role of 2-aminobutyric acid production and its significance in the rumen is not well understood. Production of 2-aminobutyric acid by M. elsdenii was first observed with strain LC1 by Wallace (1986) who determined that Thr[1] but not Ser led to its formation. The action of Thr deaminase (4.2.1.16) on Thr (Walker, 1958) results in the formation of 2-ketobutyric acid which is presumably aminated or transaminated to form 2-aminobutyric acid. The export of 2-aminobutyric acid out of the cell may prevent its deamination or a reversal of the transaminase as the 2-aminobutyric acid concentration increases. Our previous results suggested that 2-ketobutyric acid was an electron and possibly an NH_4^+ acceptor for M. elsdenii (resulting in 2-aminobutyric acid formation) but that its metabolism may not be directly coupled to catabolic transamination of other amino acids.

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[1]Abbreviations used: aspartate, Asp; asparagine, Asn; glutamate, Glu; glutamine, Gln; serine, Ser; threonine, Thr; glycine, Gly; histidine, His; arginine, Arg; tyrosine, Tyr; phenylalanine, Phe; tryptophan, Trp; alanine, Ala; valine, Val; leucine, Leu; isoleucine, Ile; methionine, Met; lysine; Lys; cysteine, Cys; proline, Pro.

This was inferred since the presence of NO_2^- or NH_4^+, which generally increased amino acid catabolism decreased 2-aminobutyric acid (and Ala) production rather than increasing their concentrations as expected.

The amount of 2-aminobutyric acid produced in the previous experiment exceeded the amount of Thr lost from the media suggesting that Thr was not the lone source of 2-ketobutyric acid. Other potential sources of 2-ketobutyric acid include the cleavage of cystathionine to form Cys and 2-ketobutyric acid and the dehydration of homoserine formed from Asp (Davis and Metzler, 1970). Cystathionine can be formed by the condensation of Ser and homocysteine; the latter being formed from either Asp, Met or Cys.

The potential sources for 2-aminobutyric acid were investigated as well as the mechanism for its production by utilizing L-cycloserine, an inhibitor of alanine transaminase (2.6.1.2). In a previous experiment in which the effects of NO_2^- and NH_4^+ on amino acid catabolism were investigated, 2-aminobutyric acid production was found to parallel that of Ala suggesting either shared enzyme systems or regulatory mechanisms. L-cycloserine, an inhibitor of alanine transaminases, was used to investigate the mechanism of 2-aminobutyric acid production in the present study.

III-2. Materials and Methods

III-2-1. Organisms. Five strains of M. elsdenii from the Agriculture Canada Research Station culture collection at Lethbridge, Alberta. were used. Strains J1 and LC1 (Elsden et al., 1951) were originally obtained

from S. O. Mann of the Rowett Research Institute, Aberdeen. Strains AW106, T81 and B159 (Bryant et al., 1958) were from Dr. M. P. Bryant of the University of Illinois, Urbana. These five cultures were maintained on media containing (per litre) 0.45 g K_2HPO_4, 0.45 g KH_2PO_4, 0.9 g $(NH_4)_2SO_4$, 0.9 g NaCl, 0.09 g $MgSO_4$, 0.09 g $CaCl_2$, 0.03 g $FeSO_4$, 10 g Bacto-casamino acids (Difco Laboratories, Detroit, Mich.), 2.5 g yeast extract (Difco), 0.001 g resazurin, and 6 mL of 60% sodium lactate. Following adjustment of the pH of the above mixture to 6.5, it was dispensed in 300 mL aliquots to 500 mL round-bottomed flasks, and 15 mL of 8% Na_2CO_3 (w/v) was added to each. All subsequent reduction procedures were performed anaerobically under O_2-free CO_2 (obtained by passing Coleman or Instrument grade CO_2 through a copper furnace heated to approximately 350°C). The medium was brought to boiling, 0.3 g crystalline cysteine-HCl was added and the solution was brought to boiling again and then allowed to cool for 5 min. The solution was then bubbled for 10 min with CO_2, distributed, sealed and autoclaved for 20 min at 120°C.

Anaerobic culture techniques were essentially those of Hungate (1950) as modified by Bryant and Burkey (1953).

III-2-2. Experimental Protocol. In order to determine alternate amino acid precursors of 2-aminobutyric acid, 0.1 mL of aqueous solutions of Thr, Ser, Met or Asp (or sterile H_2O as a control) were added separately to 5 mL of lactate medium to increase their respective concentrations by approximately 1.5 mM. Each of the five strains (T81, B159, LC1, AW106 and J1) were incubated in triplicate with each of the above amino acid treatments at 39°C for 24 h.

To determine an effective concentration of L-cycloserine to use with M. elsdenii, strain LC1 (the type strain) was initially incubated in triplicate in 5 mL lactate media at 39°C for 24 hrs with 0, 1, 2, 4 and 8 mM L-cycloserine. Subsequently, all five strains were incubated in triplicate in lactate media with and without 1 mM L-cycloserine for 24 hrs at 39°C.

At the end of the incubation periods, cell-free supernatants were obtained by centrifugation of the cultures at approximately 12,000xg for 15 min at -4°C. Supernatant aliquots were frozen at -40°C until required for amino acid analysis and aliquots for volatile fatty acid (VFA) analysis were mixed in a 5:1:1 ratio of supernatant:25% phosphoric acid:internal standard and stored at 4°C.

Each of the above experiments was run once.

III-2-3. Methods of Analysis. Analyses for VFAs were performed using a temperature- programmed Varian 3700 Gas Chromatograph (GC) (Varian Canada, Inc., Georgetown, Ont.) equipped with a flame ionization detector. Columns used were: OV 351 (Supelco Canada Ltd., Oakville, Ont), (30 m x 0.25 mm I.D. with a 0.25 µm film thickness) with N_2 as a carrier gas. Data were processed by a Hewlett-Packard 3350 data system (Hewlett-Packard (Canada) Ltd., Mississauga, Ont), 10% SP1000/1% H_3PO_4 on 100/120 Chromosorb W (Supelco) AW (6' x 0.125", glass) with He as carrier gas and a Shimadzu Chromatopac C-R1B data system (Shimadzu Corporation, Kyoto, Japan). Neither system allowed separation of 2-methylbutyric from 3-methylbutyric (isovaleric) acid. Internal standard was 2-ethylhexanoic acid.

Prior to analyses, the o-phthaldialdehyde (OPA) derivatives of the

amino acids were prepared according the method of Jones and Gilligan (1983). Analyses were done on a Varian 5000 or a Varian 5560 high performance liquid chromatograph (HPLC) using a Varian 2070 Spectrofluorometer or a Fluorichrom detector at an excitation wavelength of 340 nm and emission wavelength of 450 nm. Data were processed by a Hewlett-Packard 3350 data system. The column used was a Supelcosil 3 µm LC-18 reverse phase (4.6 x 150 mm; Supelco) equipped with a guard column (4.6 x 50 mm) packed with LC-18 reverse phase packing (20-40 µm; Supelco). Ethanolamine was the internal standard. A two-solvent gradient system based on the method of Jones and Gilligan (1983) was used for elution. Pro and Cys were not measurable by this method. The standard used was a commercially available preparation (AA-S-18; Sigma) to which Asn, Trp and Gln were added.

Data were analyzed using t-tests and the Least Square Differences multiple range test of the GLM procedure of the SAS System (SAS Institute Inc., 1985).

III-3. Results

Appendices 6 and 7 show the effect of amino acid supplementation on the final amino acid concentration and VFA production by the five strains of M. elsdenii in the media. Growth problems were encountered with strain B159 as evidenced by the lack of VFA production and low Thr utilization relative to the previous studies. Problems were also encountered with strain J1. VFA production and amino acid concentrations were comparable to the other strains but the source

culture was found to be contaminated. A new vial of the freeze-dried strain was obtained. It is not known if this contamination was present in this experiment and if this caused the lack of significant change in 2-aminobutyric acid levels in response to Thr supplementation. This part of the experiment was not rerun with the newly obtained J1 source although it was used in the following L-cycloserine experiment.

The amino acid concentrations for the unsupplemented control were generally lower than the supplemented treatments regardless of amino acid supplemented for strains T81, LC1 and AW106 which grew normally. The reason for this is unclear. Of the three remaining strains, Asp appeared to inhibit strains T81 and AW106 whereas LC1 appeared to be unaffected. Ser and Thr were utilized almost completely while Asp and Met did not appear to be metabolized at all.

Strains T81, LC1 and AW106 showed significant increases in 2-aminobutyric acid production in response to Thr supplementation whereas strain LC1 also had a significant increase with Met supplementation. Relative to the other supplemented amino acids, Thr and Met supplementation of strain LC1 caused significant decreases in Ala, Val, Leu and Lys production. Thr supplementation of strain AW106 caused a significant decrease in Ala and Glu production.

There were numerous significant changes in VFA production but no pattern emerged in response to amino acid supplementation.

The effects of increasing levels of L-cycloserine on amino acid metabolism by strain LC1 can be seen in Appendix 8. Significant increases in Asp, Val, Ile, and Leu concentration were observed as well as decreases in Ala, 2-aminobutyric acid and Phe concentration in the

presence of L-cycloserine.

Appendix 9 shows the effect of 1 mM L-cycloserine on amino acid metabolism of all five strains of M. elsdenii. Due to missing values in both sets of triplicates (with and without L-cycloserine) of strain LC1, the significance of the observed effects could not be determined due to insufficient degrees of freedom but the changes observed were similar to those reported above for strain LC1 treated with 1 mM L-cycloserine (Appendix 8) except that the degradation of the branched-chain amino acids was much greater in this experiment. Growth of strain B159 was inhibited by the administration of 1 mM L-cycloserine.

Decreases in both Ala and 2-aminobutyric acid production were seen with strains LC1 and J1 but the degree of significance of the change with LC1 could not be determined. Strain J1 also had decreased utilization for Val, Ile and Leu which corresponds to the changes observed with strain LC1 in this treatment as well as with LC1 treated with a range of L-cycloserine concentrations.

L-cycloserine decreased propionate production (Appendix 10) for strains T81, AW106 and J1 and butyrate production for strains LC1, AW106 and J1. Both isobutyrate and isovalerate concentrations were lower in the presence of 1 mM L-cycloserine for strains LC1 and J1 corresponding to their decreased utilization of the branched-chain amino acids. Valerate concentrations were affected differently between strains; increased for strains T81 and J1 but decreased for strains LC1 and AW106. Acetate production was also decreased for strains LC1 and AW106.

III-4. Discussion

Supplementation with Thr for the three strains which grew normally resulted in an increase in 2-aminobutyric acid (Figure III-1) confirming the role of Thr in 2-aminobutyric acid production as earlier demonstrated by Wallace (1986). Met also stimulated 2-aminobutyric acid production by strains T81 and LC1 but apparently not via formation of 2-ketobutyric acid from Met since the concentration of Met in the media was not decreased. The presence of Met may have had a catalytic effect on 2-aminobutyric acid production but there is no evidence to support this possibility or obvious biochemical hypothesis to explain this effect.

The concentration of 2-aminobutyric acid increased by approximately 1-1.8 mM for strains T81, LC1 and AW106 with Thr supplentation. This amount can be attributed to the catabolism of Thr to 2-ketobutyric acid and subsequent amination or transamination to 2-aminobutyric acid. However, levels of 2-aminobutyric acid in treatments without supplemented Thr were greater than the amount of Thr available. This may have been caused by Thr present in peptides that M. elsdenii can utilize but which would not be quantitated as Thr during HPLC analysis. These may have been present in small amounts in Bacto-casamino acids (Difco) and/or Yeast Extract (Difco).

With strains T81, LC1 and AW106, Ala and 2-aminobutyric acid production was inversely related suggesting that pyruvate and 2-ketobutyric acid may be utilizing NH_4^+ from the same source pool. Also directly correlated to the changes in Ala and inversely correlated

to the changes in 2-aminobutyric acid are the changes in branched-chain amino acid concentrations (Table III-2) suggesting some linkage between these two groups. Threonine supplementation could potentially increase Ile production since 2-ketobutyric acid is a precursor to Ile biosynthesis but this only occurred with strain J1. Catabolized Thr appears to have gone to 2-aminobutyric acid production with stains T81, LC1 and AW106 while J1, which did not increase 2-aminobutyric acid production, appeared to use some of the 2-ketobutyric acid produced from Thr for Ile production.

In the presence of increasing levels of the alanine transaminase inhibitor L-cycloserine, the concentrations of 2-aminobutyric acid, Ala and Phe were lower while the concentration of the branched-chain amino acids and Asp were greater. As above, it appears that decreased Ala and 2-aminobutyric acid results in increased branched-chain amino acid concentrations demonstrating an inverse relationship.

In the presence of L-cycloserine, the concentration of Asp was greater. Some clostridial species such as C. welchii contain a decarboxylase which converts Asp to Ala (Gottschalk, 1979). The reverse reaction may be possible if the Ala concentration was sufficiently high and if this enzyme is present in M. elsdenii. A more likely source is by the transamination of oxaloacetate which may have taken up the role of pyruvate and 2-ketobutyric acid as an NH_4^+ acceptor in transamination thereby forming Asp. Oxaloacetate can be formed from the degradation of Asp and Asn, but Asn is present in the media in insignificant amounts and Asp could not be an increasing source for itself. Therefore, it is likely that oxaloacetate is produced from 2-ketoglutarate possibly via

the glyoxalate cycle. Possible sources of 2-ketoglutarate include Glu, Gln, Pro, Arg and His. Arg and His are not significantly utilized and Gln was not present in the media. Since Glu is produced by strain LC1, Pro is the probable source. Unfortunately, Pro was not detected by the method used to analyze amino acids since the derivatizing agent does not react with secondary amines. Therefore our data provides no direct evidence for the above hypothesis. However, Cotta and Russell (1982) demonstrated that 15% of the Pro present in media was utilized by strain T81 while Wallace (1986) demonstrated an increase in Pro concentration with strain LC1 under continuous culture conditions. Their studies confirm the ability of M. elsdenii to metabolize Pro thereby making the above hypothesis feasible.

Strains T81, LC1, AW106 and J1 all exhibited decreases in Ala and 2-aminobutyric acid concentration in the presence of L-cycloserine but only strain J1 achieved significance at the 0.05 probability level. The significance of the changes with LC1 could not be determined but would likely have been significant at least with 2-aminobutyric acid considering the extent of the decrease. These results support the assumption that 2-aminobutyric acid is formed by a transamination reaction. Also, since there appears to be an inverse relation between 2-aminobutyric acid and the branched-chain amino acids, it is likely that the branched-chain amino acids act as the electron and NH_4^+ donor and 2-ketobutyric acid acts as the acceptor. As well, since the changes within strains between Ala and 2-aminobutyric acid were very similar in magnitude and because of their structural similarity, it is possible that the formation of Ala and 2-aminobutyric acid are performed by the

same enzyme.

Strain J1 also showed the same pattern between the branched-chain amino acids and Ala and 2-aminobutyric acid as strain LC1. This further supports the existence of a relationship between the branched-chain amino acids and Ala or 2-aminobutyric acid. Pyruvate and 2-ketobutyric acid are likely co-substrates with the branched-chain amino acids for transamination.

If 2-ketobutyric acid and a branched-chain amino acid undergo a transamination reaction to form 2-aminobutyric acid and the corresponding keto acid, the supplementation of Thr would be expected to cause an increase in transamination assuming complete conversion of Thr to 2-ketobutyric acid. A decrease in concentrations of the branched-chain amino acids is observed with strains T81, LC1, AW106 and J1 (except for Ile with strain J1) supporting the above hypothesis.

Serine supplementation could also increase transamination activity resulting in increased Ala and decreased branched-chain amino acids. Alanine concentrations appear not to have increased with Ser supplementation except with strain T81. However, this only appears to be an increase relative to decreases in Ala concentration caused by treatments which increased 2-aminobutyric acid production. As well, branched-chain amino acid concentrations did not decrease with Ser supplementation. This suggests that the additional pyruvate may have been used in anapleurotic or pathways other than Ala production.

In response to L-cycloserine, strains T81, AW106 and J1 exhibited decreases in propionate and increases in butyrate and valerate concentrations whereas strain LC1 showed an increase in acetate and a

decrease in both butyrate and valerate concentrations. With the L-cycloserine-induced reduction in Ala and 2-aminobutyric acid production, an increase in acetate and propionate, respectively, is expected. For the three former strains, this appears to have manifested itself in the production of butyrate and valerate which are products of an acetate-acetate and an acetate-propionate condensation respectively. Strain LC1 gave an increase in acetate but no increase in propionate or valerate thereby accounting for pyruvate. This does not, however, account for the diverted 2-ketobutyric acid.

L-cycloserine has been reported to chemically cause decarboxylation of 2-keto acids (Perez-Sala et al., 1986). This could account for the decrease in Ala and 2-aminobutyric acid production but not the decrease in acetate and propionate since the decarboxylation of pyruvate and 2-ketobutyric acid should increase the amount of acetate and propionate in the media. The increase in Asp and Glu production (with strain LC1 at increasing levels of L-cycloserine) should also be reduced since oxaloacetate and 2-ketoglutarate should also be decarboxylated. The production of branched-chain amino acids (formed by transamination of their respective branched chain 2-keto acids) by LC1 in this experiment was also unaffected confirming the results of Hutson et al., (1988) who observed no decarboxylating effect of L-cycloserine on the branched-chain 2-keto acids.

From these results, it can be concluded that for M. elsdenii, Thr is the only amino acid source of 2-ketobutyric acid from which 2-aminobutyric acid is assumed to be formed. Met may indirectly stimulate 2-aminobutyric acid production but this was only demonstrated

with strains T81 and LC1 and the mechanism by which this occurred is unknown. As well, the data provides evidence that the enzyme system responsible for the production of 2-aminobutyric acid is regulated similarily to that which produces Ala. Enzymes specific for only 2-aminobutyric acid have not yet been isolated but because of the similarity in structure and response to treatments between Ala and 2-aminobutyric acid, and since 2-aminobutyric acid has no known role in peptides or proteins or a specific role in metabolism, it is suggested that 2-ketobutyric acid and pyruvate are competing substrates for the same transaminase system. 2-Ketobutyric acid may also play a role in metabolism similar to pyruvate acting as an NH_4^+ acceptor in transaminase reactions and may also be a substrate for the pyruvate oxidoreductase producing propionate as an end product. Furthermore, these two 2-keto acids appear to be electron and NH_4^+ acceptors for the transamination of the branched-chain amino acids either directly or via another transaminase system such as the 2-ketoglutate/glu system.

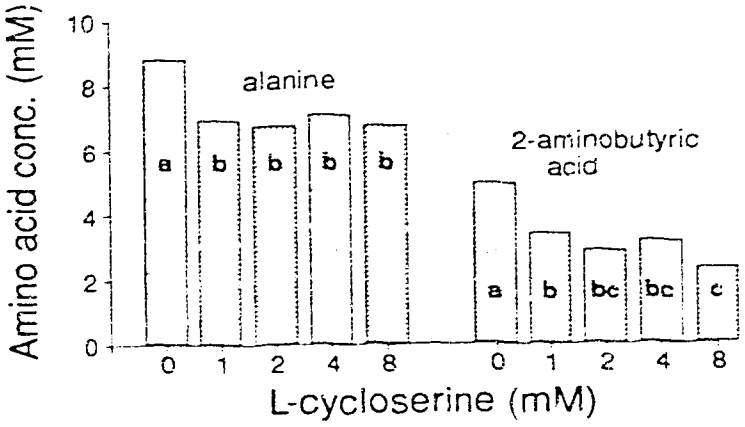

Figure III-1. Effect of increasing concentrations of L-cycloserine on 2-aminobutyric acid and alanine concentrations (mM) in incubations with four strains of M. elsdenii.*

(Refer to Appendix 6 for statistical data)

* Bars bearing different letters are significantly different at the 0.05 level.

Table III-1. Effect of threonine, serine, methionine, or aspartate supplementation on final concentration (mM) of 2-aminobutyrate, alanine, valine, leucine and isoleucine.[a,b]

Strain and Treatment	2-amino-butyrate	Alanine	Valine	Leucine	Iso-leucine
T81					
None	4.73c	8.90d	4.84	4.61c	3.55c
Threonine	6.23a	9.94bc	5.18	4.92bc	3.78bc
Serine	5.51b	11.21a	6.12	5.98a	4.60a
Methionine	5.18b	9.36cd	5.08	4.71c	3.72c
Aspartate	5.15bc	10.60ab	4.50	5.47ab	4.16b
S.E.	0.06	0 09	0.23	0.09	0.06
LC1					
None	2.47d	5.13c	2.78c	2.40c	2.01c
Threonine	5.23a	9.16b	4.90b	4.46b	3.64b
Serine	3.35c	10.23a	5.86a	6.58a	4.12ab
Methionine	4.22b	9.03b	5.21b	5.01b	3.87ab
Aspartate	3.77c	10.04a	5.92a	6.32a	4.33a
S.E.	0.06	0.11	0.05	0.10	0.07
AW106					
None	3.98b	7.17c	3.03c	2.80b	2.18c
Threonine	5.26a	8.95b	4.02b	4.32a	3.04b
Serine	4.42b	10.71a	4.53ab	5.23a	3.60a
Methionine	4.58b	10.18a	4.53ab	4.85a	3.40ab
Aspartate	3.98b	10.68a	4.60a	5.07a	3.70a
S.E.	0.08	0.15	0.07	0.13	0.07
J1					
None	3.25	6.73ab	3.75ab	5.02ab	2.68ab
Threonine	2.79	5.15b	3.07b	4.28b	2.19b
Serine	3.22	7.65a	4.52a	6.16a	3.24a
Methionine	3.25	7.63a	4.57a	5.92ab	3.12ab
Aspartate	3.24	8.10a	4.85a	6.66a	3.50a
S.E.	0.11	0.27	0.17	0.25	0.13

[a] Values bearing different letters within a column within strains are significantly different at the 0.05 level.

[b] n = 3 for all means except AW106-Threonine addition where n = 2.

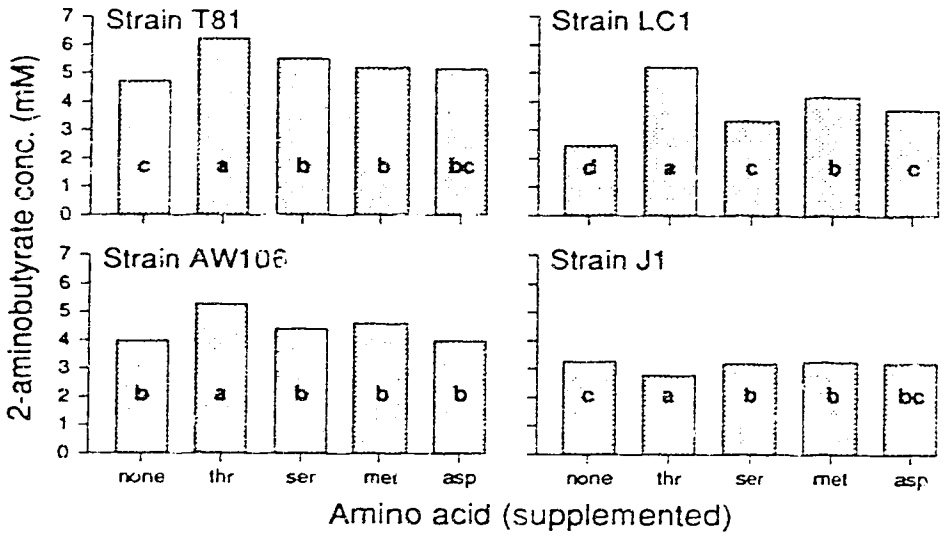

Figure III-3. Effect of threonine, serine, methionine, or aspartate supplementation on final concentration (mM) of 2-aminobutyric acid and alanine in incubations with M. elsdenii LC1.*

(Refer to Appendix 3 for statistical data)

* Bars bearing different letters are significantly different at the 0.05 level.

Table III-2. Effect of 1 mM L-cycloserine (CS) on final concentration(mM) of 2-aminobutyric acid, alanine, valine, leucine and isoleucine.[a]

Strain	CS	2-Aba	Ala	Val	Leu	Ile
T81	-	3.64	5.53	1.79	1.74	1.22
	+	3.33	4.53	2.16	2.30	1.34
S.E.		0.06	0.10	0.29	0.27	0.22
Prob[b]		0.244	0.02	0.67	0.52	0.85
B159	-	1.86	6.80	3.88	3.72	2.90
	+	0.24	4.05	3.11	4.25	2.26
S.E.		0.05	0.34	0.18	0.22	0.13
Prob		0.01	0.06	0.17	0.36	0.13
LC1	-	4.00	6.46	0.89	1.36	0.55
	+	1.98	5.43	3.58	4.69	2.60
S.E.		n.c.[c]	n.c.	n.c.	n.c.	n.c.
Prob		n.c.	n.c.	n.c.	n.c.	n.c.
AW106	-	3.96	6.44	3.78	3.48	2.74
	+	3.21	5.06	3.69	3.64	2.66
S.E.		0.15	0.31	0.22	0.25	0.16
Prob		0.13	0.16	0.87	0.78	0.84
J1	-	3.03	5.41	1.62	2.03	1.18
	+	1.63	4.18	2.77	3.52	1.94
S.E.		0.01	0.04	0.07	0.10	0.05
Prob		0.01	0.04	0.08	0.10	0.09

[a] n = 3 for all means except for T81-, LC1-, LC1+ and J1+ where n = 2.

[b] Prob = probability.

[c] n.c. = not calculated.

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General Conclusion and Discussion

In anaerobic systems, electron disposal is a critical problem as the electron carriers are present in catalytic amounts and must be reoxidized for metabolism to continue. The growth substrate is the electron source and is often used as the electron sink as well such that intermediates formed during metabolism of substrate are used as acceptors for electrons obtained from the oxidation of the same substrate. The introduction of an alternate electron acceptor to an anaerobic system generally causes a shift in the fermentation end products resulting in an increased production of oxidized products. Greater energy yield may result due to a greater proportion of the substrate proceeding through the energy-yielding oxidation processes as well as possibly allowing a greater amount of substrate to be metabolized if electron acceptors were limiting. Use of inorganic electron acceptors should theoretically allow more organic substrate to be oxidized.

Numerous compounds may act as inorganic alternate electron acceptors including the nitrogen oxyanions (de Vries et al., 1974; Farra and Satter, 1971; Yamamoto et al., 1982; Alaboudi and Jones, 1985; Allison and MacFarlane, 1988). Nitrate is present in varying amounts in plant materials. When ingested by the ruminant into the reticulo-rumen, an essentially anaerobic environment, NO_3^- is rapidly reduced to NO_2^-. Subsequently, NO_2^- is reduced to NH_4^+ but at a much slower rate such that NO_2^- accumulates in the rumen (Pfander et al., 1957; Wang et al., 1961). Nitrite is absorbed into the ruminant's bloodstream where it can oxidize

hemoglobin. High levels of NO_2^- can impair O_2 transport sufficiently to be fatal to the animal (Deeb and Sloan, 1975).

Nitrite, while toxic, also has the potential to serve as an alternate electron acceptor. Cheng et al., (1988) screened various rumen microbial strains for the ability to reduce NO_2^- and demonstrated that Megasphaera elsdenii (as well as several other strains) were able to rapidly reduce NO_2^-. This anaerobic Gram-negative cocci (Elsden et al., 1951; Scheifinger et al., 1976) plays an important role in the rumen acting as a secondary fermentor utilizing the lactate and amino acids produced by the starch fermentors and thereby aiding starch digestion by metabolizing the starch digestion end products. Also, it produces branched chain fatty acids, essential to the growth of some cellulolytic strains (Hungate, 1966), which are produced from the metabolism of the corresponding branched chain amino acids. Because of its importance in the rumen ecosystem and its ability to degrade NO_2^-, this organism was chosen for this study.

The first investigation was to observe the effects of NO_2^- on both lactate and amino acid metabolism of M. elsdenii. The presence of NO_2^- in culture with M. elsdenii did not yield the expected results. At the level used, NO_2^- did not result in a shift to more oxidized products. The effect of NO_2^- appeared to be primarily that of an inhibitor of metabolism and secondarily that of an alternate electron acceptor. Only strain LC1, when grown with glucose in the presence of NO_2^-, showed increased CO_2 production. This may reflect a greater resistance to the inhibitory effects of NO_2^- relative to the other strains. However, a decrease in H_2 production when M. elsdenii was grown on lactate was

observed consistent with the expected results.

Generally, amino acid catabolism was stimulated by the presence of NO_2^-. Nitrite, as an inhibitor of the pyruvate:ferredoxin oxidoreductase or as an alternate electron acceptor, could be responsible for this increase. Inhibition of the pyruvate:ferredoxin oxidoreductase could cause a shift from carbohydrate metabolism to amino acid metabolism as the primary source of energy. Alternatively, the presence of NO_2^- could allow greater oxidative deamination and subsequent metabolism of the resulting carbon skeleton. However, the effect on VFA production appears to be caused primarily by NO_2^-'s action as an inhibitor. The decrease in Ala and 2-aminobutyric acid production in the presence of NO_2^- suggests a role for these amino acids as electron sinks for M. elsdenii

The added NO_2^- was not detectable after the incubation period therefore detoxification had to occur. This was probably by reduction to NH_4^+ which is a process requiring six electrons which would come from substrate oxidation.

The data presented offers no evidence to conclude if the reduction of NO_2^- is an enzyme-linked or chemical process or, assuming the presence of a NO_2^- reductase, if NO_2^- reduction is an energy-yielding process. These issues warrants further investigation.

The second investigation was to determine the source of 2-aminobutyric acid whose production we had observed in the previous investigation as well as its possible role in electron disposal in M. elsdenii. The production of 2-aminobutyric acid in M. elsdenii was observed by Wallace (1986) but no role in metabolism was suggested for

this amino acid. 2-Aminobutyric acid is found in animals, plants and other microbes but has not been found to be a constituent of any protein studied. Its only identified roles are as a substituent of opthalmic acid for the L isomer and as a substituent of staphylomycin S for the D isomer (Meister, 1965).

Because of the structural similarity between 2-aminobutyric acid and alanine (Ala) as well as the lack of a defined role in metabolism in M. elsdenii, it was speculated that 2-ketobutyric acid, the precursor of 2-aminobutyric acid, formed by the degradation of threonine may act as a substrate for the enzyme(s) responsible for the production of Ala from pyruvate. In the presence of L-cycloserine, an inhibitor of alanine transaminases, both Ala and 2-aminobutyric acid production was decreased. Considering also the parallel response of Ala and 2-aminobutyric acid to NO_2^- and NH_4^+ treatments, the experimental evidence supports this hypothesis. In the presence of L-cycloserine, when 2-aminobutyric acid and Ala production decreased, branched-chain amino acid disappearance also decreased suggesting that puruvate and 2-ketobutyric acid may act as electron and NH_4^+ acceptors in the deamination of the branched-chain amino acids possibly in a transamination system. Further evidence supporting the hypothesis that 2-ketobutyric acid and pyruvate are substrates for the same enzyme(s) is observed with threonine supplementation of strain LC1 and AW106. While threonine supplementation increased 2-aminobutyric acid production, Ala production decreased. The probable explanation is that 2-ketobutyric acid is competing against pyruvate as a substrate for NH_4^+ and electrons. As NH_4^+ was not limiting, it is unlikely two different

enzyme systems each responsible for the production of either 2-aminobutyric acid or Ala are competing for NH_4^+. Definitive proof of this hypothesis cannot be obtained until it demonstrated that either a specific 2-aminobutyric acid transaminase is present or that all the 2-aminobutyric acid-producing activity is associated with the alanine transaminase.

Some Clostridia spp. have been shown to produce 2-aminobutyric acid via 2-ketobutyric acid from methionine (Mead, 1971). Results from the supplementation experiments with threonine, serine, methionine and aspartic acid (Asp) suggested that only threonine was a source for 2-aminobutyric acid production but with strain LC1, Met may also play some regulatory role as its presence increased 2-aminobutyric acid production without Met itself being metabolized.

In conclusion, NO_2^-'s effects appeared to be due to possible inhibition of carbohydrate metabolism which stimulated amino acid metabolism as well as an alternate electron acceptor affecting Ala and 2-aminobutyric acid production. Further study utilizing lower concentrations of NO_2^- is necessary to determine if NO_2^- can act as an effective electron acceptor without possibly inhibiting carbohydrate metabolism.

The existence of a NO_2^- reductase, especially considering the lack of a NO_3^- reductase in M. eladenii, also requires further investigation and, if present, it needs to be determined whether NO_2^- reduction is an energy-yielding process.

The presented investigations suggest that 2-aminobutyric acid is produced by a transaminase, possibly alanine transaminase, and may be

linked to the deamination of the branched-chain amino acids. The role of 2-aminobutyric acid needs further study, especially the possibility that it may be present in a protein or peptide yet unstudied.

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Appendix 1. Effect of NO_2^- (3.57 mM NO_2^--N) on volatile fatty acid concentration and H_2[a] (mL) and CO_2[b] (mL) production by five strains of M. elsdenii grown on glucose (33.3 mM).[c]

		Strain				
	NO_2^-	T81	B159	LC1	AW106	J1
Acetate	-	9.77	5.21	7.17	16.72	31.27
	+	11.29	6.95	10.86	21.50	26.93
S.E.		0.43	0.65	1.09	1.30	1.52
Prob.		0.144	0.172	0.144	0.130	0.171
Propionate	-	0.50	0.00	0.17	0.33	23.64
	+	0.17	0.67	0.17	0.17	18.81
S.E.		0.17	0.00	0.00	0.00	0.33
Prob.		0.334	0.022	0.510	0.325	0.011
Isobutyrate	-	2.16	2.70	3.24	2.43	3.64
	+	1.89	1.48	3.24	2.02	3.24
S.E.		0.13	0.40	0.13	0.13	0.13
Prob.		0.198	0.153	0.923	0.178	0.098
Butyrate	-	4.99	2.43	2.83	13.36	10.53
	+	7.02	0.67	4.86	17.68	12.69
S.E.		0.27	0.13	0.13	0.13	0.13
Prob.		0.036	0.015	0.010	0.001	0.008
Isovalerate	-	3.40	4.20	4.20	4.77	5.33
	+	1.02	0.68	4.43	4.08	5.45
S.E.		0.11	0.11	0.11	0.11	0.11
Prob.		0.004	0.002	0.285	0.03	0.763

Appendix 1. Continued.

	NO_2^-	Strain				
		T81	B159	LC1	AW106	J1
Valerate	-	1.25	1.36	1.82	1.70	12.71
	+	1.13	1.36	1.93	1.36	11.80
S.E.		0.00	0.23	0.00	0.11	0.11
Prob.		0.347	0.924	0.598	0.075	0.028
Caproate	-	3.23	0.78	1.66	5.97	0.78
	+	0.98	0.29	1.76	3.82	0.98
S.E.		0.20	0.00	0.00	0.10	0.10
Prob.		0.009	0.018	0.150	0.006	0.178
H_2	-	1.64	0.11	0.80	12.92	1.03
	+	0.58	0.00	0.76	12.61	1.25
S.E.		0.06	0.01	0.03	0.18	0.06
Prob.		0.007	0.008	0.469	0.329	0.125
CO_2	-	9.00	1.46	4.67	20.77	14.37
	+	4.99	-2.13	5.87	20.56	14.99
S.E.		0.32	0.12	0.27	0.26	0.12
Prob.		0.013	0.002	0.090	0.624	0.071

[a] Hydrogen gas production determined from mL of total gas produced multiplied by % H_2 in headspace (not corrected to STP).

[b] CO_2 production was determined by subtracting mL of H_2 produced from total gas produced (not corrected to STP).

[c] n = 3 for all means.

Appendix 2. Effect of NO_2^- (3.57 mM NO_2^--N) on volatile fatty acid concentration and H_2[a] (mL) and CO_2[b] (mL) production by five strains of M. elsdenii grown on lactate (56.6 mM).[c]

	NO_2^-	Strain				
		T81	B159	LC1	AW106	J1
Acetate	-	32.14	38.00	27.80	47.77	25.41
	+	1.09	0.00	34.96	43.65	26.71
S.E.		0.87	0.65	1.09	1.30	1 09
Prob.		0.002	0.001	0.048	0.152	0.477
Propionate	-	29.96	44.44	39.95	32.96	31.79
	+	1.33	1.00	38.62	25.47	24.97
S.E.		0.17	0.47	0.50	1.83	0.17
Prob.		0.000	0.000	0.158	0.105	0.001
Isobutyrate	-	2.02	2.97	3.10	2.16	3.10
	+	0.81	0.54	2.70	1.75	3.10
S.E.		0.13	0.00	0.13	0.00	0.13
Prob.		0.024	0.001	0.124	0.014	0.840
Butyrate	-	11.47	8.10	10.26	6.75	13.36
	+	0.94	0.27	9.31	12.69	15.52
S.E.		0.40	0.00	0.13	2.02	0.13
Prob.		0.002	0.000	0.032	0.170	0.003
Isovalerate	-	5.45	7.60	7.49	5.22	7.04
	+	0.45	0.45	7.04	3.86	7.26
S.E.		0.11	0.00	0.11	0.00	0.11
Prob.		0.002	0.000	0.116	0.003	0.351

Appendix 2. Continued.

	NO_2^-	Strain				
		T81	B159	LC1	AW106	J1
Valerate	-	14.07	10.10	15.66	8.28	16.57
	+	0.79	0.57	11.91	8.74	16.79
S.E.		0.23	0.00	0.45	0.23	0.23
Prob.		0.001	0.000	0.032	0.328	0.647
Caproate	-	0.69	0.29	0.59	0.49	0.78
	+	0.20	0.39	0.39	0.59	1.17
S.E.		0.00	0.00	0.10	0.00	0.00
Prob.		0.013	0.039	0.237	0.308	0.007
H_2	-	2.02	3.63	0.87	8.38	2.12
	+	0.00	0.00	0.55	7.20	1.52
S.E.		0.06	0.03	0.02	0.14	0.05
Prob.		0.002	0.000	0.006	0.027	0.012
CO_2	-	8.98	10.70	7.16	12.29	8.48
	+	-0.90	-0.07	5.69	11.50	7.01
S.E.		0.33	0.36	0.14	0.12	0.14
Prob.		0.002	0.002	0.018	0.042	0.018

[a] Hydrogen gas production determined from mL of total gas produced multiplied by % H_2 in headspace (not corrected to STP).

[b] CO_2 production was determined by subtracting mL of H_2 produced from total gas produced (not corrected to STP).

[c] n = 3 for all means.

Appendix 3. Calculation of electron balance from VFA production by *M. elsdenii*

	UNITS	A[a]	P	IB	B	IV	V	C	H2	TOTAL
Glucose substrate										
T81	mg/mL[b]	0.07	-0.02	-0.02	0.15	-0.21	-0.01	-0.23	-1.06	
	µmol	23.33	-5.41	-4.55	34.09	-41.18	-1.96	-39.66	-44.07	
	e⁻ PAIRS[c]	46.67	N.D.[d]	-9.09	102.27	-82.35	-1.96	-158.62	44.07	-59.01
LC1	mg/mL	0.17	0.00	0.00	0.15	0.02	0.01	0.01	-0.04	
	µmol	56.67	0.00	0.00	34.09	3.92	1.96	1.72	-1.66	
	e⁻ PAIRS	113.33	N.D.	0.00	102.27	7.84	1.96	6.90	1.66	233.96
AW106	mg/mL	0.22	-0.01	-0.03	0.32	-0.06	-0.03	-0.22	-0.31	
	µmol	73.33	-2.70	-6.82	72.73	-11.76	-5.88	-37.93	-12.89	
	e⁻ PAIRS	146.67	N.D.	-13.64	218.18	-23.53	-5.88	-151.72	12.89	192.97
Lactate substrate										
LC1	mg/mL	0.33	-0.08	-0.03	-0.07	-0.04	-0.33	-0.02	-0.32	
	µmol	110.00	-21.62	-6.82	-15.91	-7.84	-64.71	-3.45	-13.30	
	e⁻ PAIRS	220.00	21.62	-13.64	-47.73	-15.69	-64.71	-13.79	13.30	99.36
AW106	mg/mL	-0.19	-0.45	-0.03	0.44	-0.12	0.04	0.04	-1.18	
	µmol	-63.33	-121.62	-6.82	100.00	-23.53	7.84	1.72	-49.05	
	e⁻ PAIRS	-126.67	121.62	-13.64	300.00	-47.06	7.84	6.90	49.05	298.04
J1	mg/mL	0.06	-0.41	0	0.16	0.02	0.02	0.04	-0.6	
	µmol	20.00	-110.81	0.00	36.36	3.92	3.92	6.90	-24.94	
	e⁻ PAIRS	40.00	110.81	0.00	109.09	7.84	3.92	27.59	24.94	324.19

[a] Abbreviations used: A, acetate; P, propionate; IB, isobutyrate; B, butyrate; IV, isovalerate; V, valerate; C, caproate.

[b] Difference in VFA concentration - (with NO_3) - (without NO_3).

[c] Assuming the following production of µmole e⁻ pairs per µmol product produced: one per valerate, two per acetate, isobutyrate and isovalerate, three per butyrate, four per caproate, and the utilization of one per propionate and H_2.

[d] N.D. - not determined

Appendix 4. - Effect of NO_3^- (3.57 mM NO_3^--N) and/or NH_4^+ (13.63 mM NH_4^+-N) on amino acid disappearance (%) by five strains of *M. elsdenii*.[a,b]

Strain and additions	Gly[c]	Ala	Val	Leu	Ile	Ser	Thr	Met	Asp	Asn	Glu	Arg	Lys	His	Tyr	Phe	Trp
T81																	
NONE	-19.51a	135.75a	31.66	38.47	33.39	75.87ab	69.50a	-0. 4a	13.03	52.00a	-5.90a	-23.03a	-1.40a	-2.44a	8.16a	-29.25a	-7.63a
NO_3^-	10.36b	-79.99b	42.95	46.52	40.16	81.09ab	86.10a	25 75b	31.73	50.13a	11.39ab	8.28b	20.57ab	23.08b	32.15b	13.97b	13 92b
NH_4^+	20.49b	-77.36b	42.76	41.07	40.75	88.61b	128.51b	28.13b	38.34	40.03b	17.99b	12.82b	23.97b	28.58b	36.19b	4.04b	20.32b
NO_3^- + NH_4^+	17.45b	-47.54c	34.70	38.93	33.97	57.18a	81.78a	28.75b	31.86	28.99c	-1.06ab	9.08b	25.66b	27.96b	37.34b	18.69b	23.84b
S.E.	2.98	3.26	3.96	2.26	3.64	3.67	4.75	2.90	4.35	0.63	3.21	2.51	3.13	2.67	2.98	3.98	2.19
B159																	
NONE	-63.17a	-74.74a	18.54a	33.80a	20.10a	89.73a	57.18b	4.60	34.52a	37.84ab	-13.97a	-12.13ab	0.47ab	3.25	-11.51	-0.71ab	-8.87
NO_3^-	-10.50c	-27.18c	-6.44b	1.32c	1.42b	37.33b	2.09a	-4.18	-15.21b	45.08a	4.34b	-32.94a	-1.81a	-4 62	-6.59	-8.31a	-2 30
NH_4^+	-40.86b	-57.54b	15.69a	25.63b	13.83a	88.29a	92.57c	6.01	34.26a	30.08b	-2.57ab	-5.19b	8.68b	4.54	0.36	4.12b	-3.93
NO_3^- + NH_4^+	-9.90c	-7.54c	-5.53b	-0.87c	-4.55b	1.87c	4.37a	-3.70	59.90c	18.86c	-16.97b	-15.37ab	2.34ab	-2.03	-12.54	-3.92ab	-3.25
S.E.	2.36	1.90	1.68	0.87	1.27	4.24	3.80	1.90	3.01	1.28	2.39	3.84	1.33	1.85	5.28	1.60	41.41
LC1																	
NONE	3.46a	-45.04b	47.98ab	75.50a	47.06a	89.93	81.20a	13.05ab	-24.47a	44.81a	-3.55b	-5.10ab	8.31a	5.59b	2.79b	-5.50	-3.84a
NO_3^-	0.13ab	-51.63b	44.50ab	75.47a	38.47ab	93.84	98.17ab	6.79b	-28.45a	55.61a	5.68a	-11.20ab	6.08a	-5.31ab	-14.99a	-8.81	-9 62a
NH_4^+	21.24c	-23.24c	49.79b	70.18a	48.02a	88.14	128.91c	26.99a	23.20c	9.38b	-5.29b	7.78b	19.32a	25.11c	16.87c	10.60	if. 86b
NO_3^- + NH_4^+	-15.21a	-81.12a	32.93a	59.11b	15.71b	87.67	113.48ab	-9.00c	-6.25b	2.38b	-38.65c	-29.52a	-19.60b	-16.24a	-17.75a	-1.67	-7.06a
S.E.	2.49	2.81	1.75	1.51	3.68	0.58	3.76	2.10	2.99	2.97	0.89	3.55	2.79	2.02	1.54	8.02	1.86
AW106																	
NONE	-8.78a	-83.31a	15.34	49.76ab	20.20	89.76	78.56a	-6.68a	-28.02ab	23.38ab	-39.57a	0.87	-2.82a	-9.13	-9.12	-23.31	-12.71
NO_3^-	2.70bc	-77.43ab	22.85	57.31b	26.59	94.40	99.28ab	10.57b	38.19ab	38.76a	21.11b	-6.56	6.49ab	21.42	19.61	-13.52	-4.89
NH_4^+	13.71c	-57.85bc	25.02	44.88eb	23.71	89.05	128.91c	14.98b	47.34a	-18.34c	7.65b	-2.07	13.98b	13.66	24.88	-10.60	-1.76
NO_3^- + NH_4^+	-5.61ab	-54.52c	17.26	35.74a	19.94	87.71	112.95bc	2.31ab	-40.21b	16.95b	-30.07a	-10.65	-2.04a	1.66	17.94	-17.70	-12.78
S.E.	1.59	2.51	2.19	2.12	2.99	1.84	3.44	1.85	10.35	2.20	3.20	1.72	1.90	4.46	4.54	2.35	2.51
J1																	
NONE	2.81a	-32.16a	28.88ab	26.67a	29.55b	87.09	79.83a	6.56a	52.08a	12.27	12.60ab	-3.61a	8.92a	-2.17a	15.23a	-2.00a	-1.97
NO_3^-	35.93b	18.83b	50.04c	48.88b	47.50a	95.74	98.76ab	37.25b	82.70c	15.85	43.43b	34.28b	38.92b	33.90b	37.05b	32.78b	22.06
NH_4^+	8.86a	-33.88a	23.74a	19.42a	17.41c	88.26	128.29c	7.98a	51.58a	-44.03	-6.03a	-3.52a	7.80a	2.51a	18.32a	8.58a	-1.00
NO_3^- + NH_4^+	16.65ab	-19.60a	40.32bc	42.87b	26.05bc	89.39	113.48bc	19.06ab	66.59b	-15.76	-0.58ab	-1.79a	18.81ab	19.39ab	27.24ab	12.69ab	17.26
S.E.	3.16	4.65	2.09	2.19	1.45	1.47	3.83	3.20	1.22	12.64	6.98	4.57	3.48	3.17	1.82	3.25	3.84

[a] Values bearing different letters within a column per amino acid per strain are significantly different at the 0.05 level.

[b] n = 3 for all means except for means of T81-NONE, AW106-NONE and AW106-NO_3^- + NH_4^+ where n = 2.

[c] Abbreviations used: Gly, glycine; Ala, alanine; Val, valine; Leu, leucine; Ile, isoleucine; Ser, serine; Thr, threonine; Met, methionine; Asp, aspartate;; Asn, asparagine; Glu, glutamate; Arg, arginine; Lys, lysine; His, histidine; Tyr, tyrosine; Phe, phenylalanine; Trp, tryptophan.

Appendix 5. Effect of NO_2^- (3.57 mM NO_2^--N) and/or NH_4^+ (13.63 mM NH_4^+-N) on volatile fatty acid concentration (mM) by five strains of *M. elsdenii*.[a,b]

STRAIN	A[c]	P	IB	B	IV	V	C
T81							
NONE	35.61ab	34.29ab	0.40	9.31	2.50a	11.91ab	0.39
NO_2^-	39.96b	40.61b	0.40	10.66	2.38a	13.39b	0.29
NH_4^+	29.30a	25.97a	0.40	9.04	2.50a	11.01a	0.29
NO_2^- + NH_4^+	33.52ab	33.29ab	0.00	9.18	0.68b	10.33a	0.20
S.E.	1.09	1.50	0.13	0.27	0.11	0.23	0.10
B159							
NONE	49.73a	54.26a	0.00	5.80a	1.02a	4.43a	0.00a
NO_2^-	20.85b	17.48b	0.00	1.08b	0.00b	0.57c	0.20ab
NH_4^+	45.60a	48.10a	0.00	5.67a	0.79a	4.20b	0.10a
NO_2^- + NH_4^+	4.34c	2.83c	0.00	0.40c	0.00b	0.23d	0.88b
S.E.	1.30	1.17	0.00	0.13	0.00	0.00	0.10
LC1							
NONE	36.05	46.60ab	0.40	7.42	3.40	7.04a	0.29
NO_2^-	36.48	48.44b	0.40	7.02	3.52	7.26a	0.00
NH_4^+	31.27	41.28a	0.81	6.21	3.06	5.90b	0.10
NO_2^- + NH_4^+	31.92	43.11ab	0.81	6.75	3.06	6.13b	0.00
S.E.	0.65	0.83	0.13	0.27	0.11	0.11	0.00
AW106							
NONE	45.39	31.79a	0.27	10.80a	2.04ab	9.08a	0.39a
NO_2^-	46.04	35.62a	0.13	10.66a	2.16b	6.81ab	0.20b
NH_4^+	39.74	30.63a	0.27	9.04b	2.27b	8.06b	0.20b
NO_2^- + NH_4^+	39.52	24.13b	0.13	10.93a	1.13a	8.40ab	0.29ab
S.E.	1.52	0.83	0.00	0.13	0.11	0.23	0.00

Appendix 5. Continued.

STRAIN	A[c]	P	IB	B	IV	V	C
J1							
NONE	35.83ab	41.78a	0.13	10.66a	0.91a	8.62ab	0.10
NO_2^-	38.44b	42.28a	0.13	11.07a	0.57a	8.74b	0.10
NH_4^+	37.13ab	38.28b	0.13	9.72b	0.91a	7.60a	0.10
NO_2^- + NH_4^+	30.18a	37.28b	0.40	10.80a	1.25b	8.96b	0.10
S.E.	1.09	0.33	0.00	0.13	0.00	0.11	0.00

[a] Values bearing different letters within a column per amino acid per strain are significantly different at the 0.05 level.

[b] n = 3 for all means except for means of T81-NONE, AW106-NONE and AW106-NO_2^- + NH_4^+ where n = 2.

[c] Abbreviations used: A, acetate; P, propionate; IB, isobutyrate; B, butyrate; IV, isovalerate; V, valerate; C, caproate.

Appendix 6. Effect of threonine, serine, methionine and aspartate supplementation on amino acid concentration (final concentration - mM) by five strains of *M. elsdenii* [a,b]

Strain	Addition	Gly[c]	Ala	Val	Leu	Ile	2-Aba	Ser	Thr	Tyr	Met	Asp	Asn	Glu	Arg	Lys	His	Phe	Trp
T81	none	3.30c	8.90d	4.84	4.61c	3.55c	4.73c	0.19b	0.00	0.85c	1.46c	3.63d	0.04b	10.38d	1.63c	4.89c	1.21c	2.53c	0.21c
	Thr	3.74b	9.94bc	5.18	4.92bc	3.78bc	6.23a	0.20b	0.00	0.96b	1.61bc	4.10c	0.04b	11.81bc	1.85b	5.33b	1.26bc	2.51ab	0.23bc
	Ser	4.15a	11.21a	6.12	5.98a	4.60a	5.51b	0.32a	0.00	1.08a	1.80b	4.53b	0.07ab	12.91a	2.01a	6.03a	1.39ab	3.03a	0.25a
	Met	3.37c	9.37cd	5.08	4.71c	3.72c	5.18b	0.22b	0.00	0.91bc	3.68a	3.95cd	0.06ab	11.05cd	1.77bc	5.15bc	1.30abc	2.72bc	0.22bc
	Asp	3.69b	10.61ab	4.50	5.47ab	4.16b	5.15bc	0.18b	0.00	1.00ab	1.75b	7.38a	0.08a	12.24ab	2.01a	5.25bc	1.42a	2.71bc	0.24ab
	S.E.	0.04	0.09	0.23	0.09	0.06	0.06	0.01	0.00	0.01	0.03	0.06	0.00	0.12	0.02	0.06	0.02	0.04	0.00
B159	none	2.49	4.46	3.49	4.80	2.44	0.00	0.15	2.23b	0.50	1.19b	3.37b	0.13b	8.12	1.27	3.79	0.93	1.68	0.16
	Thr	2.23	4.24	3.23	4.87	2.40	0.00	1.18	3.91a	0.49	1.19b	3.36b	0.20a	7.82	1.21	3.48	0.90	1.65	0.16
	Ser	2.11	4.08	3.22	4.69	2.34	0.00	0.14	2.07b	0.46	1.16b	3.14b	0.20a	7.65	1.29	3.18	0.84	1.62	0.15
	Met	2.13	4.04	3.26	4.97	2.45	0.00	0.15	2.07b	0.47	2.90a	3.15b	0.16ab	7.92	1.22	3.58	0.81	1.62	0.14
	Asp	2.23	4.26	3.29	4.90	2.43	0.00	0.11	2.18b	0.46	1.19b	5.14a	0.21a	7.97	1.20	3.39	0.86	1.67	0.16
	S.E.	0.05	0.13	0.09	0.11	0.06	0.00	0.19	0.09	0.02	0.03	0.13	0.01	0.25	0.06	0.13	0.03	0.04	0.00
LC1	none	1.77b	5.13c	2.78c	2.40c	2.01c	2.47d	0.12c	0.04d	0.50b	0.87d	2.24d	0.07b	6.57b	0.62c	2.64c	0.72b	1.48b	0.13c
	Thr	3.33a	9.16b	4.90b	4.46b	3.64b	5.23a	0.17b	0.17c	0.97a	1.57c	3.76c	0.09a	11.52a	0.86ab	5.20b	1.38a	2.84a	0.20b
	Ser	3.54a	10.23a	5.86a	6.58a	4.12ab	3.35c	0.25a	0.23ab	0.99a	1.86b	4.46b	0.07b	12.42a	0.70bc	6.13a	1.48a	2.89a	0.23a
	Met	3.35a	9.03b	5.21b	5.01b	3.87ab	4.22b	0.18b	0.19bc	0.98a	3.91a	4.02c	0.10a	12.09a	0.91a	5.41b	1.43a	2.83a	0.20b
	Asp	3.63a	10.04a	5.93a	6.32a	4.33a	3.77c	0.27a	0.25a	1.04a	1.81b	7.15a	0.06b	12.60a	0.69bc	6.24a	1.54a	2.89a	0.22ab
	S.E.	0.04	0.11	0.05	0.10	0.07	0.06	0.01	0.01	0.01	0.03	0.06	0.00	0.16	0.03	0.08	0.02	0.03	0.00
AW106	none	2.62c	7.17c	3.03c	2.80b	2.18c	3.98b	0.13	0.00b	0.61b	1.29d	3.22d	0.19c	9.03c	1.44b	3.85b	1.03b	2.46c	0.19c
	Thr	3.51b	8.95b	4.02b	4.32a	3.04b	5.26a	0.16	0.06a	0.79a	1.61c	4.25c	0.22b	11.25b	1.92ab	5.25a	1.36a	3.07b	0.22b
	Ser	3.68a	10.71a	4.53ab	5.23a	3.60a	4.42b	0.21	0.04ab	0.97a	1.77bc	5.15b	0.25ab	12.89a	2.27a	6.12a	1.39a	3.59a	0.25a
	Met	3.76ab	10.18a	4.53ab	4.85a	3.40ab	4.58b	0.17	0.02ab	0.86a	4.17a	4.67bc	0.27a	12.55a	2.24a	5.48a	1.45a	3.31ab	0.24ab
	Asp	3.82ab	10.68a	4.60a	5.07a	3.70a	3.98b	0.18	0.00b	0.88a	1.80b	7.84a	0.25ab	12.78a	2.30a	5.32a	1.48a	3.48ab	0.25a
	S.E.	0.05	0.15	0.07	0.13	0.07	0.01	0.01	0.01	0.02	0.02	0.09	0.00	0.12	0.03	0.13	0.04	0.06	0.00
J1	none	2.95ab	6.73ab	3.75ab	5.02ab	2.68ab	3.25	0.15ab	0.00	0.69a	1.43bc	3.24bc	0.17a	10.26ab	1.79a	4.35ab	1.09a	2.40a	0.17bc
	Thr	2.21b	5.15b	3.07b	4.28b	2.19b	2.79	0.10b	0.00	0.50b	1.15c	2.66c	0.12b	7.76b	1.18b	3.53b	0.76b	1.70b	0.14c
	Ser	3.18a	7.65a	4.52a	6.16a	1.24a	3.22	0.18a	0.00	0.71a	1.63b	3.86b	0.18a	11.18a	1.74a	4.86ab	1.16a	2.39a	0.20ab
	Met	3.21a	7.63a	4.57a	5.92a	3.12ab	3.25	0.18a	0.00	0.71a	3.86a	3.73b	0.19a	11.28a	1.71a	4.86ab	1.18a	2.41a	0.20ab
	Asp	3.27a	8.10a	4.85a	6.66a	3.50a	3.24	0.18a	0.00	0.75a	1.73b	6.97a	0.20a	12.03a	1.79a	5.62a	1.28a	2.49a	0.21a
	S.E.	0.11	0.27	0.17	0.25	0.13	0.11	0.01	0.00	0.03	0.06	0.14	0.01	0.38	0.07	0.20	0.04	0.06	0.01
Uninoc.		2.42	4.33	3.40	4.99	2.55	0.00	3.24	2.37	0.50	1.26	4.10	0.27	8.39	1.28	3.74	0.96	1.77	0.17

[a] Values bearing different subscripts within a column per amino acid measured per strain are significantly different at the 0.05 level.

[b] n = 3 for all means except AW106-Thr addition where n = 2

[c] Abbreviations used: Gly, glycine; Ala, alanine; Val, valine; Leu, leucine; Ile, isoleucine; Ser, serine; Thr, threonine; Met, methionine; Asp, aspartate; Asn, asparagine; Glu, glutamate; Arg, arginine; Lys, lysine; His, histidine; Tyr, tyrosine; Phe, phenylalanine, Trp, tryptophan; 2-aba, 2-aminobutyrate

Appendix 7. Effect of threonine, serine, methionine and aspartate supplementation on volatile fatty acid concentration by 5 strains of M. elsdenii.[a,b]

Strain and Additions	A[c]	P	IB	B	IV	V	C
T81							
none	31.70b	33.95c	0.40b	10.26a	3.63c	9.64c	0.49a
Thr	36.48ab	36.28bc	0.81a	12.28a	4.54ab	11.46b	0.49a
Ser	38.65ab	37.95ab	0.27bc	13.63a	3.97bc	13.39a	0.59a
Met	39.96a	39.28a	0.54ab	13.36a	4.99a	13.05a	0.39a
Asp	10.64c	10.32d	0c	3.24b	0.34d	0.34d	0.20b
S.E.	1.09	0.33	0.00	0.54	0.11	0.11	0.00
B159							
none	6.51b	1.50c	0b	0b	0b	0.11c	0.10bc
Thr	2.82c	1.50c	0b	0.27b	0.11b	0.34bc	0c
Ser	4.99bc	3.99b	0b	0.67b	0.23b	0.45b	0.20ab
Met	7.82b	1.33c	0b	0.13b	0b	0c	0c
Asp	26.71a	36.45a	0.54a	7.56a	4.20a	8.85a	0.29a
S.E.	0.43	0.33	0.00	0.13	0.11	0.00	0.00
LC1							
none	36.70bc	45.77ab	0.94a	10.26	5.33a	11.80	0.29c
Thr	39.31abc	54.09a	0.27c	8.77	2.04c	10.10	0.69ab
Ser	36.26c	46.77ab	0.54b	9.18	2.84bc	10.10	0.78a
Met	41.26ab	51.76a	0.54b	10.26	3.52bc	11.12	0.59abc
Asp	41.48a	41.28b	0.27a	9.58	4.31ab	8.85	0.49bc
S.E.	0.65	1.33	0.00	0.27	0.23	0.45	0.00
AW106							
none	50.81a	41.61a	0.81ab	13.23a	5.45a	11.12b	0.59ab
Thr	46.25b	37.12b	0.67b	13.77a	4.88ab	11.12b	0.59ab
Ser	43.65b	37.12b	0.54b	9.99b	4.43ab	8.74c	0.39c
Met	47.12ab	37.95b	0.67b	13.63a	4.99ab	11.23b	0.69a
Asp	28.01c	32.96c	1.35a	14.30a	3.06b	13.62a	0.59bc
S.E.	0.65	0.33	0.13	0.27	0.23	0.23	0.00

Appendix 7. Continued.

Strain and Additions	A	P	IB	B	IV	V	C
J1							
none	35.61	38.62	0.81	18.22	1.70	16.45a	0.78ab
Thr	31.70	33.95	0.67	18.08	1.59	16.91a	0.78a
Ser	29.75	34.29	0.54	15.11	1.36	14.18ab	0.59c
Met	33.66	39.45	0.94	17.41	1.93	15.55ab	0.69bc
Asp	30.62	33.29	0.54	14.71	1.25	12.71b	0.59c
S.E.	1.09	1.17	0.00	0.54	0.11	0.45	0.00

[a] Values bearing different letters within a column per amino acid per strain are significantly different at the 0.05 level.

[b] n = 3 for all means except AW106-Thr addition where n = 2.

[c] Abbreviations used: A, acetate; P, propionate; IB, isoboutyrate; B, butyrate; IV, isovalerate; V, valerate; C, caproate.

Appendix 8. Effect of L-cycloserine on amino acid concentration (final concentration - mM) by M. elsdenii LC1.[a,b]

	L-cycloserine concentration						Uninoc.	
	0 mM	1 mM	2 mM	4 mM	8 mM	S.E.	media	S.E.
Gly[c]	3.01	3.06	2.98	3.17	2.97	0.03	2.07	0.13
Ala	8.81a	6.94b	6.76b	7.10b	6.76b	0.08	4.34	0.04
Val	3.04a	3.78b	3.69b	4.16b	4.25b	0.04	3.15	0.11
Leu	2.96a	4.50b	5.01bc	5.38bc	6.03c	0.10	4.60	0.03
Ile	2.25a	2.87b	2.82b	3.14b	3.02b	0.04	2.32	0.04
2-Aba	4.95a	3.42b	2.87bc	3.17bc	2.31c	0.07	0.00	0.00
Ser	0.16	0.18	0.19	0.20	0.18	0.01	2.58	0.09
Thr	0.00	0.00	0.00	0.01	0.00	0.00	1.77	0.03
Tyr	0.64	0.60	0.55	0.55	0.50	0.00	0.36	0.06
Met	1.61	1.60	1.70	1.72	1.68	0.01	1.16	0.01
Asp	4.11a	4.65ab	4.61ab	4.80ab	5.10b	0.07	3.54	0.01
Asn	0.09	0.05	0.11	0.09	0.03	0.01	0.13	0.01
Glu	11.75	12.07	11.76	13.40	12.22	0.18	8.04	0.29
Arg	1.74	1.65	1.57	1.90	1.78	0.04	1.22	0.07
Lys	5.23	5.24	5.24	5.53	5.20	0.04	3.70	0.09
His	1.33	1.29	1.31	1.44	1.34	0.02	1.01	0.08
Phe	2.94a	2.59ab	2.47b	2.71ab	2.52ab	0.03	1.63	0.03
Trp	0.23	0.20	0.21	0.20	0.21	0.00	0.14	0.01

[a] Means bearing different letters within a row are significantly different at the 0.05 level.

[b] n = 3 for 0, 1 and 4 mM cycloserine, n = 2 for 2 and 8 mM cycloserine and the uninoculated media.

[c] Abbreviations used: Gly, glycine; Ala, alanine; Val, valine; Leu, leucine; Ile, isoleucine; Ser, serine; Thr, threonine; Met, methionine; Asp, aspartate; Asn, asparagine; Glu, glutamate; Arg, arginine; Lys, lysine; His, histidine; Tyr, tyrosine; Phe, phenylalanine; Trp, tryptophan; 2-aba, 2-aminobutyrate

Appendix 9. Effect of 1 mM L-cycloserine (CS) on amino acid concentration (final concentration - mM) by five strains of *M. elsdenii*.[a]

Amino Acid	CS	Strain T81	B159	LC1[b]	AW106	J1	Initial Concentration
Gly[c]	-	1.67	3.00	2.62	2.44	2.49	2.62
	+	1.68	2.18	2.86	2.42	2.17	0.02
S.E.		0.04	0.14	n.c.[d]	0.14	0.05	
Prob.[e]		0.941	0.101	n.c.	0.955	0.208	
Ala	-	5.53	6.80	6.46	6.44	5.41	5.05
	+	4.53	4.05	5.43	5.06	4.18	0.05
S.E.		0.10	0.34	n.c.	0.31	0.04	
Prob.		0.0147	0.055	n.c.	0.156	0.043	
Val	-	1.79	3.88	0.89	3.78	1.62	3.97
	+	2.16	3.11	3.58	3.69	2.77	0.05
S.E.		0.29	0.18	n.c.	0.22	0.07	
Prob.		0.671	0.168	n.c.	0.872	0.084	
Leu	-	1.74	3.72	1.36	3.48	2.03	5.62
	+	2.30	4.25	4.69	3.64	3.52	0.08
S.E.		0.27	0.22	n.c.	0.25	0.10	
Prob.		0.523	0.356	n.c.	0.779	0.098	
Ile	-	1.22	2.90	0.55	2.74	1.18	2.91
	+	1.34	2.26	2.60	2.66	1.94	0.04
S.E.		0.22	0.13	n.c.	0.16	0.05	
Prob.		0.852	0.133	n.c.	0.844	0.091	
2-Aba	-	3.64	1.86	4.00	3.96	3.03	0.16
	+	3.33	0.24	1.98	3.21	1.63	0.00
S.E.		0.06	0.05	n.c.	0.15	0.01	
Prob.		0.244	0.004	n.c.	0.134	0.014	
Ser	-	0.18	0.29	0.19	0.15	0.17	3.67
	+	0.26	1.73	0.19	0.16	0.19	0.03
S.E.		0.00	0.09	n.c.	0.01	0.01	
Prob.		0.066	0.015	n.c.	0.481	0.46	
Thr	-	0.00	1.65	0.01	0.00	0.20	2.63
	+	0.02	1.98	0.01	0.00	0.78	0.03
S.E.		0.00	0.12	n.c.	0.00	0.13	
Prob.		0.016	0.306	n.c.	n.c.	0.290	
Tyr	-	0.35	0.37	0.36	0.42	0.14	0.46
	+	0.33	0.32	0.35	0.38	0.13	0.01
S.E.		0.01	0.03	n.c.	0.01	0.00	
Prob		0.507	0.458	n.c.	0.218	0.172	
Met	-	1.06	1.18	1.20	1.21	1.17	1.43
	+	1.08	1.10	1.32	1.23	1.06	0.02
S.E.		0.04	0.06	n.c.	0.07	0.01	
Prob.		0.835	0.572	n.c.	0.893	0.145	
Asp	-	2.61	1.98	4.58	3.01	3.34	4.65
	+	3.01	3.12	4.48	3.58	2.92	0.37
S.E.		0.08	0.15	n.c.	0.20	0.06	
Prob.		0.278	0.064	n.c.	0.302	0.181	

Appendix 9. Continued.

Amino Acid	CS	Strain T81	B159	LC1	AW106	J1	Initial Concentration
Asn	-	0.00	0.08	0.10	0.14	0.15	0.16
	+	0.00	0.04	0.16	0.14	0.12	0.00
S.E.		0.00	0.00	n.c.	0.01	0.00	
Prob.		0.500	0.026	n.c.	0.910	0.205	
Glu	-	7.61	8.18	9.96	8.53	8.82	10.41
	+	7.84	7.30	9.61	8.78	7.65	0.78
S.E.		0.17	0.41	n.c.	0.55	0.15	
Prob.		0.649	0.394	n.c.	0.836	0.180	
Arg	-	1.31	1.34	1.45	1.17	0.34	1.62
	+	1.28	1.19	1.39	1.21	0.33	0.04
S.E.		0.03	0.07	n.c.	0.07	0.00	
Prob.		0.785	0.333	n.c.	0.791	0.024	
Lys	-	3.53	3.85	4.38	3.72	[illegible]14	4.42
	+	3.48	3.32	3.93	3.86	[illegible]58	0.11
S.E.		0.10	0.20	n.c.	0.18	0.06	
Prob.		0.886	0.320	n.c.	0.746	0.145	
His	-	0.90	1.09	1.13	0.98	1.09	1.19
	+	0.90	0.87	1.11	0.87	0.92	0.00
S.E.		0.02	0.06	n.c.	0.02	0.02	
Prob.		0.966	0.204	n.c.	0.139	0.150	
Phe	-	2.16	1.78	2.45	1.88	1.95	1.97
	+	2.38	1.47	1.94	1.99	1.69	0.04
S.E.		0.00	0.09	n.c.	0.10	0.04	
Prob.		0.008	0.220	n.c.	0.628	0.209	
Trp	-	0.20	0.22	0.25	0.23	0.21	0.24
	+	0.18	0.18	0.22	0.20	0.16	0.00
S.E.		0.00	0.01	n.c.	0.01	0.00	
Prob.		0.265	0.264	n.c.	0.282	0.075	

[a] n = 3 for all means except for T81 -, LC1 -, LC1 + and J1 + where n = 2.

[b] T-tests could not be run on LC1 because of 0 degrees of freedom due to missing data.

[c] Abbreviations used: Gly, glycine; Ala, alanine; Val, valine; Leu, leucine; Ile, isoleucine; Ser, serine; Thr, threonine; Met, methionine; Asp, aspartate; Asn, asparagine; Glu, glutamate; Arg, arginine; Lys, lysine; His, histidine; Tyr, tyrosine; Phe, phenylalanine; Trp, tryptophan; 2-aba, 2-aminobutyrate.

[d] Prop = probability.

[e] n.c. = not calculated.

Appendix 10. Effect of 1 mM L-cycloserine (CS) on volatile fatty acid concentrations (mM) of five strains of M. elsdenii.*

	CS	Strain				
		T81	B159	LC1	AW106	J1
Acetate	-	56.89	103.58	56.68	111.83	59.50
	+	29.28	61.45	73.83	103.37	54.72
S.E.		1.16	0.20	0.59	0.84	1.14
Prob.		0.423	0.000	0.051	0.036	0.181
Propionate	-	57.42	112.68	86.05	81.06	70.74
	+	54.93	34.95	82.22	64.08	64.41
S.E.		0.22	0.38	3.48	1.07	1.08
Prob.		0.02	0.000	0.718	0.015	0.098
Isobutyrate	-	6.75	3.24	11.61	3.37	9.18
	+	6.75	1.89	2.43	3.10	3.91
S.E.		0.69	0.05	0.07	0.30	0.34
Prob.		0.96	0.005	0.010	0.715	0.016
Butyrate	-	24.83	16.73	26.72	27.26	33.60
	+	28.34	4.45	18.62	36.84	37.38
S.E.		0.90	0.13	0.41	0.45	0.22
Prob.		0.21	0.001	0.070	0.009	0.079
Isovalerate	-	13.96	6.69	22.69	8.17	17.59
	+	14.86	0.68	3.06	7.83	8.40
S.E.		1.27	0.07	0.13	0.44	0.71
Prob.		0.75	0.001	0.009	0.678	0.023
Valerate	-	33.81	14.64	38.01	22.35	41.87
	+	40.05	2.95	27.69	29.96	45.61
S.E.		0.98	0.16	0.65	0.42	0.35
Prob.		0.08	0.001	0.089	0.012	0.034
Caproate	-	1.47	0.59	1.47	1.57	1.76
	+	2.06	1.08	0.69	2.15	1.96
S.E.		0.10	0.08	0.17	0.05	0.05
Prob.		0.11	0.094	0.270	0.028	0.144

* n = 3 for all means except LC1 + where n = 2.