TESIS DE DOCTORADO

UNDERSTANDING THE EFFECT OF KEY OPERATIONAL CONDITIONS ON AMINO ACID ACIDIFICATION FOR A KNOWLEDGE-DRIVEN PROTEIN FERMENTATION

Riccardo Bevilacqua

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D. Riccardo Bevilacqua

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Understanding the effect of key operational conditions on amino acid acidification for a knowledge-driven protein fermentation

Dña. Marta Carballa Arcos, Profesora Contratada Doctora
D. Miguel Mauricio Iglesias, Profesor Ayudante Doctor

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ABSTRACT

The depletion of fossil fuels and the growing need for a more sustainable, circular economy is paving the way to the concepts of biorefinery and resource recovery. In the biorefinery framework, the anaerobic digestion for the production of biogas and biomethane is an already established technology. However, the application of those products is generally limited to energy generation and the associated profitability is quite low. The carboxylate platform approach poses an interesting alternative, allowing to convert organic wastes to volatile fatty acids, which are then further refined to a variety of end-products (pharmaceuticals, bioplastics, etc). Volatile fatty acids are produced in mixed-culture fermentation processes, whose application has been thoroughly studied for sugar and carbohydrates-rich substrates. Conversely, the knowledge concerning the anaerobic fermentation of proteins is limited and sometimes contradictory. In particular, little information is available on how the operational conditions specifically affect the consumption and transformation of the different amino acids and their interactions.

Therefore, the goal of this PhD thesis is to understand the effect of key operational conditions on the conversion of amino acids to volatile fatty acids. The focus is placed on the effect of substrate composition, pH value and micronutrients supplementation on the process. Beyond the fermentation of proteins, the opportunities of using carbohydrates as a cosubstrate are explored together with the feasibility of chain elongation processes leading to medium chain fatty acids.

The results obtained highlight protein composition as one factor determining both the overall conversion of the amino acids to volatile fatty acids and the resulting products spectra. In fact, preferential consumption was identified during casein and gelatin fermentation, probably as a result of amino acids interactions. Their redox roles appear to be influential in determining the outcome of the process, as a surplus in electron donor amino acids seems to promote the overall acidification. In conjunction with protein composition, pH is identified as a key parameter as well. Neutral conditions are the most favourable for the microbial community, as protein conversion is maximised regardless of the amino acid profile. High pH values are associated with increased acetic acid formation, whereas acid conditions lead to a more diverse product spectrum. In particular, the production of longer chain volatile fatty acids is promoted at low pH, partially as a result of chain elongation processes. Interestingly, the effect of pH depends on the protein composition, as casein fermentation was influenced more strongly than in gelatin case. The supplementation of micronutrients promotes the overall conversion of amino acids to volatile fatty acids only at neutral pH, favouring the occurrence of secondary processes such as chain elongation reactions and the isomerisation between linear and branched forms of the carboxylic acids. The supplementation of sugars is another viable strategy to steer the selectivity of protein fermentation towards the production of longer chain volatile fatty acids, such as n-butyric and especially n-valeric acid. Moreover, protein conversion is not affected for sugar-to-protein ratio equal or lower than 1. Conversely, higher sugar loadings appear to hinder amino acids consumption while favouring the production of

short chain volatile fatty acids and secondary metabolites (e.g. ethanol). Interestingly, these changes are reversible given that lowering the sugar-to-protein ratio allows to progressively return to the previous products distribution. Chain elongation processes based on amino acid consumption were also detected during casein monofermentation at acid conditions and the metabolic pathways associated with these processes were conceptualised and described. It is hypothesised that chain elongation reactions were used by the microbial community as a way to reduce the toxicity exerted by the acid equivalents at low pH by taking advantage of the excess of reducing power generated by casein conversion. Micronutrients supplementation enables chain elongation occurrence for different proteins (i.e. gelatin) and different pH values (i.e. 7), while the cofermentation of proteins with sugars further promotes the process by providing a surplus of electron donor compounds (i.e. ethanol and lactate) and reducing power.

In conclusion, the knowledge acquired in this thesis is useful for the design and optimisation of mixed-culture fermentation processes using protein-rich streams, as it will help to define the operational strategies to steer proteins fermentation towards the desired outcome.

Keywords: amino acid acidification, biorefinery, mixed-culture fermentation, operational conditions impact, volatile fatty acid production

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ABBREVIATIONS AND ACRONYMS

AA	Amino acid
Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
ATP	Adenosine 5'-triphosphate
BMP	Biochemical methane potential
CE	Chain elongation
Co	Cobalt
COD	Chemical oxygen demand
CSTR	Continuous stirred tank reactor
Cu	Copper
Cys	Cysteine
Fe	Iron
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
HAc	Acetic acid
HBut	n-Butyric acid
HiBut	Iso-butyric acid
HiCapr	Iso-caproic acid
His	Histidine
HiVal	Iso-valeric acid

HPr	Propionic acid
HRT	Hydraulic retention time
HVal	n-Valeric acid
Ile	Isoleucine
ISR	Inoculum-to-substrate ratio
Leu	Leucine
Lys	Lysine
MCF	Mixed-culture fermentation
MCFA	Medium chain fatty acids
Met	Methionine
NADH	Nicotinamide adenine dinucleotide
Ni	Nickel
OD	Optical density
OLR	Organic loading rate
Pro	Proline
Se	Selenium
Ser	Serine
SIR	Substrate-to-inoculum ratio
SRT	Solids retention time
STP	Sugar-to-protein ratio
TAN	Total ammonia nitrogen
Thr	Threonine
TKN	Total Kjeldahl nitrogen
Val	Valine
VFA	Volatile fatty acid
Zn	Zinc

RESUMO

Capítulo 1. Introdución e contexto

A crecente escaseza de combustibles fósiles está empuxando a investigación cara a busca de métodos alternativos de obtención de enerxía e produtos químicos. Neste contexto, o concepto de biorrefinaría substitúe o da refinaría tradicional na produción dos ácidos graxos volátiles. Estes compostos son intermedios obtidos mediante fermentación en condicións anaerobias, e precursores na elaboración dunha multitude de produtos coma bioplásticos, cosméticos, biocombustibles, etc. Porén, a aplicación da fermentación anaerobia é limitada pola falta de coñecemento sobre os mecanismos involucrados e a necesidade de optimización do proceso en termos de conversión do substrato e selectividade dos produtos, sobre todo cando o proceso se leva a cabo por parte dunha comunidade microbiana mixta.

A literatura previa indica que tanto a composición do substrato empregado coma as condicións operacionais son os factores que máis determinan o resultado da fermentación en cultivo mixto. Moitos estudos centráronse ata agora na conversión de compostos modelo, principalmente azucres coma glicosa, ou residuos reais ricos en azucres. Pola contra, pódese atopar escasa información sobre a fermentación de proteínas; ademais, a pouca que está dispoñible consiste en experimentos con resultados contraditorios.

A conversión de proteínas, ou máis ben dos aminoácidos que as compoñen, é descrita habitualmente mediante reaccións Stickland, reaccións redox que emparellan dous ou máis aminoácidos onde un actúa como aceptador de electróns e o outro como dador de electróns. Ademais, cada aminoácido ten rutas metabólicas definidas, dando lugar a ácidos graxos volátiles específicos de acordo coa súa estrutura. Por iso, non cabe dubida de que o perfil de aminoácidos sexa unha variable das máis importantes no proceso de fermentación de proteínas. Porén, hai outras variables operacionais que potencialmente poden alterar ditas rutas metabólica, polo que resulta interesante entender coma se poden manipular para dirixir o proceso cara os resultados desexados. O pH é un dos parámetros operacionais destacados xa que pode ser controlado na operación e dá lugar a cambios na conversión e na selectividade da fermentación acidoxénica. Valores neutros normalmente coinciden co óptimo dos cultivos mixtos, maximizando a produción de ácidos graxos volátiles. Condicións alcalinas adoitan favorecer a hidrólise abiótica das proteínas pero limitando a conversión dos aminoácidos. Valores baixos de pH tamén reducen a acidificación, aínda que favorezan compostos máis atractivos có ácido acético coma o ácido butírico ou valérico. Axustar o tempo de retención hidráulica e a velocidade de carga orgánica tamén resulta importante debido á necesidade dun compromiso entre favorecer a hidrólise das proteínas, máis lenta cá dos azucres, e evitar a metanizacion, aínda que o seu efecto sobre a selectividade é moito máis reducido có do pH. Pola contra, a temperatura é un dos parámetros que menos se considera para dirixir o proceso, posto que a súa influencia está máis enfocada na velocidade de degradación. Malia seren importantes no proceso de dixestión anaerobia, o efecto da presenza e ausencia dos micronutrientes non se investigou detidamente, limitando a información dispoñible sobre o tema. Existen tamén outras alternativas máis alá da manipulación dos parámetros operacionais para dirixir o proceso de fermentación das proteínas. Algúns estudos centráronse na posibilidade de aumentar a conversión dos residuos mediante a mestura de varias correntes,

nomeadamente de residuos proteicos e ricos en carbohidratos. Os resultados experimentais encontrados na literatura evidencian tendencias contraditorias, entre o antagonismo e a sinerxía, dependendo das proporcións de carbohidratos e proteínas no substrato global. Outra alternativa son os chamados procesos de "alongamento de cadea", unha evolución dos procesos acidoxénicos, para os cales non hai apenas resultados previos no uso de proteínas malia a súa importancia como fonte de carbono de orixe residual.

Polo tanto, o obxectivo principal desta tese é entender como os parámetros operacionais máis relevantes afectan a fermentación das proteínas, en termos de conversión do substrato e selectividade. Este coñecemento servirá para optimizar o deseño e desenvolvemento de procesos de fermentación que teñan como substrato residuos ricos en proteínas, maximizando a súa acidificación e potencialmente dirixindo a distribución dos produtos cara ao resultado desexado.

En particular, esta tese intenta entender mellor o proceso buscando respostas ás seguintes preguntas:

- **Cal é a influencia do perfil en aminoácidos sobre o proceso de fermentación de proteínas?** Hai diferenzas en termos de conversión e espectro de produtos entre proteínas diferentes? Como se explican estas diferenzas? Pódese predicir o resultado do proceso?
- **Como inflúen o pH e a suplementación de micronutrientes na conversión e selectividade do proceso?** Pódense axustar para dirixir o proceso cara o resultado desexado? Como se ven afectados as rutas metabólicas e os mecanismos asociados?
- **Que oportunidades hai para o tratamento dos residuos proteicos a parte da monofermentación?** É a cofermentation con azucres unha alternativa para facer máis atractivo o proceso? Son posibles os proceso de alongamento de cadea durante a fermentación de proteínas? Pódense mellorar e/ou intensificar?

Capítulo 2. Materiais, métodos e equipos

Neste capítulo descríbense as composicións dos substratos utilizados, a preparación do inóculo e as diferentes configuracións experimentais empregadas durante o desenvolvemento da tese. Ademais, inclúense todos os métodos analíticos empregados e os cálculos necesarios para a análise dos datos.

Para a preparación das alimentacións usáronse dúas proteínas diferentes en termos de composición en aminoácidos, caseína e xelatina. Tamén se usou glicosa para simular a fracción orgánica dos azucres nos experimentos de cofermentation.

As fermentacións leváronse a cabo en biorreactores continuos de mestura completa. Tras acadar o estado estacionario, realizáronse experimentos en descontinuo para estudar o proceso fermentativo en ausencia de limitacións cinéticas. A monitorización dos experimentos incluíu análises de nutrientes, produtos catabólicos, substrato non consumido e biomasa producida.

Capítulo 3. A composición das proteínas determina o consumo preferente dos aminoácidos durante a fermentación en cultivo mixto

Tal e como se describiu no **Capítulo 1**, a fermentación das proteínas estudouse en menor medida cá dos azucres. Sábese que a maioría das reaccións de conversión dos aminoácidos a ácidos graxos volátiles é do tipo Stickland, pero a estequiometría proposta para describilas asume consumo completo dos aminoácidos sen considerar se o balance redox pode ser atinxido. Pola contra, hai moitos exemplos na literatura que contradín dita estequiometría fixa. Por iso, este capítulo analiza como a composición das proteínas afecta o consumo e as interaccións entre aminoácidos e, consecuentemente, o espectro de produtos e a conversión global a ácidos orgánicos.

Dous reactores foron alimentados en continuo con caseína e xelatina por separado tras a inoculación con biomasa acidoxénica, chegando a máis de 100 días de operación a pH neutro. O tempo de retención hidráulica, fixado a 1 – 1,5 días, permitiu inhibir eficazmente a metanoxénese dos ácidos producidos. O grao de acidificación dos dous reactores en estado estacionario alcanzou diferentes resultados debido á diferente composición das proteínas. O reactor alimentado con caseína chegou a un valor de 50%. A conversión da xelatina foi máis baixa, cerca do 40%. A selectividade dos produtos tamén resultou diferente. Agás no caso do ácido acético, o produto maioritario da fermentación das dúas proteínas, a caseína estivo relacionada coa produción de ácido n-butírico mentres que a xelatina converteuse preferentemente a ácido propiónico. Tamén se observaron outras diferenzas nas fraccións molares dos ácidos graxos volátiles secundarios. Os experimentos en descontinuo non só confirmaron estas tendencias senón que permitiron estimar os parámetros cinéticos asociados coa fermentación das dúas proteínas.

Analizando a composición de aminoácidos no reactor concluíuse que o seu consumo individual non é homoxéneo e varía dependendo da composición das proteínas. Por exemplo, a glicina convértese completamente durante a fermentación de caseína mentres que na xelatina só se alcanza un consumo do 50%. Formulouse a hipótese de que este consumo preferente pode considerarse coma unha estratexia para compensar o balance redox xeral, aínda que un exceso de aminoácidos dadores de electróns parece favorecer o proceso fermentativo, como acontece para a fermentación da caseína. A monitorización do consumo de aminoácidos tamén permitiu establecer balances molares entre substrato (aminoácidos) e produtos (ácidos graxos volátiles) indicando que a estequiometría das reaccións Stickland é consistente coa produción dos ácidos i- e n-butíricos pero non coa dos ácidos i- e n-valéricos suxerindo que o modelo de fermentación anaerobia das proteínas previamente proposto non é suficiente para describir o resultado do proceso.

Capítulo 4. Dirixindo a conversión de residuos proteicos en ácidos graxos volátiles a través do control do pH

A influencia do pH sobre o proceso de fermentación anaerobia é ben coñecida no que se refire aos azucres. Pola contra, apenas hai resultados sobre o papel deste parámetro na fermentación de proteínas. De acordo coa estequiometría aceptada previamente a esta tese**,** o pH non inflúe no consumo dos aminoácidos nin sobre a distribución dos ácidos orgánicos producidos. Porén, varios estudios experimentais demostran que cambios de pH poden limitar o proceso

de fermentación e/ou alterar a súa selectividade, de maneira similar ao caso dos azucres. Este capítulo pretende resolver estas contradicións investigando o efecto do pH sobre o consumo de aminoácidos durante a fermentación de caseína e xelatina tanto en condicións ácidas como alcalinas.

A fermentación de caseína e xelatina estudouse en biorreactores de tanque axitado a pH 5, 7 e 9. Para as dúas proteínas, a conversión a ácidos graxos volátiles foi máxima a pH neutro (40-50%) mentres que, a pH ácido, baixou a valores comprendidos entre 20 e 30%. O efecto do pH alcalino dependeu da composición da proteína. No caso da caseína, o grao de acidificación non variou, permanecendo cerca do 50%. Pola contra, a conversión da xelatina vese afectada negativamente (< 20%).

En termos de selectividade de produtos, puidéronse apreciar diferenzas significativas entre o rango ácido e o alcalino. A pH 9 favoreceuse especialmente a produción de ácido acético que é, en todo caso, o produto maioritario da fermentación de proteínas en todas as condicións testadas. Porén, no rango ácido, o espectro de produtos vólvese máis equilibrado, favorecéndose a produción de ácidos graxos volátiles de cadea máis longa coma os ácidos iso- e n-valéricos e o ácido n-butírico. Estas tendencias xerais apreciáronse nas dúas proteínas ensaiadas pero con intensidades diferentes, xa que as variacións do espectro de produtos da caseína foron máis relevantes ca no caso da xelatina, suxerindo que o consumo dos aminoácidos segue dependendo tamén da composición das proteínas, aínda que a súa biodispoñibilidade varíe de acordo co pH do medio.

En conclusión, neste capítulo demostrouse como axustar o valor do pH pode ser unha estratexia viable para dirixir o proceso de fermentación das proteínas. Ademais, confirmouse que o modelo previo de estequiometría fixa de degradación das proteínas non é suficiente para describir o comportamento dos aminoácidos durante procesos de fermentación anaerobia.

Capítulo 5. Efecto da adición de elementos traza sobre a produción de ácidos graxos volátiles a partir de proteínas

A suplementación de elementos traza nos procesos de dixestión anaerobia adoita ter un efecto positivo sobre a produción de biogás. De feito, moitos estudos subliñan a necesidade de engadir elementos traza (ferro, cobre, níquel, cobalto e zinc, entre os máis importantes) a certas correntes residuais ao non estaren en concentracións suficientes o que limita ou restrinxe o fluxo de certas rutas metabólicas. O coñecemento sobre o efecto da suplementación na fermentación anaerobia está sobre todo centrado no uso de azucres, demostrando a súa potencialidade para dirixir o resultado do proceso. Polo tanto, neste capítulo avalíase dita potencialidade no caso das proteínas, estudando o efecto da presenza e ausencia dos elementos traza a diferentes pH e substratos proteicos.

Dado o interese demostrado no **capítulo 4** polo espectro de produtos obtido a pH ácido, decidiuse operar biorreactores a pH 5 e 7 tanto con caseína como con xelatina, permitindo unha comparación apropiada cos resultados dos capítulos anteriores. A suplementación de elementos traza aumentou a conversión do substrato a pH neutro, sobre todo polo que se refire á fermentación da xelatina. Porén, non se rexistrou efecto ningún operando os reactores a pH

ácido, polo que se formulou a hipótese de que a presión exercida por este parámetro operacional supera o beneficio suposto pola presenza dos micronutrientes. En case todos os casos estudados, a suplementación ten un efecto positivo sobre o crecemento da biomasa bacteriana, probablemente debido a un incremento da eficiencia na extracción de enerxía dende o substrato proteico.

En condicións neutras, o consumo de aminoácidos creceu de acordo coa súa abundancia relativa na composición de cada proteína, sen favorecer de maneira especifica o consumo duns sobre outros. Tanto a pH ácido como neutro, a presenza de elementos traza favoreceu especialmente a conversión da xelatina grazas ao seu efecto positivo sobre o consumo de glicina, un dos compoñentes principais desta proteína, cun consumo que aumentou dun 50% ata case o 100%. Ao ser a glicina precursora da produción de ácido acético, o seu incremento de consumo xustifica case completamente a maior produción do ácido acético na fermentación de xelatina, un cambio que non se apreciou no caso da caseína. En xeral, os micronutrientes favoreceron a formación dos ácidos valéricos para ambas proteínas, mentres que outras variacións foron máis específicas. No caso da caseína, foron favorecidas as producións de ácido propiónico e o alongamento de cadea para dar ácido caproico. Pola contra, a xelatina estivo asociada cun incremento de ácido iso-butírico na distribución dos produtos. Cómpre destacar que estas variacións non só se deben ao incremento do consumo dos aminoácidos senón tamén a procesos secundarios como o alongamento de cadea e á isomerización entre ácidos lineais e ramificados. Os balances molares entre consumo de aminoácidos e produción de ácidos graxos volátiles axudaron a identificar estes procesos, subliñando o seu impacto sobre a formación dos ácidos butíricos e valéricos.

Capítulo 6. Dirixir o proceso de cofermentación a través de cambios na relación entre azucres e proteínas

A cofermentación de proteínas con azucres é unha alternativa atractiva á monofermentación por varios motivos. A mestura de diferentes correntes residuais pode favorecer a produción de ácidos graxos volátiles xa que se equilibran as relacións carbono-nitróxeno, se aportan maiores cantidades de micronutrientes, se dilúen compostos potencialmente tóxicos e inhibitorios e/ou se incrementa a velocidade da hidrólise. Porén, hai moita variabilidade na intensidade destes efectos beneficiosos e pódense atopar antagonismos entre as dúas fraccións orgánicas cando son mesturadas de maneira inadecuada. Para entender como evitar os antagonismos e favorecer as sinerxías, neste traballo estúdase a influencia da relación azucres-proteínas sobre as interaccións entre as dúas fraccións orgánicas e, en consecuencia, sobre o resultado do proceso cofermentativo coa fin de axustar dita relación para dirixir o proceso cofermentativo.

Parte da biomasa adaptada a monofermentación de caseína (**Capítulo 5**) foi usada para operar un reactor de cofermentación, no cal se alimentaron concentracións crecentes de glicosa mantendo unha concentración constante de caseína. Desta maneira, variouse a relación glicosa-caseína entre 0,25 e 2,00. Operouse o reactor a pH 7, mentres que todas as outras condicións operacionais mantivéronse similares ós outros experimentos descritos nesta tese

(p.ex. HRT 1,5 días). Cómpre destacar que a presenza da glicosa, substrato de máis fácil degradación, levou a necesidade de engadir un inhibidor de metanoxénese. O composto elixido foi a sal sódica do ácido bromoetansulfónico (BES). En total, o reactor operouse durante máis de 400 días, alcanzando estados estacionarios para cada relación glicosa-caseína.

A conversión da proteína non se viu afectada sempre que a relación glicosa-caseína se mantivese por debaixo de 1,00. Pola contra, aplicando un valor superior (2,00), o seu consumo baixou do 70 ao 55%. Esta tendencia negativa reflectiuse nas variacións do consumo especifico dos aminoácidos, que polo xeral diminuíron de acordo coa amonificación. O grao de acidificación tamén se viu afectado, pasando de 60 a 40%, dado que se xeraron metabolitos secundarios coma o etanol e quedou caseína sen consumir no reactor. En termos de selectividade, o incremento da concentración da glicosa na alimentación favoreceu progresivamente a produción de ácidos graxos de cadea longa ata chegar a unha relación glicosa-caseina igual a 1,00. O incremento na produción de ácido n-butirico e n-valérico suxire a ocorrencia de alongamento de cadea. Formulouse a hipótese de que a presenza da glicosa favorece este tipo de proceso pola súa conversión en etanol e lactato, ambos compostos dadores de electróns e a miúdo asociados co alongamento de cadea. Porén, relacións maiores a 1,00 parecen inhibir ditos procesos á vez que se promove a produción de ácido acético e propiónico. Tamén aparecen lactato e etanol nos efluentes debido a que sen alongamento de cadea estes compostos non se consumen. Demostrouse que estes cambios de selectividade son reversibles en certa medida, xa que a produción de ácidos graxos volátiles de cadea máis longa se puidorecuperar mediante a redución da relación glicosa-caseina na alimentación.

En xeral, neste capítulo estudáronse con éxito as interaccións entre azucres e proteínas, demostrando como axustar a relación entre as dúas fraccións en valores menores ou iguais a 1,00 pode ser unha estratexia viable para dirixir a cofermentación cara á produción de acido n-butírico e n-valérico sen inhibicións no consumo do substrato.

Capítulo 7. O alongamento de cadea pode ocorrer durante a fermentación en cultivo mixto de proteínas sen suplementación de compostos dadores de electróns

A maioría dos estudos sobre o alongamento de cadea fixéronse con azucres, engadindo compostos dadores de electróns (p.ex. etanol) para fomentar o proceso. No caso dos aminoácidos, o alongamento de cadea só se demostrou en cultivos puros. Polo tanto, este capítulo ten como obxectivo revisar o potencial das proteínas para dar lugar a procesos de elongación de cadea durante a súa (co-) fermentación en cultivo mixto, reunindo os resultados obtidos nos capítulos anteriores (**Capítulo 4, 5 e 6**) e inferindo as condiciones operacionais requiridas que favorecerían este proceso.

Grazas ós datos xerados no **Capítulo 4**, observouse que o proceso pode ocorrer nos reactores continuos de fermentación de proteínas en cultivo mixto. En particular, a ocorrencia do alongamento de cadea restrinxiuse á operación no rango ácido de pH e soamente usando caseína como substrato. Ao seren compostas tanto por aminoácidos dadores como aceptadores de electróns, as proteínas poden ser o único substrato sen necesitar suplementacións externas

de dadores de electróns como lactato ou etanol. Os balances molares indican que o ácido n-valérico é o principal produto de elongación, debido a que só o consumo do aminoácido asociado (prolina) non xustifica a súa produción. Tampouco se descartou a posibilidade de que os ácidos n-butírico e iso-valérico foran outros produtos de elongación, aínda que a contribución do alongamento da cadea na súa formación quedou menos clara. A hipótese é que a comunidade microbiana usa o proceso como estratexia de disipación tanto do exceso de poder redutor asociado coa caseína coma dos equivalentes ácidos xerados pola súa fermentación. De feito, reducir o número de grupos ácidos na solución resulta particularmente importante a pH baixo debido a que os ácidos graxos volátiles, na forma non disociada, poden difundirse a través das paredes e membrana celulares máis facilmente causando problemas de toxicidade no interior das células.

Para comprobar a ocorrencia do proceso e entender o efecto da dispoñibilidade do substrato, realizáronse experimentos en descontinuo a pH 5 usando caseína coma substrato a diferentes concentracións iniciais. Os resultados axudaron na identificación das condicións necesarias para o alongamento de cadea. Tanto o ácido acético coma o propiónico son consumidos polo proceso para producir n-valérico, usando aminoácidos coma dadores de electróns. Ademais, observouse que o proceso se ve favorecido cando os dous substratos necesarios para o alongamento están presentes ó mesmo tempo de xeito prolongado. Esta tendencia xustificouse pola competición entre as comunidades fermentativas e as comunidades alongadoras polo consumo dos aminoácidos que pode haber no caso de que haxa unha concentración limitada de substrato inicial.

Para estudar o rol dos ácidos de cadea corta, levouse a cabo outro experimento en descontinuo engadindo ácido acético no substrato inicial. Deste xeito, pretendeuse demostrar a posibilidade de fomentar o alongamento de cadea evitando a competición previamente mencionada. O ácido acético foi efectivamente consumido e a súa suplementación inicial favoreceu o seu alongamento a ácido n-butírico. Ademais, a selectividade do proceso viuse desviada cara a formación de ácido iso-valérico, reducindo a contribución que tiña na produción de n-valérico.

A partir de todo o coñecemento xerado neste capítulo, foi posible conceptualizar os mecanismos do alongamento de cadea das fermentacións de proteínas en cultivo mixto, subliñando o papel que os aminoácidos xogan tanto na produción dos ácidos de cadea corta coma dos intermedios de reacción (acetil-CoA e propionil-CoA) e na creación do poder redutor. Integrando esta información coa dos **Capítulos 5 e 6**, tamén se puideron entender as vantaxes xeradas pola suplementación dos elementos traza e pola cofermentación con azucres de cara a fomentar o proceso de alongamento de cadea. En particular, a presenza de micronutrientes estende a idoneidade dos procesos de alongamento de cadea a proteínas que non necesariamente teñen un exceso de aminoácidos dadores de electróns, aínda que tanto a selectividade coma intensidade do proceso dependan da composición da proteína usada. Ademais, amplían o rango de pH no cal se pode observar o proceso a valores neutros, xa que os balances molares poñen de manifesto o alongamento da cadea tanto no reactor de caseína coma de xelatina a pH 7. A presenza de azucres pode favorecer a produción selectiva de ácido

n-valérico sempre que a súa concentración na mestura de substratos sexa inferior ou igual a da proteína, confirmando que a cofermentación é outra estratexia operacional adecuada para dirixir a selectividade do proceso de monofermentación de proteínas. Nestes dous casos, formulouse a hipótese de que a forza impulsora do proceso de alongamento segue sendo a presión exercida polos equivalentes ácidos, que son producidos en maiores concentracións precisamente grazas á suplementación tanto de micronutrientes coma de glicosa.

Capítulo 8. Discusión xeral e conclusións

Neste capítulo intégranse e discútense conxuntamente todos os resultados obtidos ao longo do desenvolvemento da tese, intentando contestar as preguntas expostas na introdución sobre a fermentación anaerobia das proteínas. **Cal é a influencia do perfil en aminoácidos sobre o proceso de fermentación de proteínas? Como inflúen o pH e a suplementación de micronutrientes na conversión e selectividade do proceso? Que oportunidades hai para o tratamento dos residuos proteicos á parte da monofermentación?**

Todo o coñecemento xerado ao longo da presente tese pretende proporcionar ferramentas e estratexias para un mellor deseño e operación dos procesos de fermentación anaerobia que teñan as proteínas como unha das compoñentes máis relevantes do substrato (**Capítulo 8**). Coñecer a composición en aminoácidos dunha corrente residual rica en proteínas é fundamental para seleccionar as condición de operación necesarias para atinxir o resultado desexado. No caso de que a selectividade non sexa unha prioridade, deberían seleccionarse condicións de pH neutro para maximizar a produción de ácidos graxos volátiles, engadindo micronutrientes en concentracións suficientes para estimular o proceso e promover a ocorrencia do alongamento de cadea. No caso de querer dirixir o proceso cara a produción dun ácido determinado, sobre todo de cadea máis longa, axustar o pH no rango ácido é unha das opcións principais. Aínda que engadir micronutrientes non aumenta a conversión do substrato nese rango, a súa suplementación é recomendable para ampliar a aplicabilidade do alongamento de cadea ás diferentes proteínas. Outra alternativa é a cofermentación con azucres nos valores neutro de pH e con relacións azucres-proteínas menores ou iguais a 1.00. De feito, a presenza dos azucres intensifica o proceso de alongamento visto durante a monofermentación, favorecendo a produción selectiva de ácido n-butírico e sobre todo de ácido n-valérico.

Por último, descríbense diferentes posibilidades para investigacións futuras sobre esta temática. Entre elas, cómpre destacar o interese que pode ter o estudo do impacto destas condicións e estratexias operacionais sobre a etapa de hidrólise ou a busca dunha estratexia operacional capaz de incrementar a conversión das proteínas a pH baixo. Tamén tería interese o estudo da fermentación dos aminoácidos individuais, para refinar o coñecemento sobre as súas rutas metabólicas. Suplementacións especificas de micronutrientes poderían avaliarse coma unha estratexia para promover a produción dos ácidos desexados. A sinerxía na cofermentación poderíase estudar tanto en outros rangos de pH coma con outros substratos (lípidos) para determinar se a presenza dos azucres pode favorecer a produción de ácidos graxos volátiles en diferentes condicións operacionais. O estudo do alongamento de cadea

poderíase refinar a través dunha análise especifica do rol do ácido propiónico e avaliando con máis detalle os mecanismos asociados, por exemplo marcando os posibles substratos do proceso con isótopos do carbono. Finalmente, o estudo da composición microbiana permitiría entender mellor se os cambios causados no proceso polas estratexias operacionais se deben a variacións na biodiversidade e/ou a cambios nas rutas metabólicas.

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CHAPTER 1

INTRODUCTION AND CONTEXT

1.1. CARBOXYLATE PLATFORM

Concerns over the environmental impacts associated to the use of fossil fuels has stimulated the research, development and implementation of sustainable alternatives to satisfy the growing global need for chemical compounds and energy. Coincidentally, the increasing quantity of waste and wastewaters generated by our societies can be seen as an opportunity to tackle both issues at once (Agler et al., 2011). In fact, the biorefinery concept (Fernando et al., 2006) pretends to substitute the traditional oil-based one by using streams which are rich in organic matter to produce a wide range of products and fuels. Biorefineries tend to encompass several conversion steps, from organic substrates to intermediates and then to valuable products. This structure provides flexibility to the range of products that can be obtained, with the intermediates acting as the network link between substrates and products. These intermediates are referred to as platforms and serve to classify the different biorefineries.

Even though different platforms exist in the resource recovery framework, nowadays the focus has shifted towards studying the application of the carboxylate platform (Agler et al., 2011). In this biorefinery scheme (Figure 1.1), organic waste and wastewaters of various origins are initially converted to short chain carboxylates, commonly known as volatile fatty acids (VFAs, Table 1.1), organic solvents (e.g. ethanol) and gaseous hydrogen (H_2) by anaerobic microbial populations (Lee et al., 2014). These compounds are then adequately purified, if needed, and used as feedstocks for the production of pharmaceuticals, bioplastics, food flavourings, biofuels, etc. In fact, they can be refined via conventional methods (e.g. Fischer esterification) or further processed by biological means (e.g. polyhydroxyalkanoates formation (Bathia et al., 2021)) to obtain the desired end product. Thus, the production of these VFAs plays a central role in the carboxylate platform, meaning that studying the process mechanisms and understanding how they can be optimised and selectively produced should be a research priority (Lee et al., 2014).

VFAs are produced during an intermediate step of the anaerobic digestion process (Khanal et al., 2008). Initially, complex organic molecules such as carbohydrates and proteins are hydrolysed to their constituting monomers, e.g. simple sugars and amino acids (AAs). These monomers are then converted into a mix of carboxylic acids with five or less carbon atoms (Bathia & Yang, 2017) in the fermentation step. The resulting products composition and the conversion efficiency depend on several factors, among which the initial substrate composition and the environmental conditions play an important role. As acetogenesis and methanogenesis transform and consume VFAs to ultimately produce methane (CH_4), both phases should be inhibited to preserve them.

Given their wider range of applications beyond energy generation, VFA production appears to be more profitable than CH_4 generation (Lee et al., 2014; Moscoviz et al., 2018). To become economically viable and competitive, however, the anaerobic fermentation process should be optimised due to the variability of the products distribution and the limited anaerobic biodegradability of certain organic feedstocks (Bathia & Yang, 2017). Designing an anaerobic process to yield the desired products would ideally need to consider how the reactor

configuration, operational conditions and feedstock composition determine the fermentation outcome (Atasoy et al., 2018). For this reason, further studies on their impact on the process are required.

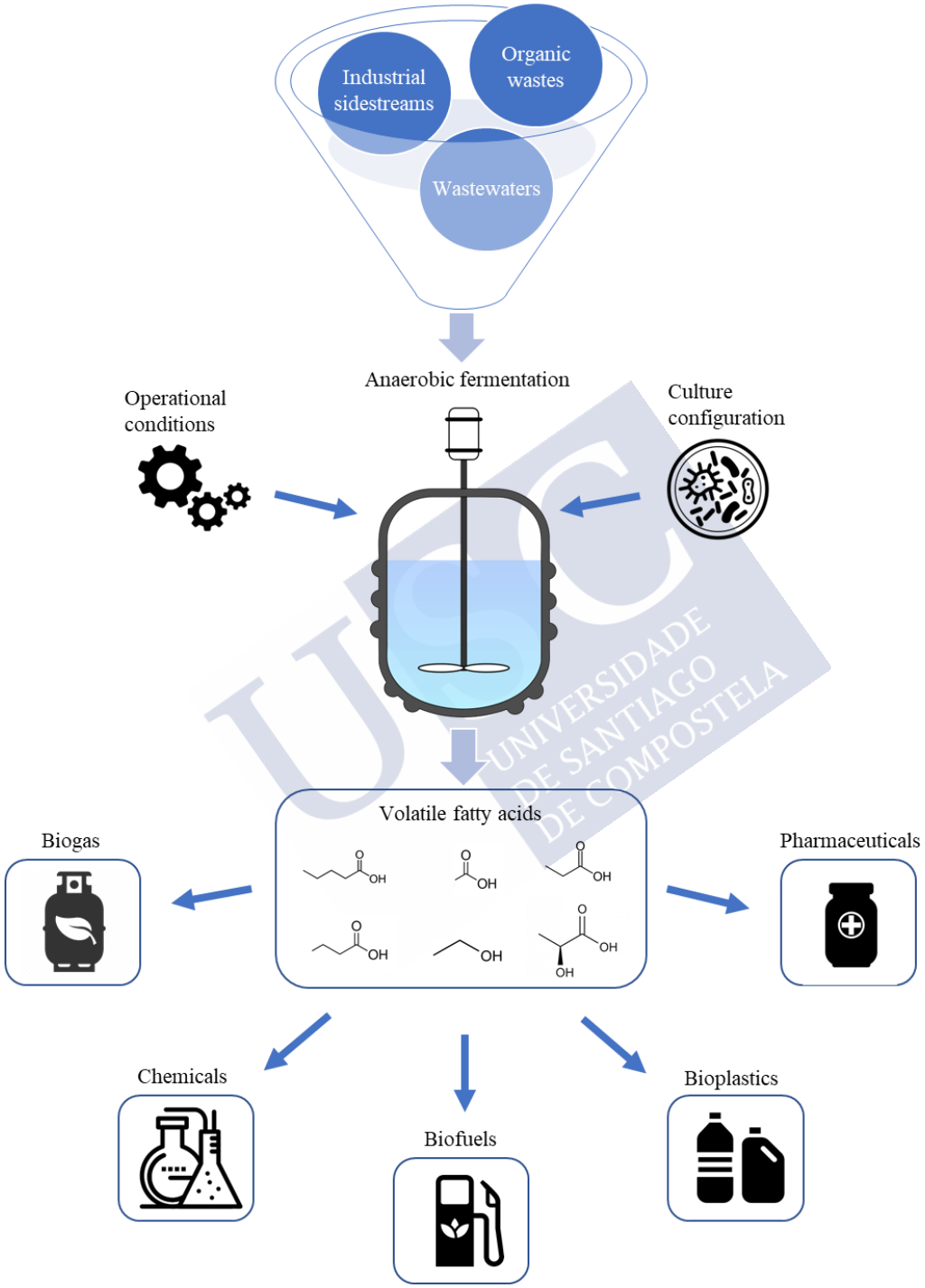

Figure 1.1. Carboxylate platform schematisation. Waste, sidestreams and wastewaters act as suitable substrate for the fermentation process and are converted to a mix of carboxylic compounds. Then, these compounds are further processed into added-value products.

Table 1.1. VFA chemical formulas and relevant parameters. Chemical Oxygen Demand (COD) ratio is used to calculate the equivalent VFA concentrations, making them comparable.

VFA	Chemical formula	Molar mass (g/mol)	COD ratio (g COD/g)	pKa
Acetic acid	$C_2H_4O_2$	60.05	1.07	4.76
Propionic acid	$C_3H_6O_2$	74.08	1.51	4.88
Iso-butyric acid	$C_4H_8O_2$	88.11	1.82	4.86
n-Butyric acid	$C_4H_8O_2$	88.11	1.82	4.82
Iso-valeric acid	$C_5H_{10}O_2$	102.13	2.04	4.80
n-Valeric acid	$C_5H_{10}O_2$	102.13	2.04	4.82

1.2. ANAEROBIC FERMENTATION

The production of VFA and organic solvents via anaerobic fermentation can be performed via different bacterial culture configurations, which determine the yields, the purity and also the costs of the process. In pure cultures, involving individual microorganism strains, targeted products are obtained at high concentration in either batch or fed-batch reactors (Stowers et al., 2014). The downsides of these systems are the high costs of pure inocula and feedstock sterilisation to avoid external contamination. Besides, microbial populations are to be chosen accordingly to the feedstock used as substrate, which needs to be readily fermentable (i.e. low versatility), and to the desired product (Coral et al., 2008). Likewise, the substrate must be relatively homogeneous, which limits the use as feedstock of residues and many low-grade biomass sources. Therefore, these systems are feasible for small productions of high purity compounds with a high market price. Provided that their metabolism is well known and compatible, two or more specifically selected bacterial species and/or strains might also be combined in pure co-cultures (cross-feeding), in which one microorganism converts the initial feedstock into a suitable substrate for the other (Selder et al., 2020). While this option improves the flexibility of the fermentation process in comparison with pure mono-cultures, the downsides remain the same since the growth media and the equipment must be sterilised and the strains must be maintained pure.

The second major alternative involves using (open) mixed cultures. Provided that it is sufficiently diverse, the initial inoculum composition is not relevant, given that the microbial population evolves constantly inside a continuous reactor (Carballa et al., 2015). Moreover, the lack of sterilisation procedures determines constant external species immigration which, to a smaller extent, might contribute to the overall diversity. The resulting microbiome is more resilient and suitable for the fermentation of multiple substrates, allowing the bulk production of carboxylic acids from even the most complex industrial sidestreams (e.g. paper mill wastewaters). The microbiome resilience also allows continuous feeding, which greatly increases the process productivity and, therefore, economic viability. The main downside of

this configuration is related to the variability of products concentration, fundamentally depending on microbial composition and operational conditions (Domingos et al., 2017). Mixed cultures can also be bioaugmented with pure bacterial strains in order to favour either specific selectivities (Atasoy et al., 2020) and/or secondary process, such as chain elongation (CE) ones. However, the chosen microorganisms (e.g. *Clostridium kluyveri*) might be outcompeted by the autochthone ones, limiting the effectivity of the bioaugmentation strategy (Zagrodnik et al., 2020). Overall, microbiology can be engineered only to a limited extent, meaning that operational parameters and especially substrate composition are the important factors to consider when designing a mixed-culture fermentation (MCF) process (Bathia & Yang, 2017).

1.3 ANAEROBIC FERMENTATION OF PROTEINS

While many studies focused on either sugar or complex streams conversion to VFAs (Gujer & Zehnder, 1983; Pavlostathis & Giraldo-Gomez, 1991; Skiadas et al., 2000, Temudo et al., 2007; González-Cabaleiro, 2015; Garcia-Aguirre et al., 2017; Dahiya et al., 2018), limited literature is available on proteins, regardless of their relevance for the process itself. Lipids fermentation is not well known either, but it is considered to be less attractive due to their recalcitrant nature and the fact that their conversion is almost only limited to acetic acid (Mackie et al., 1991; Sousa et al., 2008; Alves et al., 2009).

In the anaerobic fermentation process, proteins are first hydrolysed to either oligopeptides or monopeptides, the AAs, by specific enzymes called proteases. These monomers are then converted to VFAs, ammonia, carbon dioxide, hydrogen and reduced sulphur with net ATP production (catabolism) or assimilated for biomass growth (anabolism). Proteins present high compositional variability (Regueira et al., 2020) depending to their origin and structure, and from a metabolic point of view should be considered as a mix of twenty different substrates, i.e. the AAs (Figure 1.2; Table 1.2). These characteristics make the fermentation mechanisms more complex than in the case of sugar monomers.

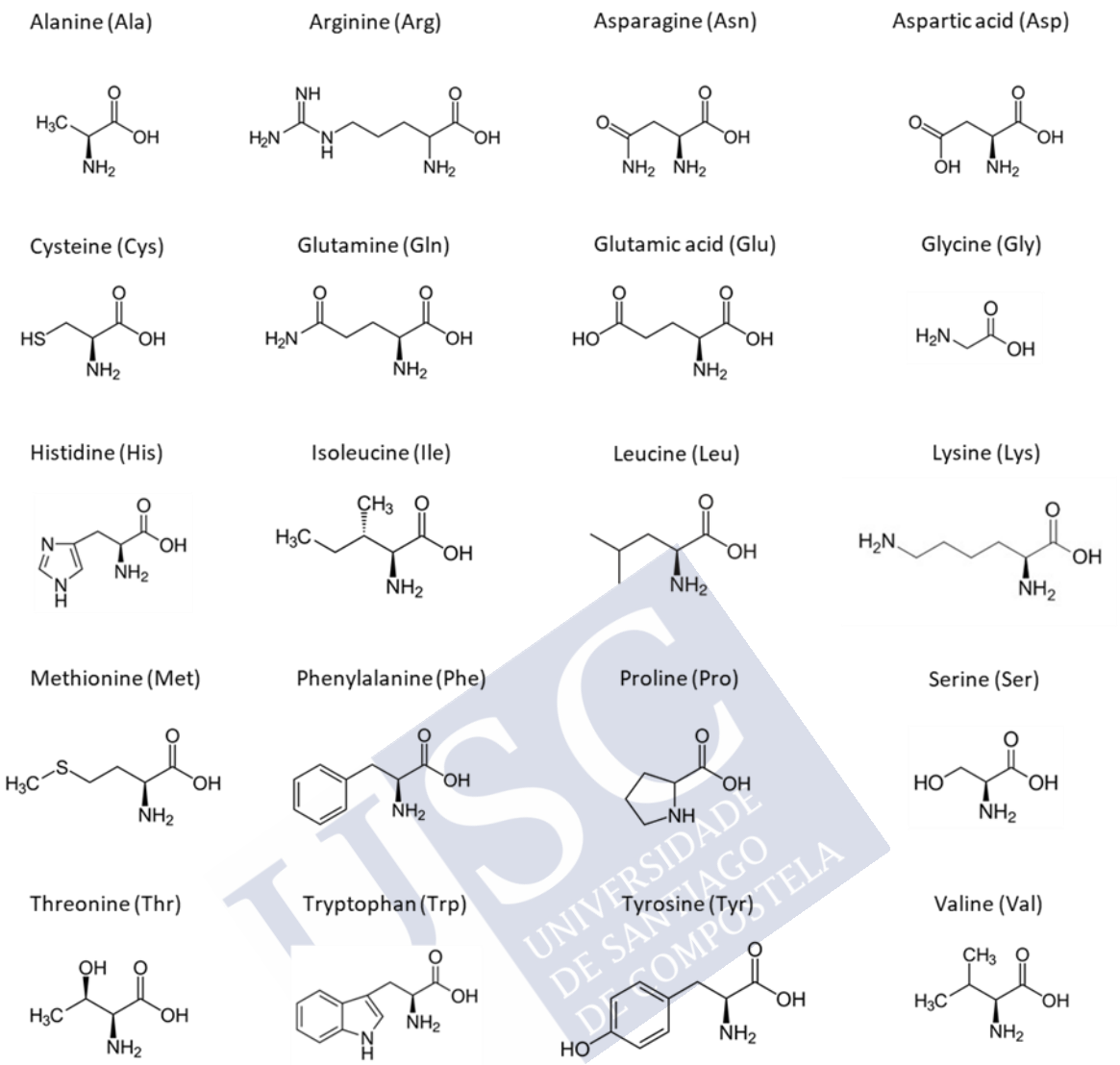

Figure 1.2. Amino acids nomenclature and chemical structures

Table 1.2. Amino acid characteristics and relevant parameters. The pKa 1, 2 and 3 values respectively refer to the carboxyl group, the amino group and the side chain.

AA	Polarity	Isoelectric point	pKa 1	pKa 2	pKa 3
Ala	Non-polar	6.00	2.34	9.69	-
Arg	Positively charged	10.8	2.17	9.04	12.5
Asn	Uncharged polar	5.41	2.02	8.80	-
Asp	Negatively charged	2.77	1.88	9.60	3.65
Cys	Non-polar	5.07	1.96	8.18	-
Gln	Uncharged polar	5.65	2.17	9.13	-
Glu	Negatively charged	3.22	2.19	9.67	4.25
Gly	Non-polar	5.97	2.34	9.60	-
His	Positively charged	7.59	1.82	9.17	6.00
Ile	Non-polar	6.02	2.36	9.60	-
Leu	Non-polar	5.98	2.36	9.60	-
Lys	Positively charged	9.74	2.18	8.95	10.5
Met	Non-polar	5.74	2.28	9.21	-
Phe	Non-polar	5.48	1.83	9.13	-
Pro	Non-polar	6.30	1.99	10.6	-
Ser	Uncharged polar	5.68	2.21	9.15	-
Thr	Uncharged polar	5.60	2.09	9.10	-
Trp	Non-polar	5.89	2.83	9.39	-
Tyr	Non-polar	5.66	2.20	9.11	-
Val	Non-polar	5.96	2.32	9.62	-

The accepted anaerobic protein metabolism indicates that AAs feature two main fermentation pathways (Nagase & Matsuo, 1982; Ramsay & Pullammanappallil, 2001). They can be oxidised to VFA with hydrogen release, though this route is limited as it is not bioenergetically favoured and generally requires the presence of hydrogen-consuming bacteria (e.g. hydrogenotrophic methanogens). Alternatively, they are coupled in Stickland redox reactions (Figure 1.3), in which one AA acts as the electron donor compound while one or more AAs act as electron acceptors (Ramsay & Pullammanappallil, 2001). The oxidised AA is converted to a VFA with a carbon atom less, generating ATP and releasing both ammonia and carbon dioxide. The reduced AAs are converted to VFAs with equal number of carbon atoms, release of ammonia and without ATP generation. Stickland reactions can account up to the 90% of AA conversion, assuming a balanced redox roles composition (Nagase and Matsuo, 1982). However, this accepted paradigm of protein fermentation cannot

explain reactions changes when the protein amino acid composition is not balanced, or upon the variation of operational conditions (e.g. pH).

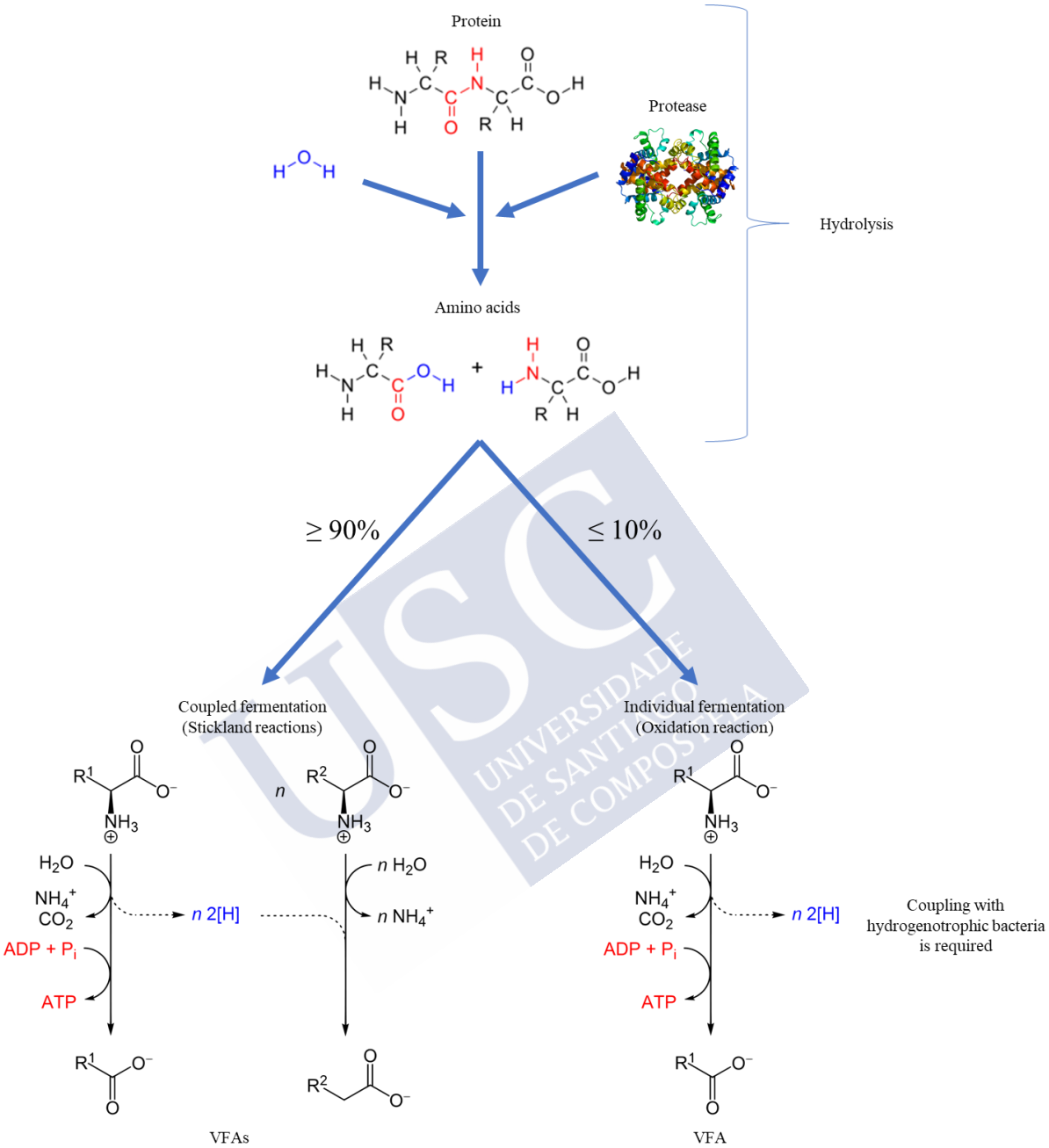

Figure 1.3. Representation of the reactions involved in the protein conversion into VFAs. During Stickland reactions, one amino acid is oxidised to a VFA with a carbon atom less (bottom left), generating ATP, NH_4^+, CO_2 and reducing power. One or more amino acids are then deaminated and reduced to a VFA (bottom centre).

1.4 THE IMPORTANCE OF PROTEIN COMPOSITION

As previously mentioned, proteins should be considered as a combination of twenty individual substrates, the AAs, each with its own metabolic pathways leading to one or more VFA (Barker, 1981). For example, isoleucine can only generate iso-valeric acid, while leucine

can be degraded to either iso-valeric or iso-caproic acid depending on the overall NADH balance (Britz & Wilkinson, 1987) Hence, knowing the protein composition should help predict the outcome of the anaerobic fermentation process. Ramsay and Pullammanappallil (2001) proposed a degradation model (Table 1.3) for this purpose, based on several assumptions: i) the vast majority of the conversion reactions are indeed of the Stickland type (Nagase & Matsuo, 1982); ii) a fixed stoichiometry, built considering the most likely reactions to happen, is sufficient to describe VFA production; iii) different protein compositions do not affect how the individual AAs are fermented; iv) AAs are equally and completely consumed. However, there are several studies contradicting some of the abovementioned assumptions. For example, gelatin was not completely consumed during the operation of an anaerobic continuous stirred tank reactor (Breure & van Andel, 1984) neither in an upflow anaerobic sludge blanket reactor (Yu and Fang, 2003). Interestingly, this incomplete acidification appears to not be related with hydrolysis nor kinetic limitations as free AAs can be found in the mixed liquor at the end of batch fermentation of proteins (Duong et al., 2019). The fact that the proposed model does not take into account the interactions between different AAs as they might not be balanced in terms of redox roles also questions its validity. Finally, the fixed stoichiometry might not be sufficiently accurate at describing the fermentative behaviour of AAs as some of them can either act as electron donors or acceptors (e.g. arginine) depending on the overall protein composition. Due to these controversies, a better insight in the anaerobic fermentation of AAs is required.

Table 1.3. AA stoichiometry adapted from Ramsay & Pullammanappallil (2001).

AA	Products	Reaction type	Reference
Ala	$HAc + NH_3 + CO_2 + 2H_2 + ATP$	Stickland	Andreesen et al. (1989)
Arg	$2HAc + 4NH_3 + 2CO_2 + 3H_2 + 2ATP$	Stickland (oxidation)	Andreesen et al. (1989)
Arg	$½HAc + ½HPr + ½HVal + 4NH_3 + CO_2 + H_2 + ATP$	Stickland (reduction)	Mead (1971)
Asp	$HAc + NH_3 + 2CO_2 + 2H_2 + 2ATP$	Either	Brock & Modigan (1991)
Cys	$HAc + NH_3 + CO_2 + H_2S + ½H_2 + ATP$	Stickland	Barker (1961)
Glu	$HAc + ½HBut + NH_3 + CO_2 + 2ATP$	Stickland	Hardman & Stadtman (1960)
Glu	$HAc + NH_3 + CO_2 + H_2 + 2ATP$	Non-Stickland	Barker (1961)
Gly	$HAc + NH_3$	Stickland	Seto (1980)
Gly	$¾HAc + NH_3 + ½CO_2 + ¼ATP$	Non-Stickland	Lebertz & Andreesen (1988)
His	$CH_3ON + HAc + ½HBut + 2NH_3 + CO_2 + 2ATP$	Stickland	Barker (1961)
His	$HAc + NH_3 + CO_2 + H_2 + 2ATP$	Non-Stickland	Barker (1961)
Ile	$HiVal + NH_3 + CO_2 + 2H_2 + ATP$	Stickland	Elsden & Hilton (1978)
Leu	$HiVal + NH_3 + CO_2 + 2H_2 + ATP$	Stickland (oxidation)	Elsden & Hilton (1978)
Leu	$HiCapr + NH_3$	Stickland (reduction)	Elsden & Hilton (1978)
Lys	$HAc + HBut + 2NH_3 + ATP$	Either	McInerney (1988)
Met	$HPr + CO_2 + NH_3 + CH_4S + H_2 + ATP$	Stickland	Wiesendanger & Nisman (1953)
Phe	Aromatic VFA + NH3 + CO2 + 2H2 + ATP	Stickland (oxidation)	Elsden & Hilton (1976)
Phe	Aromatic VFA + NH3	Stickland (reduction)	Elsden & Hilton (1976)
Phe	Phenol + HAc + NH3 + CO2 + H2 + ATP	Non-Stickland	Elsden & Hilton (1976)
Pro	$½HAc + ½HPr + ½HVal + NH_3$	Stickland	Elsden & Hilton (1979)
Ser	$HAc + NH_3 + CO_2 + H_2 + ATP$	Either	Carter & Sagers (1972)
Thr	$HAc + ½HBut + NH_3 + ATP$	Stickland	Hardman & Stadtman (1960)
Thr	$HPr + NH_3 + CO_2 + H_2 + ATP$	Non-Stickland	Tokushigo & Hayaishi (1972)
Trp	Aromatic VFA + NH3 + CO2 + 2H2 + ATP	Stickland (oxidation)	Elsden & Hilton (1976)
Trp	Aromatic VFA + NH3	Stickland (reduction)	Elsden & Hilton (1976)
Trp	Indole + HAc + NH3 + CO2 + H2 + ATP	Non-Stickland	Elsden & Hilton (1976)
Tyr	Aromatic VFA + NH3 + CO2 + 2H2 + ATP	Stickland (oxidation)	Elsden & Hilton (1976)
Tyr	Aromatic VFA + NH3	Stickland (reduction)	Elsden & Hilton (1976)
Tyr	Cresol + HAc + NH3 + CO2 + H2 + ATP	Stickland (oxidation)	Elsden & Hilton (1976)
Val	$HiBut + NH_3 + CO_2 + 2H_2 + ATP$	Stickland	Elsden & Hilton (1978)

1.5. THE IMPACT OF OPERATIONAL CONDITIONS ON PROTEIN MCF

Knowing how operational conditions affect the conversion of proteins to VFA would also help to better design MCF processes applied to protein-rich sidestreams and wastewaters. The following subsections will show the available knowledge on the impact of most relevant operational conditions on acidification degree and VFA selectivity during protein MCF.

1.5.1. pH

Among the different operational conditions, pH appears to be one of the most influential. In fact, it shapes both the microbial composition (Atasoy et al., 2018) and the bioenergetics of substrates and VFAs transportation between the cells and the environment (González et al., 2015). Its impact on glucose fermentation has been thoroughly studied (Zoetemeyer et al., 1982b; Horiuchi et al., 2002; Temudo et al., 2007; Mohd-Zaki et al., 2016), observing that the product selectivity is especially affected. The production of butyrate, acetate and gaseous hydrogen, favoured at low pH (4-6.5) is, in fact, progressively replaced by acetate, ethanol and formate generation at neutral and slightly alkaline values (6.5-8.5). In general, low pH values promote a more diverse VFA composition from the fermentation of sugar-rich substrates (Jankowska et al., 2017; Garcia-Aguirre et al., 2017).

Conversely, very limited and controversial information is available on the effect pH has on the conversion of proteins and AAs to carboxylic acids. Considering that some of AAs are transformed to pyruvate (e.g. alanine) as an intermediate step (Regueira et al., 2020), pH might be indeed a key parameter in determining which VFAs are produced. However, as proteins are a mix of twenty different AAs, not all the involved reactions might be affected by pH in the same way. Ramsay and Pullammanappallil (2001) assumed that, similarly to glucose, proteic substrates are completely consumed and fermented to specific VFAs regardless of the operational conditions applied to the process. On the contrary, several studies (Breure & van Andel, 1984; Breure et al., 1986; Yu & Fang, 2003, Duong et al., 2019) not only observed incomplete acidifications of proteins, as mentioned in section 1.3, but also variations in the VFA selectivity depending on the operational pH. Either propionic (Breure & van Andel, 1984) and/or valeric acid (Duong et al., 2019) production is promoted while acetic acid one is reduced when operating at acid conditions (≤6). No information is available on the alkaline range, with the exception of its positive effect on the hydrolysis step (Wang et al., 2017; Wang et al., 2019). Considering that pH is one of the few operational parameters which can be controlled during fermentation processes, gathering further knowledge on the subject might help to understand how to potentially use pH to steer the fermentation of protein-rich substrates towards desired productivity and/or selectivity.

1.5.2. Temperature

Temperature appears to be less relevant than pH for the fermentation process (Kim et al., 2013), as acclimation and careful selection of the inocula can offset the limitations of relatively low and high temperature ranges. Mesophilic range appears to be the most productive (Cavinato et al., 2017) and the most widely used (Lee et al., 2014), as it is not as energy intensive as the thermophilic nor imposes hydrolysis rate limitations as the psychrophilic (Poirrier, 2005). In general, temperature progressively increases the acidification degree while the effect on VFA selectivity is substrate-dependant (Garcia-Aguirre et al., 2017). For example, papermill wastewaters are more easily fermented at thermophilic conditions, maintaining the same VFA spectrum, while winery wastewaters conversion at 55°C is more selective towards n-butyric acid.

Information on the specific effect of temperature on protein fermentation is quite limited, indicating that increasing temperature leads to higher acidification degrees and faster conversion rates in the thermophilic range compared to mesophilic conditions, but with a similar product spectrum (Yu & Fang, 2003).

1.5.3. Micronutrients

Micronutrients, such as iron (Fe), cobalt (Co) and zinc (Zn), are generally required by the microbial community as enzyme cofactors for many reactions involved in anaerobic processes, with stimulatory concentrations ranging from as little as 0.006 mg/L up to more than 10 mg/L (Choong et al., 2016). While they can be normally found in municipal wastewaters (Sung et al., 1986), many industrial streams might not have sufficient concentrations of these compounds, limiting the application of VFA-based resource recovery processes (Speece, 1996; Rittmann and McCarty, 2001; Zandvoort et al., 2006; Fermoso et al., 2008). In those cases, micronutrients might be either absent or present at levels which are below stimulatory threshold (Choong et al., 2016), meaning that their supplementation to the cultivation feedstock should positively affect the fermentation process. For example, Fe, Co and nickel (Ni) addition favours the hydrolysis of macromolecules, consequently increasing VFA production (Kim et al., 2003). Knowing how specific AAs fermentation pathways are affected by micronutrients could also allow to selectively steer the fermentation towards target products, as Dahiya et al. (2020) demonstrated by enhancing the production of propionic acid from glucose with Co and Zn.

The vast majority of the information available in literature deals with the effect of micronutrients on either glucose and carbohydrates or complex substrates. In contrast, there are no specific studies on the impact of micronutrients on protein fermentation, even whether the process could be still performed without them (or when they are beneath stimulatory concentrations). Given that some AAs are transformed into pyruvate before being converted to VFAs (Regueira et al., 2020), the supplementation of micronutrients involved in glucose fermentation should be beneficial for the acidification of proteins as well. Besides, it is known that certain compounds are specifically required for the conversion of some AAs to VFAs. For example, Dürre and Andreesen (1981) observed that selenium is necessary for the *Clostridium* genus to produce glycine reductase, which is involved in glycine conversion to acetic acid. Hence, the absence or low concentration of selenium might prove inhibitory to the fermentation of those proteins rich in glycine.

1.5.4. Organic loading rate and hydraulic retention time

Organic loading rate (OLR) is generally considered an important operational condition for the anaerobic digestion process, as suboptimal values might hinder methane production due to VFA accumulation (Mao et al., 2015; Braz et al., 2018). Beyond preventing VFA conversion to methane, manipulation of the OLR often plays a small role in anaerobic fermentation. Besides, the extent of its effect is strongly related to the hydraulic retention time (HRT). High OLR values determined by extremely low HRTs might lead to decreased acidification degrees due to kinetic limitation, especially in those cases where solid retention

time is coupled (e.g. CSTR operation). Conversely, high OLR values obtained at high substrate concentration should not be an issue for the fermentation processes, given that acidogenic bacteria can withstand high titres of both VFAs and ammonia (Shi et al., 2016; Domingos et al., 2017). Low OLRs are not generally considered to be viable for anaerobic fermentation processes. Avoiding kinetic limitations by applying high HRTs leads to at least a partial loss of the produced VFAs due to their methanisation (Khanal et al., 2008). Regardless of the high conversion efficiencies achieved, the use of diluted substrates is not attractive on the other hand because the titres and productivities are low, making the separation step particularly energy-intensive and expensive (Simonetti et al., 2020).

Protein fermentation seems to be particularly affected by low HRTs, as many authors indicate the hydrolysis as the limiting step of the process (Breure & van Andel, 1984; Fang & Yu, 2002). However, more recent studies demonstrated that protein cannot be completely acidified even without kinetic limitations (Yin et al., 2016; Duong et al., 2019). In fact, inert free AAs were detected at the end of batch tests, regardless of the experiment duration, suggesting that the acidification can be the limiting step of protein fermentation instead. In general, OLR and the HRT do not seem to be particularly influential for the selectivity of the process, as the VFA molar fractions are mostly stable regardless of their value (Fang & Yu, 2002). Thus, adjusting OLR and HRT does not appear to be a viable strategy to steer protein fermentation, albeit they should be carefully chosen to avoid conversion limitations and methanisation.

1.6. OPPORTUNITIES AND ALTERNATIVES TO PROTEIN MCF

Apart from operational conditions adjustment, there are alternative ways of making the protein fermentation more appealing and economically viable. One such strategy is the cofermentation with sugars, as multiple studies suggest that synergism in the degradation process occurs (Fang et al., 2020), for example during the hydrolysis step, subsequently favouring the conversion of proteins to VFAs. Yet, certain limitations were also found in terms of blending proportions between the different feedstock fractions, suggesting the need for further investigations on the subject. Beyond anaerobic fermentation, CE can be promoted to upgrade the regular AA fermentation, being a process which contributes to the production of longer-chain acids with four or more carbon atoms, of greater commercial interest (Moscoviz et al., 2018; Candry & Ganigué, 2021). As this kind of process requires specific environmental conditions to occur, a specific study is required to understand its feasibility using proteins as substrate.

1.6.1. Co-fermentation of proteins and sugars

Mixed-culture cofermentation processes can be seen as an opportunity to improve the overall VFA production by mixing streams with different composition altogether (Fang et al., 2020). In fact, the ratio between the three different fractions (sugars, proteins and lipids) can be potentially manipulated in order to steer the process towards desired product compositions (Ma et al., 2017). Still, the interaction between the individual substrates is not well known and

both protein and lipids have not been studied as thoroughly as sugars. For these reasons, the focus has been principally placed on sugar and protein cofermentation.

The conversion of these organic fractions is rarely independent from one another. For instance, sugars are generally associated with greater biomass yields (Batstone et al., 2002) than proteins. In turn, greater biomass concentrations might favour the overall degradation of the feedstock. However, there are some factors which should be taken into account when opting for a cofermentation process. One such factor is the feedstock ratio between sugars and proteins (Fang et al., 2020). Increasing sugar-rich streams loading seems to promote proteins consumption and subsequent transformation into VFAs (Ma et al., 2017) while allowing to steer the process towards acetic and butyric acid production. Yet, most studies on the subject indicate that the sugar proportion in the feedstock should not exceed the protein fraction as this generally lowers its conversion to VFAs. Breure et al. (1986) identified 1:1 (COD basis) as a critical proportion threshold, as protein hydrolysis decreased together with AA acidification, whereas Tommaso et al. (2003) detected a decrease in the protein degradation rate even at the slightest sugar addition. Another key parameter is HRT. Sugars are rapidly fermented even at the lowest retention values (Temudo et al., 2007), exhibiting priority over proteins (Breure et al., 1986). More specifically, increasing concentrations of sugars seems to determine an inhibition of proteases activity, severely hindering the hydrolysis of proteins. The pressure exerted by acid pH values can prioritise the fermentation of sugars in detriment of protein conversion as well (Fra-Vazquez et al., 2020).

As the knowledge on the subject is at times controversial (Fang et al., 2020), with both synergic and antagonistic interactions being detected during the cofermentation, further investigations on how proteins and sugars interact are needed. Gaining more insight on the role the feedstock ratios play in determining the overall conversion into VFAs should be a priority, as it would help to understand how to effectively blend different substrates to maximise the process output and target desired products composition. Besides, specific information on the impact of sugars on AA consumption will potentially help in choosing the most suitable feedstock ratio between protein-rich and sugar-rich streams, also based on their composition, avoiding unnecessary antagonisms between organic fractions. The knowledge gathered on the subject would not only apply to the cofermentation of feedstocks mixtures but also to most organic sidestreams and wastes as they are themselves complex mixtures of carbohydrates and proteins. For example, cheese whey usually features high concentrations of lactose (sugar) but can also contain a relevant proteic fraction in the form of immunoglobulins and albumins (Carvalho et al., 2013), making it an appealing fermentation feedstock (Domingos et al., 2017; Asunis et al., 2019).

1.6.2. Targeting longer-chain fatty acids

The production of longer-chain VFAs and medium chain fatty acids (MCFA), such as caproic acid, is often detected as a secondary process called CE, which can occur during the MCF of several industrial sidestreams and wastes (e.g. food waste, Owusu-Agyeman et al., 2020). Such process takes place when a VFA and an electron donor compound (e.g. ethanol

or lactate) are consumed together with reducing power to yield a new fatty acid with a longer carbon chain, with four or more carbon atoms. Many studies have investigated its potential application as a full-fledged process to produce longer chain carboxylic acids as they have interesting applications and greater market prices in comparison with short chain VFAs (Angenent et al., 2016). Moreover, their lower solubilities facilitate the downstream processing, as the separation from the mixed-culture broth is easier when compared to shorter chain VFAs (Candry & Ganigué, 2021). Recently, a lot of information on the subject has been gathered, with plenty of studies focusing on either the mechanisms (reverse β-oxidation) or the suitability of certain feedstocks for the CE. A lot of attention has been also paid to the supplementation of external electron donor compounds (Han et al., 2019).

While the feasibility of CE has been mainly investigated using sugar-rich substrates (Xu et al., 2018; De Groof et al., 2019; Duber et al., 2020), limited insight is available on the suitability of protein-rich streams. In fact, to the best of our knowledge, there are only two existing studies on the subject (Wallace et al., 2003; Wallace et al., 2004) which focused on pure cultures of *Eubacterium pyruvativorans*. This microorganism is capable of using amino acids both to perform regular fermentation and to elongate acetic, propionic and butyric acid to butyric, valeric and caproic acid respectively. The metabolic pathway used is similar to the reverse β-oxidation reaction performed by *Clostridium kluyveri*.

However, no evidence of CE occurring during protein MCF was ever found. Knowing whether this kind of process can coexist with the anaerobic conversion of AAs to VFAs can increase the appeal of fermenting protein-rich substrates or their mixing in cofermentation processes.

1.7. OBJECTIVES AND STRUCTURE

In this section, the main objective of the present PhD thesis is presented together with the surrounding research questions, which are then translated into research objectives. Lastly, the structure of the thesis is described.

1.7.1. Main objective

The main objective of this PhD dissertation is to better understand the underlying mechanisms of protein MCF by focusing on the influence of AA profile and the effect the operational conditions. This knowledge will help to optimise and/or steer the fermentation protein-rich wastewaters and industrial sidestreams towards desired products compositions.

1.7.2. Research questions

To fulfil the main objective of the thesis and filling in the gaps of the existing knowledge on protein MCF, several research questions were devised:

- **What is the role of amino acid profiles on protein MCF outcome?** Does the fermentation of different proteins lead to different conversion efficiencies and VFA selectivities? How can these different behaviours be explained? Can the process outcome be predicted based on this knowledge?

- **How do pH and micronutrients supplementation affect the process conversion efficiency and products distribution?** Can they be manipulated to obtain the desired result? How do the metabolic pathways and associated mechanisms are affected by these changes in operational conditions?
- **What are the opportunities for protein streams beyond monofermentation?** Is the cofermentation of proteins with carbohydrates a viable alternative? Are there limitations or which are the bottlenecks? Are CE processes feasible during protein MCFs? Which are the operational conditions required for the elongation to occur? Are they selective towards specific products or comparable with sugar-based CE?

1.7.3. Research objectives

After a solid state-of-the-art analysis and review on the knowledge gaps on protein MCF, all these research questions were translated into more concrete research objectives:

- To understand the influence of protein composition, pH and micronutrients on the AAs conversion mechanisms
- To identify the bottlenecks in the cofermentation of proteins with sugars
- To verify the feasibility of CE processes during protein MCF

1.7.4. Thesis structure

The organisation and structure of the thesis (Figure 1.4) highlights the strong connections existing among several aspects of protein MCF process.

The impact of AA profile on protein MCF processes is investigated in **Chapter 3.** The conversion of different proteins is compared to understand how the interactions between AA can affect the process outcome in terms of overall acidification and product selectivity. Moreover, the mechanisms responsible for the detected differences are evaluated.

The influence of pH on the conversion of AAs to VFAs is then evaluated in **Chapter 4.** A wide range of values, from acid to alkaline conditions, are tested in order to understand how this operational condition can steer protein fermentation. Moreover, the effect of AA composition on the extent of its influence is also evaluated.

Chapter 5 is centred on understanding whether micronutrients presence is fundamental for the AA fermentation, as many industrial sidestreams and waste sometimes do not feature them at stimulatory concentrations. Micronutrients supplementation is tested with different proteins and at different pH conditions to discern whether its effect depends on AA composition and/or the pH value.

Chapter 6 is focused on understanding the interaction between AAs and glucose during their cofermentation. The critical ratio between the two substrates concentration is investigated in order to identify potential process bottlenecks.

Chapter 7 evaluates the feasibility of CE processes during protein MCF based on all the evidence found in the previous chapters. In fact, protein fermentation is mostly associated

with acetic acid production which, being the least economically attractive VFA, reduces its overall profitability. Promoting CE processes during protein MCFs could solve this issue through the upgrade of short chain VFAs to longer chain ones (C_4+). Hence, the focus is placed on understanding how CE mechanisms are related to the operational conditions, especially protein composition, pH levels and the influence of sugars presence.

Finally, **Chapter 8** jointly analyses the results described in the previous chapter identifying the greatest novelties and advances that this thesis achieved on the subject of protein-based MCFs. With the knowledge and insight generated, several operational strategies are highlighted as useful in steering the process towards the desired outcome. Suggestions for future research are also provided based on the identified research gaps and opportunities.

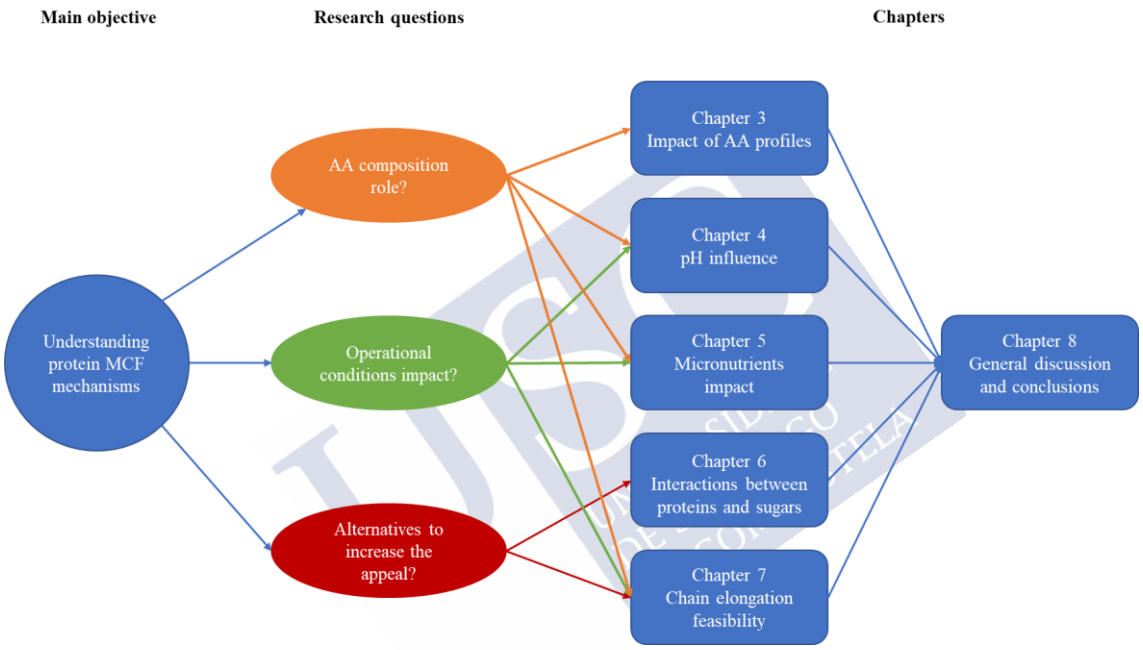

Figure 1.4 Thesis structure and connections among all the aspects related to protein MCF.

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CHAPTER 2

MATERIALS, METHODS AND EQUIPMENT

SUMMARY

In this chapter, the different experimental set-ups used during this PhD thesis are described together with the analytical methods and calculations applied. The composition of the feedstock used is also provided, both in terms of amino acid composition of the proteic carbon sources (casein and gelatin) and nutrients supplementation (micro and macro).

2.1. FEEDSTOCK PREPARATION

Since the focus is placed on better understanding the mechanisms of protein conversion to VFAs also in their interaction with sugars presence, the feedstocks used in the experiments were prepared by carefully controlling the overall composition. For this reason, AA profiles of the chosen proteins were analysed, the sugar used in the cofermentation experiments, glucose (D(+)-Glucose anhydrous, 131341.1211 PanReac), is a well-studied substrate in terms of degradation mechanisms (González-Cabaleiro et al., 2015) and distilled water was preferred over tap one for all the necessary dilution.

2.1.1. Protein composition

Two proteins with different AA composition, casein and gelatin, were used. Both casein (A2208,0500 PanReac) and gelatin (70,951-1 KG-F Sigma-Aldrich) were added to the feedstock as hydrolysates (peptones), given that the focus of the experiments was placed on the acidogenic step. Their concentration in the feedstock was of 7.5 and 7.6 g/L, respectively, in order to achieve a comparable COD concentration in the influent equal to 8 g COD/L. Their AA profiles were also analysed and are reported in Table 2.1.

Table 2.1. Amino acid composition of casein and gelatin (molar fraction in %).

Amino acid	Casein	Gelatin
Alanine (Ala)	7.64	13.5
Arginine (Arg)	3.83	5.11
Aspartic acid (Asp)[a]	2.54	2.03
Cysteine (Cys)	0.00	0.00
Glutamic acid (Glu)[a]	15.0	7.07
Glycine (Gly)	2.80	34.5
Histidine (His)	1.96	0.80
Isoleucine (Ile)	5.94	1.33
Leucine (Leu)	9.46	3.07
Lysine (Lys)	6.96	3.02
Methionine (Met)	0.93	0.49
Phenylalanine (Phe)	4.56	1.73
Proline (Pro)	13.9	16.1
Serine (Ser)	7.12	4.32
Threonine (Thr)	5.83	2.87
Tryptophan (Trp)	0.00	0.00
Tyrosine (Tyr)	2.74	0.55
Valine (Val)	8.79	3.44

[a] Glu and Asp also include the fraction related to Glutamine and Asparagine, respectively.

2.1.2. Macro and micronutrients

Macronutrients were always supplemented in the continuous and the batch experiments with the concentrations shown in Table 2.2, as adapted from Temudo et al. (2007).

Table 2.2. Macronutrients concentrations (g/L) in the feedstock.

Macronutrient	Concentration
KH_2PO_4	0.780
$MgCl_2 \cdot 6H_2O$	0.120
NaCl	0.292
Na_2SO_4	0.057
NH_4Cl	0.530

Selenium (0.1 mg/L SeO_2) was also supplemented in all the experiments described in the present thesis to ensure that glycine fermentation could occur, as its presence is required for the formation of the required enzyme complex, glycine reductase (Dürre & Andreesen, 1981).

Other micronutrients were only supplied in the experiments described in **Chapter 5** and **6** at the concentrations shown in Table 2.3, as adapted from Temudo et al. (2007).

Table 2.3. Micronutrients concentrations (mg/L) in the feedstock

Micronutrient	Concentration
$CaCl_2$	0.6
$CoCl_2 \cdot H_2O$	0.6
$CuCl_2 \cdot 2H_2O$	2.2
$FeSO_4 \cdot 7H_2O$	3.1
H_3BO_4	0.1
$MnCl_2 \cdot 4H_2O$	2.5
$Na_2MoO_4 \cdot 2H_2O$	0.1
$NiCl_2 \cdot 6H_2O$	0.5
$ZnSO_4 \cdot 7H_2O$	3.2

2.2. INITIAL INOCULUM PREPARATION

A parent reactor made of glass and with a working volume of 4 L was inoculated with a mix of anaerobic sludges coming from a sewage sludge digester (Guillarei, Spain) and from an anaerobic digester treating brewery wastewaters (Estrella Galicia, A Coruña, Spain) to ensure that the resulting microbiome was as diverse as possible.

The reactor was then fed with a synthetic medium composed of three different carbon sources: glucose (sugars), casein (proteins) and sodium oleate (lipids). Macronutrients were supplemented according to Temudo et al. (2007). This feedstock composition was chosen to guarantee that the microbial population would be able to retain all the metabolic functionalities and the capacity of converting all the different types of substrate.

The reactor was operated at room temperature (approximately 25°C) with an HRT of 2 days and at mildly acid conditions (pH 5.7 ± 0.2) in order to limit the proliferation of methanogenic microorganisms and progressively wash them out, while allowing acidogenic-only biomass to thrive. When steady-state conditions were finally achieved, the reactor effluent was used for the inoculation of the CSTRs used in **Chapter 3**. No further inoculation was required as the experiments described in **Chapter 4 and 5** were performed by operating the same reactors of **Chapter 3**, while the cofermentation reactor (**Chapter 6**) was inoculated with effluents from the casein reactor operated at pH 7 (**Chapter 5**).

2.3. EXPERIMENTAL DESIGN

Both continuous reactors operation and batch tests were performed to understand proteins fermentation in open systems. Through the operation of continuous reactors, steady-state conditions were achieved and maintained in order to gather information on the average acidification, product spectra and AA consumption. Batch tests helped to determine the kinetics parameters, the conversion limitations and more specific mechanisms which are involved during protein conversion to VFAs (e.g. chain elongation).

2.3.1. Continuous reactors

Two glass vessels of 1 L of working volume (Figure 2.1) were used as continuous stirred tank reactors (CSTRs) to perform the continuous experiments. They were inoculated with an in-reactor biomass concentration of approximately 1.0 g VSS/L and then always operated as CSTR at 25°C in a temperature-controlled room. HRT was mostly set at 1.5 days, resulting in a constant OLR of 5.3 g COD/L. This only changed during the cofermentation experiments (**Chapter 6**). A multiparametric analyser (CHEMITEC, Italy), connected to Hamilton probes, was used to continuously monitor pH and control it through addition of either HCl (3M) or NaOH (3M). The mixing of the liquid phase was provided by magnetic stirrers set at 200 rpm and by nitrogen sparging (approximately 10 mL/min), which also ensured that anaerobic conditioned were maintained and that hydrogen saturation was minimised.

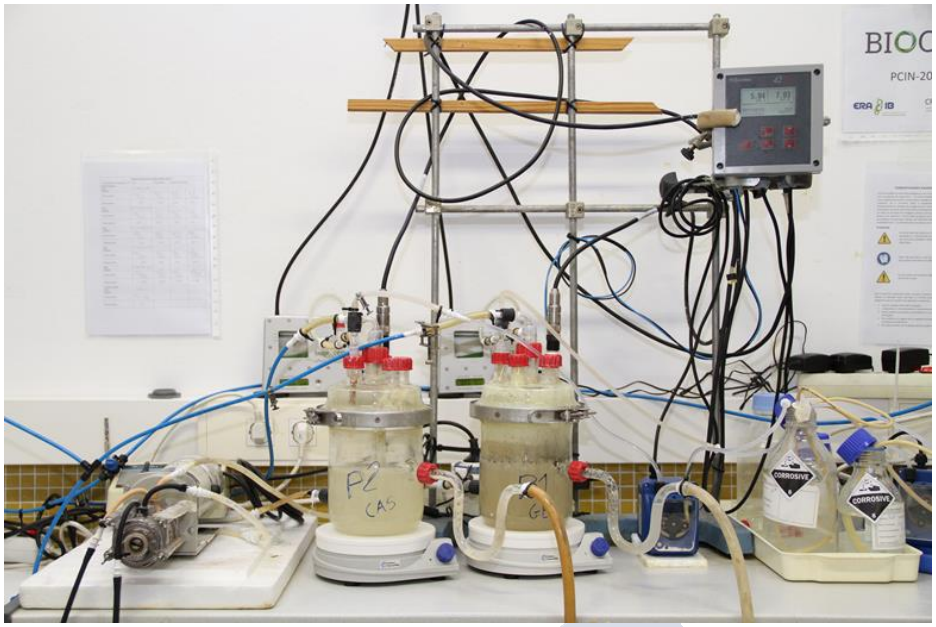

Figure 2.1. Setup of the continuous stirred tank reactors.

The reactors operation was mainly monitored through standard analytical methods (APHA, 2017). COD concentrations of the feedstock (total) and of the effluent (total and soluble) were measured once to three times per week depending on the experimental phase. VFA concentrations and total ammonia nitrogen (TAN) were measured twice to three times per week. Total and suspended solids concentrations were determined once a week.

Some occasional measurements were also performed, such as lactate, ethanol and glucose concentrations, when their presence in the reactor effluent was suspected (e.g. decrease in VFA production, unknown soluble COD fraction) and the composition of the gas phase to verify whether total COD decreases in the effluent were due to either hydrogen production or methanisation. Finally, mixed liquor samples were taken from the reactors once per week during steady-state operation and frozen for AA content analysis.

2.3.2. Batch experiments

Two type of batch tests were also set up to better understand the anaerobic degradation of proteins: Biochemical Methane Potential (BMP) tests and acidification tests. BMP tests were employed to determine the maximum conversion threshold of the proteic substrates (casein and gelatin). Acidification tests were performed to evaluate the kinetics of proteins conversion to VFA overtime, thus determining the associated kinetic parameters, as well as the occurrence of secondary metabolic processes (i.e. chain elongation).

2.3.2.1. Biochemical Methane Potential tests

The BMP tests were performed as described by Holliger et al. (2016). Bottles of 0.5 L total volume (0.375 L of working volume) with rubber stoppers were used. The bottles were inoculated with anaerobic biomass coming from a mesophilic lab-scale reactor fed with sewage sludge. Inoculum (8.0 g VS/L) and substrate (4.0 g/L) concentrations were selected in order to achieve a non-inhibitory inoculum-to-substrate ratio (ISR) of 2. A blank assay (only

inoculum) was also included to monitor residual biogas production from the inoculum. The tests were conducted by triplicates at 37.5°C in an orbital shaker for 16 days. Biogas production and composition were monitored daily.

2.3.2.2. Acidification tests

Glass bottles of 0.5 L total volume (0.375 L of working volume) with rubber stoppers or 2 L glass vessels (1 L of working volume) were used for the acidification tests (Figure 2.2). The chosen operational conditions were comparable to the ones applied to the continuous reactors (25°C, N2 sparging, pH value). The vessels were inoculated with biomass from the continuous reactor: effluent samples were centrifuged to separate the VFA-rich supernatants, and the obtained biomass pellets were resuspended in the test bottles to achieve an initial biomass concentration of 0.5 g VSS/L. The proteic substrate was added according to the selected substrate-to-inoculum ratio (SIR), 10 g COD substrate/g VSS, while macro and micronutrients were supplemented with the same concentrations as in the reactors' feedstock. 10 to 20 mL samples were taken at increasing time intervals (initially 2-3 hours). Half of the sample volume was centrifuged and filtered for several determinations (mainly TAN, VFA, soluble COD), with the surplus being frozen for AAs analysis. The remaining 5 mL were used for optical density determination. VSS concentration was only measured at the end of the tests.

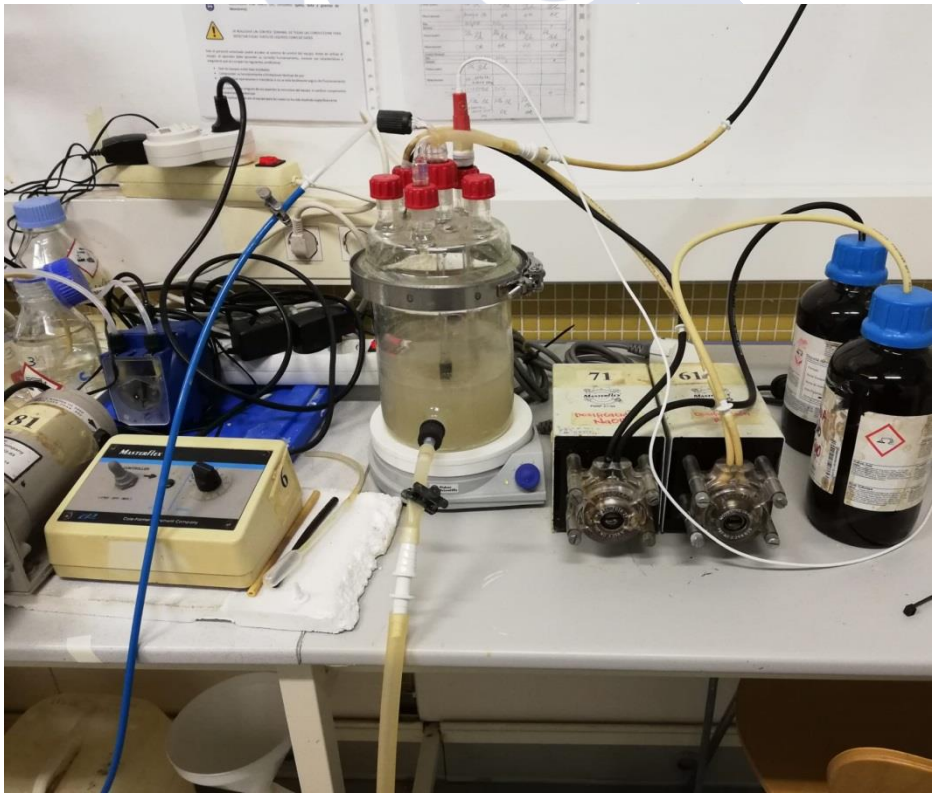

Figure 2.2. Setup of the batch tests.

2.4. ANALYTICAL METHODS

As previously mentioned, several analytical methods were applied to samples from both the continuous experiments and the batch tests. While most of them were routinely performed to monitor the operation of the reactors (e.g. COD method), others were more situational (e.g. AA analysis) and depended on achieving steady-state conditions or a stabilisation in substrate conversion to VFAs.

Most of the methods described in the following sections were performed according to Standard Methods (APHA, 2017). The more specific ones are described according to appropriate references.

2.4.1. Total and soluble chemical oxygen demand (COD)

COD is defined as the concentration of oxygen, expressed in g/L, required to completely oxidise the organic compounds found in a liquid volume. Hence, its measurement is performed to determine the concentration of organic matter in a given sample. Knowing the composition of a substrate allows to stoichiometrically calculate it, as the measured values approximate quite well the theoretical ones.

The method chosen to perform both total and soluble COD measurements is the closed reflux titrimetric one, with slight modifications from Standard Methods 5220C (APHA, 2017) and a detection range between 0.09 and 0.9 g COD/L. The required equipment is composed of a block heater (VELP Scientifica, Italy), digestion vessels of 10 mL capacity made of borosilicate, a magnetic stirrer and a microburet for the titration phase. The reagents required are listed below:

- Digestion solution: 10.216 g of $K_2Cr_2O_7$ (previously dried at 105°C) and 33 g of $HgSO_4$ are initially dissolved in 500 mL of distilled water, then 167 mL of concentrated H_2SO_4 are added to the resulting solution, which is finally diluted to a volume of 1000 mL.
- Catalytic solution: 10.7 g of Ag_2SO_4 are dissolved in 1 L of concentrated H_2SO_4
- Ferroin indicator: 1.485 g of $C_{18}H_8N_2 \cdot H_2O$ (phenanthroline monohydrate) and 0.695 g of $FeSO_4 \cdot 7H_2O$ are dissolved in 100 mL of distilled water
- Ferrous ammonium sulphate (FAS) titrant (0.035N): 13.72 g of $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$ are initially dissolved in distilled water, to which 20 mL of concentrated H_2SO_4 are added. The solution is then diluted to 1 L with distilled water
- Potassium dichromate standard solution (0.05N): 1.2258 g of $K_2Cr_2O_7$ are dissolved in 500 mL of distilled water

As the first step, each digestion vessel is filled with 2.5 mL of the sample. The sample can be either whole for the total COD measurement or pre-filtered at 0.45 µm for the soluble COD determination. Given the method range (0.09 and 0.90 g/L), the samples are to be diluted accordingly. Then, 1.5 mL of digestion solution and 3.5 mL of the catalytic solution are added. The reagents addition is to be performed carefully as the second solution should not be mixed before placing the digestion vessels inside the block heater. Blanks are prepared

as well, with 2.5 mL of distilled water instead of the sample and the same volumes of reagent solutions. Overall, the digestion tubes preparation is performed in either duplicates or triplicates.

Once the digestion vessels are prepared, they are sealed with Teflon and either bakelite or plastic caps. The mix of the liquid is performed manually, before placing the tubes in the block heater (heated at 150°C) for two hours.

After the digestion time has passed and the vessels have cooled down to room temperature, the liquid is transferred and magnetically stirred in a 50 mL glass Erlenmeyer flask, to which a few drops of ferroin indicator are added. Titration is performed by carefully adding FAS solution to the liquid via the microburet until the end point is reached: a colour change from light blue to red-brown.

A similar procedure is performed to determine FAS normality. 5 mL of potassium dichromate standard solution are mixed with 5 mL of distilled water and 3.5 mL of catalytic solution. When cool, the liquid is titrated with FAS as in the sample measurements.

All the consumed FAS volumes are noted down to be used for the calculations, as shown below:

$$COD\ (g/L) = \frac{(\mathrm{B} - \mathrm{A}) \times N_{FAS} \times 8}{2.5} \times \mathrm{DF} \quad (1)$$

Where B and A respectively stands for the volume (mL) of FAS consumed to titrate the blank tubes and the volume (mL) of FAS consumed to titrate the digestion vessels containing the samples. DF stands for the dilution factor of the samples, when applicable.

N_{FAS} is the normality of the FAS solution, which is determined, as previously described, with a standard solution of potassium dichromate to be titrated with the FAS solution itself. The related equation is the following one:

$$N_{FAS} = \frac{0.025}{C} \quad (2)$$

Where C stands for the volume (mL) of FAS consumed to titrate the solution to the red-coloured end point.

2.4.2. Total Kjeldahl nitrogen (TKN)

The TKN method is used to quantitatively determine the nitrogen content of the organic fraction and of ammonia in a given sample, as its procedure involves complete digestion of amino compounds and ammonia to ammonium, whose concentration can be lately measured with different appropriate methods (e.g. titration, colorimetry, etc). Its application is particularly useful with protein-rich substrates as measuring the nitrogen content allows to estimate the global protein concentration.

The SM4500-N_{org}.B (APHA, 2017) was the chosen TKN method during this PhD thesis. After a distillation step, the nitrogen concentration was measured with the SM4500-NH_3.C titrimetric method (APHA, 2017). The two methods combined allowed to characterise the

nitrogen content of the two selected proteins, casein and gelatin. The required equipment is composed of a digestion unit (Gerhardt, Germany) capable of providing temperature control between 375 and 385°C, with capacity for 800 mL digestion tubes, and a distillation unit (Vapodest Gerhardt, Germany) which automatically performs the required separation process, provided that the required reagents are correctly added. A microburet was also used for the titration step. The complete list of reagents is the following one:

- Digestion step:
    - Digestion solution: 134 g of K_2SO_4 and 7.3 g of $CuSO_4$ are dissolved in approximately 800 mL of distilled water. Then, 134 mL of concentrated H_2SO_4 are added. Finally, the solution is diluted to 1 L.
- Distillation step:
    - Alkaline solution: 500 g of NaOH and 25 g of $Na_2S_2O_3 \cdot 5H_2O$ are dissolved in 1 L of distilled water
    - Mixed indicator solution: 200 mg of methyl red indicator are dissolved in 100 mL of ethanol (95%). 100 mg of methylene blue are dissolved in 50 mL of ethanol (95%). Then, the two solutions are mixed together to obtain the complete reagent, which is to be used within a month
    - Indicating boric acid solution: 20 g of H_3BO_3 are dissolved in distilled water, to which 10 mL of mixed indicator solution are added. The solution is then diluted to a final volume of 1 L
- Titration step:
    - Standard sulfuric acid titrant (0.02N): first, 2.8 mL of concentrated H_2SO_4 are diluted to 1 L with distilled water. Then, 200 mL of the first solution are once more diluted with distilled water to reach a final volume of 1 L. The reagent is standardised with a Na_2CO_3 solution (0.05N) as described in SM2320B (APHA, 2017).

The procedure starts with the selection of the sample volume/weight, with the values chosen based on the assumed nitrogen content of the sample itself. The samples are placed in 800 mL digestion tubes and diluted if necessary.

50 mL of digestion solution is added to the final sample volume and the resulting liquid is thoroughly mixed. Then, the tubes are placed in the digestion unit, which should be precautionary placed under a hood regardless of having an integrated ejection equipment for the toxic fumes produced during the digestion process. The samples are boiled at high temperature until they are reduced to 25-50 mL and white/gray fumes are generated. After 30 more minutes, the digestion is stopped, and the tubes are left to cool down. When room temperature is reached, the liquid left is diluted to 300 mL with distilled water and then mixed.

The tubes are then fitted into the distillation unit, which automatically doses the alkaline solution required to transform all the ammonium obtained during the digestion step into ammonia. The liquid is then distilled and condensed inside a flask containing 50 mL of

indicating boric acid solution (absorbent solution), which converts the ammonia into ammonium. The resulting distillate presents a brilliant green colour, due to the boric acid solution, and is ready to be titrated.

The final nitrogen measurement is performed, as previously mentioned, with a titration method. More specifically, the 0.02N solution of H_2SO_4 is progressively added to the distillate until the turning point (lavender colour). Blanks are also required, and they are prepared by applying the same steps of the procedure except for the digestion one.

The volumes of titrant solution used both for the sample distillates and the blanks are noted down and used to calculate the nitrogen content as shown in the following equation:

$$TKN\ (mgN/L) = \frac{(\mathrm{A} - \mathrm{B}) \times 280}{mL\ sample} \times \mathrm{DF} \quad (3)$$

Where A and B respectively stands for the volume (mL) of titrant solution added to the blanks and the volume (mL) of titrant solution added to the distillates. DF stands for the dilution factor of the samples, when applicable. DF stands for the dilution factor of the samples, when applicable.

2.4.3. Total ammonia nitrogen (TAN)

The TAN method measures the concentration of nitrogen in ammonia/ammonium form found in a given sample. Determining the TAN value is useful during protein fermentation processes since the concentration of NH_3/NH_4^+ can be used as a proxy for substrate degradation. In fact, the conversion of AA to VFA is always related with the generation of one or more NH_3/NH_4^+ molecules. Hence, knowing the nitrogen content of a proteic substrate and comparing it with the TAN value allows to evaluate its overall consumption (see section 2.5.3 for the related calculation).

Different ways of measuring the TAN are available, ranging from titration to selective electrode determination. The method of choice was adapted from SM4500-NH3.F (APHA, 2017) and from the one described by Weatherburn (1967). Being a colorimetric method, it is based on the formation of a blue compound, indophenol, through the reaction between ammonia/ammonium, phenol and hypochlorite, catalysed by sodium nitroprusside. Given that turbidity can interfere with the absorbance measurements, the samples should be filtered in advance. Another potential interference is found in calcium and magnesium ions presence, which can be offset by adding EDTA to the reagents mix.

The only required equipment is a spectrophotometer (Shimazdu UV-1800) set a wavelength equal to 635 nm, as the sample preparation and its mixing with reagents can be performed in regular 10 mL assay plastic tubes. The reagents involved in the TAN determination are prepared as follows:

- Phenol-nitroprusside solution (R1): 0.05 g of $Na_2[Fe(CN)_5NO]\cdot 2H_2O$ (sodium nitroprusside) and 15 g of C_6H_5OH (phenol) are dissolved in 250 mL of buffer solution, containing 30 g of $Na_3PO_4\cdot 12H_2O$ (trisodium phosphate), 30 g of $Na_3C_6H_5O_7$

(trisodium citrate) and 3 g of EDTA. The solution is finally diluted to 1 L with distilled water.

- Oxidising solution (R2): 15 mL of commercial-grade NaClO (sodium hypochlorite) is mixed with 200 mL of NaOH 1N solution. The mix is then diluted to 500 mL with distilled water.

As previously mentioned, the procedure starts with the 0.45 µm filtration of the sample, to eliminate the potential interference of suspended solids. Given the sensibility of the method (0 – 1 mgN-NH_4^+/L), the sample should be diluted accordingly. After the dilution step, the assay tubes are filled with 2.5 mL of samples in duplicates, plus a single blank tube with the same volume of distilled water. 1 mL of the phenol-nitroprusside reagent is then added to each assay tube, plus 1.5 mL of the oxidising solution. Thorough mixing of the resulting liquid is required, which can be performed by using a vortex appliance for several seconds. The time required for the coloration reaction to be concluded is of approximately 45 minutes, after which the indophenol produced is stable for approximately 1 hour. In this time interval, the samples absorbance should be measured, taking into account the blank measurements against distilled water.

The TAN value is then calculated from the equation correlating the absorbance at 635 nm with the ammonia/ammonium nitrogen concentration found in the samples, obtained through a calibration curve with standard solutions of either ammonium chloride or ammonium sulphate.

$$TAN\ (mgN/L) = ((\mathrm{X} \times \mathrm{Abs}) + \mathrm{Y}) \times \mathrm{DF} \quad (4)$$

Where Abs stand for the absorbance detected at 635 nm through the spectrophotometer, while DF stands for the dilution factor of the samples, when applicable.

2.4.4. Solids concentration

Depending on which kind of solids concentration is determined, the related information allows to understand different aspects of the fermentative process. Total and volatile solids (TS and VS) are measured to identify the solid fraction of the fermentation broth and which part of it is made of organic matter. Moreover, the difference between TS and VS can be used to estimate the total inorganic compounds of a liquid sample, whose concentration is generally identified as salinity. On the other hand, total and volatile suspended solids (TSS and VSS) are determined to identify the organic fraction of the suspension/turbidity found in a given sample. When the substrate used is mostly liquid (i.e. the organic matter is mostly soluble), VSS values generally represent the concentration of biomass in the fermentation broth, while the difference between TSS and VSS can be used to estimate whether the biomass is accumulating inorganic compounds inside the cell walls (transport phenomena) or outside (adsorption).

The Standard Methods used to determine all four kind of solids concentrations are the SM2540B, D and E (APHA, 2017). The equipment required includes an oven set at 105-110°C, a muffle furnace set at 550°C, alumina ceramic crucibles, filtration Erlenmeyer flasks

and glass fibre filters (Whatman GF/C, 4.7 cm diameter). Crucibles and filters are dried in the oven before use.

For TS and VS determination, crucibles weight (tare) is measured before being filled with samples of known weight and placed in the oven for at least 1 hour. Longer times might be required when the sample matrix is complex and/or is rich in organic matter. Afterwards, their dried weight is measured, and they are transferred to the muffle furnace for the calcination procedure for a minimum of 20 minutes. As in the case of the oven drying, the time required might increase with the complexity and the number of samples processed at the same time. Finally, the crucibles are weighed post ignition at 550°C.

TSS and VSS determination is similar to TS and VS one, with the exception that the crucibles are replaced by the glass fibre filters as vessels for the liquid samples of known weight. In fact, the dried filters are weighted before the filtration, after the drying and after the calcination.

The weights measured at each step of the procedure are then used to calculate the final concentrations as follows:

$$Total\ (suspended)\ solids\ (g/L) = \frac{\mathrm{A} - \mathrm{B}}{L \times 1000} \quad (5)$$

Where A stand for the weight (in g) of the oven-dried vessels and B for the weight (in g) of their tare. L represents the weight (in g) of the initial liquid sample.

$$Volatile\ (suspended)\ solids\ (g/L) = \frac{A - C}{L \times 1000} \quad (6)$$

Where A stand for the weight (in g) of the oven-dried vessels and C for their weight (in g) after the calcination at 550°C. L represents the weight (in g) of the initial liquid sample. To facilitate the conversion to g/L, the density of the liquid sample was assumed to be equal to 1.

2.4.5. Optical density (OD600)

Monitoring bacterial growth is a key aspect of batch experiments, as understanding the kinetics involved and the biomass yield of given substrates can help to improve and optimise the process design. While VSS determination is reliable in representing the biomass concentration of certain fermentative broths, as described in section 2.4.4, the related method is often inadequate for rapid sample processing. A valid alternative can be the optical density determination, a common spectrophotometric technique used in microbiology which is based on the amount of light scattered by the microbial cultures rather than the absorbance.

The required equipment is composed of a spectrophotometer set at a wavelength of 600 nm, a centrifuge, plastic vessels for centrifugation and cuvettes. A 0.7% w/w solution of NaCl is also used during sample preparation.

The procedure starts with the collection of a known volume of sample and its centrifugation (≤10000 rpm). Afterwards, the supernatant is discarded, and the biomass pellet is resuspended with the NaCl solution to the original known volume in order to inhibit further

microbial growth. The absorbance, or rather light scatter, is then measured with the spectrophotometer at 600 nm, taking into account blank measurement against distilled water. If the reading exceeds an absorbance equal to 1.000, the sample is diluted with the same NaCl solution.

OD measurements can be combined with VSS determination, obtaining a calibration curve which correlates the absorbance at 600 nm with the biomass concentration (in g/L), allowing to indirectly estimate the value by spectrophotometric reading. The following calibration curve was obtained and successfully used during the present thesis:

$$Biomass\ concentration\ (g\ VSS/L) = ((0.8815 \times \text{Abs}) - 0.1856) \times \text{DF} \qquad (7)$$

Where Abs stand for the absorbance detected at 600 nm through the spectrophotometer, while DF stands for the dilution factor of the samples, when applicable.

2.4.6. Volatile fatty acids (VFA)

The VFAs are those monocarboxylic acids whose composition includes 2 to 5 carbon atoms, from acetic to n-valeric acid. While they are considered intermediate products of the anaerobic digestion for the production of biogas, VFAs are undoubtedly the main output of MCFs. As such, measuring their concentration is of key importance when monitoring the outcome of fermentative processes.

The determination can be performed via multiple methods, of which GC was the chosen one (AGV-DB1 method). It should be also noted that this method allow to measure specific medium chain fatty acids (MCFA), namely the two caproic acid isomers and heptanoic acid. The equipment used was an Agilent 6850 with a flame ionization detector. The column used was a DB-Wax, from Agilent Technologies (30 m × 0.250 mm x 0.25 μm). The injector had a temperature of 200 °C while the detector was set at 300 °C. The carrier gas was nitrogen, while hydrogen and synthetic air (N_2/O_2 mix) were used as auxiliary gases. The samples were filtered (0.45 μm) and then acidified with 10 μM of concentrated H3PO4 (85%) prior to analysis.

2.4.7. Amino acids

Understanding how AA are consumed and then converted to VFAs is essential to evaluate the mechanisms of protein MCF. Hence, determining both the composition of the substrate used and the residual concentrations found in the reactors' effluents is vital, as they provide insight on how the individual AA are consumed depending on the operational conditions and allow to establish a balance with the VFA production in order to verify the stoichiometry of the process.

Only total AA determination was performed, as the free ones are included in the detected concentrations. Samples first undergo filtration at 0.45 μm and then acid hydrolysis for 24 h at 110°C using HCl 6 N. AccQ-Tag method is used to convert them to stable fluorescent derivatives (Cohen et al., 1993) which are finally analysed through HPLC with a Waters 2695 equipped with a fluorescence detector (Waters 2475).

Except for sample collection, preparation and storage (-20°C), the analysis was performed in an external analysis centre (CEntro TEcnolóxico da CArne, Ourense, Spain).

2.4.8. Ethanol, lactic acid, formic acid determination

While VFAs are the main products of MCFs, secondary metabolites can be formed during the process, the most important of which are ethanol, lactic acid and formic acid. Their determination has been performed through HPLC using the GLEFG1 method. The equipment involved was a Hewlett Packard 1100, equipped with an infrared HP1047A detector. The column used was an AMINEX HPX-87H (300 × 7.8 mm) using H2SO4 (5 mM) as an isocratic eluent. The set temperature for the column was 30°C while for the detector was 35°C. The samples were prepared in the same way as for VFA determination.

2.4.9. Glucose determination

Monitoring the glucose concentration is important as well when it is part of feedstock, as in the case of the cofermentation experiments. Its determination was performed by using two different methods: HPLC and kit measurement.

In the first case, the sample was processed and analysed as described in section 2.4.8. On the other hand, the kit measurement (Glucose TR – 1001190, Spinreact) is a colorimetric analysis. This method is based on glucose oxidase promoting the oxidation of glucose to gluconic acid, with parallel hydrogen peroxide formation. The peroxide, in turns, reacts with phenol and 4-aminophenazone to produce a red compound, quinone, with peroxidase as catalyst. The colour intensity of the resulting solution is proportional to the glucose concentration found in the sample.

The equipment required is a spectrophotometer set at 505 nm wavelength and 1 mL cuvettes.

The working reagent is reconstituted by mixing two starting reagents (R1 and R2) already prepared and found in the kit, obtaining a solution composed of:

- TRIS pH 7.4 (92 mmol/L)
- Phenol (0.3 mmol/L)
- Glucose oxidase (15000 U/L)
- Peroxidase (1000 U/L)
- 4-Aminophenazone (2.6 mmol/L)

Initially, the samples are filtered at 0.45 μm to remove the interference of turbidity. They should also be diluted to feature a final glucose concentration lower than 1 g/L, as this value correspond to the standard solution used to calibrate the method. 10 μL of samples and standard solution are added to the cuvettes together with 1 mL of working reagent and the resulting liquid is thoroughly mixed. Blank cuvettes are also required, containing only 1 mL of working reagent. After 20 minutes of reaction at room temperature, the red colour will have developed and its absorbance at 505 nm can be measured, taking into account the blank measurements against distilled water.

The values obtained are then used to calculate the glucose concentration of the samples:

$$Glucose\ (g/L) = \frac{\text{Abs} \times \text{St}}{AbsSt} \times \text{DF} \quad (8)$$

Where Abs and AbsSt respectively stands for the absorbance of the sample-containing cuvette and the absorbance of the standard-containing cuvette. St represents the concentration of the standard glucose solution (1 g/L), while DF stands for the dilution factor of the samples, when applicable.

2.4.10. Gas composition analysis

The composition of the gas mix found in the headspace of the vessels used for the experiments provides different information depending on the process performed. For example, during the BMP test its analysis allows to determine the production of methane and, consequently, the overall anaerobic biodegradability of the substrate used. Conversely, measuring the composition of the gases produced during a MCF can be used to detect potential methanisation episodes, which are to be avoided for a correct evaluation of the process.

The chosen method to determine the gas composition (N_2 (air), CH_4 and CO_2) is GC, performed with a HP 5890 Series II with a thermal conductivity detector. The 2 m-long column is maintained at 35°C, while the temperature of both the injector and the detector is set at 110°C. He was used as carrier gas at a flow rate equal to 15 mL/min. Gas syringes of 1 mL were used to extract the gaseous samples through silicon rubber septa attached to the outflow gas tubes of the reactors and through the bottle rubber stoppers.

2.5. CALCULATIONS

2.5.1. Anaerobic biodegradability

Anaerobic biodegradability was calculated in COD basis as a ratio between the methane produced and the total substrate used in the test, as described in the following equation:

$$Anaerobic\ biodegradability\ (\%) = \frac{M_{CH4}}{M_{pr}} \times 100 \quad (9)$$

where M_{CH4} stands for the total production of methane (in g COD-CH_4) and M_{pr} for the initial protein mass (in g COD).

Combining Stickland stoichiometry (Ramsay and Pullammanappallil, 2001) and the measured anaerobic biodegradability, it was possible to differentiate the methane produced from aliphatic VFA, aromatic ones and hydrogen.

2.5.2. Acidification degree

Acidification degree was the parameter chosen to describe substrate conversion (in COD basis), based on the concentration of measured VFA (in this case aliphatic VFA) and expressed as:

$$Acidification\ degree\ (\%) = \frac{\Sigma\ C_{VFA}}{C_{pr}} \times 100 \quad (10)$$

where C_{VFA} stands for the total concentration of the measured VFAs (in g COD-VFA/L) in the reactor effluent and C_{pr} for the total protein concentration (in g COD/L) in the reactors' feedstock.

2.5.3. Ammonification

Ammonification was also used as a proxy to monitor protein conversion to VFA, as amino acid fermentation is always related to NH_4^+ release. It was expressed as follows:

$$Ammonification\ (\%) = \frac{C_{TAN,effluent} - C_{TAN,feeding}}{C_{TAN,maximum}} \times 100 \quad (11)$$

Where $C_{TAN,effluent}$ is the concentration of ammonium nitrogen (mg N-NH_4^+/L) measured in the reactor effluent and $CT_{AN,feeding}$ is the concentration of ammonium nitrogen (mg N-NH_4^+/L) in the reactor feeding derived from the macronutrients supplementation and $C_{TAN,maximum}$ is the maximum concentration of ammonium nitrogen (mg N-NH_4^+/L) achieved if complete degradation of proteins occurs. $C_{TAN,maximum}$ was estimated based on TKN measurements of both proteins, which yielded a mg N-NH4+/g protein ratio of 171.94 and 201.94 for casein and gelatin, respectively.

2.5.4. AA consumption and VFA production balances

To better understand how protein conversion stoichiometry is affected by the operational conditions and strategies, balances between AA consumption and VFA production were established. AA consumption was calculated from the measured AA molar concentrations in the influent and effluent of the reactors. To link AA consumption and VFA production, the stoichiometry of AA conversion proposed by Ramsay and Pullammanappallil (2001) was used. Corrections were also made based on Regueira et al. (2020). Based on the abovementioned literature, iso-butyric is related to valine degradation alone, whereas n-butyric acid is produced from glutamic acid, histidine, lysine and either threonine or methionine depending on the protein composition. Iso-valeric acid is strictly linked to isoleucine and leucine consumption. Leucine dissimilation can also lead to the production of iso-caproic acid via a fermentative pathway (Britz & Wilkinson, 1981). On the other hand, n-valeric acid is only associated with proline conversion. Acetic and propionic acid balances were limitedly described as these VFAs can be produced by most AAs via the pyruvate pathway (Regueira et al., 2020).

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CHAPTER 3

PROTEIN COMPOSITION DETERMINES THE PREFERENTIAL CONSUMPTION OF AMINO ACIDS DURING ANAEROBIC MIXED-CULTURE FERMENTATION

SUMMARY

The aim of this chapter was to investigate how protein composition, i.e. the amino acid (AA) profile, affects the individual consumption of amino acids and, consequently, the outcome of the process. Mixed-culture fermentations were performed with two model proteins (casein and gelatin) using continuous and batch reactors at neutral pH values and 25 °C. The acidification was incomplete for both proteins, with casein achieving a higher value than gelatin. Albeit dominated by acetic acid, product spectra were different as well, with n-butyric acid as the second major product for casein and propionic acid for gelatin. The preferential consumption of amino acids was demonstrated, which interestingly depends on protein composition. The previously accepted stoichiometry accurately describes iso and n-butyric acid production, but it fails for propionic, iso and n-valeric acid generation. Overall, this chapter offers a better understanding of protein fermentation mechanisms, which will help to improve degradation models and to design fermentation processes, based on optimal substrate selection.

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3.1 INTRODUCTION

Regardless of being a relevant fraction of many industrial sidestreams and wastes, proteins and their fermentation mechanisms have not been studied so thoroughly. Their main complexity lies in the fact that they can be considered as a mix of some 20 different substrates, i.e. the amino acids (AAs), as opposed to glucose, which is only one substance.

Stickland coupled redox reactions are currently the accepted metabolic pathway for AAs fermentation (Ramsay & Pullammanappallil, 2001), accounting for up to 90% of the overall degradation process (Nagase & Matsuo, 1982). Based on these reactions, Ramsay and Pullammanappallil (2001) proposed a fixed stoichiometry to describe MCF of AAs. This means that each AA is converted to specific VFAs, regardless of the protein composition or the operational conditions. Therefore, this degradation model does not take into account possible imbalances between AA redox roles, related to the protein composition, excluding alternative metabolic pathways that fermentative bacteria might opt for if a surplus of either electron donor or acceptors accumulates in the reactor. In addition, the flexibility of some AAs, which can potentially act both as electron donor or acceptor (e.g. leucine and arginine), is not compatible with the proposed fixed stoichiometry. Moreover, the experimental evidence of incomplete protein consumption, both in continuous and batch experiments (Breure & van Andel, 1984; Duong et al., 2019), might indicate the preferential consumption of some AAs due to bioenergetics motivation (Regueira et al., 2020), refuting the assumption of the proposed fixed stoichiometry, by which all AAs are completely and equally degraded.

From the abovementioned hypotheses and model limitations, the main aim of this chapter is to assess how AA composition of proteic substrates affects their consumption and interaction, and consequently, the VFA selectivity and productivity of the process. The gathered knowledge contributes to the understanding of protein degradation mechanisms during anaerobic MCF.

3.2 MATERIALS AND METHODS

The AA composition of the chosen proteins (section 2.1.1), casein and gelatin, was integrated with the assumed stoichiometry and the individual redox roles as reported in literature (Table 3.1).

Table 3.1. Measured AA composition (molar fraction in %) of casein and gelatin, redox roles (RR) and VFA produced according to the stoichiometry proposed by Ramsay and Pullammanappallil (2001). D/A are AAs that can act as both donor and/or acceptor. Uncoupled AAs are not involved in Stickland reactions.

AA	Casein	Gelatin	RR[1]	RR[2]	Stickland-related VFA(s)
Ala	7.64	13.5	Donor	Donor	Acetic
Arg	3.83	5.11	Donor	D/A	Acetic/propionic/n-valeric
Asp*	2.54	2.03	Donor	Acceptor	Acetic
Cys	0.00	0.00	Donor	Donor	Acetic
Glu*	15.0	7.07	Donor	Donor	Acetic/n-butyric
Gly	2.80	34.5	Acceptor	Acceptor	Acetic
His	1.96	0.80	Uncoupled	Donor	Acetic/n-butyric
Ile	5.94	1.33	Donor	Donor	Iso-valeric
Leu	9.46	3.07	D/A	D/A	Iso-valeric
Lys	6.96	3.02	Donor	Donor	Acetic/n-butyric
Met	0.93	0.49	Donor	Donor	Propionic
Phe	4.56	1.73	D/A	D/A	Aromatic VFA
Pro	13.9	16.1	Acceptor	Acceptor	Acetic/propionic/n-valeric
Ser	7.12	4.32	Donor	Donor	Acetic
Thr	5.83	2.87	D/A	Donor	Acetic/n-butyric
Trp	0.00	0.00	D/A	D/A	Aromatic VFA
Tyr	2.74	0.55	D/A	D/A	Acetic/aromatic VFA
Val	8.79	3.44	Donor	Donor	Iso-butyric

[1] Redox roles according to Ramsay (1997). [2] Redox roles according to De Vladar (2012). *Glu and Asp also include the fraction related to Glutamine and Asparagine, respectively.

The maximum biodegradability of the chosen proteins was then measured by performing BMP tests as described in section 2.3.2.1.

The reactors used were inoculated at an initial in-reactor biomass concentration of around 1.0 g VSS/L with acidogenic biomass obtained from the parent reactor (section 2.2) and then

operated with an HRT of 1.0 d and an OLR of 8.0 g COD/L·d. On day 44, the HRT of the gelatin reactor was increased to 1.5 d (OLR of 5.3 g COD/L·d) in order to surpass possible kinetic limitations.

Analysis of the influents and the effluents of the reactors were performed according to the methods and the calculations described in **Chapter 2.** COD (total and soluble) and VFA concentrations were determined three times per week, while Total Ammonia Nitrogen (TAN), secondary metabolites and solids concentrations were measured once a week. Headspace gas composition was occasionally analysed to discard the occurrence of methanisation processes AA content was measured on selected samples from steady state periods of operation.

At steady-state operation, biomass from reactors was extracted to perform acidification batch tests (Section 2.3.2.2) to further understand the impact of protein composition on the fermentation process without potential kinetic limitations.

The kinetic parameters of protein-degrading biomass (maximum specific growth rate (μ_{max}), yield (Y), decay constant ($k_{dec,X}$) and the different VFA stoichiometric factors (F_{VFA})) in fermentative environments were estimated based on the kinetic model developed by Regueira et al. (2020). It was assumed that AA conversion to VFA was performed by a single population of microorganisms (AA degraders) and that the feeding consisted of a mixture of AAs. AA conversion was modelled following a Monod equation, with a fixed half saturation constant (1.5 g COD-AA/L). To reflect the observed non-complete consumption of AA, the model includes the possibility of converting the substrate to an inert fraction. Aromatic VFA and H_2 yields were determined based on the AA composition of the fermented protein and following the stoichiometry proposed by Ramsay and Pullammanappallil (2001), as they were not measured experimentally.

The calibration procedure was done following the non-linear least squares method (Eq. (3)) in MATLAB 9.0 (R2016a) (Mathworks Inc., Natick, MA, USA) using the lsqnonlin command (trust-region reflective algorithm). A Bootstrap methodology was followed to ensure a robust parameter estimation, as described in Gonzalez-Gil et al. (2018).

$$\hat{\theta} = \arg\min\left(\sum_{k}\left(\sum_{j}\left(\sum_{i}\left(\frac{y_{j,i}(\theta) - y_{j,i,exp}}{\sigma_{j,i}}\right)^2\right)\right)\right) \quad (12)$$

Where $\hat{\theta}$ is the set of parameters to estimate, y is the simulated concentration, yexp is the experimentally measured concentration and σ is the experimental standard deviation. The subscript i refers to the different compounds, the subscript j refers to the different measurements over time and the subscript k refers to the different batch experiments.

3.3 RESULTS AND DISCUSSION

3.3.1 Reactors operation

Casein and gelatin reactors were continuously operated for 140 and 170 days, respectively (Figure 3.1a and b). No pH control was needed since it naturally adjusted on

neutral values (7.2-7.4) due to the joint effect of ammonia release (buffering VFA production acidification) and nitrogen sparging (partially stripping the CO_2 from the system). Biomass concentration rapidly decreased from above 1.0 g VSS/L to 0.35 – 0.40 g VSS/L in both CSTRs during the first seven days, remaining constant afterwards. No methanisation occurred during the experiment since no differences were observed between the total COD concentrations in the influent and effluent in any of the two reactors (Figure 3.1a and 3.1b). Analysis of the gas composition of the headspace volume of the reactors confirmed the absence of methane, with nitrogen (close to 100%) and carbon dioxide (up to 2%) as the only detected gases. Hydrogen was not detected either throughout the whole experiment.

Acidification degree increased over time from 31.3% (days 40-85) to 48.8% (days 100-140) in casein reactor and from less than 10% (days 15-70) to 40% (days 145-170) in gelatin reactor. In the latter case, different strategies were adopted in order to improve substrate conversion. The addition of selenium dioxide (1 µM on day 91), to satisfy the requisite for glycine reductase production (D**ü**rre & Andreesen, 1981), an enzyme especially relevant to gelatin degradation because of its high content in glycine (Table 3.1), and an increase in HRT from 1.0 to 1.5 days (day 44) were not successful (Figure 3.1b). In contrast, cross inoculation with methanogenic biomass to increase the diversity of the microbial population inside the reactor (days 70 and 133) resulted in VFA peak production followed by a gradual decrease and stabilization with a 2-fold improvement of the acidification degree. This suggests that gelatin conversion was hindered either by a low microbial diversity and/or by the lack of some micronutrients.

Even though acidification degree appeared to be stable, product composition (VFA spectra) varied more during the experimental period (Figure 3.1c and d). Acetic acid was the major product in both reactors. However, the second major product differed between them, being n-butyric acid in casein reactor and propionic acid in gelatin reactor. Other possible fermentation products, such as lactic acid, formic acid and ethanol, were not detected during the operational period.

In order to assess the influence of protein composition on fermentation performance, the following periods, day 100-140 and day 147-170, were selected as stable periods for casein and gelatin, respectively. Steady-state periods were identified as those where the variability of the VFA relative molar fractions was lower than 15% (measured as the coefficient of variation).

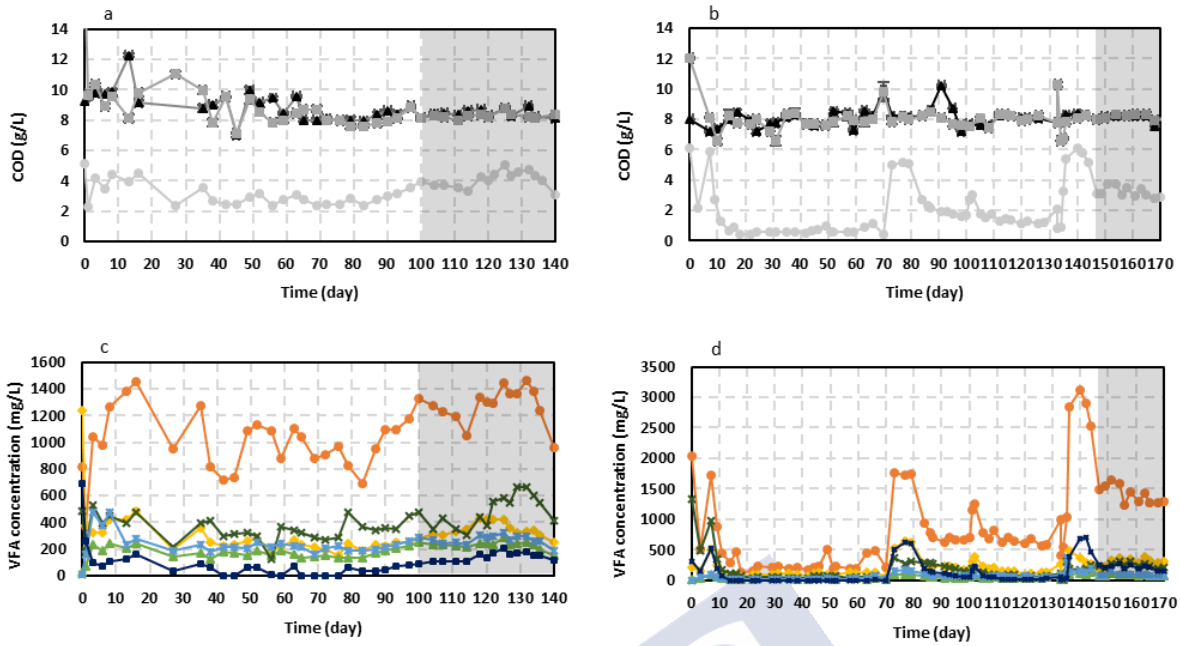

Figure 3.1. COD balance (a, casein; b, gelatin: ▲ Influent total COD; ■ Effluent total COD; ● VFAs COD) and individual VFA concentrations in the reactors (c, casein; d, gelatin: ● Acetic; ◆ Propionic; ▲ Iso-butyric; x n-Butyric; * Iso-valeric; ■ n-Valeric), with the shadowed areas corresponding to the identified steady-state periods

3.3.2 Influence of protein composition on acidification degree and selectivity

Protein composition affects acidification degree, with higher values being achieved for casein (around 50%) than for gelatin (around 40%). This, combined with the lower HRT applied in the casein reactor, derived in higher productivities (4.1 ± 0.5 g COD-VFA/L·d; 11.6 ± 3.0 g COD-VFA/g VSS·d) when compared to the values obtained for gelatin (2.1 ± 0.2 g COD-VFA/L·d; 5.1 ± 1.1 g COD-VFA/g VSS·d). In general, lower values were reported in literature for casein fermentation (30% of the influent carbon), though the HRT was lower as well (0.4 d, Ramsay, 1997). In contrast, literature data indicate higher acidification of gelatin (around 50%) regardless of the HRT applied (Breure & van Andel, 1984; Breure et al., 1986). This difference in conversion efficiencies might be attributed to a number of factors (e.g. protein composition, inocula type), among which micronutrients presence seems to be the most influential one.

To explain the limited conversion achieved during continuous experiments, biochemical methane potential tests of the chosen proteins were performed to evaluate their maximum anaerobic biodegradability. The results showed that the conversion of both proteins to methane is very similar, with values close to 90%. According to the fixed stoichiometry (Ramsay & Pullammanappallil, 2001), 73-83% of the methane produced is related to aliphatic VFA, while non-measured products (aromatic VFAs and hydrogen) only account for approximately 5-15%. There are different and possibly concurrent explanations to explain these higher values compared to the acidification degrees achieved in the reactors: the higher temperature applied to the batch tests (37.5 against 25°C), the presence of micronutrients in

the inoculum, higher microbial diversity covering all possible metabolic niches, absence of product (VFA) inhibition due to their conversion to methane and longer reaction time. Temperature role was discarded as similar acidification degrees were obtained regardless of its value (Yu & Fang, 2003).

To verify whether product inhibition and/or limited reaction time were responsible for the incomplete CSTR conversions, three casein batch experiments were carried out varying the substrate-to-inoculum ratios (SIR) from 5 to 20 (g COD protein/g VSS inoculated), while the gelatin batch test was only performed at a value of 10. The acidification degree of casein was close to 50% in all the three cases (Figure 3.2a) after 96 h, as also observed in the continuous CSTR operation, highlighting the SIR and HRT values as being uninfluential on the outcome. Given that the actual VFA concentration at the end of the tests was 1, 2 and 4 gCOD-VFA/L, respectively, potential product inhibition was ruled out as well as the cause of the limitation in substrate conversion. On the contrary, the acidification degree of gelatin after 192 h was double than the one achieved in the continuous reactor (Figure 3.2b) meaning that reaction time might play a more important role in this case.

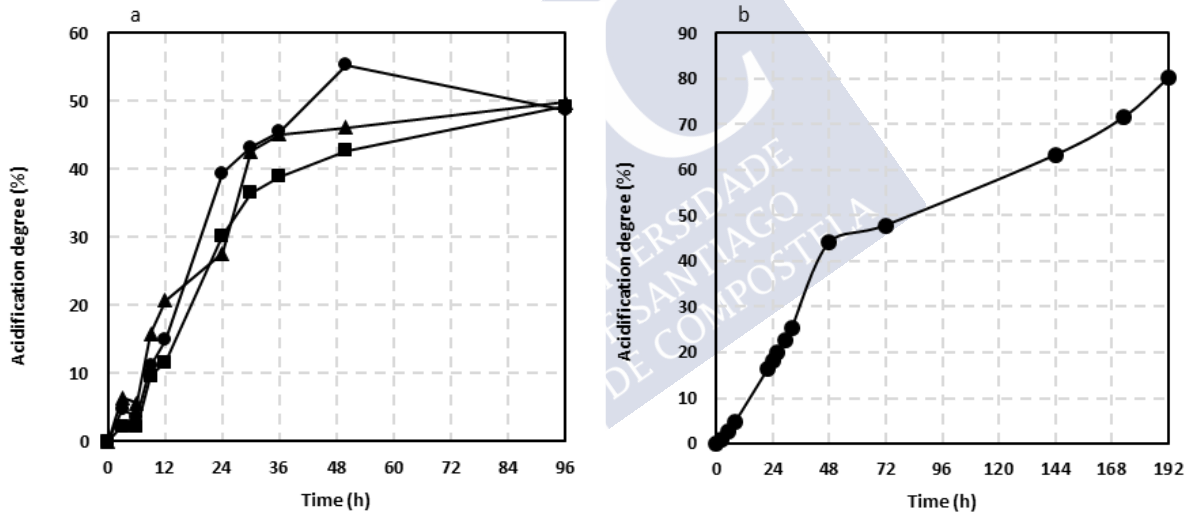

Figure 3.2. Acidification degree evolution over time for casein conversion (a, at three substrate-to-inoculum ratios, ■ SIR20; ● SIR10; ▲ SIR5) and gelatin (b, only ● SIR10)

Protein composition determined process selectivity as well (Figure 3.3). In all cases, acetic acid was the main product, followed by either propionic acid (in gelatin) or n-butyric acid (in casein). Iso-butyric and iso- and n-valeric acids were minor products accounting for less than 10% of the total VFA molar percentage. The main difference between casein and gelatin was that more reduced products were obtained from casein in detriment of acetic acid. No significant differences were observed between continuous and batch experiments (Figure 3.3b and 3.3c). These results are comparable to those previously described in literature (Breure & van Andel, 1984; Ramsay, 1997).

This VFA selectivity can be explained taking into account the AA composition of the two proteins (Table 3.1). The large proportion of glycine in gelatin, a precursor of acetic acid, is

likely responsible for the predominance of this acid in all the gelatin tests. Similarly, valine is the sole responsible of iso-butyric acid production and it is more abundant in casein than in gelatin.

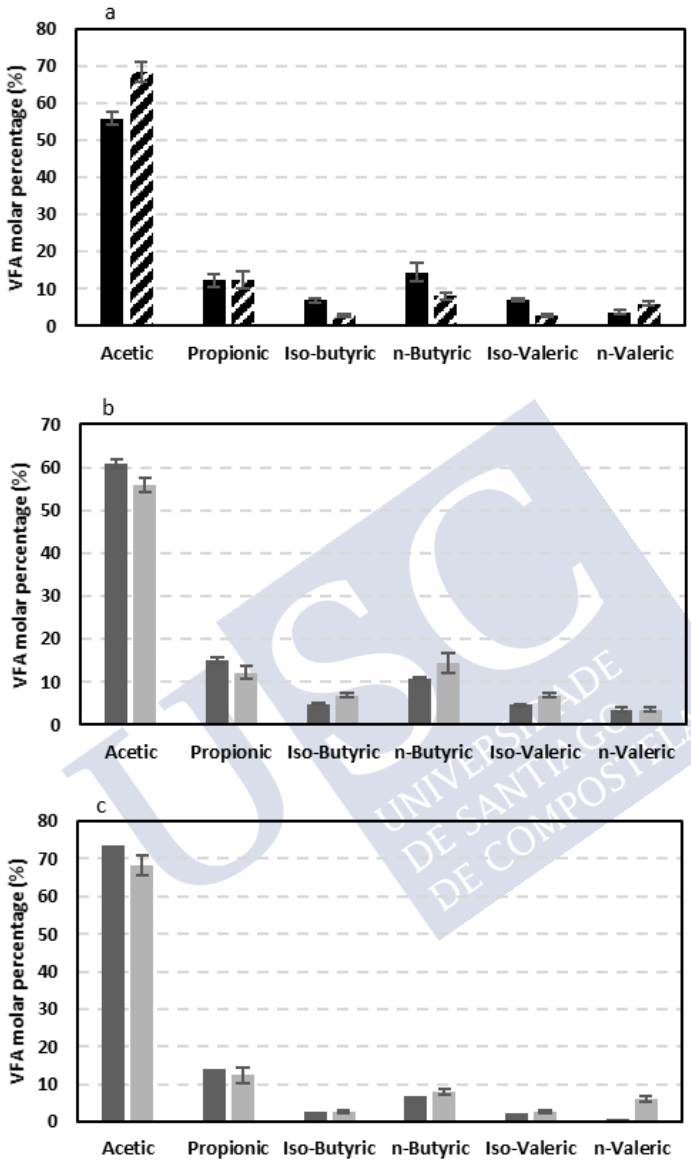

Figure 3.3. Comparison of VFA spectra between casein (■) and gelatin (▨) during CSTR operation (a) and between batch (■) and continuous (■) operation (b, casein; c, gelatin)

As protein composition affects both the acidification degree and the VFA selectivity, knowing the average AA profile of different suitable substrates will be interesting/crucial when designing and/or operating a VFA recovery installation from proteinaceous sidestreams and wastewaters because it will enable the definition of the most suitable feedstock composition to achieve the desired goal (greater yields and/or required product distribution).

3.3.3 Influence of protein composition on amino acid consumption

The previous section showed that the protein acidification was incomplete and varied depending on the substrate composition. The consumption of individual AAs is evaluated in this section, both for continuous (Figure 3.4a) and batch (Figure 3.4b) experiments, as the root cause for the different observed acidifications.

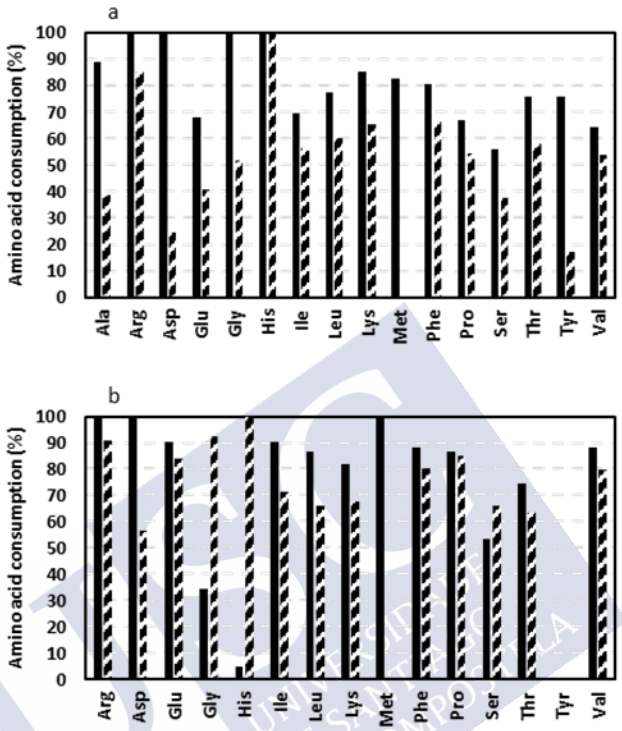

Figure 3.4. Comparison of amino acid consumption during casein (■) and gelatin (▨) fermentation in continuous reactor (a) and batch tests (b). No data of alanine consumption is available for the batch tests. Arg: Arginine; Ala: Alanine; Asp: Aspartic acid; Glu: Glutamic acid; Gly: Glycine; His: Histidine; Ile: Isoleucine; Leu: Leucine; Lys: Lysine; Met: Methionine; Phe: Phenylalanine; Pro: Proline; Ser: Serine; Thr: Threonine; Trp: Tryptophan; Tyr: Tyrosine; Val: Valine.

First of all, it can be observed that the amino acid degradation differs from the acidification degrees of casein (50%) and gelatin (40%). During casein fermentation, the majority of the AAs were largely consumed (≥70%), with some even reaching the complete utilisation (e.g. Arg and Asp). The least consumed AA was Ser, whose conversion only reached 55%. In comparison, gelatin fermentation led to generally lower and more variable consumptions: Arg and His were extensively converted (≥80%), while the other AAs were consumed between 40 and 60%. The only exceptions were Asp and Tyr, whose limited fermentation only reached the 20%, together with Met being not even metabolised by the mixed culture. Comparing the two reactor configurations, the batch system did not extensively alter the consumption patterns observed in the CSTR operation, only with overall higher values, especially for gelatin fermentation due to the greater acidification degree achieved during the discontinuous test. The failed conversion of Tyr in both gelatin and casein

batch test fermentation was considered as a strategy to avoid further accumulation of aromatic VFAs (more toxic than the aliphatic ones), while the lower consumptions of Gly and His during batch fermentation of casein were probably related to experimental noise, given their limited abundance in casein composition (Table 3.1).

From these results, it can be concluded that AAs are not consumed evenly, and that the preferential consumption depends on the protein composition. Recognising Stickland reactions as the main route for AA conversion into VFA (Nagase and Matsuo, 1982; Ramsay and Pullammanappallil, 2001) leads to the hypothesis that AA redox roles (i.e. electron donors and/or acceptors) should be equilibrated. It was consequently hypothesised that the preferential consumption of AA might respond to a strategy to compensate the overall redox balance. Indeed, the redox balances calculated from measured AA consumptions (Table 3.2), expressed as mmoles of hydrogen equivalents per C-mmole of degraded protein, are close to zero for both proteins, thus supporting the aforementioned hypothesis. Still, a surplus of electron donor AA, as in the case of casein (Table 3.3), might prove beneficial to the overall conversion to VFA due to both higher acidification degree and AA consumptions than in the case of gelatin.

Table 3.2. Experimental reducing power (H_2 equivalents) balance from amino acid degradation in casein and gelatin reactors, based on the fixed stoichiometry proposed by Ramsay and Pullammanappallil (2001) and assuming fixed AAs redox roles.

Amino acid	H_2 mole/consumed AA mole	Casein H_2 mmoles	Gelatin H_2 mmoles
Alanine (Ala)	2	4.9794	5.5816
Arginine (Arg)	-1	-1.4067	-2.3109
Aspartic acid (Asp)	2	1.8616	0.5340
Cysteine (Cys)	0.5	0.0000	0.0000
Glutamic acid (Glu)	0	0.0000	0.0000
Glycine (Gly)	-1	-1.0269	-9.4557
Histidine (His)	0	0.0000	0.0000
Isoleucine (Ile)	2	3.0450	0.7971
Leucine (Leu)	2	5.3937	1.9703
Lysine (Lys)	0	0.0000	0.0000
Methionine (Met)	1	0.2834	0.0000
Phenylalanine (Phe)	2	2.6928	1.2204
Proline (Pro)	-1	-3.4354	-4.6597
Serine (Ser)	1	1.4626	0.8570
Threonine (Thr)	-1	-1.6244	-0.8858
Tryptophan (Trp)	2	0.0000	0.0000
Tyrosine (Tyr)	1	0.7615	0.0505
Valine (Val)	2	4.1340	1.9586
	Sum	17.1206	-4.3427
	H_2 mmoles/protein C-mmole	0.1207	-0.0405

Table 3.3. Protein composition in terms of electron donor and acceptor AAs, according to the stoichiometry proposed by Ramsay and Pullammanappallil (2001).

Role	Casein	Gelatin
e^- acceptor (%)	26.4	58.6
e^- donor (%)	71.7	40.6
Uncoupled (%)	1.96	0.80

3.3.4 Balancing AA consumption with VFA production

Balancing the AA consumption with the VFA produced is a manner to understand better the transformation routes and how these may change as a response to the substrate composition. To do this, the stoichiometry proposed by Ramsay and Pullammanappalil (2001) is taken as a starting point and other possible routes are discussed. Acetic acid was left out of this analysis as it is yielded by many AAs, hindering the identification of the metabolic pathways.

Iso-butyric production, being linked only to valine degradation, was well described by this stoichiometry during both casein and gelatin fermentation (Figure 3.5a and b). The same applied to n-butyric acid production which appears to be correctly related to the degradation of four specific AAs, namely glutamate, threonine, histidine and lysine (Figure 3.5c and d).

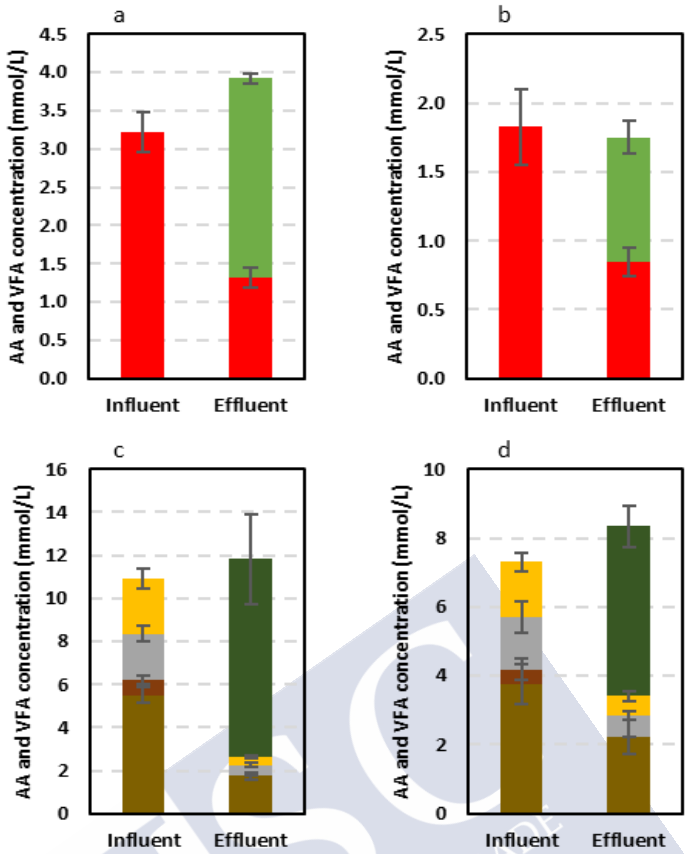

Figure 3.5. Iso-butyric (a, casein; b, gelatin: ■ Valine; ■ Iso-Butyric acid) and n-butyric (c, casein; d, gelatin: ■ Glutamic acid; ■ Histidine; ■ Threonine; ■ Lysine; ■ n-Butyric acid) acid balance in the continuous reactors.

However, discrepancies arose with propionic, iso- and n-valeric balances (Figure 3.6). Iso-valeric was produced to a lower-than-expected extent (1:1 molar ratio) in comparison with the degradation of the related AAs, isoleucine and leucine (Figure 3.6a and b), especially in casein case. In the case of propionic and n-valeric acids, both acids should be produced at equal molar ratios from arginine and proline (1 mmol AA = 0.5 mmol Pr + 0.5 mmol nVal), although propionic acid is also generated from methionine (1:1 molar ratio). Even though the balance appears to be closed (Figure 3.6c and d), the production of n-valeric acid was much lower than the one of propionic acid.

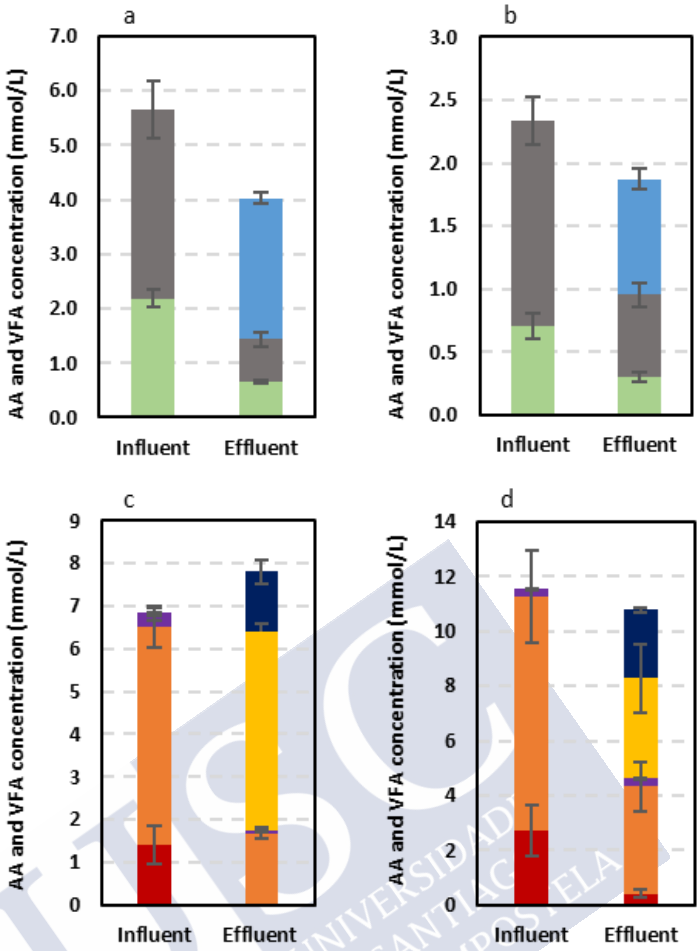

Figure 3.6: Iso-valeric (a, casein; b, gelatin: Isoleucine; Leucine; Iso-Valeric acid), propionic and n-valeric (c, casein; d, gelatin: Arginine; Proline; Methionine; Propionic acid; n-Valeric acid) acid balance in the continuous reactors.

To explain the abovementioned discrepancies, it was hypothesised that either the stoichiometric coefficients are incorrect or other unknown metabolic pathways should be considered. For example, leucine can also be converted to iso-caproic acid (Regueira et al., 2020), though it was rarely detected and only at low concentrations in this study. Also, arginine can be converted to alanine and acetyl-CoA (Fonknechten et al., 2010) and, ultimately, to acetic and propionic acid rather than going through 5-aminovalerate route (Barker et al., 1987); and, both aspartic acid, via fumarate (Unden et al., 2016), and threonine (Sawers, 1998) could potentially generate propionic acid. As a general recommendation, energetic criteria appear to be the most effective ones to identify the routes linking AA and VFA (Regueira et al., 2020).

3.3.5 Kinetic parameters of protein degrading microorganisms

The data gathered during the fermentation batch tests were used to obtain kinetic parameters for casein and gelatin fermentation (Table 3.4). Gelatin root-mean-squared error (RMSE) was 5.3% and it lied between 5.5 and 7.6% for the three casein experiments, showing the good validity of the estimated parameters. Moreover, in the case of casein, the parameters have shown to be suitable for the different SIR applied in the batch experiments.

Table 3.4: Estimated kinetic parameters (average [estimated confidence interval with a = 0.05]) for protein-degrading biomass (BM)

Parameter	Casein	Gelatin
μ_{max} (h^{-1})	0.034 [0.030, 0.039]	0.019 [0.017, 0.021]
Yield (g_{COD} BM/g_{COD} AA)	0.192 [0.170, 0.225]	0.165 [0.146, 0.188]
k_{decay} (h^{-1})	$6 \cdot 10^{-3}$ [$5 \cdot 10^{-3}$, $9 \cdot 10^{-3}$]	$9 \cdot 10^{-4}$ [0, $2 \cdot 10^{-3}$]
F_{Ac} (g_{COD} Ac/g_{COD} AA)	0.338 [0.327, 0.350]	0.571 [0.556, 0.587]
F_{Pro} (g_{COD} Pro/g_{COD} AA)	0.141 [0.134, 0.148]	0.177 [0.167, 0.187]
F_{But} (g_{COD} But/g_{COD} AA)	0.223 [0.214, 0.233]	0.131 [0.121, 0.140]
F_{Val} (g_{COD} Val/g_{COD} AA)	0.136 [0.128, 0.145]	0.076 [0.068, 0.084]
Inert AA (%)	45.2 [42.7, 47.4]	13.9 [8.4, 19.0]

The values of the estimated parameters show significant differences depending on the fermented protein. Casein fermenters have maximum growth rates almost two times higher than gelatin fermenters, while the biomass yield in both cases is comparable. These values are higher than the few available data in literature (Ramsay, 1997) and are similar to values reported for sugar fermenters (Batstone et al., 2002). The ATP production per gram of COD of some AAs was determined and, in fact, is comparable to the ratio found in glucose fermentation (data not shown). Decay values are 20% and 5% with respect to the μ_{max} value for casein and gelatin, respectively, which are usual values for anaerobic biomass. The stoichiometry coefficients show that the selectivity on the different VFA is influenced by the fermented protein, in agreement with the results of section 3.3.2. Overall, acetic acid dominates the product spectra in both cases but to a greater extent in gelatin fermentation. Consequently, in casein fermentation the yields of the secondary VFA have greater values than in the case of gelatin. Finally, gelatin was converted almost completely to VFA and biomass and only 13.9% was converted to inert substrate while almost half of the casein was converted to inerts, underlying the differences in acidification degree depending on the substrate composition. This difference cannot be attributed to different batch test duration since in both experiments VFA concentrations were stable. The effect of the test length is

reflected in the different μmax values, instead. In consequence, to properly design processes centred on VFA production, models need trustworthy kinetic parameters specifically estimated in fermentative environments and considering substrate composition.

3.4 CONCLUSIONS

To the best of our knowledge, this chapter investigated for the first time the impact of protein composition on individual amino acid consumption in mixed-culture anaerobic fermentations, linking it to VFA production. In particular, the main findings are:

- Protein fermentation results in an incomplete acidification, which depends on protein composition.
- A balanced AA composition, in terms of redox roles, does not guarantee a higher acidification.
- Acetic acid is the major product, regardless of the protein composition, but casein fermentation results in a higher fraction of reduced products than gelatin fermentation.
- Preferential consumption of AAs was demonstrated, which interestingly depends on protein composition.
- The known stoichiometry accurately describes iso and n-butyric acid production, but fails for propionic, iso- and n-valeric acids. Therefore, further studies are required to upgrade it.

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CHAPTER 4

STEERING THE CONVERSION OF PROTEIN RESIDUES TO VOLATILE FATTY ACIDS BY ADJUSTING PH

SUMMARY

This chapter investigates the influence of pH on protein conversion into volatile fatty acids by anaerobic mixed-culture fermentation, a topic that, in contrast to glucose fermentation, only has scarce and contradictory information available. Several experiments were performed with two model proteins (casein and gelatin) at three different pH values (5, 7 and 9) using chemostats and batch tests. Highest conversion was reached at neutral pH although complete acidification was never achieved. Longer chain carboxylates production was favoured at low pH, while acetic acid was the main product at pH 7 and 9. Amino acids preferential consumption also varied with pH and protein composition. In fact, protein conversion stoichiometry is mainly driven by energetic yields and amino acid molecular configuration. Overall, this work identified pH adjustment as a way to steer volatile fatty acid production during mixed-culture fermentation of proteins.

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4.1 INTRODUCTION

A fixed stoichiometry to describe and predict the conversion of amino acids (AAs) to VFAs was proposed by Ramsay and Pullammanappalil (2001) and then revised and improved by Regueira et al. (2020), highlighting Stickland reactions as the main degradation pathway (**Chapter 1**). As these reactions involve the redox coupling between two or more AAs (Nagase & Matsuo, 1982), protein composition plays an important role in determining both the selectivity and the conversion efficiency of the MCF. In fact, the preferential consumption of AA depends on their interactions (**Chapter 3**), which, in turns, could be potentially affected by pH. However, the information on the subject is contradictory. While the degradation model of Ramsay and Pullammanappallil (2001) discards pH influence on the process, experimental results demonstrated its relevance (Breure & van Andel, 1984; Breure et al., 1986; Yu & Fang, 2003; Duong et al., 2019). Acidification degree appears to be favoured at neutral conditions, whereas either propionic (Breure & van Andel, 1984) or n-valeric acid (Duong et al., 2019) productions are favoured over acetic acid generation at acid pH values. No data on alkaline fermentation of proteins is available.

Therefore, the main aim of this chapter is to understand how protein degradation is affected by pH changes during anaerobic MCF, focusing on its impact on acidification degree and VFA selectivity. As a novel approach to the subject, AA analysis will help to understand how the conversion mechanisms vary according to pH. Given the lack of information and the available inconclusive results, determining the link between AA consumption and VFA production at different pH conditions will broaden the available knowledge on protein MCF as well as denoting how the process can be steered towards desired product compositions.

4.2 MATERIALS AND METHODS

The two CSTRs, used in **Chapter 3** to investigate the impact of protein composition on MCF processes, were maintained operational without the need for additional inoculation. Both of them were set at a hydraulic retention time of 1.5 d and an organic loading rate of 5.3 g COD/L·d, with continuous nitrogen sparging and magnetic stirring.

The tested pH values, 5 and 9, were selected to be representative of each pH condition, while pH 7 results (**Chapter 3**) were already obtained from the previous experiments. The 2-points steps from pH 7 were chosen in order to ensure different environmental conditions for the microbial populations while avoiding extreme pressure of more marked acid and alkaline environments. Initially, the reactors were operated at neutral pH values (7.2 - 7.4) without the need for chemical control for 140 days (casein reactor) and 170 days (gelatin reactor). Afterwards, the pH was shifted to the alkaline range (i.e. 9), and the reactors were operated at this pH for 160 days each. Before applying the acid conditions, both reactors were acclimated again at pH 7 for at least 30 days, to avoid environmental shocks which could negatively affect the microbiome. Finally, the reactors were operated at pH 5 for 320 and 110 days respectively. Each pH condition was maintained for at least 100 days to ensure that steady-state operation was achieved.

Analysis of the influents and the effluents of the reactors were performed according to the methods and the calculations described in **Chapter 2.** VFA and Total Ammonia Nitrogen (TAN) concentrations were determined twice per week, while COD (total and soluble), secondary metabolites and solids concentrations were measured once a week. Headspace gas composition was occasionally analysed to detect potential methanisation episodes. AA analysis was performed on selected number of samples from steady state periods of operation.

When steady state was achieved at each pH value, batch fermentation tests (section 2.3.2.2) were performed in order to verify whether the absence of kinetic limitations might affect the incomplete substrate acidification and/or the selectivity of the process.

4.3. RESULTS AND DISCUSSION

4.3.1 Influence of pH on substrate conversion

Casein and gelatin reactors were continuously operated for 700 and 470 days, respectively. Methanisation was discarded in both reactors throughout the experimental period since no differences were observed between influent and effluent total COD concentrations (Figure 4.1) and no methane was ever detected in the collected headspace gas samples. Biomass concentrations were similar in both reactors and varied slightly with pH, with lower concentrations (0.20 – 0.25 g VSS/L) being detected under acid conditions compared to neutral and alkaline ones (0.30 – 0.40 g VSS/L).

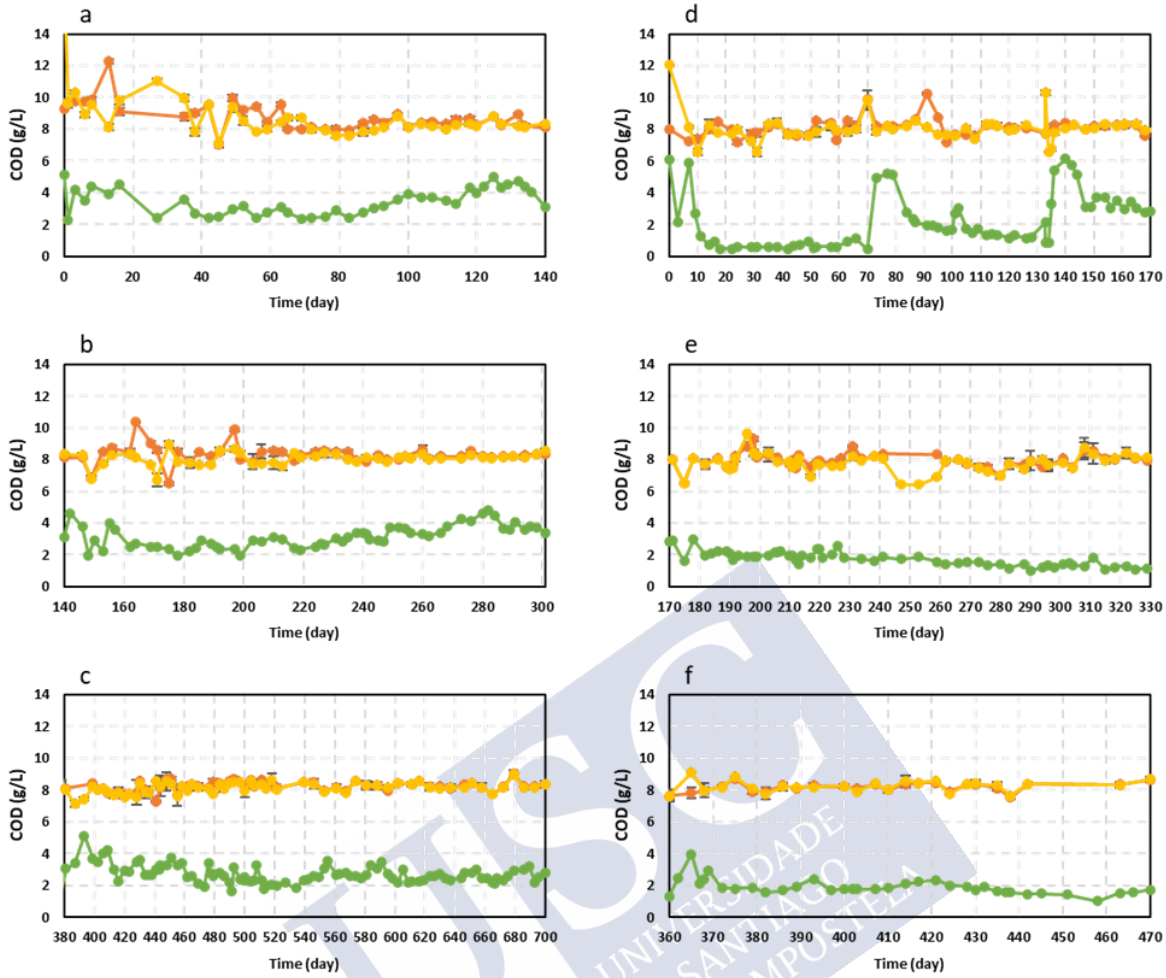

Figure 4.1. COD balance of the continuous casein (a, b, c) and gelatin (d, e, f) reactors at different pH values: 7 (a, d; **Chapter 3**), 9 (b,e) and 5 (c, f). ■ Influent total COD; ■ Effluent total COD; ■ VFAs COD. The days between 300 and 380 for casein and between 330 and 360 for gelatin correspond to transition periods at pH 7.

Substrate conversion to VFAs was monitored through acidification degree and ammonification. Their comparison allowed to check whether unmeasured products were being generated during the process. The two parameters were comparable (Figure 4.2), indicating that the production of aromatic VFAs (e.g. phenylacetic acid) and other secondary products was limited or null. Besides, no ethanol, lactate or formic acid were detected, regardless of being potential fermentation products.

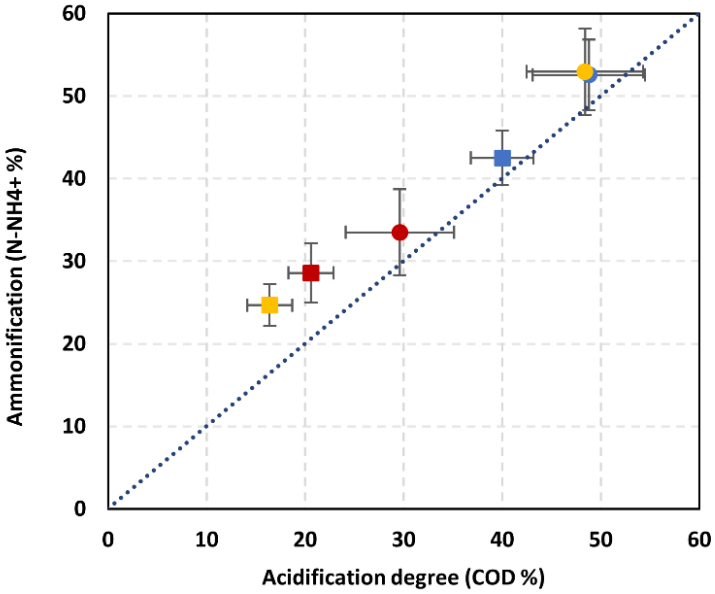

Figure 4.2. Comparison between acidification degree (x-axis) and ammonification (y-axis) during continuous fermentation of casein (● pH 5; ● pH 7 (**Chapter 3**); ● pH 9) and gelatin (■ pH 5; ■ pH 7 (**Chapter 3**); ■ pH 9).

Casein was acidified to a greater extent than gelatin at all tested pH. It was consequently hypothesised that the higher overall conversion of casein was due to a more balanced amino acid profile, whereas gelatin composition partially hindered the process, due to the dominance of glycine (Gly) in its profile (**Chapter 2**). Regardless of the difference in AA profile, the highest acidification degrees were achieved at neutral conditions (40-50%) while acid pH values negatively affected protein conversion to VFAs (20-30%). The effect of alkaline conditions differed depending on the substrate: casein degradation was similar as in neutral conditions while gelatin conversion was strongly hindered (<20%). All these results are consistent with literature, as other authors (Breure & van Andel, 1984; Yu & Fang, 2003) observed lower acidification degrees at acid conditions (≤ 5.5). No specific information was found regarding alkaline pH effect on protein fermentation.

Trying to explain the abovementioned results, batch experiments were conducted to assess pH effect on the acidification degree of casein and gelatin, with a minimum run time of 96 hours. Overall, a greater protein conversion to VFAs was achieved with the batch configuration compared to the CSTR results (Figure 4.3). The only exceptions were casein batch fermentation at pH 7 and 9, as the final values were comparable with what was observed for the continuous experiments (Figure 4.3a and b). While these differences might be justified by kinetic limitations, it appears that they are not only determined by the pH value but also by the protein composition. It was consequently hypothesised that the consumption of some AAs might be especially susceptible to the variations in environmental conditions. Besides, complete substrate conversion was never achieved, meaning that the limitations are not exclusively kinetic. It is unclear whether this was due to micronutrients deficiency and/or the limited biomass concentration (≤ 0.4 g VSS/L). Product inhibition, related to either VFA

and/or ammonia toxicity at high pH, was excluded considering previous results (**Chapter 3**) and the fact that fermentative bacteria can withstand both higher VFA titres and ammonification values (Shi et al., 2016; Domingos et al., 2017).

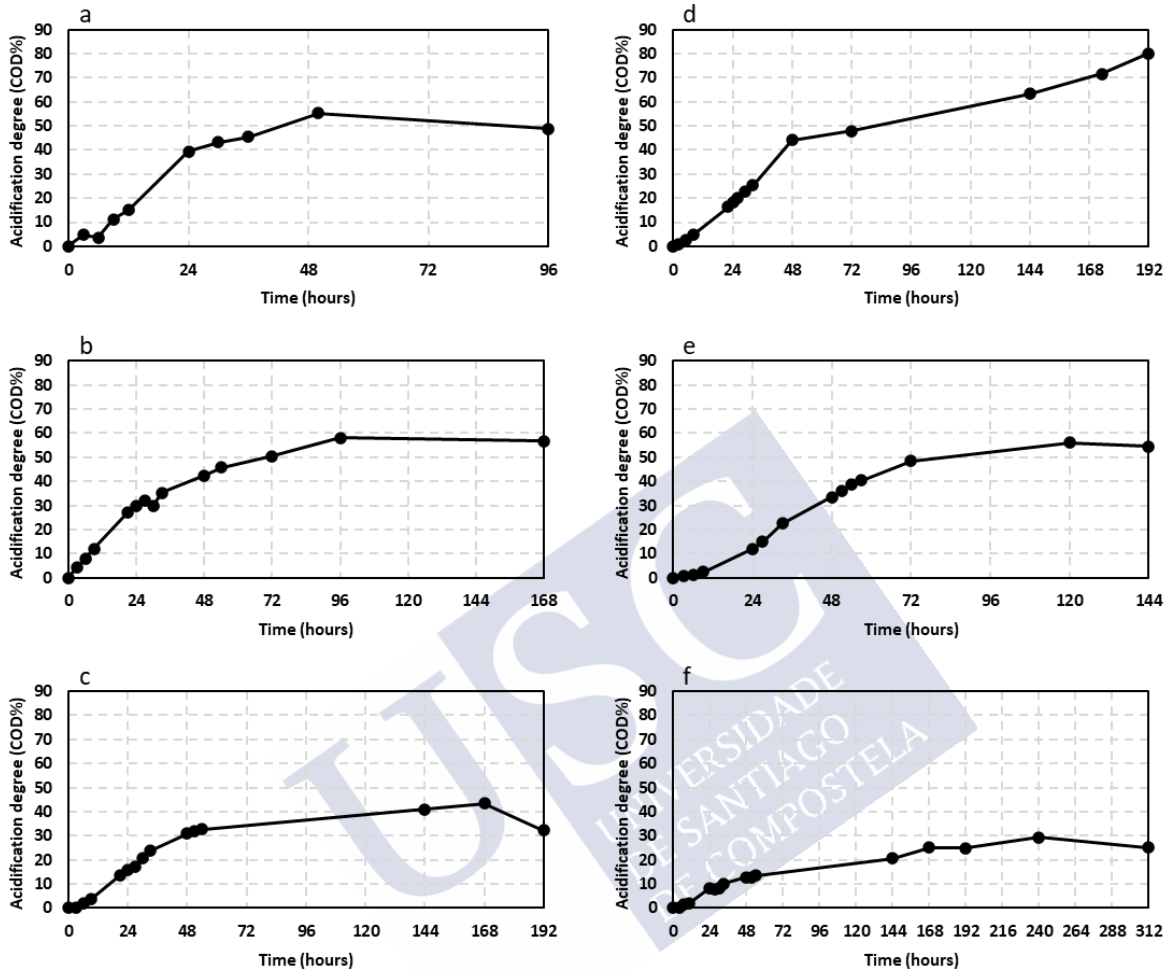

Figure 4.3. Acidification degree evolution over time during batch fermentation tests (a: casein pH 7 (**Chapter 3**); b: casein pH 9; c: casein pH 5; d: gelatin pH 7 (**Chapter 3**); e: gelatin pH 9; f: gelatin pH 5)

4.3.2 Influence of pH on VFA selectivity

Individual VFA production showed greater fluctuations in comparison with the acidification degree, especially during the first days after pH change (Figure 4.4 and 4.5). Acetic acid was the major product regardless of the protein composition and pH value, with the exception of casein fermentation at acid conditions (Figure 4.4c and f), which led to comparable concentrations of n-butyric acid and both forms of valeric acid. To evaluate pH influence on VFA selectivity, at least the last 40 days of each experimental phase were selected as stable periods. The main criterion used for this selection was that the coefficients of variation of the VFA relative molar fractions must be lower than 15%.

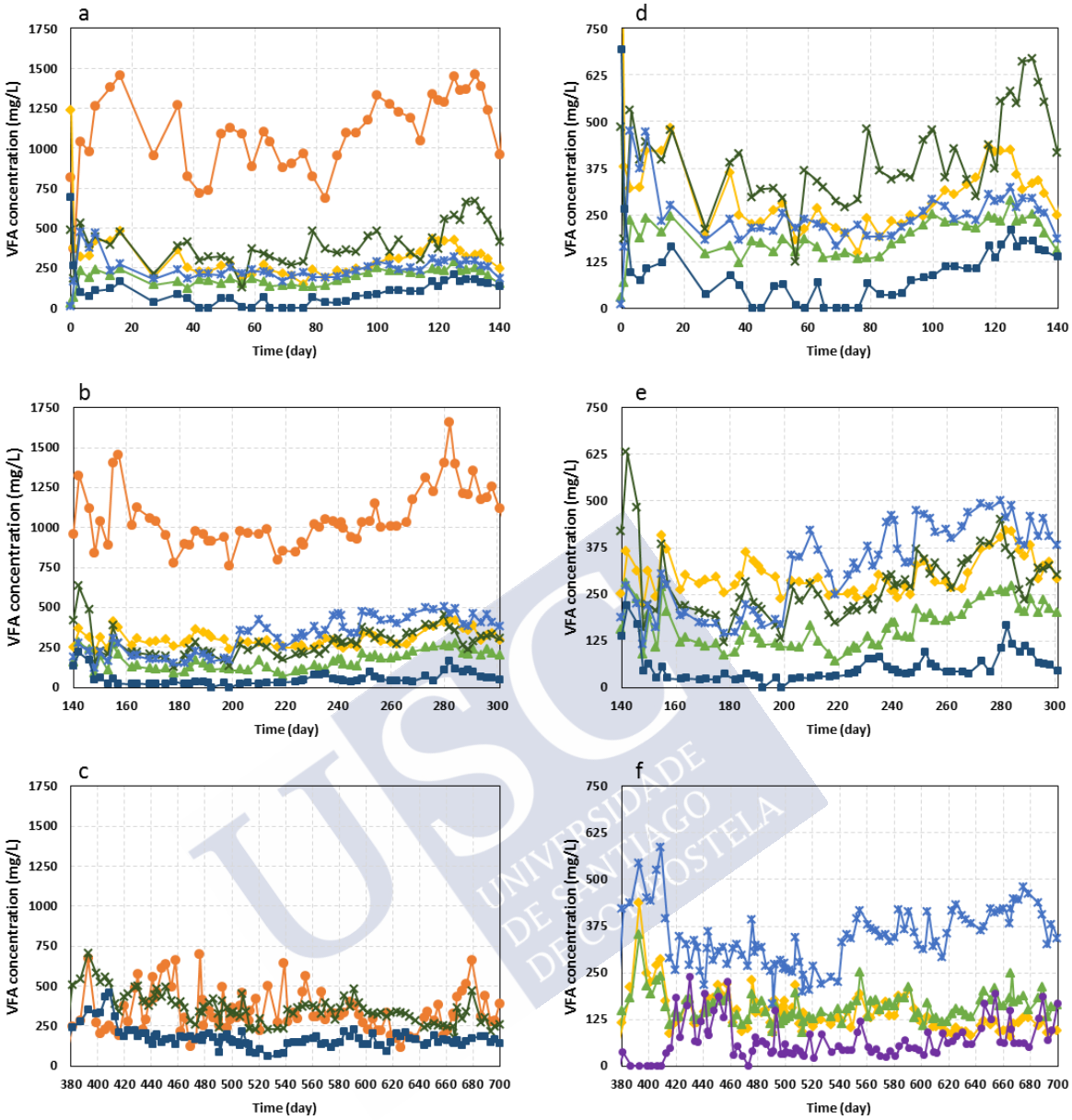

Figure 4.4. Individual VFA concentrations in the casein reactor at the different pH setpoints (a,d: pH 7 (**Chapter 3**); b,e: pH 9; c,f: pH 5). ● Acetic; ◆ Propionic; ▲ Iso-Butyric; x n-Butyric; * Iso-Valeric; ■ n-Valeric, ● Iso-Caproic. Figures on the left focus on major products (axes to 1750 mg/L) while figures on the right focus on minor products (axes to 750 mg/L). The days between 300 and 380 correspond to a transition period at pH 7.

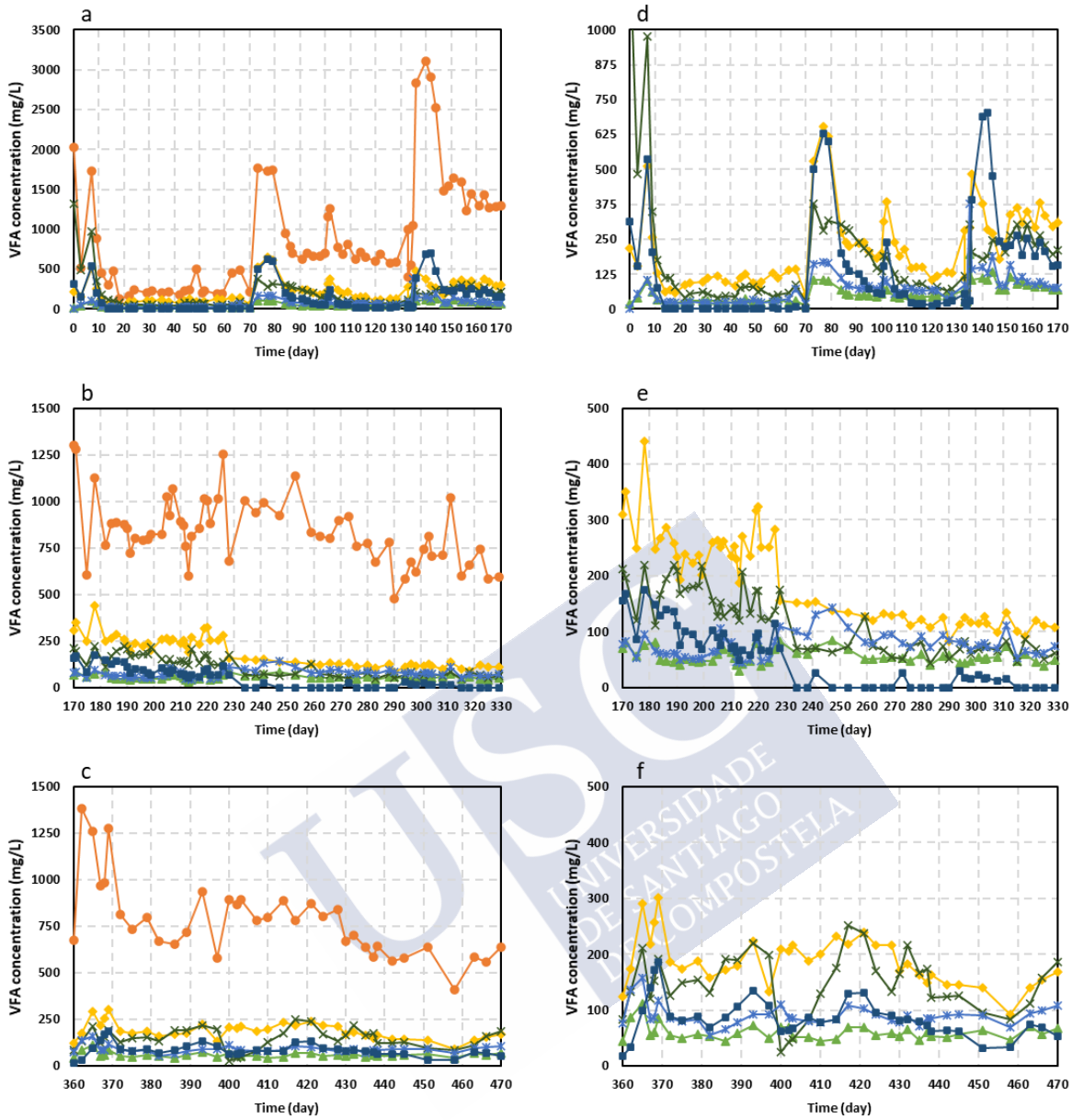

Figure 4.5. Individual VFA concentrations in the gelatin reactor at the different pH setpoints (a,d: pH 7 (**Chapter 3**); b,e: pH 9; c,f: pH 5). ● Acetic; ◆ Propionic; ▲ Iso-Butyric; x n-Butyric; * Iso-Valeric; ■ n-Valeric. Figures on the left focus on major products while figures on the right focus on minor products. The days between 330 and 360 correspond to a transition period at pH 7.

VFA selectivity (i.e. product spectra) was influenced by pH values (Figure 4.6), with acid conditions favouring the production of more reduced carboxylic acids, such as n-butyric and iso- and n-valeric acids in detriment of acetic acid, regardless of protein type. However, the extent of pH influence on the VFA selectivity depends on the protein composition itself, since the latter tendency was more pronounced in casein case, with an increase of 47% in n-butyric acid, 163% in iso-valeric acid and 133% in n-valeric acid. As a consequence, a drop of almost 50% in acetic acid was observed in comparison with the VFA spectrum at pH 7. Interestingly,

casein fermentation at low pH led to a stable production of iso-caproic acid (Figure 4.6a), which could be interpreted as a sink strategy for NADH consumption (Britz & Wilkinson, 1981), due to casein composition being dominated by electron donor AA (**Chapter 3**). In general, the greater production of reduced carboxylic acids at low pH might be justified by the fact that longer chained compounds are more easily excreted through the cell walls rather than the smaller ones (Duong et al., 2019). Another non-exclusive explanation is related to AAs uptake and consumption being affected by the change in pH. Smith and Macfarlane (1998), in fact, demonstrated how acid conditions appear to lower the uptake rates of most monopeptides while benefitting the assimilation of a selected few (e.g. serine and lysine). Still, the presented results were not always comparable with the ones found in literature. Low pH indeed tends to lower acetic acid molar fraction during gelatin fermentation, as determined in a couple of previous studies (Breure & van Andel, 1984; Yu & Fang, 2003), although the VFA benefitting the most from the shift to acid conditions was propionic acid, whose molar fraction remained stable with pH in the present study. In contrast, Duong et al. (2019) detected an increase in n-valeric production at low pH with gelatin, which in the present study was comparable in molar fraction to the one obtained at neutral conditions. Therefore, although pH might drive the consumption of AAs towards those which are related with the production of longer chain VFA, protein composition keeps playing an important role in shaping the product spectra, as it still determines the interaction between AAs (**Chapter 3**). In fact, the difference between the experimental results and the literature data might be explained by the variability of gelatin composition, as described in Regueira et al. (2020).

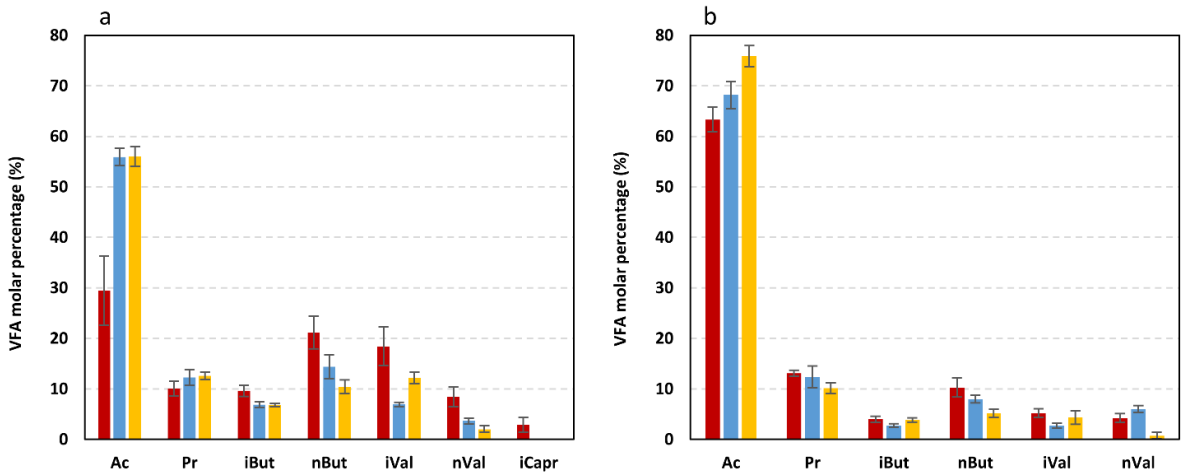

Figure 4.6. VFA spectra during casein (a) and gelatin (b) CSTR operation at different pH setpoints (■ pH 5; ■ pH 7 (**Chapter 3**); ■ pH 9). Ac: Acetic acid; Pr: Propionic acid; iBut: Iso-Butyric acid; nBut: n-Butyric acid; iVal: Iso-Valeric acid; nVal: n-Valeric acid; iCapr: Iso-Caproic acid.

The batch tests generally confirmed the selectivity tendencies related to the pH influence (Figure 4.7). Still, some specific differences between CSTR and discontinuous experiments can be detected depending on the protein. Firstly, casein batch fermentation at low pH further limited acetic acid production in favour of n-valeric acid and did not induce iso-caproic acid

generation (Figure 4.7a). It was hypothesised that n-valeric acid might have been formed through a chain elongation process, as an alternative NADH sink strategy to the iso-caproic one, given that casein fermentation is associated with the generation of an excess of reducing power due to its AA composition (**Chapter 3**). The detection of n-valeric acid condensation during the batch experiments might be related to longer reaction times and the sufficient accumulation of the required substrates (i.e. acetic and propionic acid), whose consumption was hinted between 24 and 48 hours of the batch test (Figure 4.8). Yet, the occurrence of chain elongation during the continuous experiments cannot be completely discarded, as the consumption of these short chain VFA cannot be easily detected at steady-state operation. Secondly, gelatin batch fermentation at alkaline conditions favoured acetic acid production over propionic acid (Figure 4.7b), suggesting that the greater acidification degree of the batch test in comparison with the CSTR results might have been associated with a greater consumption of AAs yielding acetic acid (e.g. Gly). Finally, n-valeric acid production was strongly hindered by the batch configuration at all tested pH, with at least a drop of 50% in molar fraction, probably due to bioenergetics constraints (Regueira et al., 2020; **Chapter 3**). In fact, one of its parent AA, arginine (Arg), can be alternatively converted to acetic acid, yielding more ATP in comparison to the metabolic pathway yielding n-valeric acid (Fonknechten et al., 2010).

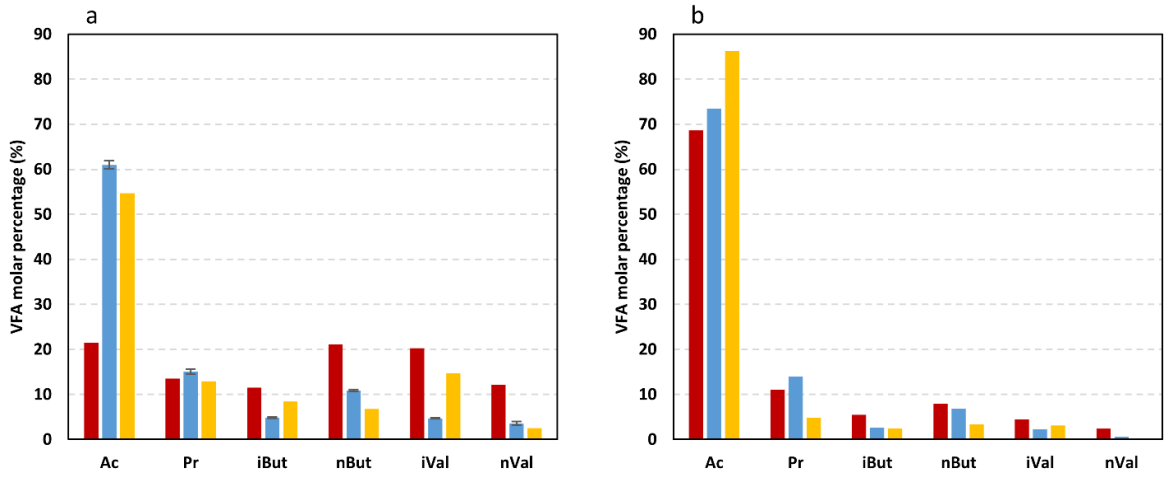

Figure 4.7. VFA spectra during batch experiments with casein (a) and gelatin (b) at different pH setpoints (■ pH 5; ■ pH 7 (**Chapter 3**); ■ pH 9). Ac: Acetic acid; Pr: Propionic acid; iBut: Iso-Butyric acid; nBut: n-Butyric acid; iVal: Iso-Valeric acid; nVal: n-Valeric acid.

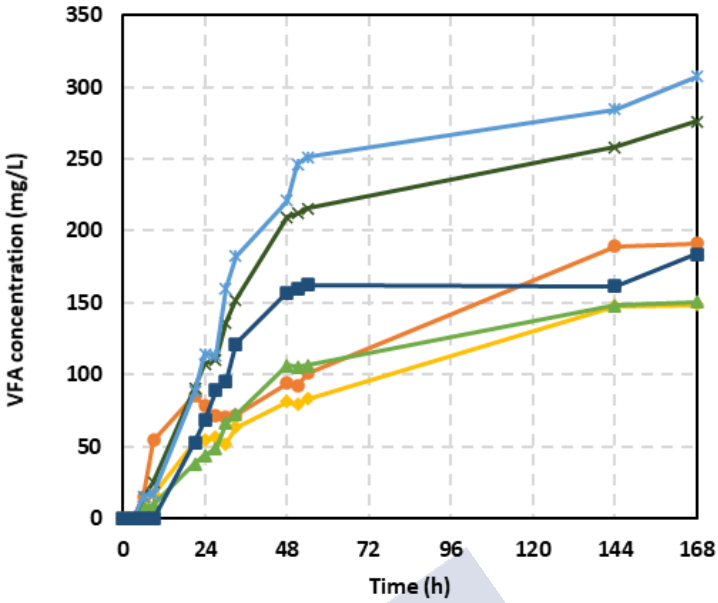

Figure 4.8. Individual VFA concentrations during casein batch fermentation at pH 5.

● Acetic; ◆ Propionic; ▲ Iso-Butyric; x n-Butyric; * Iso-Valeric; ■ n-Valeric

4.3.3 Influence of pH on specific amino acid consumption

To further understand the variations in VFA selectivity between acid, neutral and alkaline conditions, the individual AA consumptions were evaluated (Figure 4.9). Some general patterns were observed. Firstly, regardless of pH or protein composition, AA were not homogeneously consumed, with the bacteria preferring certain monopeptides over others. Secondly, AA consumptions followed the trends observed for acidification degrees, with overall higher values at neutral and/or alkaline pH. Finally, AA consumptions were generally higher for casein fermentation than for gelatin, regardless of the tested pH.

Also, some interesting specific behaviours are highlighted. Arg was the most consumed AA, taking into account the three pH values. This observation is compatible with what was observed by Smith and MacFarlane (1998) and might be justified by bioenergetics reasons (Fonknechten et al., 2010; Regueira et al., 2020). Moreover, Arg consumption followed the acidification degree variations described in section 4.3.1. Negatively charged amino acids (i.e. aspartic (Asp) and glutamic acid (Glu)) were not consumed at low pH, regardless of the considered protein. Thus, it was hypothesised that their isoelectric point, being close to pH 5, favoured their undissociated form, making them less accessible to the microbial population. Still, it is unclear why the consumption was hindered at alkaline pH during gelatin fermentation. It was also observed that tyrosine (Tyr) consumption appears to be feasible only at neutral conditions. This phenomenon might be due to its more complex nature (aromatic amino acid) and/or the related aromatic acid production, which show even higher toxicity than aliphatic VFA for anaerobic digestion microorganisms (Sabra et al., 2015).

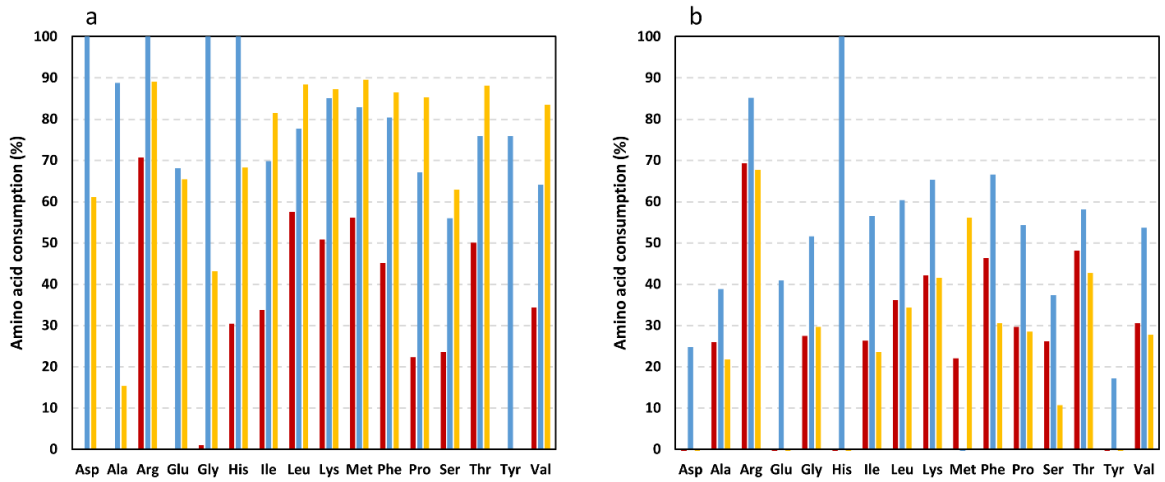

Figure 4.9. Amino acids consumption in casein (a) and gelatin (b) reactors at different pH values. (■ pH 5; ■ pH 7 (**Chapter 3**); ■ pH 9). Arg: Arginine; Ala: Alanine; Asp: Aspartic acid; Glu: Glutamic acid; Gly: Glycine; His: Histidine; Ile: Isoleucine; Leu: Leucine; Lys: Lysine; Met: Methionine; Phe: Phenylalanine; Pro: Proline; Ser: Serine; Thr: Threonine; Trp: Tryptophan; Tyr: Tyrosine; Val: Valine.

4.3.4 Influence of pH on Stickland reaction stoichiometry

Despite Stickland reactions being widely accepted as the main mechanism of AA conversion into VFA, it was already demonstrated that their fixed stoichiometry does not completely reproduce the outcome of proteins fermentation at neutral pH (**Chapter 3**). Moreover, there is some evidence suggesting that pH might affect this mechanism (Breure & van Andel, 1984; Yu & Fang, 2003). Therefore, this section analyses the influence of pH on the Stickland stoichiometry by establishing molar balances between influent and effluent concentrations of amino acids and VFA. Acetic acid was not considered as several AAs are responsible for its production.

Compatibly with what was observed for neutral conditions (**Chapter 3**), iso-butyric acid production followed the Stickland stoichiometry at pH 5 and 9, being its strict connection with valine consumption confirmed. In contrast, n-butyric acid production did not correlate with its parent amino acids (Glu, His, Thr, Lys) at pH 5 for both proteins and at pH 9 for casein (Figure 4.10). This imbalance between substrate and products at low pH might be either linked to other amino acids being converted to n-butyric acid through unconsidered metabolic pathway and/or to chain elongation between previously produced acetic acid and endogenous acetyl-CoA (Duncan et al., 2004). Conversely, alkaline conditions hindered n-butyric acid production during casein fermentation without affecting the parent AA consumption, meaning that alternative carboxylic acids might have been generated instead. Overall, n-butyric stoichiometry is not reliable outside the neutral pH range, as it underestimates its production at acid conditions and overestimates it at alkaline conditions.

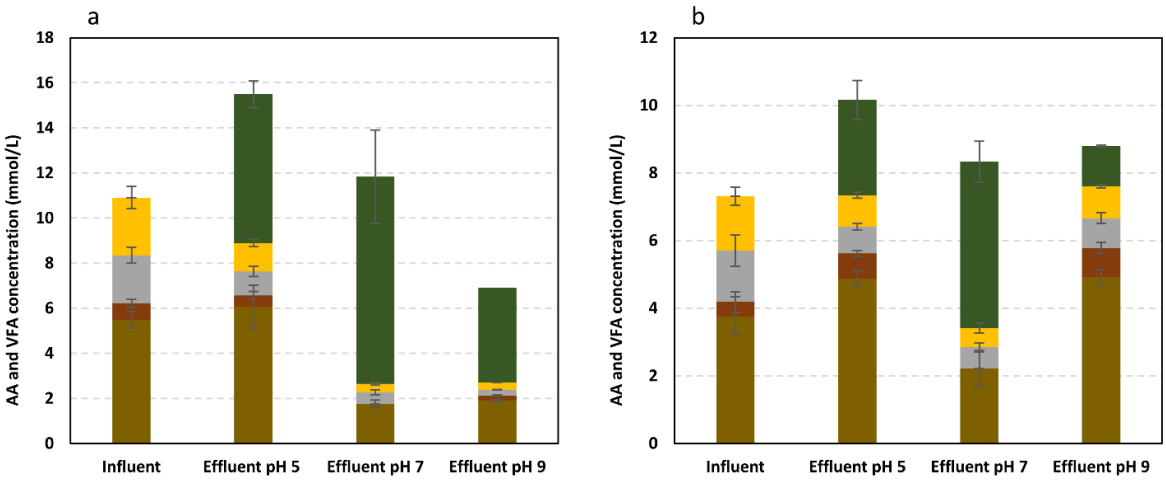

Figure 4.10. n-Butyric acid balance in the continuous casein (a) and gelatin (b) reactors. ■ Glutamic acid; ■ Histidine; ■ Threonine; ■ Lysine; ■ n-Butyric acid

Contrary to the results of pH 7, iso-valeric acid production fits Stickland stoichiometry at both pH 5 and 9 for both proteins, even when considering it together with iso-caproic acid production. However, propionic and n-valeric acid balances (jointly evaluated since both are produced at equal molar ratios from Arg and Pro, albeit propionic acid is also generated from methionine) differ depending on protein composition (Figure 4.11), although propionic acid was generally produced at higher concentrations than n-valeric acid in both cases. For casein fermentation, the results obtained at pH 5 and 9 are comparable with what was previously observed in the neutral range (**Chapter 3**), i.e. propionic acid production alone justifies amino acid consumption, suggesting that the production of n-valeric acid was potentially related to other amino acids degradation and/or to chain elongation processes (Figure 4.11a). Moreover, propionic acid production progressively increased with pH, while n-valeric acid production remained constant. Conversely, gelatin fermentation at both acid and alkaline conditions (Figure 4.11b) yielded less propionic acid than at neutral pH, and n-valeric acid production was either strongly inhibited or null, suggesting that the consumed AAs were either preferentially converted to propionic acid rather than to n-valeric acid or to different VFAs. As previously mentioned (section 4.3.2), Arg can in fact be degraded through an alternative pathway, which generates acetyl-CoA and Ala, and ultimately acetic acid (Fonknechten et al., 2010), resulting in a greater ATP yield than the one assumed by Ramsay and Pullammanappalil (2001), involving 5-aminovalerate and proline as intermediate compounds instead (Barker et al., 1987).

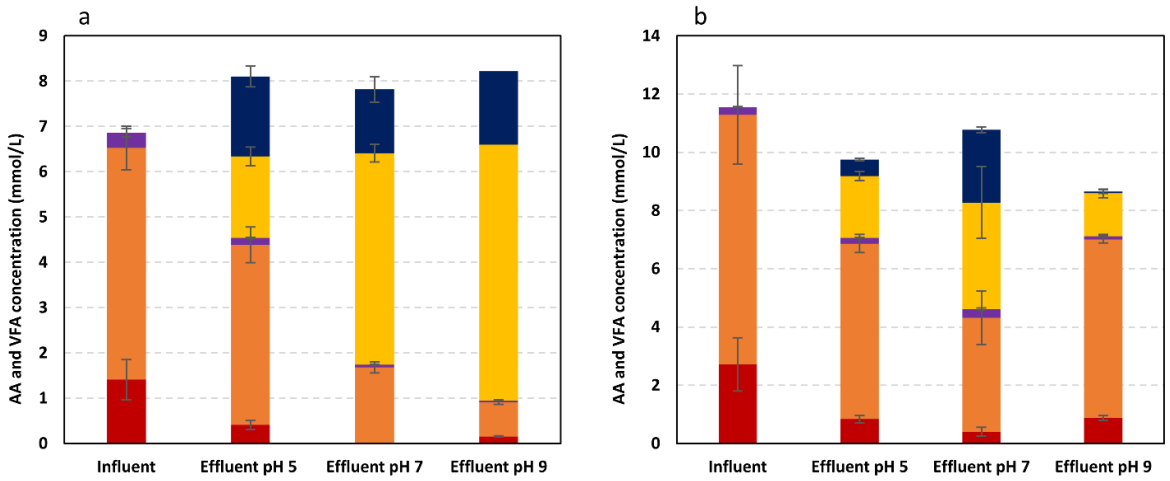

Figure 4.11. Propionic and n-Valeric acid balance in the continuous casein (a) and gelatin (b) reactors. ■ Arginine; ■ Proline; ■ Methionine; ■ Propionic acid; ■ n-Valeric acid

All these results demonstrated how the metabolic pathways of AAs fermentation into VFAs vary with protein composition and pH, with the observed deviations from the previously accepted stoichiometry being probably driven by bioenergetics constraints. A change in pH has, in fact, the potential of modifying the energetics (i.e. the ATP generated) of some amino acid pathways as described by Regueira et al. (2020), in turn affecting the interaction between them. Additionally, shifting pH might limit AAs consumption by either affecting the transportation mechanisms (Chen & Chung, 2015) and/or directly altering AA bioavailability through changes in molecular charge and subsequent precipitation (Tseng et al., 2009).

4.4 CONCLUSIONS

This work describes the effect of pH on amino acid conversion mechanisms during protein MCFs. It is demonstrated that pH affects the stoichiometry of AAs conversion into VFAs, by altering AA interactions and/or molecular configuration. Neutral pH maximises AA degradation, while a more diverse product spectrum is obtained at acid conditions, regardless of protein composition. Yet, those trends are more evident in casein fermentation. Therefore, the outcome of protein-rich substrates fermentation depends mainly on protein composition and pH.

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CHAPTER 5

UNDERSTANDING THE EFFECT OF TRACE ELEMENTS SUPPLEMENTATION ON VOLATILE FATTY ACIDS PRODUCTION FROM PROTEINS

SUMMARY

This chapter investigates the impact of trace elements supplementation on protein fermentation into volatile fatty acids. Two continuous reactors were operated with two model proteins (casein and gelatin) at two different pH conditions (5 and 7), with the addition of macro and micronutrients. The latter increased the acidification degree of both proteins to a significant extent only at pH 7, which was consistent with a greater amino acid consumption at neutral conditions. Furthermore, trace elements modified the process selectivity, promoting valeric acids production and other variations dependant on protein composition. Isomerisation and chain elongation processes were identified as a consequence of the observed deviations between amino acid consumption and VFA production. Overall, this chapter demonstrates that the supplementation of micronutrients can be useful to enhance and steer the anaerobic fermentation of protein-rich streams.

This chapter was redrafted after the following **under-review** publication:

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5.1 INTRODUCTION

In the past years, many studies have been focusing on improving the anaerobic digestion performance by adding selected micronutrients (Pobeheim et al., 2010; Schmidt et al., 2014; Wei et al., 2014), considering that many industrial sidestreams do not feature sufficient concentrations of these compounds to be suitable as feedstock for anaerobic bioprocesses (Fermoso et al., 2008; Zandvoort et al., 2006; Ezebuiro & Körner, 2017). The addition of certain trace metals appears to lead to an increase in methane yields (Choong et al., 2016). Nickel (Ni), cobalt (Co), zinc (Zn), copper (Cu) and iron (Fe) have been reported to enhance methanogenesis, being essential for archaea cell growth and metabolism (Lin et al., 1990; Zhang et al., 2003; Ezebuiro et al., 2018). However, it should be also noted that above certain threshold concentrations the same compounds can become toxic (e.g. Co $\geq$ 70 mg/L), thus inhibiting the process (Choong et al., 2016).

The impact of trace element supplementation on the fermentation step has been studied as well, generally using model carbohydrates (e.g. glucose) and sugar-rich substrates. Kim et al. (2003) demonstrated how Fe, Co and Ni addition favours the hydrolysis of synthetic sludge, thus increasing the volatile fatty acids (VFAs) production from the greater COD solubilisation. Micronutrients can be also used to selectively steer the acidogenic process, as described in Dahiya et al. (2020): supplementing Co (6.3 mg/L) and Zn (10.4 mg/L) in the culture media resulted in higher titres of propionic acid during glucose fermentation. Yu and Fang (2001) obtained comparable results fermenting dairy wastewaters with Zn concentrations lower than 10 mg/L, which not only promoted propionic acid production but also increased the overall conversion of the substrate to VFAs. Cu supplementation promoted propionic acid and ethanol production during sucrose fermentation (Han et al., 2014) in detriment of n-butyric acid formation. Contrasting results were achieved by Zheng and Yu (2004), who observed how increasing concentrations of Cu (0 – 400 mg/L) and Zn (0 – 500 mg/L) have a positive effect on n-butyric production from glucose, although slightly hindering propionic production in the process.

On the contrary, there is limited knowledge on the micronutrients requirement for the fermentation of proteins, albeit being a relevant fraction of many potential feedstocks (e.g. slaughterhouse waste (Escudero et al., 2014)). Moreover, little information is available on the role of trace elements in the conversion of amino acids (AAs) to VFAs and how they can affect the underlying mechanisms. AAs are, in fact, converted to VFA through a complex array of reactions (Ramsay & Pullammanappallil, 2001; Regueira et al., 2020), but also directly uptaken for biomass growth, requiring a wide selection of enzymes and cofactors. For example, selenium (Se) is required for the production of glycine reductase (Dürre & Andreesen, 1982), whose presence is fundamental for the conversion of this AA to acetic acid. Besides, several AAs feature pyruvate as a conversion reaction intermediate (Regueira et al., 2020), meaning that many cofactors normally associated with increased VFA production from glucose, such as Cu and Zn (Zheng & Yu, 2004), could be beneficial for protein fermentation.

Thus, the main objective of this investigation is to understand how amino acid conversion to VFA is affected by the presence of micronutrients during MCF processes. The extent of this effect is also evaluated for different protein composition (i.e. amino acid profiles) and pH levels. Molar balances were established between the production of VFA and the consumption of AAs in order to better explain the variations in acidification degree, productivity and VFA selectivity. All this knowledge is useful in determining whether trace elements supplementation can be applied as an operational strategy to optimize the fermentation of protein-rich sidestreams.

5.2 MATERIALS AND METHODS

The two CSTRs used in **Chapter 4** to study the effect of pH on protein fermentation were maintained operational at a hydraulic retention time of 1.5 d and an organic loading rate of 5.33 g COD/L·d, with continuous nitrogen sparging and magnetic stirring. Micronutrients were supplemented to both reactors as described in **Chapter 2** (Section 2.1.2.)

Analysis of the influents and the effluents of the reactors was performed according to the methods and the calculations described in **Chapter 2.** COD (total and soluble) and solids content were determined once per week while pH was continuously checked through the multiparametric analyser. Individual VFA concentrations and total ammonia nitrogen (TAN) were measured twice per week. Amino acid content of the reactors effluent was measured on selected samples from steady state periods of operation.

Biomass yield and activity were calculated to better understand how the presence of micronutrients affect the microbial communities responsible for the conversion of proteins to VFA. These two parameters were respectively expressed as follows:

$$Biomass\ yield\ (g\ CODbm/g\ CODpr\ consumed) = \frac{\mathrm{C_{biomass}}}{\mathrm{C_{pr\ consumed}}} \quad (13)$$

Where $C_{biomass}$ is the concentration of biomass (in g COD_{bm}/L) measured in the effluents, and $C_{pr\ consumed}$ is the concentration of protein consumed (in g COD_{pr}/L), estimated from the ammonification parameter.

$$Biomass\ activity\ (g\ COD - VFA/g\ VSS \cdot d) = \frac{\Sigma\ \mathrm{C}_{VFA}}{X_{\mathrm{biomass}} \times HRT} \quad (14)$$

where C_{VFA} stands for the total concentration of the measured VFAs (in g COD-VFA/L) and $X_{biomass}$ for the total biomass concentration (in g VSS/L) in the effluent of the reactors. HRT refers to the hydraulic retention time, expressed in days, which is considered to be equal to the solid retention time as a continuous stirred tank reactor was used.

Given that the AAs stoichiometry proposed by Ramsay and Pullammanappallil (2001) was found to be inaccurate depending on which VFA pathways are considered (**Chapter 3**) and on the operational conditions applied to the fermentation process (**Chapter 4**), a new prediction model for proteins was developed (Regueira et al., 2020) and consequently applied to the data analysis presented in this chapter. The catabolism of glycine was updated to include, apart

from its reduction to acetate, its conversion to CO_2 and ammonia with concomitant ATP generation by direct oxidation with NAD^+, via the glycine cleave system (Sangavai & Chellapandi, 2017). The overall stoichiometry varies according to the operational conditions and the AA profile of the protein considered (Table 5.1).

Table 5.1. Stoichiometry of AA conversion to VFAs for the casein and gelatin peptones used in the present study (Regueira et al., 2020). The stoichiometric coefficients are expressed in molar basis, assuming a value equal to 1.0 for each AA. Bold characters indicate relevant differences for the AA pathways between the two proteins.

Amino acid	Casein pathway	Gelatin pathway
Ala	**0.9 Acetic + 0.1 Propionic**	**1.0 Acetic**
Arg	1.0 Acetic + 1.0 Alanine	1.0 Acetic + 1.0 Alanine
Asp*	1.0 Propionic	1.0 Propionic
Cys	Acetic	Acetic
Glu*	1.0 Acetic + 0.5 n-Butyric	1.0 Acetic + 0.5 n-Butyric
Gly	**1.0 Acetic**	**0.8 Acetic + 0.4 CO_2**
His	1.0 Acetic + 0.5 n-Butyric	1.0 Acetic + 0.5 n-Butyric
Ile	1.0 Iso-Valeric	1.0 Iso-Valeric
Leu	1.0 Iso-Valeric	1.0 Iso-Valeric
Lys	1.0 Acetic + 1.0 n-Butyric	1.0 Acetic + 1.0 n-Butyric
Met	**1.0 n-Butyric**	**1.0 Propionic**
Pro	0.5 Acetic + 0.5 Propionic + 0.5 n-Valeric	0.5 Acetic + 0.5 Propionic + 0.5 n-Valeric
Ser	Acetic	Acetic
Thr	**Propionic**	**0.5 n-Butyric + 1.0 Glycine**
Val	1.0 Iso-Butyric	1.0 Iso-Butyric

* Aspartic and glutamic acid respectively include asparagine and glutamine fermentation.

5.3 RESULTS AND DISCUSSION

5.3.1 Influence of micronutrients on reactors performance

Casein and gelatin reactors were continuously operated for 120 and 160 days in total (Figure 5.1), with more than 40 days at each pH value, ensuring steady state was achieved. Micronutrients presence accelerated reactor stabilisation after pH shift, reducing the acclimation time from 100+ days (**Chapter 4**) to approximately 15 days (10 HRT times). In order to evaluate the influence of micronutrients on protein fermentation, steady-state periods were selected for each experimental phase (Figure 5.1): days 11-42 and 65-120 for casein at pH 5 and 7, respectively; and days 31-91 and 122-161 for gelatin at pH 7 and 5, respectively.

No differences between influent and effluent total COD concentrations were observed in any experimental phase (Figure 5.1a and b), discarding the occurrence of methanisation. Higher VFA production was observed at neutral conditions for both proteins (≈5.5 g COD/L) than at the acid ones (≤3.0 g COD/L), as previously observed without micronutrients supplementation (**Chapter 4**). Acetic acid was the major product, with the exception of casein fermentation at pH 5, while the concentration of the other acids depended on both the protein composition and the operational pH. For example, propionic acid was the second major product of casein fermentation at pH 7 but was the minor product at pH 5 (Figure 5.1c). A similar pattern was observed for n-valeric acid during gelatin fermentation (Figure 5.1d). Medium chain VFAs were only detected during casein fermentation at rather low and variable concentrations (≤100 mg/L). In particular, iso-caproic acid formation was observed during pH 5 operation period, whereas the linear form (n-caproic acid) was measured at neutral conditions (data not shown). No secondary metabolites were ever detected, regardless of protein composition and pH conditions.

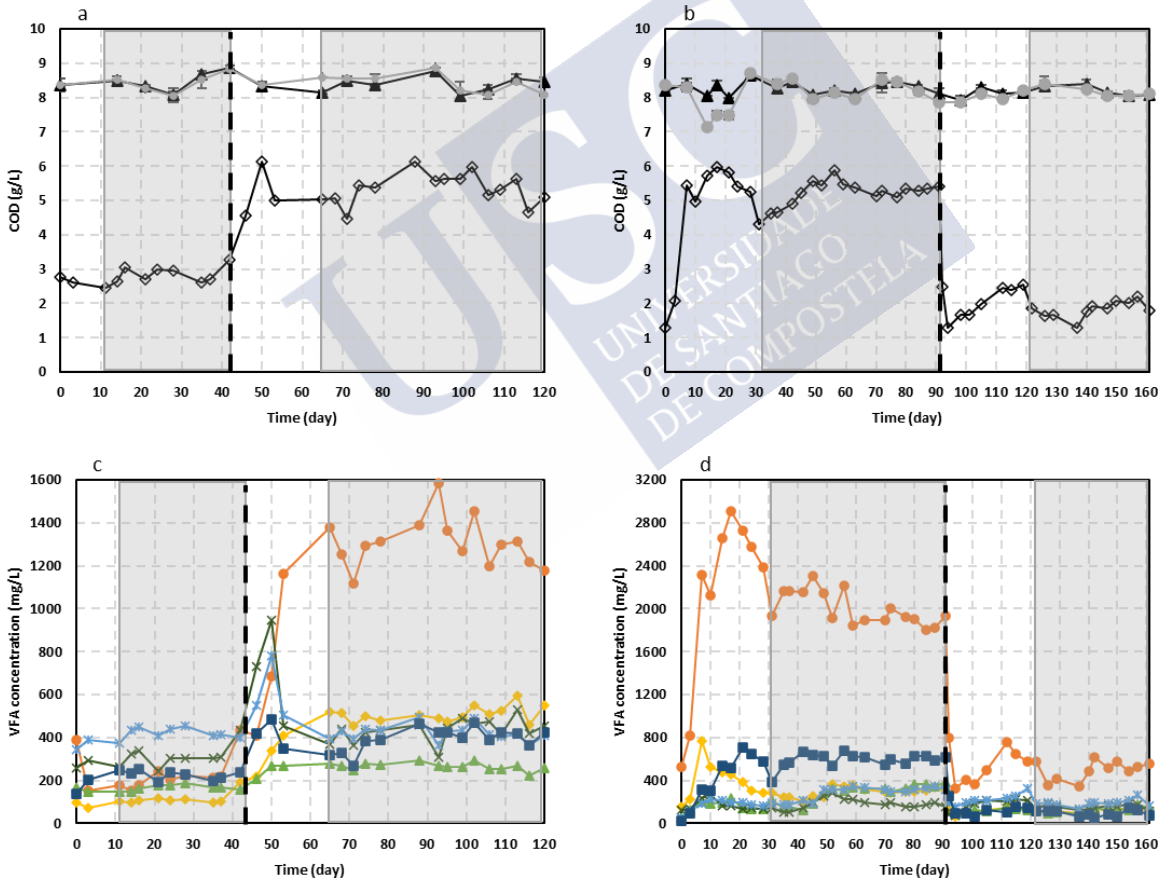

Figure 5.1. COD balance (a, casein; b, gelatin: ▲ Influent total COD; ● Effluent total COD; ◇ VFAs COD) and individual VFA concentrations (c, casein; d, gelatin: ● Acetic; ◆ Propionic; ▲ Iso-Butyric; x n-Butyric; * Iso-Valeric; ■ n-Valeric) in the reactors. The vertical segmented lines represent the pH shifts, from 5 to 7 in casein reactor (a, c) and from 7 to 5 in gelatin reactor (b, d), with the shadowed areas corresponding to the selected steady-state periods.

5.3.2 Influence of micronutrients on substrate conversion and biomass growth

The acidification degrees of both proteins increased due to the supplementation of trace elements (Figure 5.2a and b). However, this positive effect was only significant at pH 7, especially for gelatin, whose acidification degree shifted from 40 to 62%. The latter indicates that trace elements effect is conditioned by both the pH conditions and the AA composition of the substrate. In fact, the variation in acidification degree is significant only at neutral conditions, to a greater extent for gelatin than for casein fermentation. Interestingly, the inhibition exerted by the acid conditions on the reactors operation outweighs the benefit posed by the micronutrients presence, discarding their supplementation as a viable strategy to increase the VFA production at low pH. Complete conversion of the proteins to VFAs was never achieved, which is consistent with previous studies (Duong et al., 2019; **Chapter 3**). The similar pattern observed for the ammonification percentage (Figure 5.2c and d) confirms that all the consumed substrate was indeed transformed into VFAs.

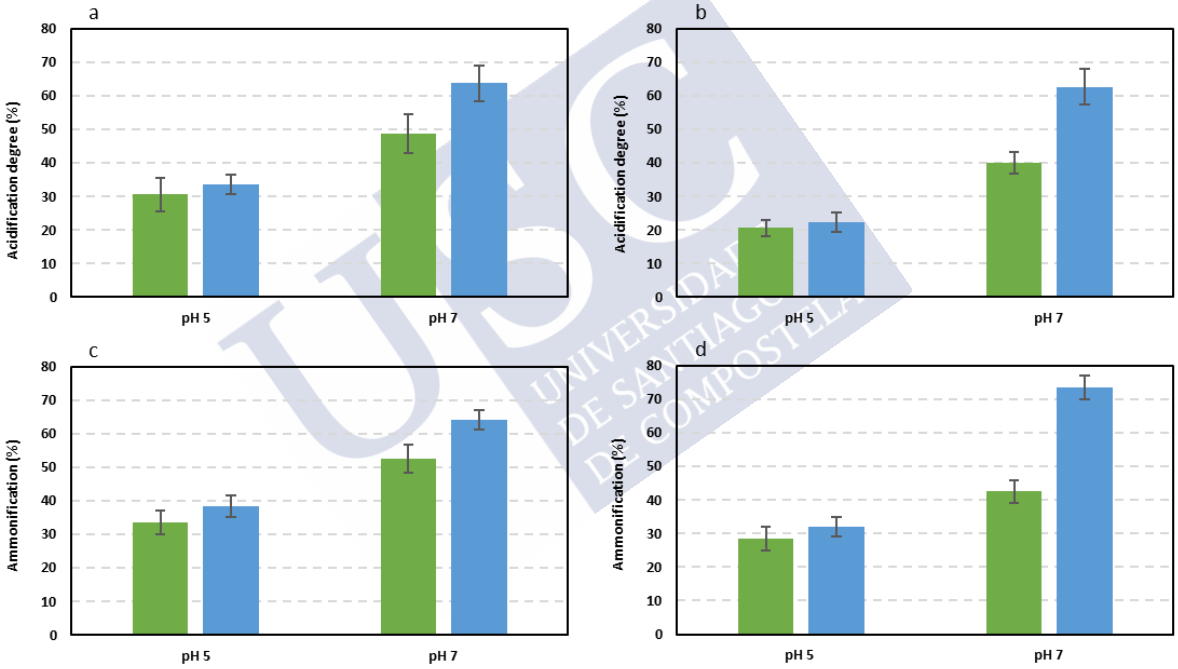

Figure 5.2. Average acidification degree and ammonification achieved during casein (a, c) and gelatin (b, d) fermentation with (■) and without (■) micronutrients.

Conversely, the effect of trace elements on the biomass growth is more generalised, as the protein composition and the operational pH does not seem to limit its extent. Micronutrients presence had a positive effect on the biomass growth (Figure 5.2a and b), with increases in biomass concentrations above 40%. The biomass yields (Figure 5.3a and b) increased compatibly, suggesting that the bacteria were more efficient at harvesting energy from the substrate for duplication purposes and/or at using the energy from catabolism in building new biomass. The only exception detected concerns gelatin fermentation at pH 7, being the values comparable with and without trace elements. In contrast, biomass activities were lower than without micronutrients supplementation (Figure 5.3c and d), since the generalised increase in biomass concentration was not met with a similar growth in VFA

production. Only gelatin fermentation seemed to maintain a comparable biomass activity at neutral conditions, which would explain the similar increase observed in biomass concentration (+55.0%) as in protein conversion to VFAs (+56.5%). This exception could be due to gelatin being almost completely consumed, leaving a residual fraction of AA whose energetic yield is too low to make their uptake and utilisation feasible.

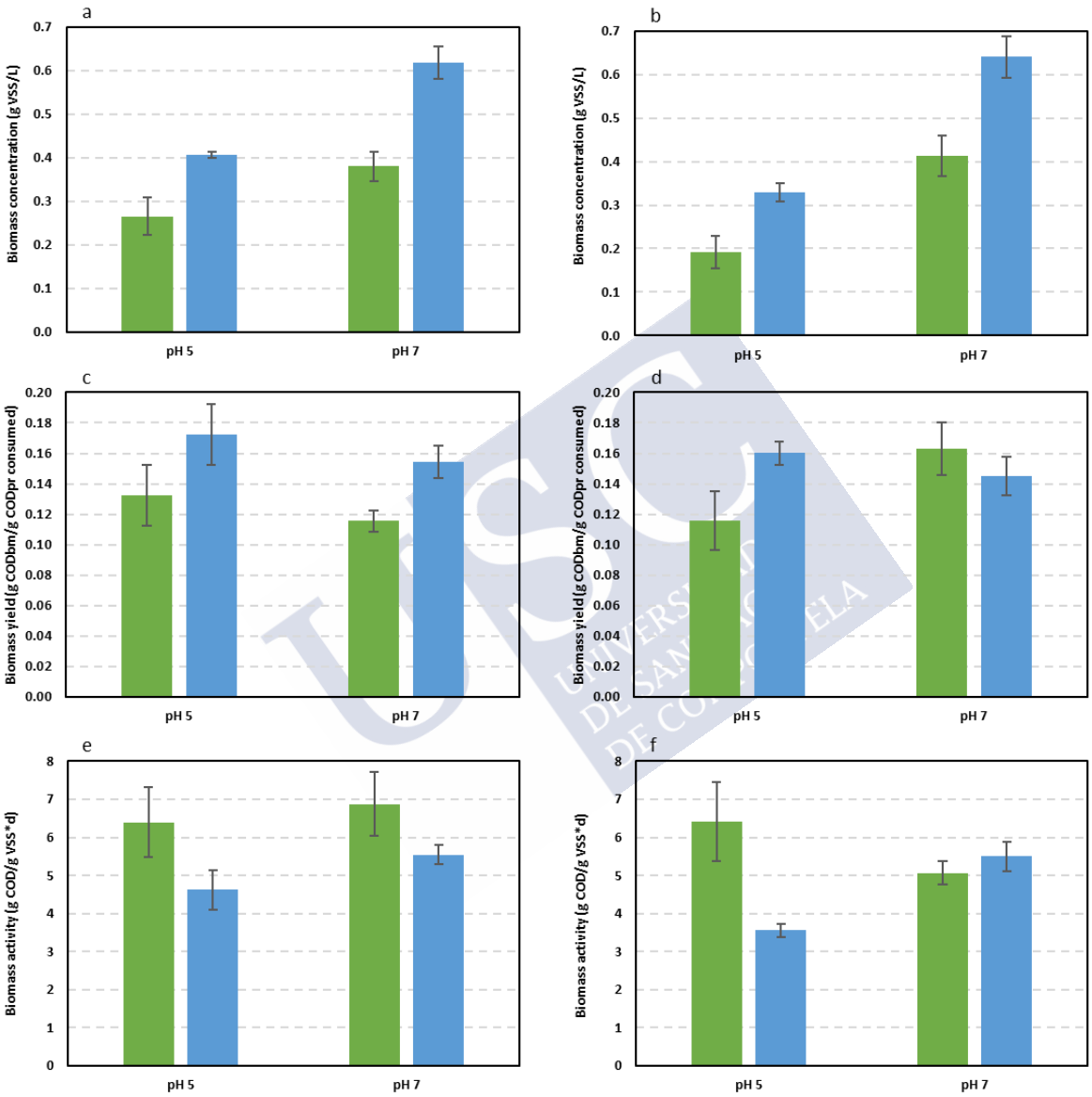

Figure 5.3. Average biomass concentration, biomass yield and biomass activity achieved during casein (a, c, e) and gelatin (b, d, f) fermentation with (■) and without (■) micronutrients supplementation.

In general, the supplementation of trace elements is more benefitial to the biomass growth rather than the production of VFA itself. Still, the two processes are interconnected, given that greater biomass concentrations can allow for greater substrate conversion and viceversa, making it difficult to discern whether the supplementation affects either the anabolism or the catabolism. More specifically, the supplementation of micronutrients might

increase the energetic yield of AA conversion by enhancing the activity of the enzymes involved in catabolism and consequently reducing the associated energetic cost of enzyme production. Likewise, a net increase of harvested energy could be then used by the biomass for replication purposes, albeit it cannot be completely excluded that the presence of micronutrients could be improving biomass yields more directly, i.e. in anabolic reactions. The exception of gelatin neutral fermentation does not contradict this hypothesis, since the conversion of the residual AAs is not energetically viable for the microbial population, as previously mentioned.

5.3.3 Influence of micronutrients on amino acid consumption and VFA selectivity

The individual AA consumption (Figure 5.4) confirmed the patterns observed in the previous section. At neutral conditions, the trace elements supplementation led to a generalised increase in AA utilisation, which was proportional to their abundance in the feedstock. As observed for the acidification degree, the effect was more relevant for gelatin (Figure 5.4b) than for casein (Figure 5.4a), as glycine (Gly) consumption experienced a 2-fold increase due to micronutrients presence. Conversely, neither significant variations nor patterns were observed in AA consumptions at low pH (Figure 5.4c and d), confirming that the limitations exerted by the acid conditions outweighs the beneficial effects associated with the presence of trace elements.

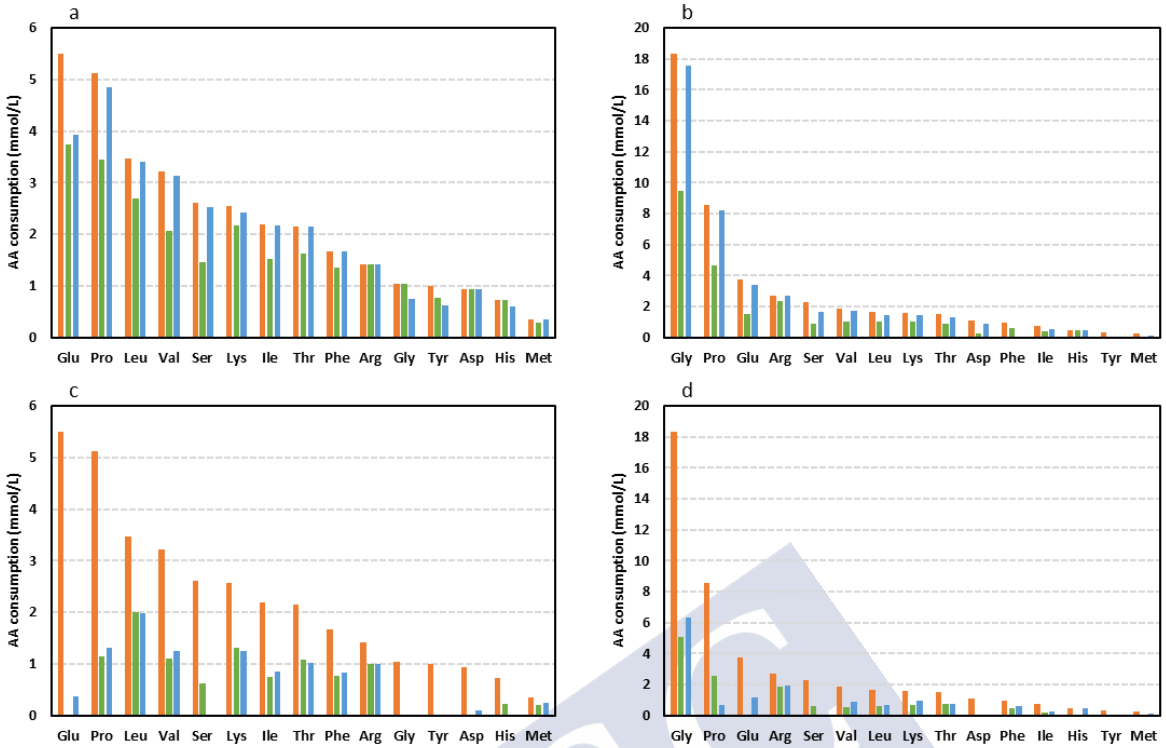

Figure 5.4. Average amino acids consumption during casein (a: pH 7; c: pH 5) and gelatin (b: pH 7; d: pH 5) fermentation with (■) and without (■) micronutrients supplementation. ■ Feedstock amino acid concentration. No data of alanine consumption is available, whereas the absence of bars indicates that no specific consumption was observed for a given amino acid. Arg: Arginine; Asp: Aspartic acid; Glu: Glutamic acid; Gly: Glycine; His: Histidine; Ile: Isoleucine; Leu: Leucine; Lys: Lysine; Met: Methionine; Phe: Phenylalanine; Pro: Proline; Ser: Serine; Thr: Threonine; Trp: Tryptophan; Tyr: Tyrosine; Val: Valine.

The VFA selectivity of the fermentation process at neutral conditions was affected as well (Figure 5.5). Trace element addition promoted the formation of both forms (i.e. branched and linear) of valeric acid (Figure 5.5c and d), which was more marked in gelatin case (Figure 5.5d). Other variations in VFA production were dependent on the protein composition. For example, acetic and iso-butyric acid formation increased only for gelatin fermentation (Figure 5.5b and d), whereas propionic and n-caproic acid production was favoured only during casein conversion (Figure 5.5c).

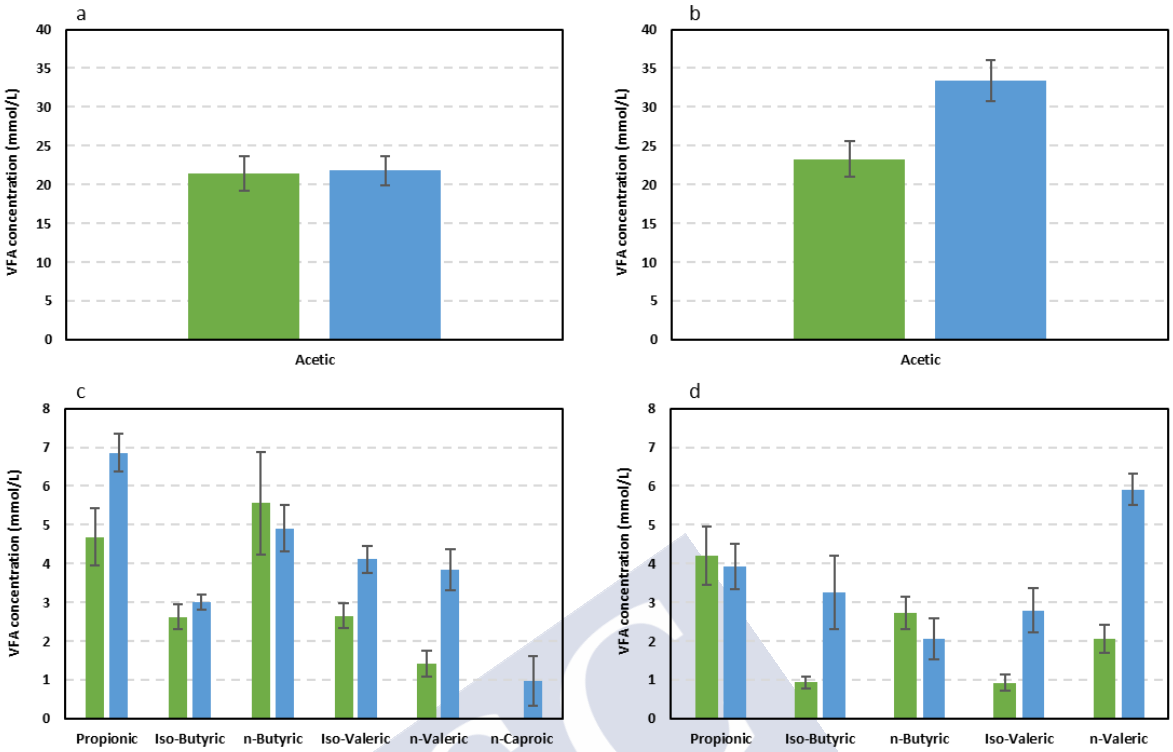

Figure 5.5. Average individual VFA production during casein (a, c) and gelatin (b, d) fermentation with (■) and without (■) micronutrients supplementation.

The positive effect of the trace elements supplementation on acetic acid production from gelatin (+10.1 mmol/L) was mainly justified by Gly increased conversion (+8.10 mmol/L), together with the contribution of glutamic acid (Glu) and proline (Pro). They also promoted the consumption of both Pro and threonine (Thr) during casein fermentation (+1.40 mmol/L), which explains the increased propionic acid formation (+1.7 mmol/L).

In contrast, the increased production of valeric acids and n-butyric acid is not justified by an increased consumption of the precursor AA (Figure 5.6). The supplementation of trace elements led to a greater iso-butyric production than what is theoretically possible from valine (Val) consumption alone (Figure 5.6a). However, the overall butyric acid balance appears to be closed, suggesting that isomerisation of n-butyric to iso-butyric acid might be occurring as a way of reducing the toxicity in the fermentative environment (Chen et al., 2017; Petrognani et al., 2020). Similarly, n-valeric acid production during casein fermentation did not match proline consumption (Figure 5.6b), while iso-valeric acid was underproduced. Interestingly, the isomerisation appears to be driving the interconversion in favour of the linear form rather than the branched one. In this case, however, the process seems to favour an equal concentration repartition between the two forms, rather than aiming to reduce the toxicity of the mixture. The fact that the trace elements supplementation promotes isomerisation is explained by a specific cofactor requirement of the associated enzymes. Both isobutyryl-CoA and isovaleryl-CoA, which catalyse the interconversion between iso and n-acids (Cracan &

Banerjee, 2012), have their activity completely depending on Co ions presence, a compound which is indeed included in the mix of micronutrients supplemented during the present study.

The pattern is different during gelatin fermentation, since both forms of valeric acid were produced to a greater extent than what was expected based on the assumed stoichiometry (Figure 5.6c). Although isomerisation cannot be completely discarded, it was hypothesised that the overproduction of these two VFAs might be due to elongation processes. This result is compatible with the n-caproic formation observed during casein fermentation, suggesting that the feasibility and the selectivity of the elongation reactions at neutral conditions depend on the trace elements supplementation and the protein composition.

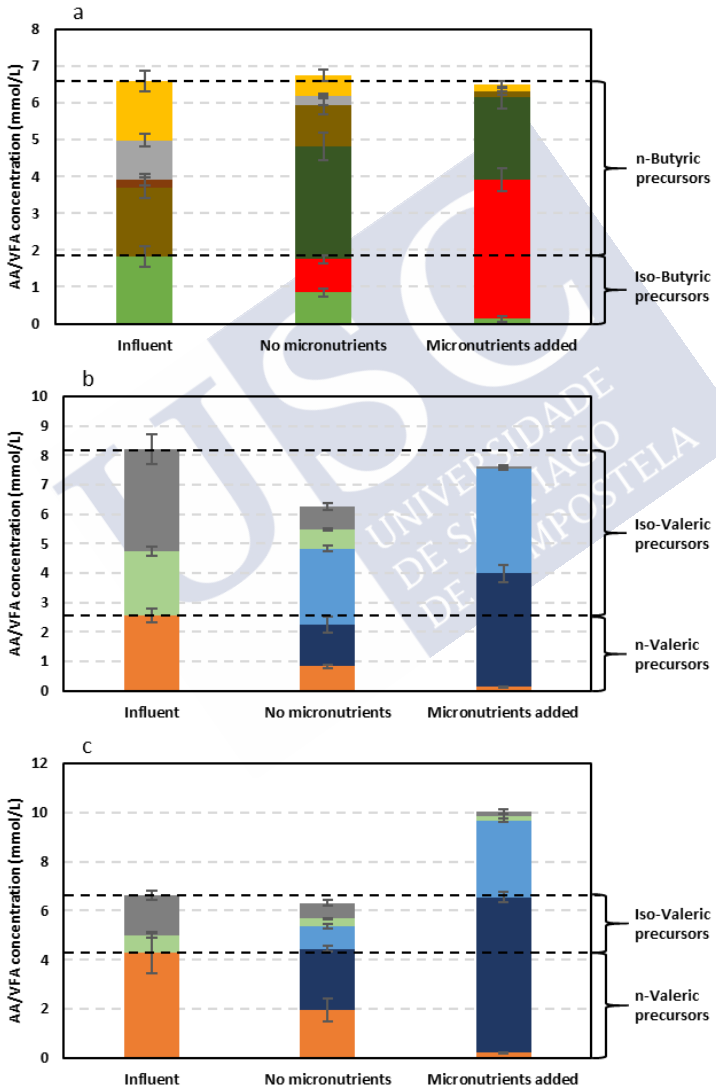

Figure 5.6. Overall butyric (a, gelatin: ■ Valine; ■ Iso-butyric acid; ■ n-Butyric acid; ■ Glutamic acid; ■ Histidine; ■ Threonine; ■ Lysine) and valeric (b, casein; c, gelatin: ■ Proline; ■ n-Valeric acid; ■ Iso-Valeric acid; ■ Isoleucine; ■ Leucine) acid balance. AA concentrations are expressed as VFA equivalents according to the stoichiometry described in section 5.2.

5.4 CONCLUSIONS

This chapter successfully demonstrated the positive effect of micronutrients supplementation on protein fermentation, especially at neutral pH conditions. The extent of this effect is, however, dependent on the amino acid profile of the protein. Interestingly, trace elements presence modifies the process selectivity, as they might enhance chain elongation and isomerisation reactions, resulting in a more flexible conversion system.

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CHAPTER 6

ENGINEERING THE OUTCOME OF COFERMENTATION PROCESSES BY ALTERING THE FEEDSTOCK SUGAR-TO-PROTEIN RATIO

SUMMARY

This chapter investigates the impact of the sugar-to-protein ratio on the outcome of their anaerobic cofermentation in terms of substrate conversion and product selectivity. For this purpose, a continuous stirred tank reactor was operated at pH 7 and fed with casein and glucose at different STP ratios (0.25, 0.50, 0.75, 1.00 and 2.00 in COD basis). Casein conversion was unaffected by glucose presence as long as the ratio was lower or equal to 1. In this range of STP ratio, n-butyric and n-valeric acid production was promoted due to the occurrence and progressive intensification of chain elongation processes. Conversely, STP ratios greater than 1 are associated with lower amino acids consumption, inhibition of the elongation metabolism and lower volatile fatty acids production due to the formation of secondary metabolites (ethanol, lactate and formate) and unidentified compounds. Interestingly, these negative effects are reversible, as lowering the sugar-to-protein ratio allows to recover protein acidification degree, process productivity and the chain elongation. Overall, this work successfully demonstrates that sugar-protein cofermentation processes can be steered by adjusting their proportions in the feedstock.

This chapter was redrafted after the following publication **in preparation**:

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6.1. INTRODUCTION

Several studies (Rughoonundun et al., 2012; Del Rio et al., 2014; Guo et al., 2015; Ma et al., 2017) highlighted the potential of mixing different substrates to enhance the production of VFAs during MCF processes. The positive effect observed during the cofermentation of proteic streams with those rich in sugars is generally associated with a better balancing of micronutrients and carbon/nitrogen proportions, dilution of potentially toxic or inhibitory compounds, and/or an increase in hydrolysis rate due to the higher biomass yields achieved (Fang et al., 2020).

Yet, attention should be paid on how the proportion between the two organic fractions affects the often-incomplete protein acidification (Duong et al., 2019; **Chapter 3; Chapter 4; Chapter 5**). Previous results in literature are not conclusive. For example, Breure et al. (1986) determined that the ratio between both fractions (in COD basis) regulates the interaction between the fermentation of gelatin with either glucose or lactose. More specifically, sugar-to-protein (STP) ratios slightly higher than 1 seems to inhibit protein hydrolysis and subsequent conversion to VFAs, whereas lower values do not show negative effects on protein fermentation. Conversely, Ma et al. (2017) determined that increasing the carbohydrate fraction in the feedstock favours the consumption of proteins, with this synergistic effect being maintained up to an STP ratio of 2, as observed during the cofermentation of sludge with potato peels residues.

Besides the conversion efficiency, sugars and proteins feature different VFA selectivity. The fermentation of glucose mainly yields acetic, propionic and butyric acid (González-Cabaleiro et al., 2015) on proportions that can be steered through pH adjustments (Temudo et al., 2007). Being a mix of 20 amino acids (AAs), proteins selectivity depends heavily on their composition (**Chapter 3**). Additionally, acetic acid tends to be the main product at neutral and alkaline conditions whereas low pH favours the conversion to longer chain VFAs (**Chapter 4**). Besides, branched chain VFAs and n-valeric acid are mostly obtained through the fermentation of specific AAs rather than from sugars (Regueira et al., 2020).

Given the specific selectivity behaviours, it was hypothesised that the cofermentation process could be steered by varying the STP ratio, leading to potential synergies between the two organic fractions. However, literature on the subject is not clear as the substrates used and the operational conditions varied among studies. For example, glucose or lactose supplementation to gelatin fermentation was associated to an increased production of n-butyric acid and ethanol (Breure et al. 1986). However, Zhou et al. (2013) observed an increase in acetic and propionic concentrations when progressively feeding greater proportions of carbohydrate-rich corn straw to the sludge-degrading reactor, whereas maintaining STP values lower than 1 favours the formation of n-valeric acid (Ma et al., 2017).

Therefore, in order to assess the impact of sugar-to-protein ratio on the outcome of their anaerobic cofermentation process, this study focuses on understanding the interactions between amino acids and glucose acidification. This knowledge will be useful to engineer the process towards the desired outcome.

6.2. MATERIALS AND METHODS

Casein peptone (A2208,0500 PanReac) and D(+)-glucose anhydrous (131341.1211 PanReac) were the model compounds used in this study. Protein concentration was fixed at 7.50 g/L throughout the experiment, while glucose concentration was progressively increased from 1.87 g/L to 14.96 g/L. The feedstock solution was supplemented with macro- and micro-nutrients, as described in **Chapter 2**, and it was maintained refrigerated throughout the experiment (4°C).

The continuous stirred tank reactor (CSTR) of 1 L used in the present study was inoculated with casein-degrading biomass from the reactor described in **Chapter 5**, with the difference of glucose being included in the feedstock at increasing concentrations in order to test several STP ratios (in COD basis): 0.25, 0.50, 0.75, 1.00, 2.00. Each resulting STP ratio (Table 6.1) was maintained for at least 40 days, in order to evaluate its impact on the cofermentation process after reaching a steady-state operation.

Table 6.1. Operational conditions of the different phases of the cofermentation reactor. STP: sugar-to-protein ratio (COD basis); OLR: organic loading rate (g COD/L·d).

Phase	STP ratio	Casein OLR	Glucose OLR
I	0.25	5.33	1.33
II	0.50	5.33	2.67
III	0.75	5.33	4.00
IV	1.00	5.33	5.33
V	2.00	5.33	10.7

Analysis of the influents and the effluents of the reactor was performed according to the methods and the calculations described in **Chapter 2.** COD (total and soluble) and solids content were determined once per week while pH was continuously checked through the multiparametric analyser. Individual VFA concentrations and total ammonia nitrogen (TAN) were measured twice per week. Amino acid content of the reactors effluent was measured on selected samples from steady state periods of operation.

It should be noted that the ammonification parameter (Equation 11, **Chapter 2**) was slightly modified to include the nitrogen uptaken by the biomass, resulting in the following equation:

$$Ammonification\ (\%) = \frac{C_{TANeffluent} - C_{TANfeeding} + C_{TANbiomass}}{C_{TANmaximum}} \times 100 \qquad (15)$$

Where $C_{TANeffluent}$ is the concentration of ammonium nitrogen (mg N-NH_4^+/L) measured in the reactor effluent, $C_{TANfeeding}$ is the concentration of ammonium nitrogen (mg N-NH_4^+/L) in the reactor feeding derived from the macronutrients supplementation, $C_{TANbiomass}$ is the concentration of ammonium nitrogen (mg N-NH_4^+/L) which was captured by biomass during

growth and $C_{TANmaximum}$ is the maximum concentration of ammonium nitrogen (mg N-NH_4^+/L) achieved if complete conversion of the protein to VFA occurs. $C_{TANbiomass}$ was calculated by multiplying the measured in-reactor biomass concentration for a biomass nitrogen ratio of 114 mgN-NH_4^+/g VSS, assuming an average biomass composition of $CH_{1.8}O_{0.5}N_{0.2}$.

6.3. RESULTS AND DISCUSSION

6.3.1. Cofermentation reactor operation

The cofermentation reactor was continuously operated for 344 days (Figure 6.1a). The first 56 days were jointly considered as a phase of start-up and acclimation to glucose presence (1.33 g COD/L·d), given that the inoculum was used to degrade only proteins (**Chapter 5**). To inhibit methanogenesis, which began to occur at day 42, sodium 2-bromoethanesulphonate (BES, 137502, SigmaAldrich) was added to the reactor feedstock at a concentration of 0.5 g/L starting from day 45. At day 344, the reactor was stopped due to Covid-19 lockdown and restrictions on research activity and its content was stored at 4°C. The operation was then resumed after two months (Figure 6.1b) by acclimating the stored biomass at the original conditions of pH, temperature and nitrogen sparging. The reactor was operated in batch mode for the first 10 days by adding a diluted feedstock pulse to the vessel, in order to safely reactivate the biomass activity. After having detected the occurrence of VFA production (Figure 6.1d), continuous feeding started at an HRT equal to 3 d (STP 1.00), to avoid potential washout of the biomass. After one week it was lowered to 2 d, and finally set at the original value of 1.5 d at day 24. On day 45, glucose concentration was increased to achieve the highest STP value (2.00). The reactor operation was then finalised at day 88.

Biomass concentration rapidly grew from 0.6 to 1.0 g VSS/L when exposed at the lowest glucose loading (STP 0.25), compatibly with the higher yields associated with sugar substrates (Batstone et al., 2002; Gerardi, 2003). Increasing the STP ratio further favoured biomass growth, reaching 1.4 g VSS/L and 2.8 g VSS/L at STP ratios of 1.00 and 2.00, respectively.

Methanisation was successfully inhibited from day 50 on, since no difference was detected between the total COD concentrations in the reactor influent and effluent (Figure 6.1a and b). The difference between total and soluble COD in the effluents matched the biomass concentrations achieved in the reactor. The overall concentration of VFA (COD basis) increased progressively with the application of higher STP ratios, peaking at approximately 10 g COD/L (STP 1.00). VFA production was 20% lower (8 g COD/L) after the reactor operation was resumed at the same conditions (Figure 6.1b), suggesting that the interruption and subsequent storage might have affected the microbial population. Soluble COD concentration was systematically higher than the VFA-COD concentration, suggesting the presence of non-converted substrate, secondary metabolites (e.g. ethanol) and/or unidentified products. As glucose could not be detected in the reactor effluents, only protein can account for the non-converted substrate.

As expected, global VFA production increased at higher STP ratios. However, the effect of STP ratio on individual VFA production was acid-dependant (Figure 6.1c and d). Acetic, n-butyric and n-valeric acids were the main products for most of the reactor original operation (≥750 mg/L), progressively increasing with the STP ratio. Interestingly, n-valeric acid production peaked at 1500 mg/L when applying an STP value of 0.75, becoming the VFA with the highest concentration. Acetic acid replaced it at STP 1.00, reaching 2000 mg/L. In comparison, n-butyric acid concentration grew more steadily, stabilising at a final concentration of 1500 mg/L at STP 1.00. Conversely, iso-butyric and iso-valeric acid production decreased from 330 to 250 mg/L and from 650 to 500 mg/L respectively when applying an STP value greater than 0.25. n-Caproic acid was only detected for a limited amount of time (STP 0.50) and only in small concentrations (≤ 150 mg/L). During the resumed operation, the increase in STP ratio especially favoured acetic and propionic production (≥2200 mg/L) in detriment of all the other VFAs, whose concentrations were equal or lower than 500 mg/L. Lactate, formate and ethanol production was not observed during the original experiment and at variable concentrations during the resumed operation (data not shown).

To assess the impact of STP ratio on casein-glucose cofermentation, several steady-state periods were identified: day 56 – 119, day 142 – 232, day 249 – 295 and day 312 – 344 for STP ratios of 0.25, 0.50, 0.75 and 1.00, respectively. For the resumed operation, the selected stable periods were day 24 – 45 and day 52 – 88 for STP ratios of 1.00 and 2.00, respectively.

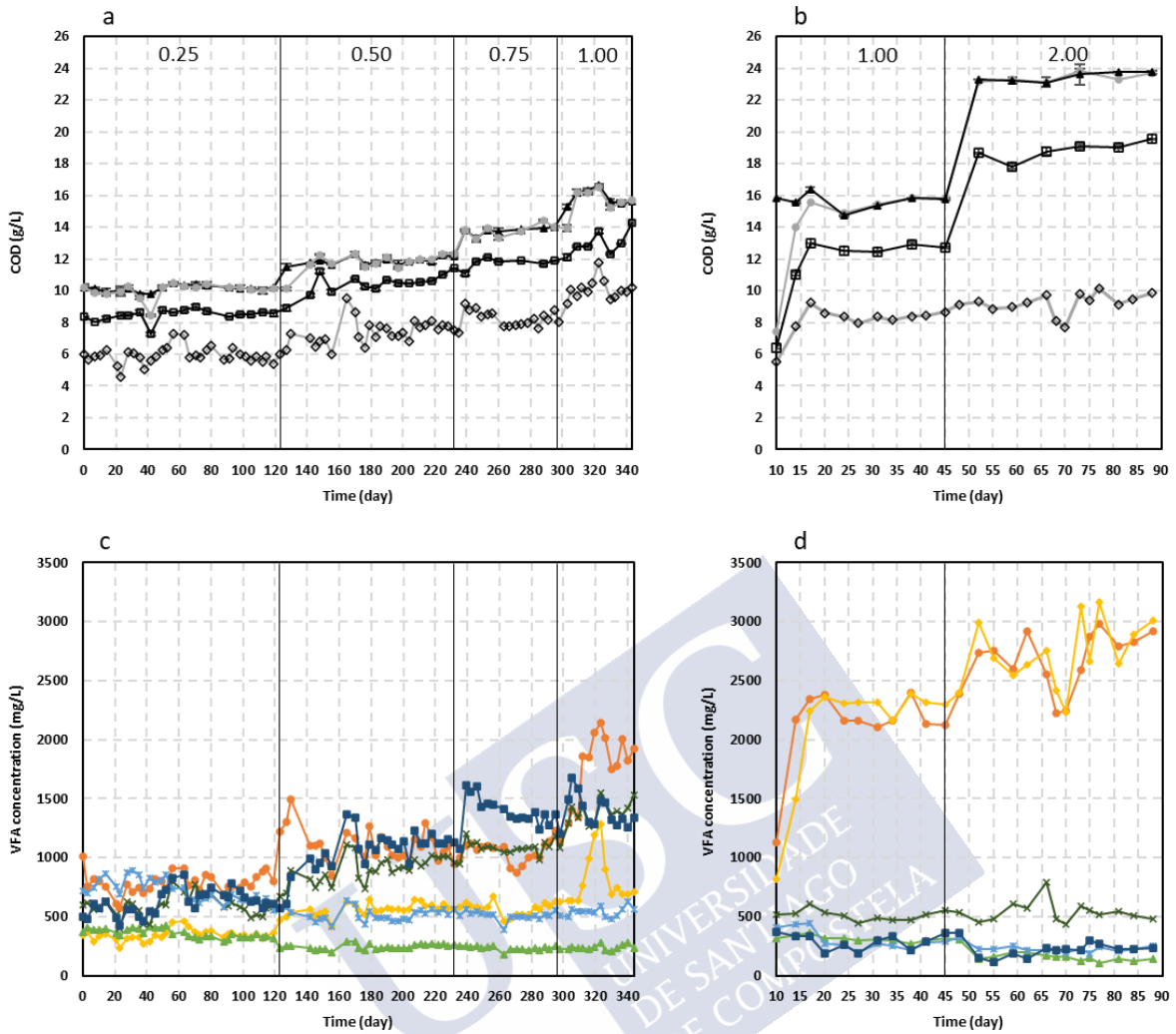

Figure 6.1. COD balance (a, original operation; b, resumed operation: ▲ Influent total COD; ● Effluent total COD; □ Effluent soluble COD; ◇ VFAs COD) and individual VFA concentrations in the cofermentation reactor (c, original operation; d, resumed operation: ● Acetic; ◆ Propionic; ▲ Iso-Butyric; x n-Butyric; * Iso-Valeric; ■ n-Valeric). The vertical black lines indicate the change in the STP ratio.

6.3.2. The influence of STP ratio on protein conversion and amino acid consumption

Glucose consumption was complete regardless of the STP ratio, while casein consumption was above 60% based on the ammonification parameter, except for the STP ratio of 2.00 (Figure 6.2). Interestingly, this value is very similar to the one achieved during casein monofermentation (STP 0.00 (**Chapter 5**) as long as the STP ratio is lower or equal to 1. The acidification degree follows the same pattern as the ammonification (Figure 6.2). Moreover, the sum between the unconverted protein concentration (2.40 – 3.68 COD/L), estimated from the ammonification, and the VFA-COD concentration mostly close the soluble COD balance, except for STP 2.00 and to a certain extent STP 0.75 (Table 6.2). In this case,

the concentrations of produced VFAs (9.20 g COD/L) and unconverted protein (3.68 g COD/L) does not account for all the measured soluble COD (18.8 g COD/L), suggesting that secondary metabolites could be acquiring more relevance in the products distribution. Indeed, ethanol together with lactate and formate became a much more relevant fraction of the soluble COD (2 – 5 g COD/L) at STP 2.00. The unidentified soluble fraction was mostly equal or lower than 1.00 g COD/L, gaining greater relevance at STP 2.00.

Table 6.2. Soluble COD fractioning at different STP ratios. All concentrations are expressed in g COD/L. UP, SC and UC respectively stand for Unconverted Protein, Secondary Metabolites and Unidentified Compounds.

STP ratio	Soluble COD	VFA COD	UP	SC	UC
0.25	8.62	6.04	2.40	0.00	0.17
0.50	10.6	7.44	2.40	0.00	0.71
0.75	11.9	8.16	2.40	0.00	1.33
1.00	13.2	10.2	2.80	0.00	0.17
1.00 (resumed)	12.7	8.33	3.20	0.50	0.63
2.00 (resumed)	18.8	9.20	3.68	2.00 -5.00	0.94 – 3.94

The effect of sugar presence on AA consumption is amino acid-dependant (Figure 6.3). Some of them (leucine, valine, isoleucine, serine and glycine) follow the same behaviour as the ammonification, while others were already inhibited by the lowest STP ratio (proline, lysine) or not affected at all even at the highest STP ratio (phenylalanine, histidine). After resuming the reactor operation, the consumptions were equal or slightly lower than the values achieved during the original experiment, probably due to the interruption having affected the microbial community and its AA degradation capacity. Only the STP ratio of 2.00 seems to have substantially hindered some AA utilisation, which is consistent with the effect observed in the ammonification and acidification degree.

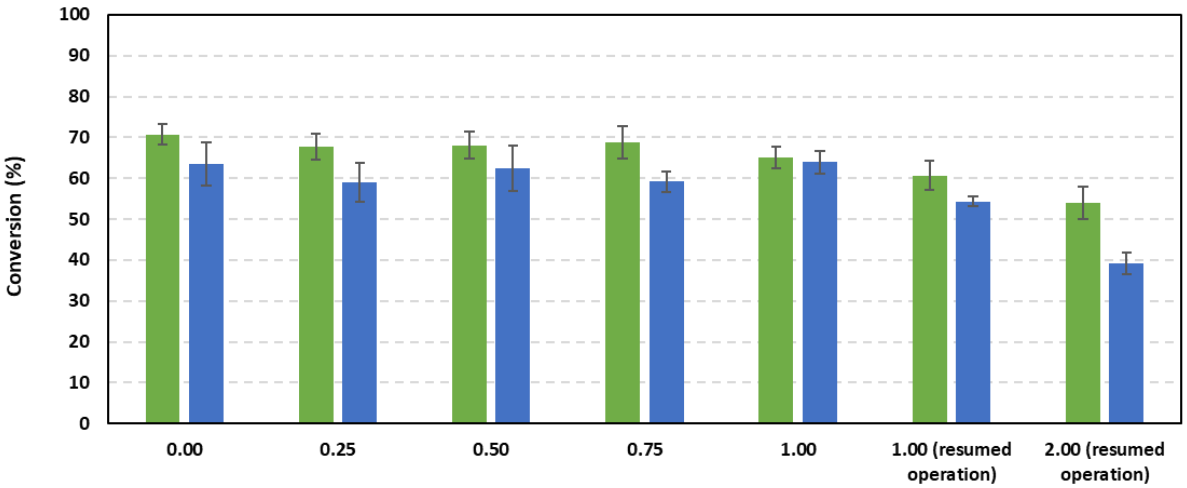

Figure 6.2. Comparison between ammonification (■) and acidification degree (■) at different sugar-to-protein ratios (0 – 2.00). The monofermentation values (sugar-to-protein ratio of 0.00) are obtained from a previous study (**Chapter 5**).

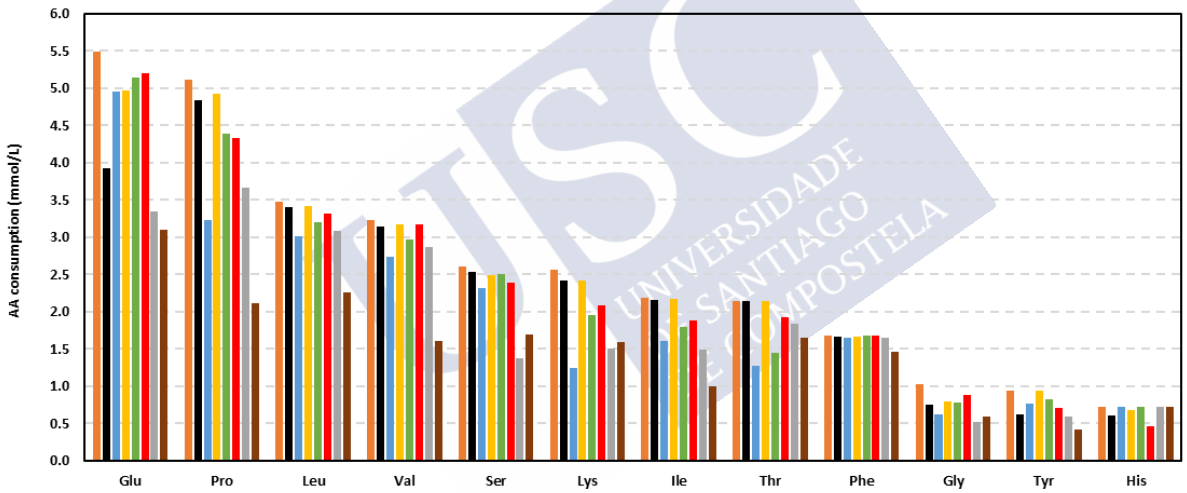

Figure 6.3. Amino acids consumption at different sugar-to-protein ratios: ■ Feedstock AA concentration; ■ 0.00 (**Chapter 5**); ■ 0.25; ■ 0.50; ■ 0.75; ■ 1.00; ■ 1.00 (resumed operation); ■ 2.00 (resumed operation). No data of alanine, arginine, methionine and tyrosine consumption is available. Glu: glutamic acid; Pro: proline; Leu: leucine; Val: valine; Ser: serine; Lys: lysine; Ile: isoleucine; Thr: threonine; Phe: phenylalanine; Gly: glycine; Tyr: tyrosine; His: histidine.

6.3.3. The influence of STP on the VFA selectivity

As seen in section 6.3.1, the influence of STP ratio on the VFA production was acid-dependant. In fact, their molar fractions showed different behaviour in response to the increase in the STP ratio (Figure 6.4). The presence of glucose even at the lowest STP decreased acetic acid relevance in the products distribution. However, values equal or greater than 1 directed the selectivity of the process towards this acid again, reaching molar fractions comparable to the monofermentation process (STP 0.00). Propionic acid fraction was mostly

stable throughout the original experiment (12-15%), becoming the second most relevant VFA after the reactor operation was resumed (40%). The behaviour of these two acids contradicts the tendencies described by Zhou et al. (2013), as acetic and propionic acid should have benefitted from glucose presence even at the lowest STP ratio. Iso-butyric and iso-valeric acid showed a similar behaviour, as their molar fractions progressively decreased in response to the STP changes. This decrease in relevance is attributed to the increase in global VFA production determined by the greater glucose concentrations in the feeding, whereas these acids, being only produced from branched-chain AA conversion (Val, Ile and Leu), are limited by the protein fixed concentration. The only exception to the tendency was observed at STP 0.25, suggesting that a secondary process (e.g. isomerisation) might be contributing to their production. Finally, n-butyric and n-valeric acid behaved in the opposite way than acetic acid, as they both progressively benefitted from the increasing STP value during the original experiment whereas their molar fractions decreased considerably after the experiment interruption. The production of n-butyric acid agrees with both the experimental results of Breure et al. (1986) and the metabolic pathways associated with glucose fermentation (Temudo et al., 2007; González-Cabaleiro et al., 2015); in contrast, n-valeric acid behaviour contradicts the fact that its production is almost only associated with proteins fermentation (Ma et al., 2017). It was consequently hypothesised that chain elongation (CE) could be progressively contributing to the formation of n-valeric acid, and possibly to n-butyric acid production as well. The decrease in n-butyric and n-valeric acid production would be then explained by the elongation process being suppressed by the interruption of the reactor operation, which could have negatively affected the microbial community responsible of this process. Yet, it is difficult to discern whether the CE suppression was solely caused by the interruption itself, as the excessive glucose loading associated with higher STP ratios might have played a role as well.

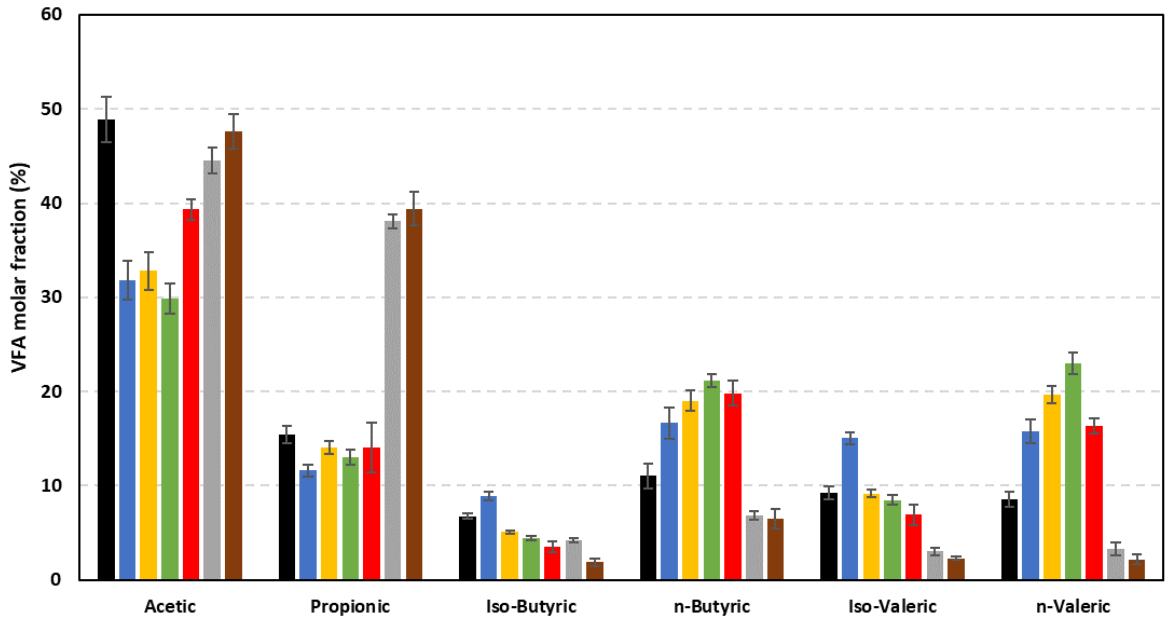

Figure 6.4. Comparison of VFA spectra at different STP ratios: ■ 0.00 (**Chapter 5**); ■ 0.25; ■ 0.50; ■ 0.75; ■ 1.00; ■ 1.00 (resumed operation); ■ 2.00 (resumed operation).

It was hypothesised that the increase in STP ratio promotes CE processes as glucose is converted into adequate electron donor compounds, such as ethanol and/or lactate (González-Cabaleiro et al., 2015; Regueira et al., 2021), while generating a surplus of reducing power (Angenent et al., 2016). These secondary metabolites are, in fact, suitable substrates for the elongation of acetic and propionic acid to n-butyric and n-valeric acid, respectively (Liang & Wan, 2015). The formation of n-butyric and n-valeric acid by CE pathways is consistent with the fact that, after the reactor operation was resumed, n-butyric and n-valeric production decreased substantially whereas lactate and ethanol started to be detected in the reactor effluents. Moreover, the lack of acetic and propionic consumption could be at least partially responsible for the concentration of these short chain VFAs. The analysis of n-butyric acid production due to CE process is not straightforward as it can be yielded by glucose alone (González-Cabaleiro et al., 2015) and by amino acids such as glutamic acid and lysine (Regueira et al., 2020). However, the combined production balance of iso and n-valeric acids (Figure 6.5) unequivocally confirms the occurrence and relevance of CE, as the production of these VFA is exclusively related to a limited number of AAs. In fact, the consumption of their precursor AAs does not justify their formation during the original operation with glucose. It also suggests that isomerisation between the two forms might be occurring in a similar way as seen for casein monofermentation (**Chapter 5**). At low STP (0.25) part of the n-valeric acid overproduction is converted to the branched form whereas at high STP (2.00) the opposite interconversion occurs, favouring the linear form. Overall, these results suggest that STP ratio can be adjusted to potentially steer the process towards the desired outcome in terms of VFA selectivity.

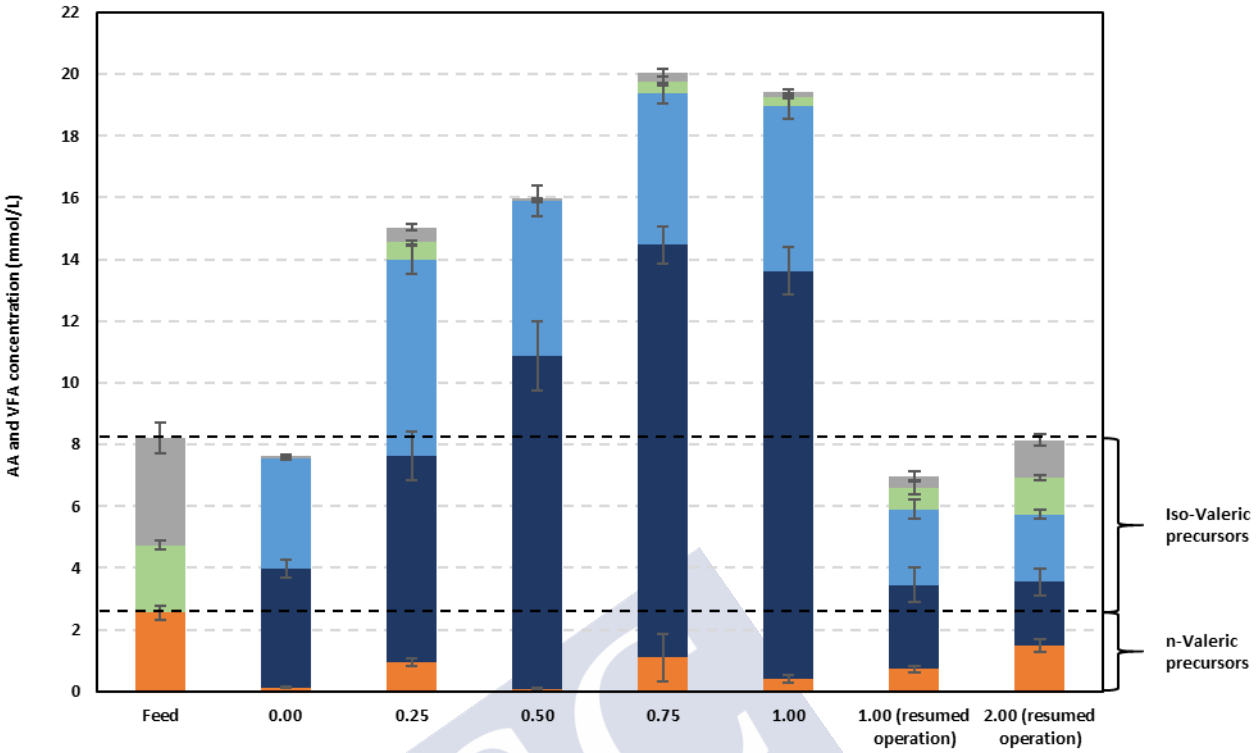

Figure 6.5. Iso and n-valeric acid balance in the cofermentation reactor: ■ Proline; ■ n-Valeric acid; ■ Isoleucine; ■ Leucine; ■ Iso-Valeric acid. AA concentrations are expressed as VFA equivalents according to the stoichiometry described by Regueira et al. (2020).

6.3.4. CE can be recovered by lowering the STP ratio

To verify whether the CE process could be recovered by lowering the glucose loading, a parallel cofermentation reactor was inoculated with biomass taken from the main reactor on day 45 of the resumed operation (STP 1.00) and operated at an STP ratio of 0.50 (Figure 6.6).

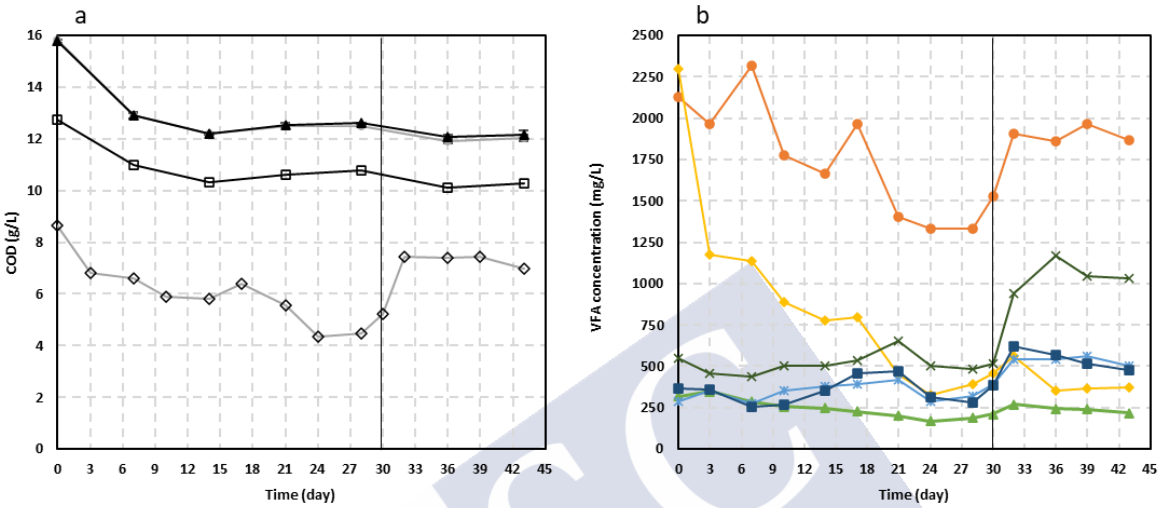

Figure 6.6. Operation of the parallel reactor at an STP ratio of 0.50 to assess CE process recovery (a, COD balance: ▲ Influent total COD; ● Effluent total COD; □ Effluent soluble COD; ◇ VFAs COD; b, VFA concentrations: ● Acetic; ◆ Propionic; ▲ Iso-Butyric; x n-Butyric; * Iso-Valeric; ■ n-Valeric). The vertical black lines separate the acclimation phase from the steady-state operation.

Both the total and the soluble COD of the reactor effluent decreased compatibly with the lower STP applied to the reactor (Figure 6.6a). Based on the VFA production (COD basis), it was possible to identify two operational periods: from the start up to day 30 (acclimation stage) and from day 30 to 43 (steady-state operation). Interestingly, the values of all COD parameters were similar to those previously obtained at STP 0.50 (Figure 6.1a), providing the first proof concerning the reversibility of excessive sugar supplementation.

In terms of VFA production (Figure 6.6b), the acclimation period was associated with a decrease in acetic and propionic acid concentrations, whereas the other VFAs remained mostly stable. In contrast, except for propionic and iso-butyric acid, VFAs production increased between day 30 and 43. In particular, n-butyric, n-valeric acid and iso-valeric generation showed a two-fold increase which, coupled with the absence of lactate and ethanol in the reactor effluents, further confirms the reversibility of the effects caused by STP ratios greater than 1.00. Also, the balance of valeric acids (Figure 6.7) indicates that CE process was recovered during the steady-state period, as proline consumption alone is not able to justify n-valeric acid production. This balance also highlights the occurrence of isomerisation from the iso to the n-form during the acclimation step.

Comparing these results with those described in the previous sections, it was hypothesised that the increased availability of glucose associated with higher STP ratios might be making further conversion of ethanol and lactate into VFAs less appealing to the microbial community due to kinetic limitations associated with high OLRs (16 g COD/L·d at STP ratio 2.00). Besides, the absence of substrate limitations might be making specialised metabolic pathways, such as CE, unnecessary. Still, the disruptive effect caused by the operation interruption cannot be completely discarded, as it might have accelerated the disappearance of the CE process at the highest STP ratios by altering the microbial community in first place. Besides, the VFA concentrations were not strictly the same as during the original experiment at STP 0.50 (Figure 6.1c), suggesting that longer operation time might be required to fully recover the previously obtained steady state.

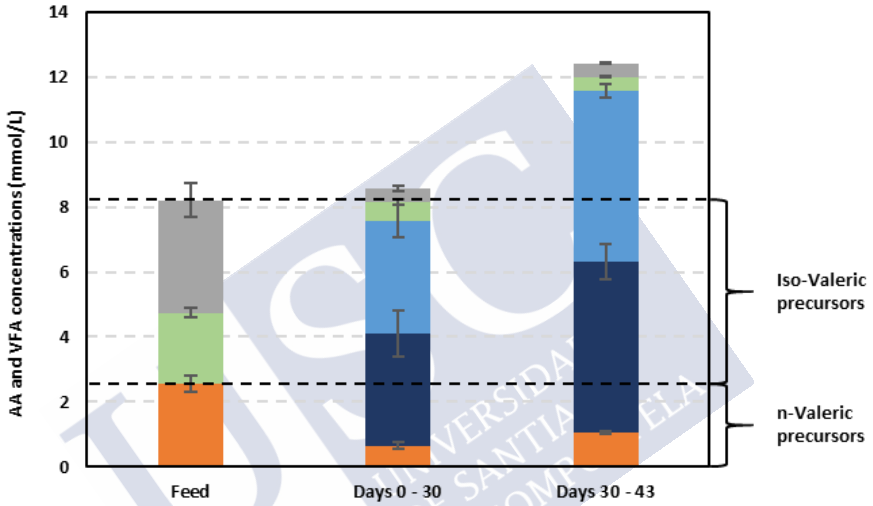

Figure 6.7. Iso and n-valeric acid balance in the parallel reactor at an STP ratio of 0.50: ■ Proline; ■ n-valeric acid; ■ Isoleucine; ■ Leucine; ■ Iso-valeric acid. AA concentrations are expressed as VFA equivalents according to the stoichiometry described by Regueira et al. (2020).

6.4. CONCLUSIONS

This study successfully investigated the interactions between amino acids and glucose during their cofermentation in order to understand the impact of the STP ratio on substrate consumption, acidification degree and product selectivity. In particular, the main findings are:

- STP ratios equal or lower than 1.00 do not affect the extent of protein conversion, but excessive sugar loading hinders AA consumption and favours the production of secondary metabolites.
- The products distribution can be steered towards the production of n-butyric and n-valeric acid by increasing the sugar proportion up to the optimal STP ratio of 1.00, which promotes the occurrence of CE processes.
- The changes produced by excessive sugar loadings are reversible, as lowering the STP ratio allows to recover the longer chain VFA production to a certain extent.

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CHAPTER 7

CHAIN ELONGATION MAY OCCUR IN PROTEIN MIXED-CULTURE FERMENTATION WITHOUT SUPPLEMENTING ELECTRON DONOR COMPOUNDS

SUMMARY

This chapter focuses on verifying the occurrence of elongation processes during protein mixed culture (co)fermentation, without the supplementation of specific electron donor compounds, and shedding some light on the underlying mechanisms.

In **Chapter 4**, experiments were performed with casein and gelatin at different pH values (5, 7, 9), showing that longer chain volatile fatty acid production increased during casein fermentation at acid conditions, which could not be completely justified by the associated AA consumption. Consequently, the occurrence of chain elongation processes was hypothesised. To verify this hypothesis, three casein batch tests, with and without acetic acid initial supplementation, were performed at pH 5. The results suggest that acetic and propionic acids are indeed consumed to selectively generate n-valeric acid through the coupling with electron donor AAs. Prolonged simultaneous availability of suitable AAs and short chain volatile fatty acids and acid equivalent pressure were identified as key parameters for the occurrence of chain elongation. The supplementation of acetic acid at the beginning of the test changed the selectivity of the elongation process, promoting n-butyric and iso-valeric production.

Based on the experiments described in **Chapter 5**, the supplementation of trace elements might also play a role on the occurrence of chain elongation process during protein mixed culture fermentation. In particular, iso-valeric acid formation is promoted during casein and gelatin fermentation at pH 5 without limiting the production of n-valeric acid. Both forms of this acid are also produced via elongation during gelatin fermentation at neutral conditions. Conversely, trace elements seem to somewhat limit the production of n-butyric acid at pH 5 for both tested proteins. The cofermentation of proteins with sugars also promotes the elongation process for the selective production of n-valeric acid, as glucose conversion provides the intermediate metabolites (i.e. ethanol and/or lactate) required (**Chapter 6**). However, this kind of chain elongation seems to be strongly dependant on the sugar-to-protein ratio in the feedstock.

This chapter was partially redrafted after the following **under-review** publication:

Bevilacqua, R., Regueira, A., Mauricio-Iglesias, M., Lema, J.M., Carballa, M. (2021). CRETUS, Department of Chemical Engineering, Universidade de Santiago de Compostela, 15782, Santiago de Compostela, Spain. Chain elongation may occur in protein mixed-culture fermentation without supplementing electron donor compounds. *Water Research*, ISSN: 0043-1354. Under review.

7.1. INTRODUCTION

Most of the chain elongation (CE) studies available in literature focuses on carbohydrates as the main substrate (Han et al., 2019), such as acid whey from the dairy industry (Xu et al., 2018; Duber et al., 2018), organic fraction of the municipal solid waste (Grootscholten et al., 2014), brewers' spent grain (Liang & Wan, 2015), thin stillage (Carvajal-Arroyo et al., 2019) or food waste (Contreras-Dávila et al., 2020). On the contrary, little is known about CE processes during anaerobic fermentation of proteins. Only two studies were found (Wallace et al., 2003; Wallace et al., 2004), which were performed with a pure culture of *Eubacterium pyruvativorans*, a bacterium isolated from bovine rumen. They observed that, supplying the amino-acid-enriched culture medium with acetic, propionic or butyric acid, the acids were consumed and elongated to n-butyric, n-valeric and n-caproic acids, using some AAs as electron donor compounds (e.g. alanine and leucine). However, there is no information about CE elongation processes during anaerobic MCF of proteins. Of the twenty most frequent AAs in proteins, some can take the role of electron donors, acceptors or even both (De Vladar, 2012). The diversity of redox roles suggests that proteins might undergo simultaneous fermentation and CE processes without the need for electron donor supplementation.

This chapter investigates the feasibility of CE elongation processes during anaerobic mixed culture fermentation of proteins, focusing on the role of protein composition (the AA profile), operational pH, trace elements supplementation and sugar presence.

7.2. MATERIALS AND METHODS

7.2.1. Continuous experiments

Two continuous stirred tank reactors (CSTR) were operated with two different proteins, casein and gelatin, at different pH values (5, 7 and 9) to understand the effect of substrate composition (**Chapter 3**) and pH (**Chapter 4**) on protein mixed culture fermentation. The same CSTRs were also supplemented with trace elements and operated at pH 5 and 7 to understand the impact these compounds have on the process (**Chapter 5**). An additional CSTR was inoculated with biomass from the casein reactor and fed with casein and glucose at varying sugar-to-protein ratios to understand the impact of sugar presence on protein conversion to VFAs (**Chapter 6**).

7.2.2. CE batch experiments

Batch experiments (Section 2.3.2.2) were conducted with casein as substrate in order to: i) confirm CE presence during anaerobic MCF of proteins, ii) understand the role of AAs in the process, and iii) verify whether the addition of shorter chain carboxylates (i.e. acetic and propionic acid) can affect CE process extension and/or selectivity.

In each assay, a 2 L sealed glass vessel (1 L of working volume) was used, and the operational conditions were set to be similar to the continuous reactors (25°C, N_2 sparging, pH 5). The inoculum was obtained from the continuous reactor and prepared targeting a 0.5 g VSS/L initial concentration. Synthetic hydrolysed protein, peptone from casein (A2208,0500

PanReac), was used as the sole carbon source. The initial casein concentration was equal to 5 g COD/L for the first test, while it was increased to 10 g COD/L for the following two tests, corresponding to a substrate-to-inoculum ratio (SIR) equal to 10 and 20 g COD/g VSS, respectively. The third batch test was initially supplemented with acetic acid at an approximate concentration of 0.5 g/L. Macronutrients were also added in all batch tests at the following concentrations (g/L): NaCl 0.292; KH_2PO_4 0.780; NH_4Cl 0.530, Na_2SO_4 0.057; $MgCl_2 \cdot 6H_2O$ 0.120.

7.2.3. Analytical methods and calculations

Analysis of the influents and the effluents of the reactors and of the batch tests was performed according to the methods and the calculations described in **Chapter 2**.

As CE leads to a higher fraction of longer-chain VFA, i.e. more reduced VFA, the degree of reduction, defined as the average COD per gram of produced aliphatic VFAs, was the proposed parameter to evaluate the presence of CE processes:

$$Degree\ of\ reduction\ (g\ COD/g\ VFA) = \frac{\Sigma\ C_{VFA-COD}}{\Sigma\ C_{VFA}} \quad (16)$$

where $C_{VFA\text{-}COD}$ stands for the total COD concentration of the measured VFAs (in g COD-VFA/L) and C_{VFA} for the total concentration of the measured VFAs (in g VFA/L) in the fermentation liquor. For VFA with 2 to 5 carbon atoms, this index varies between 1.07 g COD/g VFA corresponding to pure acetic acid and 2.04 g COD/g VFA corresponding to pure valeric acid.

7.3. RESULTS AND DISCUSSION

7.3.1. pH and AA composition determine CE feasibility during protein MCF

Longer chain VFA production is promoted during protein continuous fermentation at acid conditions (**Chapter 4**), resulting in higher average degrees of reduction than at neutral and alkaline pH values (Table 7.1), especially using casein as substrate. For this reason, it was hypothesised that CE processes might have been contributing to the observed change of selectivity, suggesting that acid conditions are required.

Table 7.1. Average degree of reduction (expressed as g COD/g VFA) achieved during continuous mixed culture fermentation of casein and gelatin at different pH values.

Protein	pH 5	pH 7	pH 9
Casein	1.72 ± 0.06	1.47 ± 0.01	1.47 ± 0.01
Gelatin	1.40 ± 0.02	1.36 ± 0.02	1.29 ± 0.02

Although it cannot be completely excluded, the formation via elongation of iso-butyric (de Leeuw et al., 2020; Petrognani et al., 2020), n-butyric (Duncan et al., 2004) and iso-valeric (Satoh et al., 1992; Filipe et al., 2001; Oehmen et al., 2005) acids was discarded in

these continuous reactors due to their production being generally consistent with the associated AA consumption. Conversely, n-valeric acid formation via elongation can be easily verified since proline is the only precursor AA (1 mmol Pro = 0.5 mmol n-Val (Regueira et al., 2020)). Interestingly, proline consumption alone could not justify n-valeric production during casein fermentation at acid conditions (Figure 7.1a), strengthening the hypothesis that low pH values are required for the protein-based CE to occur. In contrast, no excess of n-valeric acid was detected during gelatin reactor operation, regardless of the operational pH (Figure 7.1b).

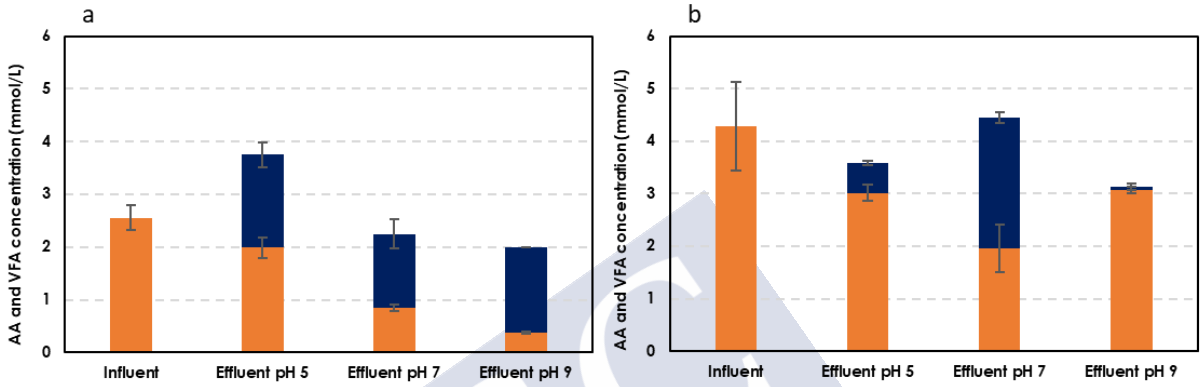

Figure 7.1. n-Valeric acid molar balance in the continuous casein (a) and gelatin (b) reactors at three different pH values**.** ■ Proline; ■ n-Valeric. Proline concentration is expressed in VFA equivalents based on the associated stoichiometry.

These results suggest that the bacterial communities could be performing CE as a mean to reduce the number of acid equivalents in the reactor bulk utilizing NADH surpluses, hence longer chain acids acting as electron sinks (Angenent et al., 2016). Indeed, at low pH there is a larger fraction of undissociated VFA that can diffuse back through the cell membrane to the intracellular space. The higher conversion of casein conversion to VFAs at pH 5 (30%) than gelatin (20%) could explain why this detoxification strategy was not observed for both proteins. Moreover, casein fermentation generates an excess of reducing power (**Chapter 3**) which could be consumed by elongating short chain VFAs, whereas gelatin composition features a higher percentage of electron acceptor AAs (58.6%) than casein (26.4%), limiting the need for CE processes to maintain the redox balance.

7.3.2. Substrate-to-inoculum ratio affects the extent of the elongation process

To verify the abovementioned hypotheses, casein batch tests were conducted at two different SIR values: 10 and 20 g COD/g VS. At SIR 10, a final acidification and degree of reduction of 43.5% and 1.76 were respectively attained. n-Butyric and iso-valeric acids were the major products, with concentrations greater than 250 mg/L (Figure 7.2b). The concentrations of acetic and propionic quickly rose during the first 24 hours, then stagnated for few hours to continue further increasing later (Figure 7.2a), while the other carboxylic acids kept being steadily produced. Notably, n-valeric acid production showed a lag phase of approximately 12 hours (Figure 7.2b). A comparable final acidification and degree of reduction were achieved at SIR 20 (45.3% and 1.80, respectively), although the duration of

the batch test was increased to 384 hours (Figure 7.2c and d), thus ruling out the occurrence of products inhibition. Once again, n-butyric and iso-valeric acids were the major products together with n-valeric acid, the three VFA reaching concentrations higher than 550 mg/L. Similarly to the previous test, acetic and propionic acid concentration increased during the first 24h and then decreased even more markedly than the trend observed for the SIR 10 test (Figure 7.2c). These stagnation periods might correspond to in-situ consumption of short chain VFAs associated with elongation processes (Kim et al., 2019).

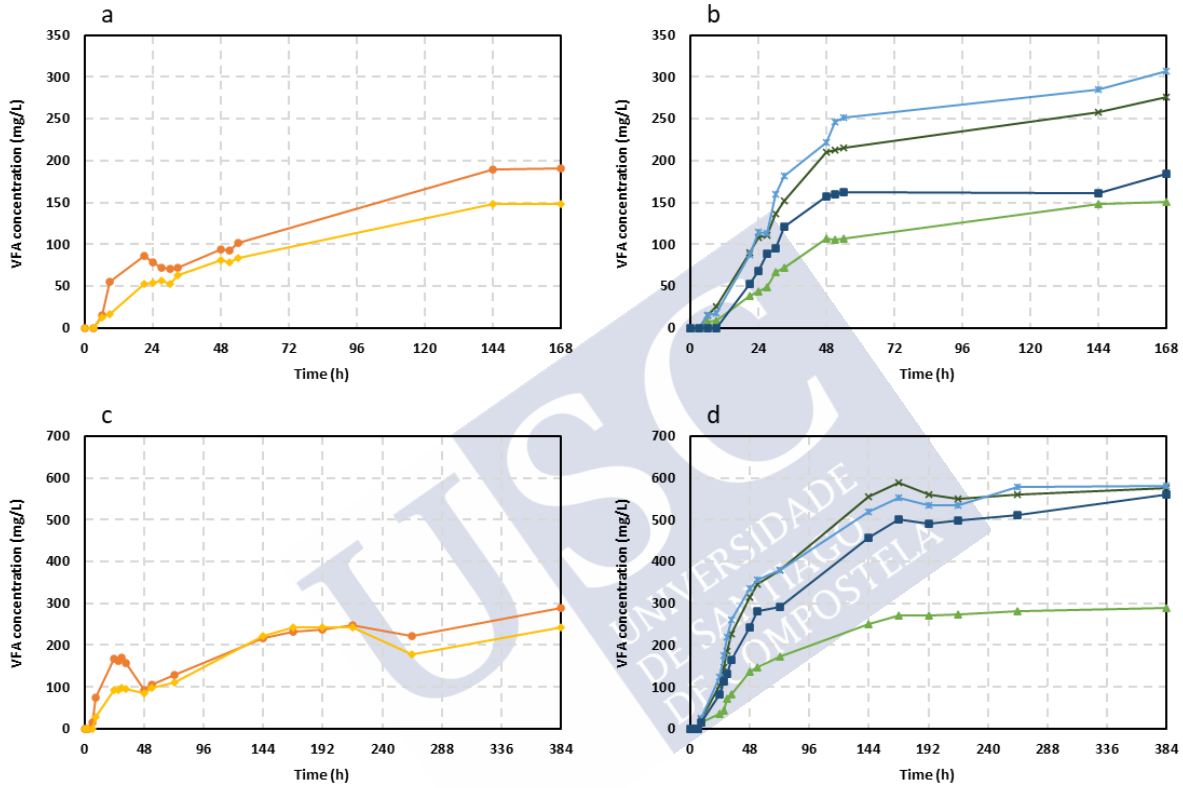

Figure 7.2. VFA production during casein batch fermentation at pH 5 and SIR 10 (a, b) and SIR 20 (c, d). ● Acetic; ◆ Propionic; ▲ Iso-Butyric; x n-Butyric; * Iso-Valeric; ■ n-Valeric

The VFA concentrations of the SIR 20 test were expected to double those of the SIR 10 one, as similar acidification degrees were achieved, thus ruling out product inhibition. However, this pattern only occurred for three VFAs (Figure 7.3): iso-butyric, n-butyric and iso-valeric acids. The ratio for acetic and propionic acid was below 2 while n-valeric acid one was higher than 3.0 (Figure 7.3), confirming the occurrence of CE processes. At SIR 10, n-valeric production (1.80 mmol/L) fitted quite well with the maximum production estimated assuming complete conversion of proline (1.60 mmol/L), the only AA assumed to yield n-valeric acid (Regueira et al., 2020), although elongation of acetic and propionic acid to n-valeric acid at SIR 10 cannot be completely excluded. Conversely, the same estimation applied to the SIR 20 test unequivocally confirms the occurrence of CE (Figure 7.4a). In fact, the SIR 20 results suggest that almost 50% of the n-valeric production could have been produced through the elongation pathway. The CE process appears to be strongly selective

towards n-valeric formation, as the iso-valeric (Figure 7.4b) and the n-butyric (Figure 7.4c) balances close quite well.

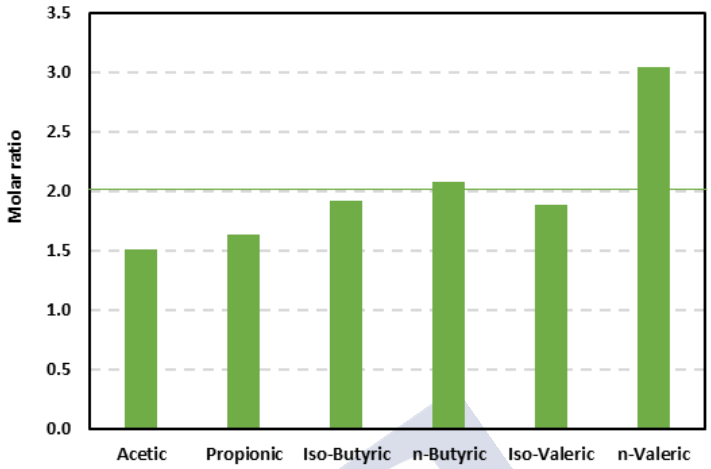

Figure 7.3. Ratios between the final VFA molar concentrations obtained during casein batch fermentation at SIR 20 and SIR 10, respectively. The horizontal line represents the proportionality threshold.

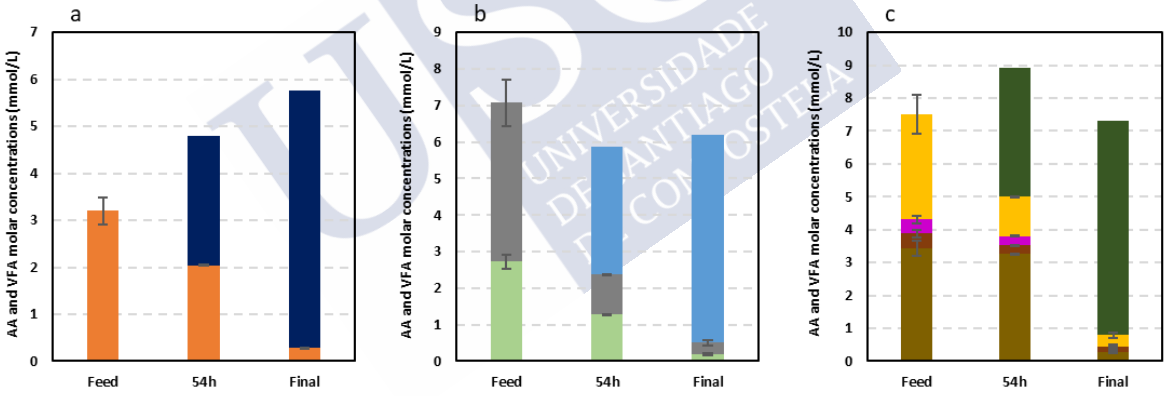

Figure 7.4. n-Valeric (a: ■ Proline; ■ n-Valeric), iso-valeric (b: ■ Isoleucine; ■ Leucine; ■ Iso-Valeric acid) and n-butyric acid (c: ■ Glutamic acid; ■ Histidine; ■ Methionine; ■ Lysine; ■ n-Butyric acid) balances in the casein batch test at SIR20 and pH 5. AA concentrations are expressed in VFA equivalents based on the associated stoichiometry.

Based on these results, apart from the pH and protein composition, the prolonged simultaneous availability of suitable electron donor AA and short chain VFAs seems to play an important role in determining the extent of the CE process. In fact, higher initial substrate concentrations in the batch test allowed the production of the short chain VFAs which were then elongated without depleting the AAs required by the CE process. In a continuous fermentation process, the continuous feeding seems to bypass this AA limitation, since CE contribution to n-valeric formation was greater during CSTR operation (68.2%, Figure 7.1a) than in the SIR 20 batch test (53.2%, Figure 7.4a).

7.3.3. Understanding the role of acetic and propionic acid during protein-based CE

A third batch test was performed with casein at SIR 20 and with the initial supplementation of acetic acid at a concentration of approximately 500 mg/L to understand whether the supplementation of short chain VFAs can promote CE processes during protein fermentation, thus avoiding the associated substrate limitations described in Section 7.3.2.

Assuming that acetic acid production and consumption occurs simultaneously, three periods can be differentiated (Figure 7.5): i) similar consumption and production rate during the first 32 h (i.e. acetic acid concentration remains mainly unchanged); ii) higher consumption rate than production during the subsequent 40 h, and, iii) higher production rate than consumption from 72 h on. Except for propionic acid, the other VFAs started to be produced only after 24 h, suggesting that the acetic acid supplementation led to an adaptation phase during which the biomass was not able to efficiently convert the substrate. Interestingly, n-valeric acid formation began only after acetic acid concentration started to decrease visibly. n-Butyric and iso-valeric acids were confirmed to be the main products of the fermentation (approximately 800 mg/L). Propionic acid did not show any sign of stagnation and its final concentration was 300 mg/L, while iso-butyric and n-valeric levels were slightly higher (400 mg/L). At the end of the test, the acetic acid consumption almost balanced its production as the final concentration is comparable to the initial one. The acidification degree (excluding the initially added acetic acid) and the degree of reduction were 50.9% and 1.86 g COD/g VFA, respectively.

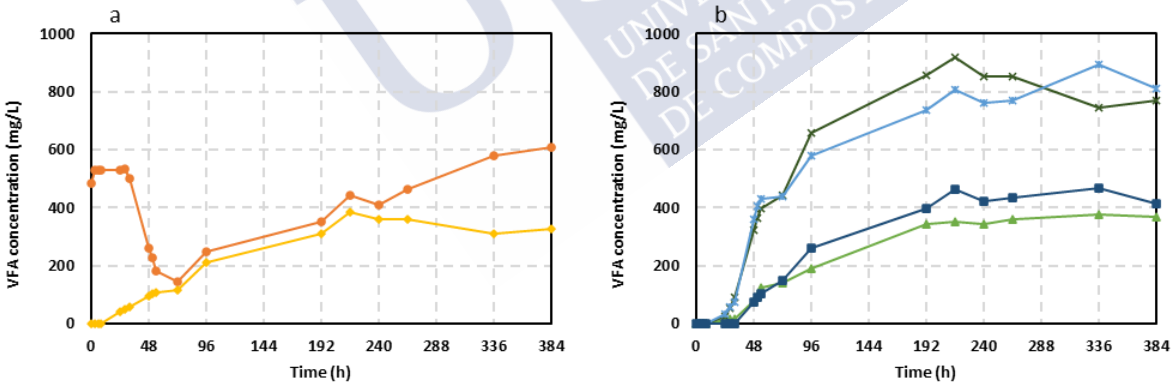

Figure 7.5. VFA production during casein batch fermentation at SIR 20 and pH 5, with initial acetic acid addition (500 mg/L). a: ● Acetic; ◆ Propionic; b: ▲ Iso-Butyric; x n-Butyric; * Iso-Valeric; ■ n-Valeric

The supplementation of acetic acid favours the production of n-butyric and iso-valeric acids but limits the formation of n-valeric acid (Figure 7.6). The final concentration of acetic acid (600 mg/L) is lower than the sum of the supplementation (500 mg/L) plus the production during the SIR 20 test (300 mg/L), indicating a lower formation of this VFA during the fermentation of casein or a higher consumption during CE processes. The increase in propionic and iso-butyric acid production is probably not significant, as it was quite limited in both cases (< 90 mg/L), and consequently attributed to experimental variability.

These results suggest that the initial availability of acetic acid can alter the selectivity of the CE process, especially promoting its elongation to n-butyric acid. Although it does not completely justify the increase in n-butyric acid final concentration (200 mg/L), the difference between expected and measured acetic acid concentration (200 mg/L) seems to be closely related. Moreover, the molar balance of this VFA (Figure 7.7a) evidenced that its four precursor AAs were not converted during the first 54 h, strengthening the hypothesis of the supplemented acetic acid being consumed to produce n-butyric acid (Duncan et al., 2004) through the coupling with acetyl-CoA from a suitable electron donor AA such as alanine (Wallace et al., 2004).

The increase of iso-valeric acid in the supplemented test corresponds to the decrease of n-valeric acid. This can be verified as the sum of iso and n-valeric acid in each test is approximately 1200 mg/L (Figure 7.6), suggesting that the acetic acid supplementation might have diverted the CE process from the production of the linear form to the branched-chain one. The global valeric acid balance (Figure 7.7b) unequivocally confirms the contribution of CE to the production of both acid forms, as their concentrations exceed the consumption of the parent AA. Interestingly, CE was initially the only responsible for n-valeric acid generation (54 hours), even before proline started being consumed.

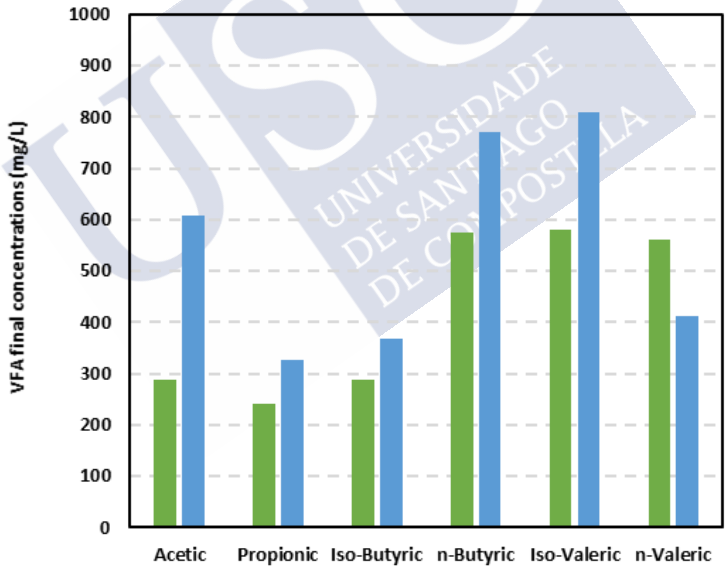

Figure 7.6. Comparison between the VFA final concentrations obtained during the batch tests at SIR 20 and pH 5 with (■) and without (■) acetic acid supplementation (500 mg/L).

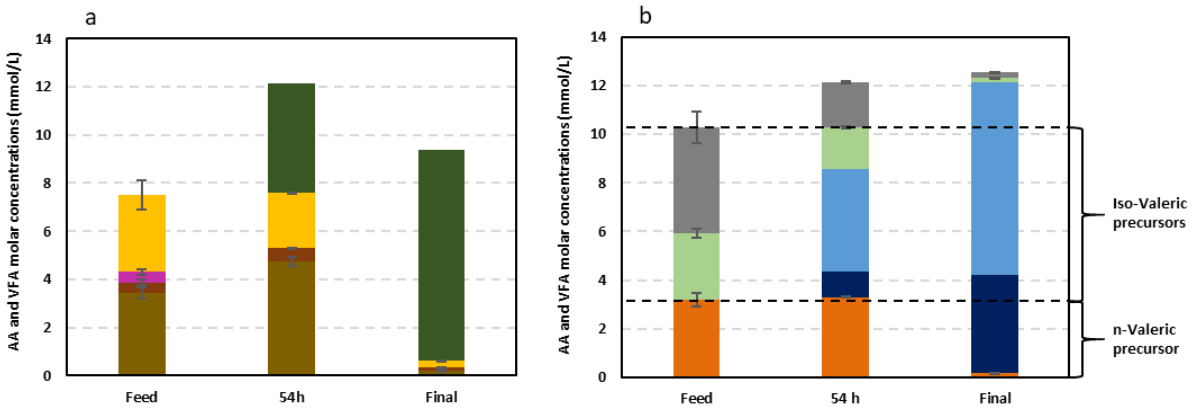

Figure 7.7. n-Butyric acid (a: ■ Glutamic acid; ■ Histidine; ■ Methionine; ■ Lysine; ■ n-Butyric acid) and global valeric (b: ■ Proline; ■ n-Valeric; ■ Isoleucine; ■ Leucine; ■ Iso-Valeric acid) balances in the casein batch test at SIR20 and pH 5 with initial acetic acid addition. AA concentrations are expressed in VFA equivalents based on the associated stoichiometry.

7.3.4. Highlighting the underlying mechanisms of protein-based chain elongation

From the abovementioned results, it can be concluded that casein-based CE is indeed feasible and occurs by using acetic or propionic acid as electron acceptor compound (Figure 7.8). Suitable electron donor AAs will supply reducing power and propionyl-CoA for CE with acetic acid, or acetil-CoA for CE with either propionic or acetic acid (Kim et al. 2019). Several AAs (alanine, arginine, aspartic acid, cysteine, glutamic acid, histidine, serine) can supply either propionyl-CoA or acetyl-CoA as they have pyruvate as intermediate product of their conversion (Regueira et al., 2020). Methionine and lysine are associated to only propionyl-CoA or only acetyl-CoA, respectively. Threonine can provide both these compounds via different pathways (Regueira et al., 2020).

In protein-based chain elongation, the process selectivity seems highly dependent on the initial availability of short chain VFA. If the process is initially limited by the availability of short chain VFAs, it becomes particularly selective towards n-valeric acid (Figure 7.8a), albeit the elongation of the other VFAs cannot be completely excluded (Section 7.3.2). Conversely, the initial supplementation of acetic acid clearly alters the selectivity of the process (Figure 7.8b), promoting the elongation of this short chain VFA to n-butyric acid while prioritising the formation of iso-valeric acid over the n-valeric (Section 7.3.3).

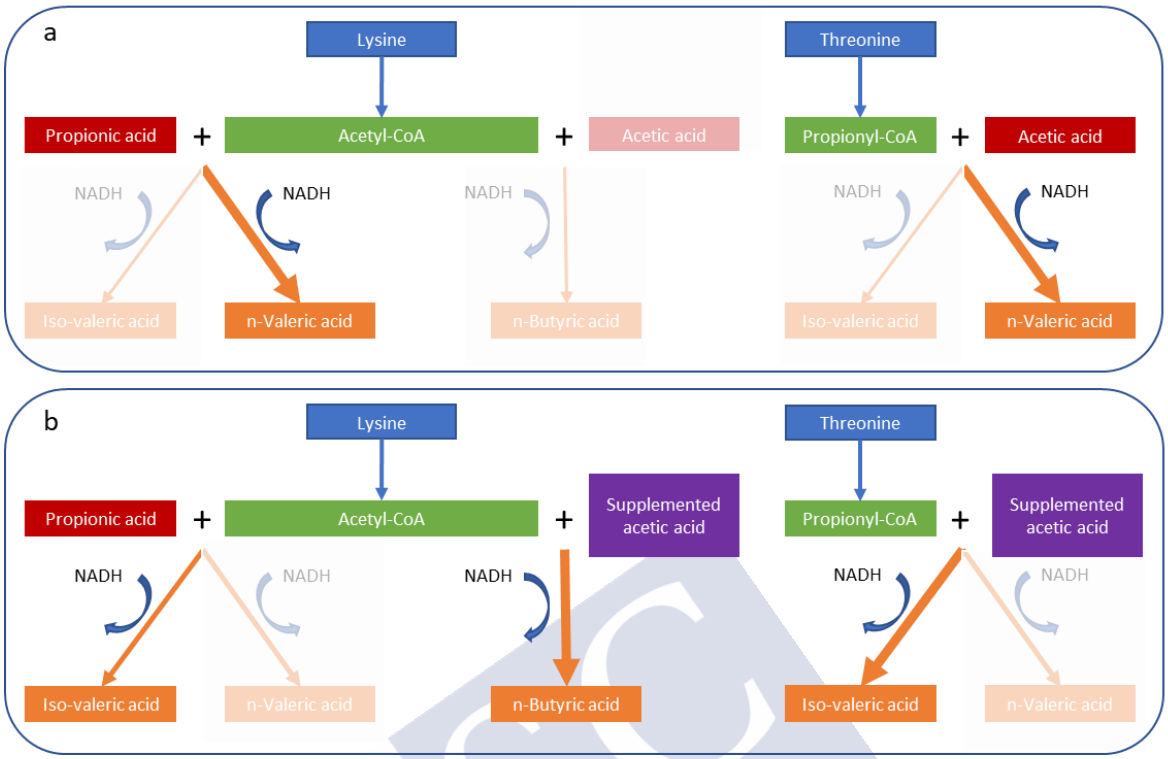

Figure 7.8. Conceptual protein-based chain elongation mechanisms based on short chain VFA and AA consumption (a: initial lack of acetic and propionic acids; b: initial availability of acetic acid). ■ short chain VFA; ■ AA; ■ intermediate metabolite; ■ Elongated product; ■ supplemented short chain VFA. The wider arrow represents the favoured elongation reactions, whereas the faded colours indicate absent or minor routes.

7.3.5. The influence of micronutrients on the chain elongation process

The presence of trace elements in the reactors feedstock facilitates CE process during gelatin fermentation at neutral pH conditions (**Chapter 5**). Both iso and n-valeric acid were hypothesised to be partially produced via elongation (Section 5.3.3) through the same mechanisms conceptualised in Figure 7.8. Also, n-caproic acid was produced via n-butyric acid elongation with acetyl-CoA provided by suitable AAs during casein fermentation at pH 7 with micronutrients. Still, it should be noted that the production of this medium chain VFA was somewhat limited (< 200 mg/L).

At acid conditions (pH 5), both proteins became suitable substrates for the CE processes due to the presence of the aforementioned trace elements. Still, some variations in terms of extent and selectivity were observed in comparison with the findings previously described in the present chapter. In particular, n-butyric acid production via elongation is partially or even completely suppressed by the supplementation of micronutrients during the fermentation of casein (Figure 7.9a) and gelatin (Figure 7.9b). Conversely, iso-valeric acid formation was promoted (Figure 7.9c and d), exceeding the combined consumption of the parent AAs, isoleucine and leucine. The appearance of iso-caproic acid during gelatin fermentation was attributed to a leucine alternative fermentation pathway (Britz & Wilkinson, 1981) rather than

being considered as a CE product. In contrast, n-valeric acid production was mostly unaffected by the micronutrients supplementation (Figure 7.9e and f), suggesting that the associated metabolic routes (regular and elongative) are not especially dependant on the presence of these compounds.

These results further help to define the requirements for protein-based CE to occur. The absence of trace elements in the feedstock seems to limit the process to MCF of proteins particularly rich in electron donor AAs (e.g. casein) when performed at acid conditions. Conversely, their presence somewhat removes the protein composition limitation, while widening the pH range at which CE can be performed by the microbiome. In fact, the supplementation of micronutrients at neutral pH could be making the CE necessary as a way of disposing of the increased acid equivalents production, without excluding that the presence of these compounds could be making viable certain elongative routes which were previously limited or unavailable to the microbial community. Taking into consideration the impact of both AA composition and pH value, these changes in metabolic pathways are conceptualised in Figure 7.10 for both proteins.

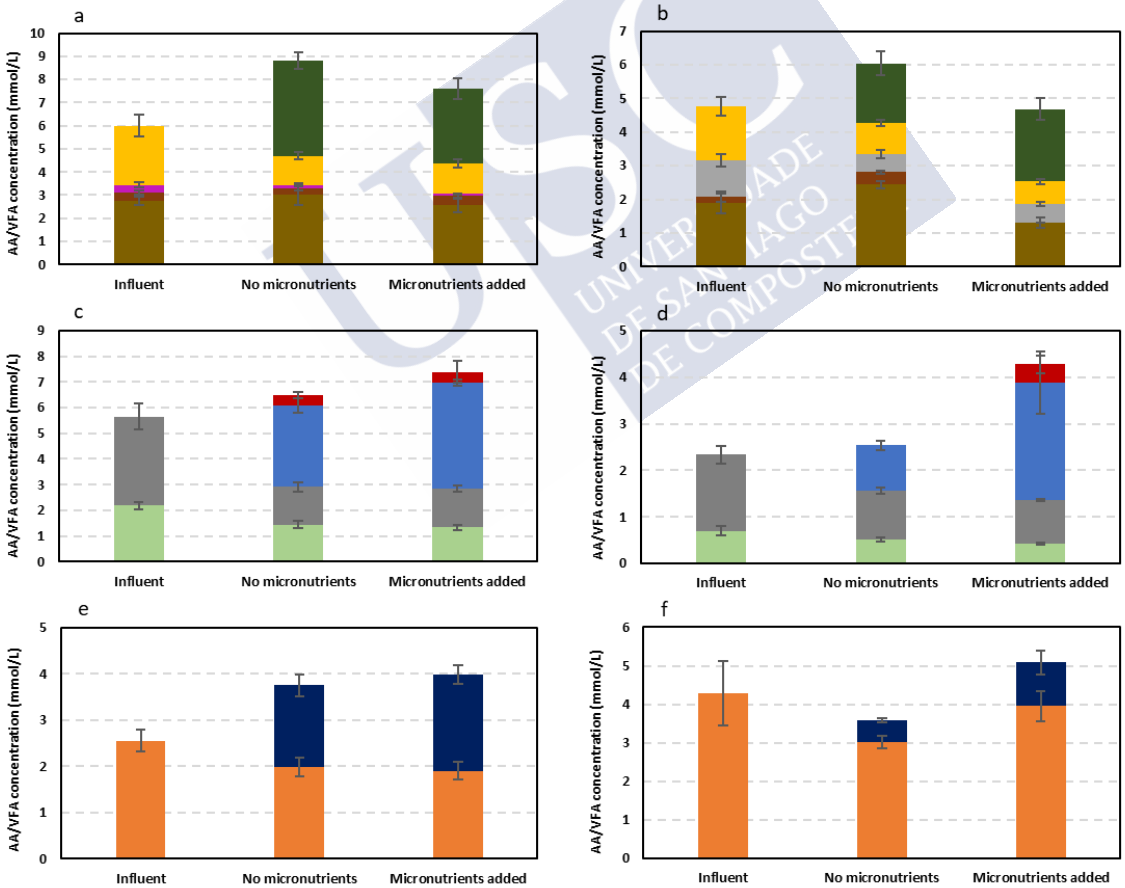

Figure 7.9. n-Butyric (a, b: ■ Glutamic acid; ■ Histidine; ■ Methionine; ■ Threonine; ■ Lysine; ■ n-Butyric acid), iso-valeric (c, d: ■ Isoleucine; ■ Leucine; ■ Iso-Valeric acid; ■ Iso-Caproic acid) and n-valeric (e, f: ■ Proline; ■ n-Valeric) acids balances during casein (a,c,e) and gelatin (b,d,f) fermentation at pH 5 supplemented with trace elements. AA concentrations are expressed in VFA equivalents based on the associated stoichiometry.

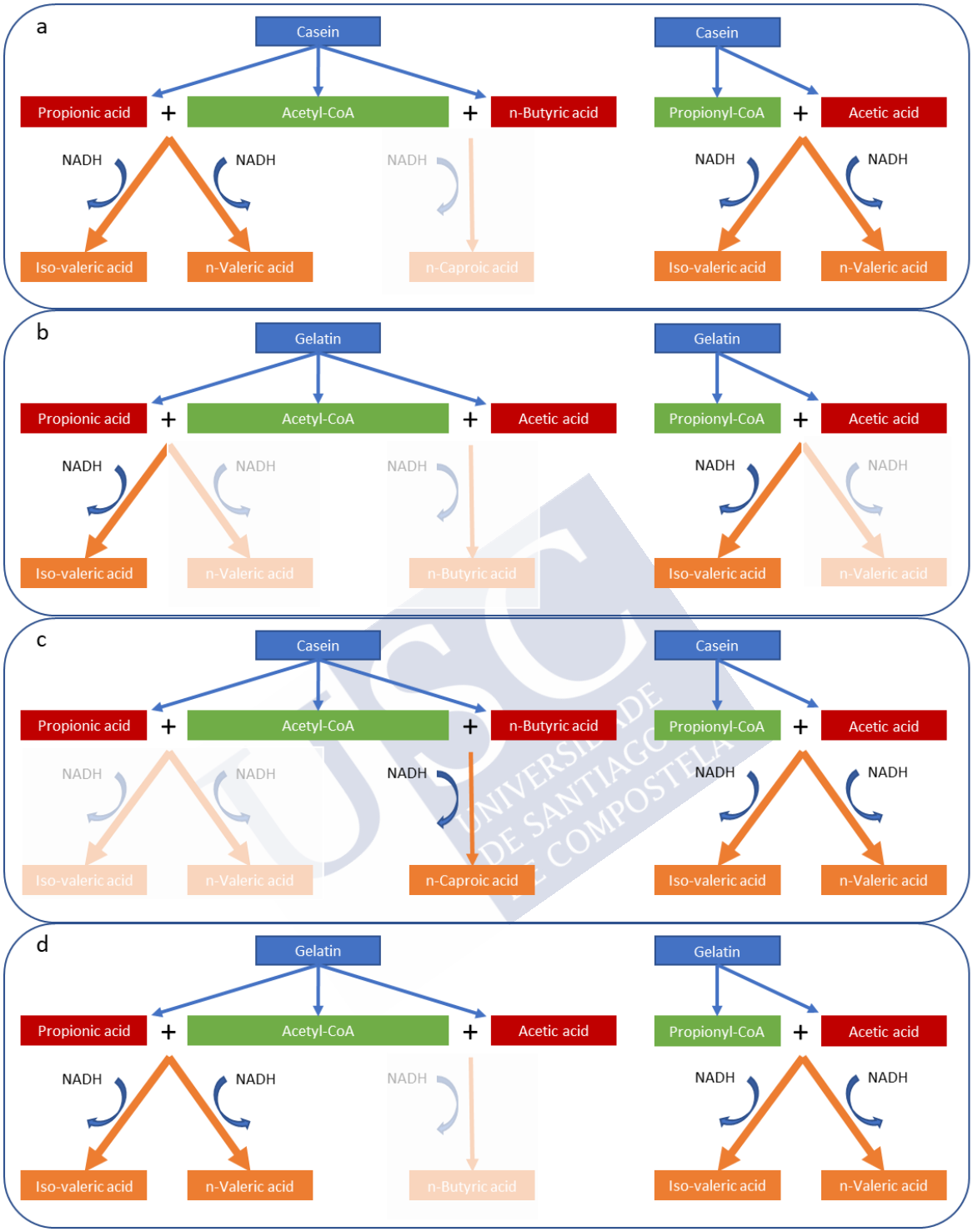

Figure 7.10. Conceptual chain elongation mechanisms based on short chain VFA, AA consumption and micronutrients presence during casein (a,c) and gelatin (b,d) fermentation (a,b: pH 5; c,d: pH 7). ■ substrate VFA; ■ protein; ■ intermediate metabolite; ■ Elongated product. The wider arrow represents the favoured elongation reactions, whereas the faded colours indicate absent or minor routes.

7.3.6 Promoting the protein-based CE through cofermentation with sugars

CE was identified during the experiments described in **Chapter 6** as well, suggesting that cofermenting protein-rich substrates with those rich in sugars might be a viable strategy to promote the occurrence of the process. The presence of glucose progressively stimulated the selective production of n-valeric acid up to a sugar-to-protein (STP) ratio equal to 1.00 Although glucose fermentation is often associated with direct n-butyric production (González-Cabaleiro et al., 2015), production of this VFA via elongation was not discarded either, given that the required substrates (acetic acid and lactate (Liang & Wan, 2015)) were present in the fermentation broth (Section 6.3.3). It should be also noted that n-caproic acid was detected as well, albeit only when applying an STP of 0.50 to the reactor operation (Section 6.3.1). The suppression of the CE process, attributed to the experiment interruption and subsequent biomass storage at 4°C, was replaced by the appearance of lactate and ethanol after the reactor operation was resumed. It should not be excluded either that STP ratios greater than 1.00 might be hindering the CE, as the process could not be recovered even after more than 80 days of resumed operation of the cofermentation reactor. Proof of the negative effect of excessive sugar loadings was obtained through the operation of a secondary reactor at a decreased STP (0.50), which allowed to recover both n-butyric and n-valeric acid production to a certain extent (Section 6.3.4).

Based on results obtained by Liang and Wan (2015) with brewers spent grain alternatively supplemented with ethanol and lactate, two main elongation mechanisms were hypothesised (Figure 7.11). Ethanol was being probably consumed together with propionic acid to generate n-valeric acid, whereas lactate was being elongated together with acetic acid to n-butyric acid. Ethanol was being consumed to be elongated with n-butyric acid to n-caproic acid as well, although this route seems a minor one as it is restricted to a specific STP (0.50). The acid equivalents pressure still appears to be the main driving force of the CE process. In fact, glucose supplementation progressively favours the production of VFAs at greater concentrations (Section 6.3.1), forcing the microbial community to use CE as a detoxification strategy. Besides, glucose fermentation further provides the system with reducing power (i.e. NADH) and ethanol/lactate, consequently magnifying the elongation process.

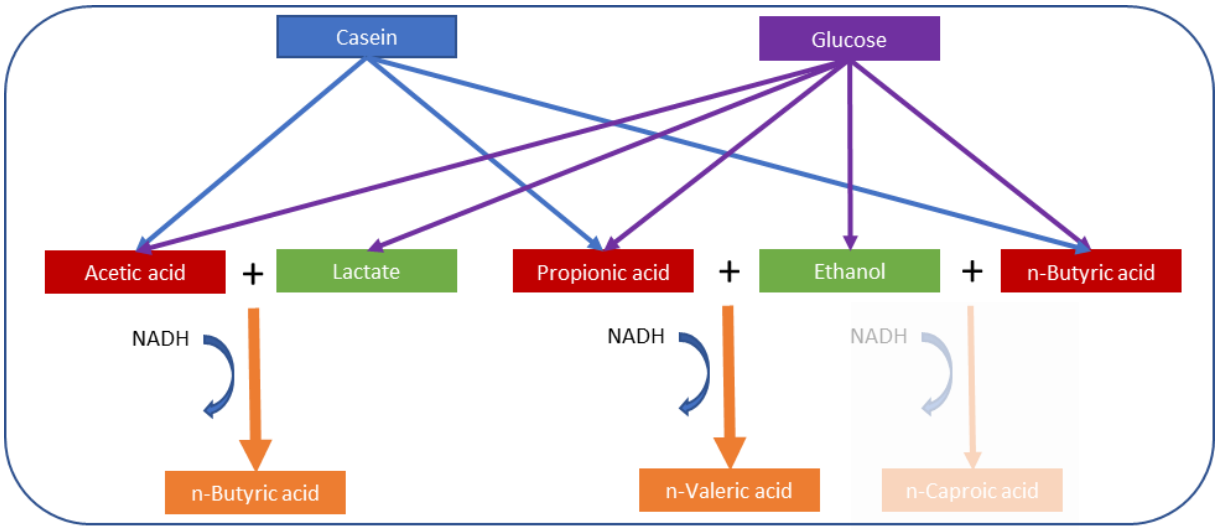

Figure 7.11. Conceptual chain elongation mechanisms during casein and glucose cofermentation. ■ Substrate VFA; ■ Protein; ■ Glucose; ■ Intermediate metabolite; ■ Elongated product. The wider arrow represents the favoured elongation reactions, whereas the faded colours indicate absent or minor routes.

7.4. CONCLUSIONS

To the best of our knowledge, this chapter identifies and targets for the first time the occurrence of chain elongation process during protein MCFs and cofermentation with sugars, with the following main conclusions:

- Acid conditions are hypothesised to be promoting protein-based CE, since this process reduces the overall acid equivalents yielded from the substrate, consequently avoiding potential toxicity constraints.
- The feasibility of CE processes during protein fermentation is significantly influenced by protein composition, with due preference for those rich in electron donor AAs.
- The occurrence of CE strongly depends on the prolonged simultaneous availability of short chain VFAs and electron donor AAs.
- The selectivity of the CE process depends on the specific availability of acetic and propionic acid.
- The supplementation of trace elements reduces the pH and protein composition limitations and changes the CE process selectivity.
- Mixing sugars with proteins up to an STP of 1.00 is an alternative way of promoting CE processes targeting n-butyric and n-valeric acid production

The knowledge generated through this work constitutes a starting point for further studies on protein-based CE aiming at an optimal integration of protein-rich (waste)waters in biorefinery frameworks, currently envisioned for carbohydrate-rich streams only.

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CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

8.1 MAIN OUTCOMES OF THE THESIS

MCF processes pose themselves as an emerging and joint solution to the need for a sustainable way of producing chemical precursors and the increasing necessity for wastewaters treatments and waste disposal (Lee et al., 2014). As such, the application of the biorefinery concept for the production of VFAs from organic matter-rich streams perfectly fits in the resource recovery framework. However, low product selectivity and concentration associated to fermentation processes need to be addressed to render the VFA biorefinery an economically viable alternative to the conventional way of producing carboxylic acids from fossil fuels (Bathia & Yang, 2017; Atasoy et al., 2018). In particular, more knowledge about the effect of the operational conditions and strategies on their underlying mechanism is required, as it could help to understand how to steer the outcome of the MCF towards the desired outcome both in terms of conversion efficiency and products selectivity.

Despite being a relevant fraction of many industrial streams and wastewaters, information on how the operational parameters affect the conversion of proteins to VFAs is limited and contradictory at times (Breure & van Andel, 1984; Ramsay & Pullammanappallil, 2001; Duong et al., 2019). This lack of insight into the fermentation mechanisms of proteins consequently limits the application of MCF to feedstocks rich in carbohydrates. Therefore, this thesis tries to provide the aforementioned insight by answering the main questions regarding proteins conversion to VFA formulated in the introduction (**Chapter 1**). A better understanding of the process is obtained by identifying and evaluating those key operational conditions which affect the acidification mechanisms of AAs. This knowledge is useful when designing MCF of protein-rich residues as it will help to understand how to steer the outcome of the process towards the desired outcome both in terms of conversion and VFA selectivity.

8.1.1 Role of AAs composition in protein MCFs outcome

As described in **Chapter 1**, the influence of AAs composition on VFAs production had already been considered by previous studies (Nagase & Matsuo, 1982; Ramsay & Pullammanappalil, 2001). However, their consumption was always assumed to be complete regardless of the overall redox balance between the AAs acting as electron acceptors and those acting as electron donors. Based on this assumption, a fixed stoichiometry was proposed to describe the conversion of AAs to VFAs suggesting that the selectivity of the process could be predicted based on AA composition.

Chapter 3 demonstrates that AA interact with each other, determining the outcome of protein MCFs. Firstly, AA profile affects protein conversion as the two model proteins used in the experiments, casein and gelatin, were acidified to different extents. Moreover, this conversion was always incomplete, regardless protein composition, as also observed by other authors (Yin et al., 2016; Duong et al., 2019). A balanced AA composition is more suitable for the conversion to VFAs, as higher acidification degrees were achieved for casein than for gelatin, which features a dominant glycine fraction (approximately a third in molar basis). It is not excluded either that an excess of reducing power, attributed to the dominance of electron donor AAs, can be also responsible for the greater acidification degree. Interestingly, AAs are

preferentially consumed, and this preferential consumption depends on amino acid profile of the protein (Figure 8.1). Given the abovementioned results, a fixed stoichiometry is not sufficient at describing the selectivity of protein MCF, suggesting that a variable stoichiometry could be more reliable (Regueira et al., 2020).

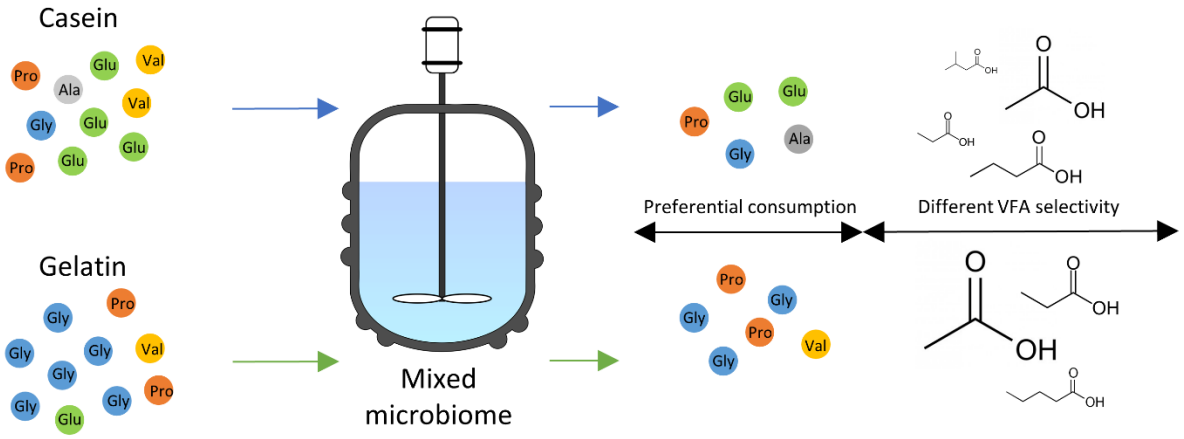

Figure 8.1 Conceptualisation of the effect of different AA composition on protein MCF

AA composition also determines the extent of pH effect on protein MCF (**Chapter 4**). In fact, the fermentation of proteins rich in those AAs with fixed or limited metabolic pathways (e.g. glycine) are potentially less affected by pH than those rich in AAs having pyruvate as an intermediate, which show similar behaviour to sugars (Temudo et al., 2007; González-Cabaleiro et al., 2015). The benefits posed by the supplementation of micronutrients depend on AA composition as well (**Chapter 5**), as the extent of the increase in their consumption at neutral pH conditions varies between different proteins. For example, proline utilisation almost doubles during gelatin fermentation whereas its consumption only increases by 37% in casein case. Finally, AA-based CE is particularly dependant on protein composition (**Chapter 7**). Proteic streams particularly rich in electron donor AA are more suitable for the elongation process, by providing the required substrates and the driving force for the process to occur (i.e. acid equivalents pressure). Interestingly, the supplementation of micronutrients seems to remove this limitation to a certain extent (**Chapter 5**), given that CE is promoted during gelatin fermentation at both acid and neutral conditions, favouring the production of iso-valeric and/or n-valeric acid.

Overall, the present thesis successfully defined the role of AA composition in the protein MCF outcome. This knowledge will not only help to verify the suitability of protein-rich wastes and sidestreams as fermentation feedstocks, but also suggest potential micronutrients supplementation based on their composition and indicate whether adjusting the pH could be effectively used to steer the process.

8.1.2 Understanding the influence of pH and micronutrients on protein MCF

The impact of some operational conditions, pH above all, has been extensively studied in the case of sugar-based MCF processes (Zoetemeyer et al., 1982b; Temudo et al., 2007; Mohd-Zaki et al., 2016; Jankowska et al., 2017), demonstrating how it can be manipulated to alter the selectivity of the process. Similarly, micronutrients can be supplemented to increase the acidification degree (Yu & Fang, 2001) and to drive these processes towards specific VFA formation (Dahiya et al., 2020). In the case of proteins, adjusting the pH value appears to be a promising strategy to steer the process as well, albeit several contradictions on the subject were highlighted in literature (Breure et al., 1984; Ramsay & Pullammanappallil, 2001; Duong et al., 2019). Conversely, no information is available regarding the impact of micronutrients on VFA production from proteins.

Chapter 4 focuses on understanding how pH affects the conversion of AAs into VFAs. Alkaline conditions were considered to be unattractive for protein fermentation, as they do not strongly affect the selectivity of the process, except for increased acetic acid production, and might limit the conversion efficiency. Conversely, neutral range is associated with the maximisation of global VFA productions. Acid conditions are attractive as well due to the more varied product spectra which they determine in comparison with neutral and alkaline conditions, although protein conversion is negatively affected. To explain these variations in selectivity and conversion, it is hypothesised that the pressure exerted by pH can be altering the bioenergetics of certain AA conversion pathways (Regueira et al., 2020), making their utilisation less attractive to the microbial community. For example, glycine is completely consumed during casein neutral fermentation and it is not converted at low pH, whereas arginine consumption is high ($\geq$ 70%) regardless of the pH range. It is not excluded either that pH might be affecting the bioavailability and subsequent uptake of AAs by altering their conformations (Chen & Chung, 2015) and/or inhibiting specific transportation mechanisms (Tseng et al., 2009). In fact, many AAs (e.g. threonine) feature an isoelectric point between pH 5 and 6, meaning that their agglutination and precipitation is more likely when operating the reactor at low pH, making them potentially inert. Despite the lowered acidification, applying low pH conditions still constitutes an appealing operational strategy through which promoting longer-chain VFA production (**Chapter 4**), mainly due to the occurrence of CE (**Chapter 7**). In fact, the process answers to the need for an acid equivalents disposal as long as the fermented proteins feature an excess of electron donor AA.

Since the lower acidification degrees detected at acid conditions are not attributed to kinetic limitations nor product inhibition (**Chapter 4 and 7**), it was hypothesised that the enzymatic complexes involved in AA fermentation might not be working properly due to the lack or limited availability of the required cofactors (i.e. trace elements). However, micronutrients supplementation was tested at pH 5 without success, as their presence does not offset the conversion limitations imposed by the operation at acid conditions (**Chapter 5**). Yet, trace elements supplementation improves protein conversion and process selectivity towards longer chain VFA at neutral pH (Figure 8.3). Other benefit of micronutrients supplementation is the removal of CE dependence on the AA composition of the substrate

(**Chapter 7**), potentially widening the application of this process to different protein-rich streams.

Overall, adjusting pH can be used as a viable strategy to steer protein MCF towards the desired outcome. If the products distribution is not of particular concern, for example in a two-stage digestion process for the production of biogas, neutral conditions should be prioritised in order to maximise the conversion of the substrate to VFAs. If the chosen feedstocks do not include trace elements at stimulatory concentrations, they should be also supplemented as a way of further promoting VFA production. Conversely, if longer chain VFA production is the goal, acid conditions should be applied to promote the production of butyric and valeric acids favouring CE processes as well.

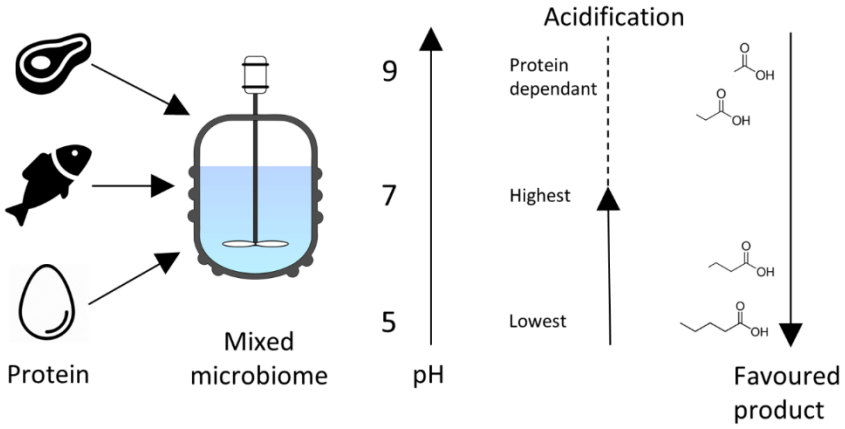

Figure 8.2 Conceptualisation of the effect of pH on protein MCF

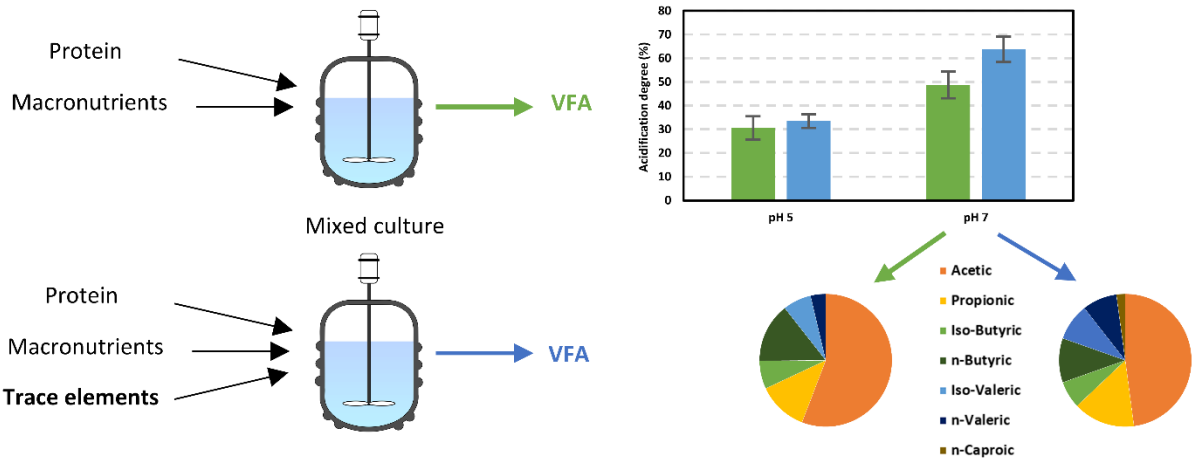

Figure 8.3 Conceptualisation of the effect of micronutrients on protein MCF

8.1.3 Identifying opportunities for and beyond the protein MCF

Given that proteins monofermentation seems to be often associated with incomplete conversion, even when not kinetically limited (**Chapter 3; Chapter 4;** Duong et al., 2019), mixing proteins with sugars in a cofermentation process can be a viable solution to increase VFA production (Ma et al., 2017). In fact, most wastes and sidestreams (e.g. food waste and acid whey) are inherently a mix of different organic fractions (Garcia-Aguirre et al., 2017), meaning that using them as fermentative substrate results in a *de facto* cofermentation process. The beneficial effect of sugar presence on protein fermentation depends on their relative abundance in the feedstock, but available information is contradictory, pointing out the need for a better understanding of the interactions between the two organic fractions during cofermentation.

Adjusting the STP ratio on values lower or equal to 1.00 appears to be a viable strategy to drive the process towards the selective production of n-butyric and n-valeric acid (**Chapter 6**), mostly through CE, highlighting synergy between proteins and sugars fermentation. Conversely, STP ratios greater than 1.00 were associated with decreased protein consumption, indicating this value as the critical threshold past which antagonism substitutes the degradation synergy (Figure 8.4). Excessive sugar loadings lead to the VFA production being partially substituted with the formation of less relevant secondary metabolites (e.g. ethanol). Still, the reversibility of the changes produced by STP manipulation suggests that the cofermentation process is quite flexible and resilient.

All these findings could be applied to the anaerobic conversion of complex protein-rich feedstocks, such as slaughterhouse solid wastes (Escudero et al., 2014), which could be adequately mixed with dairy wastewaters as a way of promoting protein consumption and overall VFA production.

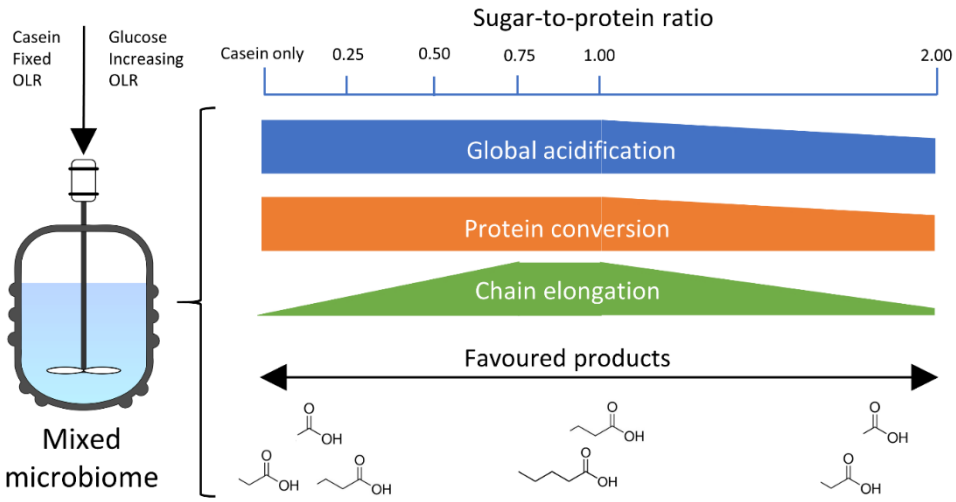

Figure 8.4 Conceptualisation of the interactions between proteins and sugar during the cofermentation

Longer-chain VFAs and MCFA have greater commercial value (Moscoviz et al., 2018) and they are more easily separated from the fermentation broth due to their lower affinity to aqueous solutions (Candry & Ganigué, 2021). Therefore, promoting the chain elongation of short chain VFAs can be seen as an opportunity to make protein monofermentation more appealing as a resource recovery process (Han et al., 2019). However, there are no previous literature examples of CE occurring in a protein MCF, as information can only be found regarding pure cultures (Wallace et al., 2003; Wallace et al., 2004).

Most chapters of the present thesis identify the occurrence of CE during the fermentation of either casein and/or gelatin depending on the operational conditions (**Chapter 7**). Yet, the underlying mechanisms are not completely characterised. Analysing the role of AAs was useful to conceptualise the associated metabolic pathways (Figure 8.5). CE occurs during protein MCF without the supplementation of electron donor compounds, such as lactate and ethanol (**Chapter 4**). Moreover, the process seems to depend on both protein composition and pH value. It is hypothesised that acid conditions could be promoting the process as a joint strategy to reduce the toxicity of the system by disposing of acid equivalents and maintain the NADH balance neutral (Angenent et al., 2016) by consuming the excess generated from the fermentation of proteins rich in electron donor AAs (e.g. casein). AA-based CE seems particularly selective towards the formation of n-valeric acid; however, supplementation of short chain VFAs (e.g. acetic acid) is not a viable strategy to intensify the elongation to n-valeric acid, as the products distribution shifts from the linear to the branched form (i.e. iso-valeric acid) while promoting the formation of n-butyric acid. This indicates that CE extent and selectivity strongly depends on the prolonged simultaneous availability of AAs and electron acceptor VFAs. Interestingly, trace elements supplementation seems to widen the occurrence of CE process not only in terms of pH, as it is identified at neutral conditions, but also in terms of AA composition (**Chapter 5 and 7**). Also supplementing sugars during protein fermentation at adequate STP ratio constitute an appealing strategy to selectively produce n-valeric acid via the CE process (**Chapter 7**),

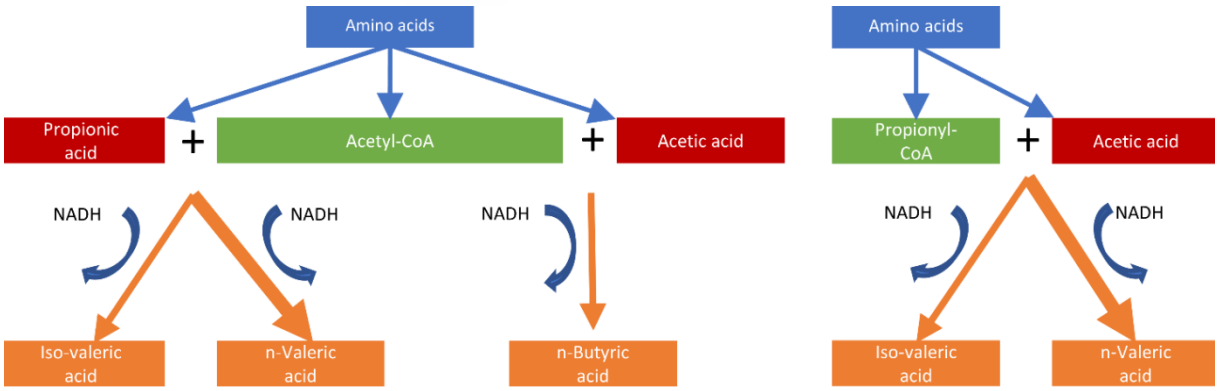

Figure 8.5 Conceptualisation of the metabolic pathways involved in protein-based CE

Overall, cofermentation and CE processes have been demonstrated as feasible and interconnected opportunities to make protein MCF more appealing both in terms of conversion efficiency and VFA selectivity. One of the most interesting aspects of the AA-based CE is the fact that it can occur without the supplementation of external electron donor compounds, as AAs can perfectly fill the role. The presence of sugars, if carefully controlled, can intensify this kind of CE by increasing the availability of electron acceptor (short chain VFAs) and electron donor compounds (ethanol and lactate) while strengthening the driving force of the process (i.e. acid equivalents pressure). All this knowledge thus constitutes a promising first step towards the design and application of CE processes to protein-rich wastes and sidestreams, incrementing their attractiveness as fermentation substrates by mixing them with suitable sugar-rich feedstocks as well.

8.2 RESEARCH GAPS AND FUTURE PERSPECTIVES

One of the aspects of protein fermentation which were not dealt with in the present work is the hydrolysis step. Although inert AAs can be found in the fermentation broth even in absence of kinetic limitations (**Chapter 3; Chapter 4; Chapter 7;** Duong et al., 2019), hydrolysis impact might not be negligible, especially when considering the nature of most organic wastes and sidestreams. Alkaline conditions are considered optimal for the fermentation of waste activated sludge due to their influence on protein configuration. In fact, high pH seems to promote both biotic and abiotic hydrolysis by unfolding their structure (Wang et al., 2019). Assuming that some peptide bonds might be more easily severed by the proteases, the resulting free AA could be preferentially fermented to different extent, as seen in **Chapter 3**. Thus, further studies on the hydrolysis impact on the proteic fraction at different pH can be a potential step forward in understanding the mechanisms regulating the outcome of protein MCFs.

Another future study might involve the search for a way of increasing the conversion efficiency of proteins at low pH, given that the increased longer chain VFA production comes at the cost of reduced acidification degrees, regardless of the presence of stimulatory concentrations of trace elements. Given that this conversion limitation is not determined by kinetic limitations either (**Chapter 4**), an alternative could be posed by the cofermentation with sugars. For example, protein conversion might benefit from increased biomass concentrations resulting from the close-to-complete sugar consumption at low pH.

For instance, studying the individual fermentation of AAs could help to better understand not only their micronutrients and pH requirements but also their interaction with each other or with different substrates (e.g. sugars). Further insight on their metabolic pathways and associated requirements will help to refine the selection of appealing feedstocks based on specific AA richness while suggesting the most suitable operational conditions for the desired fermentation outcome.

Specific additions of heavy metals have, in fact, the potential of steering the process towards the production of the desired VFA, as demonstrated by Dahiya et al. (2020) with Co and Zn applied to glucose fermentation. While this knowledge could be applied to steer the

fermentation of all those AA which have pyruvate as an intermediate product of their conversion, generating insight into the enzymatic requirements of more fixed AA metabolic pathways could potentially help to promote their conversion to the associated VFA. Such focus could be placed on the branched AA, valine, isoleucine and leucine, as their fermentation is strictly associated with iso-butyric and iso-valeric acids, which are the least produced carboxylates due to their dependency from the proteic fraction (Regueira et al., 2020).

Chapter 6 only tested the interaction between protein and sugars at neutral conditions, meaning that investigating the effect of adjusting the STP ratio at different pH value might provide combined strategies to steer the overall VFA production towards the desired outcome. This chapter also ignored other interactions, such as with lipids, albeit being a relevant organic fraction of many wastes and sidestreams. The impact of their presence could be assessed during the cofermentation with sugars and/or proteins, providing further insight on how to choose, and eventually mix, suitable feedstocks for the production of VFA.

The results presented in **Chapter 7** should be considered as a starting point for further studies on the subject of protein-based CE rather than a comprehensive guide on the application and optimisation of the process. The relevant mechanisms should be studied more in detail, as to discern which AAs are effectively involved in the process and how they interact with the short chain VFAs to produce the elongated compounds. For example, isotopically labelling carbon atoms of the relevant substrates could be an interesting method through which accomplishing this objective, as demonstrated by Kim et al. (2019). The impact of operational conditions and strategies should be further studied as well in their potential to steer the CE towards specific longer chain VFAs, as occurred with the supplementation of acetic acid (**Chapter 7**). With this perspective, propionic acid could be supplied to the fermentation broth in order to unequivocally determine whether its consumption is linked with n-valeric acid formation. In-situ product removal techniques for continuous VFA separation will not improve protein conversion as product inhibition was generally ruled out throughout this thesis.

The microbiological aspect of protein fermentation has not been dealt within the present thesis, although sample analysis is presently ongoing. Knowing the exact microbial community composition is not that relevant in open anaerobic mixed cultures given its metabolic redundancy and constant evolution (Carballa et al., 2015; Regueira et al., 2020). However, studying the microbiology of protein fermentation could help to discern whether the changes in selectivity and conversion determined by the operational strategies should be only associated with effects on the metabolic pathways or are also caused by variations in the microbial populations. Moreover, understanding how certain species are associated with isomerisation and CE could help to study those processes.

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LIST OF PUBLICATIONS

SCIENTIFIC JOURNALS

Bevilacqua, R., Mauricio-Iglesias, M., Lema, J.M., Carballa, M. (2021). Engineering the outcome of cofermentation processes by altering the feedstock sugar-to-protein ratio. **In preparation. Chapter 6** is based on this publication. **Author contribution (CRediT taxonomy):** Methodology, Investigation, Writing - original draft, Writing - review & editing.

Bevilacqua, R., Regueira, A., Mauricio-Iglesias, M., Lema, J.M., Carballa, M. (2021). Chain elongation may occur in protein mixed-culture fermentation without supplementing electron donor compounds. **Under review** by *Water Research*. Impact factor = 9.130 (2019); Subject: Water Science and Technology; Ranking: Q1. **Chapter 7** is based on this publication. **Author contribution (CRediT taxonomy):** Methodology, Investigation, Writing - original draft, Writing - review & editing.

Bevilacqua, R., Regueira, A., Mauricio-Iglesias, M., Lema, J.M., Carballa, M. (2021). Understanding the effect of trace elements supplementation on volatile fatty acids production from proteins. **Under review** by *Journal of Environmental Chemical Engineering*. Impact factor = 4.300 (2019); Subject: Chemical Engineering (miscellaneous); Ranking: Q1. **Chapter 5** is based on this publication. **Author contribution (CRediT taxonomy):** Methodology, Investigation, Writing - original draft, Writing - review & editing.

Bevilacqua, R., Regueira, A., Mauricio-Iglesias, M., Lema, J.M., Carballa, M. (2021). Steering the conversion of protein residues to short chain carboxylates by adjusting pH. *Bioresource Technology*, 320 (B), 124315. Impact factor = 7.539 (2019); Subject: Bioengineering; Ranking: Q1. **Chapter 4** is based on this publication. **Author contribution (CRediT taxonomy):** Methodology, Investigation, Writing - original draft, Writing - review & editing. https://www.elsevier.com/about/policies/copyright/permissions

Regueira, A., **Bevilacqua, R.**, Mauricio-Iglesias, M., Lema, J.M., Carballa, M. (2021). Kinetic and stoichiometric model for the computer-aided design of protein fermentation into volatile fatty acids. Published in *Chemical Engineering Journal*, Volume 406, Article 126835. Impact factor = 10.652 (2019); Subject: Chemical Engineering; Ranking: Q1 **Author contribution (CRediT taxonomy):** Investigation, Writing - review & editing.

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Regueira, A., **Bevilacqua, R.**, Lema, J.M., Carballa, M., Mauricio-Iglesias, M. (2020). A metabolic model for targeted volatile fatty acids production by cofermentation of carbohydrates and proteins. Published in *Bioresource Technology*, Volume 298, Article 122535. Impact factor = 7.539 (2019); Subject: Bioengineering; Ranking: Q1. **Author contribution (CRediT taxonomy):** Investigation, Writing - review & editing.

CONGRESS PROCEEDINGS

Oral contributions

Bevilacqua, R., Regueira, A., Mauricio-Iglesias, M., Lema, J.M., Carballa, M. Identifying chain elongation processes during the mixed-culture fermentation of proteins. International Chain Elongation Conference 2020 (ICEC2020). Wageningen (The Netherlands - virtual), October 2020.

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Poster contributions

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