Universidade Federal do Rio Grande do Sul
Instituto de Ciência e Tecnologia de Alimentos
Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos

Produção de vitamina B_{12} por *Propionibacterium freudenreichii* subsp. *shermanii* ATCC 13673 cultivada em resíduo agroindustrial de soja para enriquecimento nutricional de alimentos de origem vegetal

Dener Acosta de Assis

Porto Alegre
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Tese apresentada ao Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos como requisito para a obtenção do título de Doutor em Ciência e Tecnologia de Alimentos.

Orientador: Marco Antônio Záchia Ayub

Coorientadora: Carla Roberta Mate

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Resumo

A ingestão de vitamina B_{12} em dietas vegetais alternativas pode ser desafiadora devido à inabilidade natural das plantas em produzir este micronutriente. Porém, através de estratégias de bioprocessos, pode-se suprir esta demanda nutricional. Neste estudo foram investigadas: 1) a otimização do bioprocesso para produção de vitamina B_{12} por *Propionibacterium freudenreichii* subsp. *shermanii* ATCC 13673 utilizando resíduo agroindustrial de soja como substrato em biorreatores STR 2 L; 2) a produção de alimentos vegetais fortificados com a biomassa da linhagem e 3) a biocompatibilidade dos alimentos fortificados em modelo *in vitro* do epitélio intestinal humano (células Caco-2). A otimização das condições de bioprocessos possibilitou a obtenção de altos rendimentos de vitamina B_{12} (acima de 800 µg/g biomassa seca). A análise em sistema ICP-MS revelou a presença de resíduos de cobalto na biomassa seca (~ 3 µg/g biomassa seca), consequência da sua suplementação nos meios de cultura. Contudo, os ensaios de citotoxicidade e integridade das monocamadas de células Caco-2 (biocompatibilidade) demonstram que amostras de alimentos fortificadas com biomassa da linhagem não exerceram toxicidade sobre o modelo após digestão simulada *in vitro* (INFOGEST 2.0). No geral, os resultados demonstraram que a biomassa de *P. shermanii* ATCC 13673 cultivada em resíduo agroindustrial de soja pode ser uma fonte alternativa, segura e eficaz para fortificação de alimentos vegetais.

Palavras-chave: Vegetarianismo; Veganismo; Cobalamina, *Plant-based foods*; Sustentabilidade; Bioprocessos; Biotecnologia.

Abstract

Vitamin B_{12} intake from alternative plant-based diets can be challenging due to the natural plant's inability to produce this essential micronutrient. However, the nutritional demand can be met through bioprocess strategies. This study investigated: 1) the optimization of a bioprocess to produce vitamin B_{12} by *Propionibacterium freudenreichii* subsp. *shermanii* ATCC 13673 using soybean agro-industrial residue as substrate in 2 L STR bioreactors; 2) the production of plant-based foods fortified with the spray-dried strain's biomass; and 3) the biocompatibility of the fortified foods upon an *in vitro* model to mimic the human intestinal epithelium (Caco-2 cells monolayers). The optimization of the bioprocess conditions resulted in high yields of vitamin B_{12} (above 800 µg/g dry biomass). The ICP-MS analysis revealed the presence of cobalt residues in the spray-dried biomass (~ 3 µg/g dry biomass), a consequence of its supplementation in the culture medium. However, the cytotoxicity and integrity of Caco-2 cell monolayers assays (biocompatibility) demonstrated that food samples fortified with biomass from this strain did not exert any toxicity upon the cellular model after *in vitro* simulated digestion (INFOGEST 2.0). Overall, the results demonstrated that the biomass of *P. shermanii* ATCC 13673 grown in agro-industrial soybean residue can be an alternative, safe, and effective source for the fortification of plant-based foods.

Keywords: Vegetarianism; Veganism; Cobalamin, Plant-based foods; Sustainability; Bioprocesses; Biotechnology.

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Introdução

Alimentar 8 bilhões de indivíduos com a dieta atual está levando o planeta ao colapso ambiental (Tilman & Clark, 2014; Willett et al., 2019). Mudanças no setor alimentício são imprescindíveis para atingir as metas do Acordo de Paris sobre o Clima, assim como, para cumprir os Objetivos do Desenvolvimento Sustentável da ONU nas próximas décadas (IPCC, 2019; Willett et al., 2019). Dentre as ações necessárias, a redução substancial no consumo de derivados animais, sobretudo a carne vermelha e derivados, é fortemente endossada pela comunidade científica (Foley et al., 2011; IPCC, 2019; Poore & Nemecek, 2018a; Scarborough et al., 2014; Willett et al., 2019; Xu et al., 2021).

Além das pautas ambientais, questões éticas e relacionadas a saúde estão guiando os seres humanos em busca de alternativas aos derivados animais (Boukid, 2021). Nesse cenário, dietas flexitarianas, vegetarianas e veganas estão ganhando popularidade mundialmente. Simultaneamente, produtos análogos a hambúrguers, nuggets, frutos do mar, leite e derivados totalmente *plant-based* (feitos de vegetais) estão em ascensão no setor alimentício (Aschemann-witzel et al., 2021; Siegrist et al., 2015). Segundo a Sociedade Vegetariana Brasileira (SVB), entre 2012 e 2018 o número de vegetarianos no país cresceu 75 % chegando a 30 milhões de brasileiros (SVB, 2021). Em relação aos produtos *plant-based*, de acordo com a *Euromonitor International*, no ano de 2020 o mercado global de análogos vegetais de carne e leite movimentaram mais de US$ 20 e US$ 16 bilhões, respectivamente, e podem ultrapassar a marca de US$ 100 bilhões na próxima década (Euromonitor, 2021).

Contudo, um contraponto recorrente em relação a produtos e dietas *plant-based* é a oferta de vitamina B_{12} (Curtain & Grafenauer, 2019; Willett et al., 2019a). Peculiarmente, a vitamina B_{12} é encontrada em quantidades apreciáveis somente em derivados de animais como carne vermelha, pescados, ovos e leite (Bito et al., 2018; Chamlagain et al., 2015; Okamoto et al., 2021; Watanabe et al., 2013). Análogos *plant-based*, por sua vez, podem ser fortificados para obter conteúdo adequado de vitamina B_{12} e atender a demanda nutricional da população em dietas alternativas (Boukid, 2021).

As bactérias propiônicas ou PAB (*Propionibacterium* sp. e *Acidipropionibacterium* sp.) são os únicos microrganismos seguros para aplicação em alimentos (status GRAS e QPS) capazes de biossintetizar a forma ativa de vitamina B_{12} (Chamlagain et al., 2015; Xie et al., 2021). Recentemente, foi constatado que a bioacessibilidade da vitamina B_{12} vinculada a biomassa de *Propionibacterium freudenreichii* é tão alta quanto a bioacessibilidade da vitamina B_{12} cristalina (presente em suplementos farmacêuticos) quando adicionadas na formulação de pães de trigo (95 % vs 99 %, respectivamente) (Chamlagain et al., 2021). Portanto, estes microrganismos tem grande potencial para serem utilizadas no desenvolvimento de novos produtos *plant-based* naturalmente fortificados com vitamina B_{12} (Chamlagain et al., 2016, 2018; Xie et al., 2018, 2021).

Além disso, as PAB são versáteis e capazes de utilizar diferentes substratos para o seu desenvolvimento. Dentre eles, resíduos agroindustriais como o *Liquid Acid Protein Residue of Soybean* (LAPRS), gerado em grande volume durante o processo de isolamento da proteína de soja (Assis et al., 2020). O LAPRS é rico em carboidratos e proteínas solúveis, consequetemente, possuí elevada demanda bioquímica de oxigênio (> 20.000 mg O_2/ L) e potencial de poluição. Em estudos recentes de nosso grupo de pesquisa, a linhagem *Propionibacterium freudenreichii shermanii* ATCC 13673 demonstrou-se capaz de utilizar o LAPRS no seu crescimento e produzir em torno de ~2 mg B_{12} em condições de bioprocesso (aeração, pH, temperatura, *etc.*) não otimizadas.

2. Objetivo

Sendo assim, objetivou-se com o presente estudo otimizar a biossíntese de vitamina B_{12} por *Propionibacterium freudenreichii* subsp. *shermanii* ATCC 13673 em biorreatores STR (*Stirred Tank Reactor*) e utilizando o resíduo agroindustrial de soja (LAPRS) como substrato. E por fim, utilizar a biomassa microbiana produzida na fortificação de alimentos de origem vegetal e investigar aspectos de biocompatibilidade do micronutriente em ensaios *in vitro*.

2.1. Objetivos específicos

- Avaliar influência de parâmetros de bioprocesso sobre a cinética de crescimento da linhagem *Propionibacterium freudenreichii* subsp. *shermanii* ATCC 13673;
- Maximizar a produção de vitamina B_{12} através de planejamento experimental 2^{-K};
- Fortificar alimentos vegetais com a biomassa de *Propionibacterium freudenreichii* subsp. *shermanii* ATCC 13673;
- Avaliar biocompatibilidade da vitamina B_{12} fornecida pelos alimentos vegetais fortificados através de modelo de digestão simulada (INFOGEST) seguido por modelo do epitélio intestinal humano (células Caco-2).

3. Revisão bibliográfica

3.1. Panorama da degradação ambiental e a contribuição do setor alimentício

Neste tópico serão aprofundadas informações relevantes sobre a cenário ambiental atual, as mudanças na biosfera terrestre catalisadas pela humanidade, a participação do setor de alimentos nesse processo e a importância de inovações voltadas para sustentabilidade no setor.

3.1.1. Antropoceno: A era dos seres humanos

Em relativamente pouco tempo, inovações tecnológicas da espécie humana causaram mudanças substanciais na sociedade e na biosfera terrestre (Bar-On et al., 2018; Crutzen, 2002). A domesticação de animais, agricultura e, mais recentemente, avanços na medicina e a revolução industrial, propíciaram uma verdadeira explosão populacional humana. Entre 1950 e 2010 a população mundial saltou de 2 para 7 bilhões de indivíduos. Paralelamente, outros indicadores sócio-econômicos também explodiram (ex.: uso de água, energia, transportes, *etc.*), num fenômeno conhecido como A Grande Aceleração (Steffen, Broadgate, et al., 2015). Contudo, nesse mesmo período, a degradação ambiental aumentou de forma igualmente rápida e drástica (Steffen, Broadgate, et al., 2015).

A cobertura florestal do planeta foi reduzida em 46 % desde os primórdios da civilização humana (Crowther et al., 2015). Metade desse desmatamento aconteceu nos últimos 100 anos (Ritchie & Roser, 2021), principalmente, para expansão de terras agrícolas e produção de *commodities* (Curtis et al., 2018; Gibbs et al., 2010). No mesmo sentido, a emissão de gases de efeito estufa (Ex.: CO_2, CH_4 e N_2O) disparou (figura 1) devido a queima de combustíveis fósseis e mudanças no uso do solo, chegando a expressivos 59 bilhões de toneladas de CO_2-eq/ano em 2019 (IPCC, 2021).

Os resultados das perturbações antrópicas na biosfera terrestre são desastrosos. O planeta vive um período de aquecimento global sem precedentes em milhares de anos (IPCC, 2021). Consequentemente, eventos climáticos extremos (ex.: ondas de calor, chuvas torrenciais, alagamentos, *etc*.) estão mais frequentes e intensos, impactando a vida de milhões de indivíduos, sobretudo, os mais vulneráveis (IPCC, 2022). Paralelamente, observa-se também a perda acelerada da cobertura de gelo nos polos (IPCC, 2021), o início do processo de savanização da Amazônia (Lovejoy & Nobre, 2019), e a extinção

em massa de espécies (Steffen, Richardson, et al., 2015), fenômenos que retroalimentam o aquecimento global e seus efeitos adversos (IPCC, 2021; Lovejoy & Nobre, 2019).

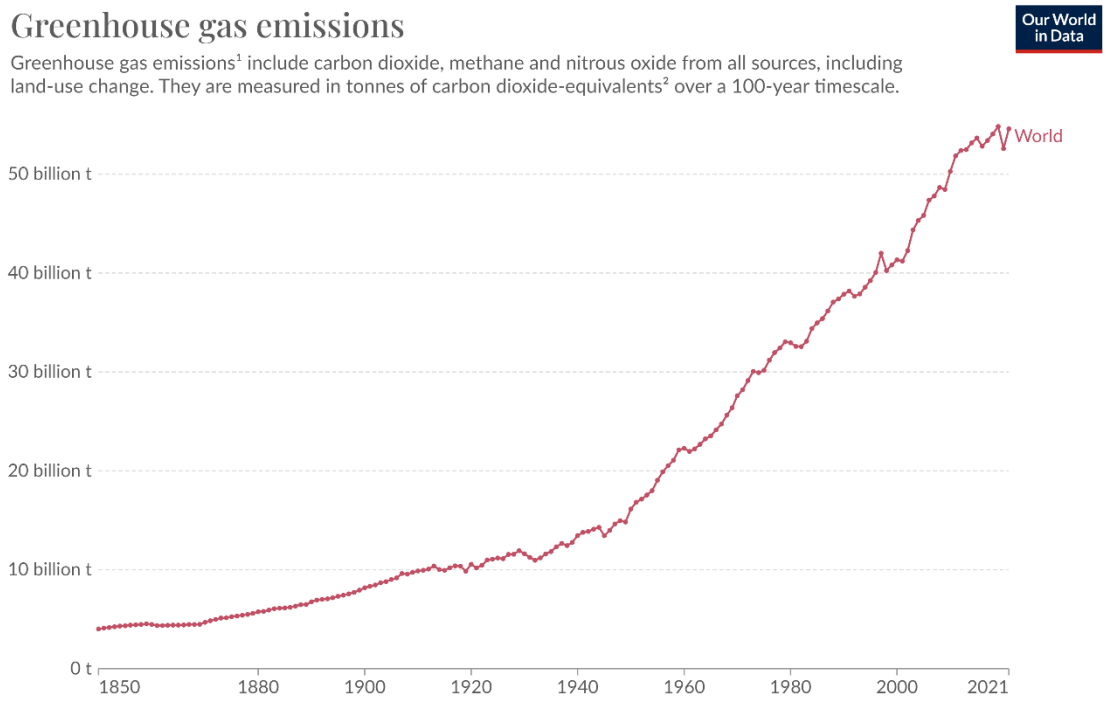

Figura 1. Evolução das emissões globais de gases de efeito estufa em bilhões de toneladas de CO_2-eq ao longo dos anos.

Fonte: Our World in Data.

Com base nas evidências, alguns pesquisadores classificam o período que estamos vivendo como: o Antropoceno, a era em que a espécie humana se tornou uma força geofísica global capaz de desequilibrar o funcionamento do planeta inteiro (Porta, 2021; Steffen, Broadgate, et al., 2015; Steffen et al., 2007; Zalasiewicz et al., 2016). Sem ação imediata, os impactos antrópicos sobre a biosfera irão se tornar ainda mais perigosos e, em alguns casos, irreversíveis (IPCC, 2021, 2022).

O *Intergovernmental Panel for Climate Change* (IPCC) é o orgão da Organização das Nações Unidas (ONU) responsável por avaliar a ciência climática mais atual e elaborar relatórios abrangentes e compressíveis para dar suporte aos tomadores de decisão (governantes) em assuntos referentes a mudanças climáticas. Em seus relatórios mais recentes, o IPCC é contundente e adverte que, para evitar os piores cenários de mudanças climáticas (aquecimento global de 1,5 °C acima da média do período pré-industrial entre 1750-1800), serão necessárias a redução de 50 % das emissões globais de gases de efeito

estufa até 2030 e atingir *net zero* até 2050. Além disso, o orgão ressalta a necessidade de ampliar áreas de manutenção de serviços ecossistêmicos (ex.: sequestro de carbono, manutenção do ciclo hidrológico dentre outros), os quais são indispensáveis para vida na Terra. Tais metas foram ratificadas por mais de 195 países, incluindo o Brasil, no Acordo de Paris em 2015 (IPCC, 2021, 2022).

Em 2007 o IPCC foi laureado com prêmio Nobel da Paz (junto ao ex-vice presidente dos EUA, "Al Gore") pelo seu trabalho excepcional e alertas relacionados aos perigos das mudanças climáticas (Marques, 2007). Vale destacar que, dentre os mais de 600 cientistas integrantes diretos da equipe do IPCC laureada com o prêmio Nobel, havia a presença de 12 pesquisadores brasileiros, sendo eles:

"Paulo Artaxo, professor do Instituto de Física da Universidade de São Paulo (USP), José Antônio Marengo, meteorologista do Centro de Previsão de Tempo e Estudos Climáticos do Instituto Nacional de Pesquisas Espaciais (Cptec/Inpe) e Pedro Dias Leite, diretor do Laboratório Nacional de Computação Científica (LNCC), no grupo I, que avaliou as bases físicas do sistema climático; Carlos Nobre, também do Cptec/Inpe, e Ulisses Confalonieri, da Fundação Oswaldo Cruz, no grupo II, que analisou os impactos, adaptações e vulnerabilidades ao aquecimento global; Emílio La Rovere, Suzana Khan e Roberto Shaeffer, da Universidade Federal do Rio de Janeiro, no grupo III, que levantou os meios de mitigar as mudanças globais. Outros três pesquisadores atuaram como revisores: Antonio Rocha Magalhães, assessor do Banco Mundial; José Roberto Moreira, professor da USP, e Philip Fearnside, do Instituto Nacional de Pesquisas da Amazônia (Inpa). Já Thelma Krug, secretária de Mudanças Climáticas do Ministério do Meio Ambiente, trabalhou como executiva do IPCC" (Marques, 2007).

3.1.2. Impactos ambientais da produção de alimentos

Nesse contexto, agir sobre a produção de alimentos é um fator crucial para atingir sustentabilidade (Willett et al., 2019).A alimentação global atual contribuiu significativamente para os impactos ambientais observados em nossa era (IPCC, 2019; Tilman & Clark, 2014; Willett et al., 2019). Seguindo o padrão atual de produção e consumo, o setor de alimentos sozinho irá esgotar o "orçamento de carbono" remanescente para humanidade nas próximas décadas (Clark et al., 2020).

O enorme impacto ambiental da produção de alimentos reside em um ponto fundamental: a criação de animais de corte em larga escala. Animais são ineficientes do ponto de vista energético e convertem menos de 10 % das calorias fornecidas à eles em alimentos para os seres humanos (XU, et al., 2021). Soma-se a esse fato a alta demanda e uma super população humana. O resultado são mais de 70 bilhões de animais abatidos todos os anos (Schuck & Ribeiro, 2018). Consequentemente, o setor exige áreas enormes de solo e recursos naturais para criação dos animais de corte (Foley et al., 2011a).

Atualmente, em torno de 3 bilhões de equitares ou mais de 50 % do solo agricultável do planeta são utilizados pelo setor através de plantações ou pastagens (FOLEY et al., 2011; POORE; NEMECEK, 2018; XU, X. et al., 2021). O mesmo promove desmatamento ou mudanças no uso do solo, emissão de gases de efeito estufa em grandes proporções (57 % das emissões relacionadas à alimentos e ¼ das emissões globais), assim como, a erosão ou esgotamento do solo (FOLEY et al., 2011; POORE; NEMECEK, 2018; XU, et al., 2021). Além disso, o setor possui elevada pegada hidríca (de 1000 à > 20.000 $m^3 \cdot ton^{-1}$ de proteína animal), alto potencial de eutrofização (criação de zonas mortas pelo escoamento de dejetos animais e fertilizantes) e retira dos ecossistemas aquáticos de 70 à 96 milhões de toneladas de pescado$\cdot ano^{-1}$ desde 1980 levando inúmeras espécies à face da extinção (FAO, 2020; Mekonnen & Hoekstra, 2012; Poore & Nemecek, 2018).

Segundo o relatório da *EAT-Lancet commision*, que conta com a análise de dezenas de especialistas renomados em diferentes áreas (ex.: agricultura, ciências políticas, medicina dentre outras), para cumprir os acordos climáticos internacionais (mencionados no tópico anterior) e evitar o colapso ambiental, o setor de produção de alimentos inteiro não deveria exceder a emissão de 5 bilhões de t de CO_2-eq$\cdot ano^{-1}$ nas próximas décadas (Tilman & Clark, 2014; Willett et al., 2019). Atualmente, apenas a produção de derivados animais emite mais de 9 bilhões de toneladas de CO_2-eq$\cdot ano^{-1}$ (Foley et al., 2011; Poore & Nemecek, 2018; Xu et al., 2021).

A produção de animais também impacta significativamente outras variáveis de controle analisadas no estudo (ex.: uso de água/fertilizantes, perda de biodiversidade, *etc*.) (Tilman & Clark, 2014; Willett et al., 2019). A análise final da comissão indicou que, para estabelecer um sistema de produção de alimentos realmente sustentável, o consumo global de derivados animais, sobretudo carne vermelha, deve ser reduzido em no mínimo 50 % nos próximos anos. Além disso, ações conjuntas em outras frentes também serão

essenciais, tais como: reduzir o desperdício de alimentos e a intensificação sustentável da produção de alimentos (Tilman & Clark, 2014; Willett et al., 2019).

3.1.3. Proteínas alternativas: produtos plant-based

Inovações na indústria de alimentos e mudanças de hábito das pessoas serão vitais para sociedade humana contemporânea (Tilman & Clark, 2014; Willett et al., 2019). Notavelmente, uma onda de novos produtos estão chegando ao mercado (Aschemann-witzel et al., 2021; Siegrist et al., 2015). São produtos que mimetizam alimentos derivados de animais, tais como: hamburguers, almôndegas, nuggets, queijos, dentre outros. Porém, produzidos com ingredientes ou processos alternativos e de baixo impacto ambiental, com intuito de auxiliar na transição sustentável do setor de alimentos (He et al., 2020). Dentre as chamadas "proteínas alternativas", destacam-se: a carne cultivada, a biomassa microbiana/produtos de fermentação (ex.: Quorn micoproteína/leghemoglobina) e os produtos feitos com proteínas vegetais ou *plant-based* (Hefferon et al., 2023).

Os produtos *plant-based* são os mais difundidos no mercado de alimentos atual. Porém, a produção e consumo de proteínas alternativas baseadas em plantas (ex.: tofu, seitan e tempeh) são milenares. O tofu ("queijo de soja") foi desenvolvido a mais de 2000 anos durante a dinastia Han na China, posteriormente, difundindo-se pela Asia (Ishaq et al., 2022). Durante a década de 1960, o processo de extrusão de matrizes vegetais proteícas (ex.: glúten de trigo, concentrado de soja) ampliou a diversidade de produtos obtidos com a proteína vegetal texturizada (PVT) (He et al., 2020).

Atualmente, está em curso uma terceira onda de inovação em relação a proteínas alternativas *plant-based* (He et al., 2020). O avanço científico e tecnológico no setor de alimentos possibilitou reproduzir com maior precisão as características dos produtos de origem animal, trazendo uma nova experiência de preparo e consumo de alimentos vegetais que se assemelham aos produtos derivados de animais. Essa nova classe de produtos é chamada de: análogos vegetais ou simplesmente produtos *plant-based* (Ishaq et al., 2022).

As proteínas de soja e/ou ervilha (concentrada e/ou isolada) estão entre os ingredientes majoritários dos produtos *plant-based*. Após a extração da proteína vegetal, as mesmas podem ser utilizadas diretamente na formulação dos produtos ou transformadas em estruturas que assemelham as fibras musculares dos produtos cárneos, processo chamado de texturização. A texturização das proteínas vegetais para o setor de

análogos *plant-based* é realizada por duas técnicas principais, sendo elas: extrusão e a tecnologia de cisalhamento (*shear cell technology*) (He et al., 2020).

Além das proteínas, também fazem parte da composição dos produtos *plant-based*: gorduras vegetais (ex.: óleo de coco ou girassol), agentes estruturantes (ex.: goma carragena e celulose), sais, especiarias, aromas e corantes (ex.: extrato de beterraba, licopeno, leghemoglobina) e, em alguns casos, vitaminas e minerais. Todos ingredientes combinados de forma precisa para recriar as propriedades físico-químicas, sensoriais e nutricionais dos produtos equivalentes de origem animal (Ishaq et al., 2022).

Inúmeras análises do ciclo de vida (LCA – *Life Cycle Assessment*) demonstram o infímo impacto ambiental dos produtos *plant-based* (Poore & Nemecek, 2018; Tilman & Clark, 2014; Xu et al., 2021). Emissões de gases de efeito estufa (CO_2.eq /g protína) para produção de proteínas de leguminosas (ex.: soja, ervilha), vastamente utilizadas nos produtos *plant-based*, podem ser até 250 vezes menor quando comparadas as emissões relacionadas a proteínas de animais ruminantes (ex.: bovinos e ovinos) (Tilman & Clark, 2014).

Em relação ao uso do solo, a produção dos análogos vegetais requer apenas uma fração de terra (de 3 a 36 %) para fornecer a mesma quantidade de proteína, em comparação aos produtos derivados de animais. O solo liberado com a mudança de hábitos alimentares viabilizaria ações de reflorestamento, preservação da biodiversidade e aprisionamento de carbono, com potencial de remover 26 Gt CO_2.eq anualmente, algo em torno de metade das emissões atuais (The Good Food Institute, 2023).

3.1.4. Vitamina B_{12} microbiana

Na dieta convencional, a vitamina B_{12} está presente em derivados animais, tais como: carne vermelha, pescados, ovos e leite, mas ausente nos produtos de origem vegetal (Bito et al., 2018; Chamlagain et al., 2015; Okamoto et al., 2021; Watanabe et al., 2013). Entretanto, os animais não são capazes de produzi-la, apenas bioacumulando a vitamina B_{12} oriunda da dieta (fortificação) ou produzida *in situ* por fermentação (ex.: animais ruminantes) (Girard et al., 2009). Os análogos *plant-based*, por sua vez, também podem ser fortificados para obter conteúdo adequado de vitamina B_{12} e atender a demanda nutricional da população em dietas alternativas (Boukid, 2021).

A vitamina B_{12} (figura 2) é um composto orgânico, corrinóide, hidrossolúvel, essencial para manutenção de funções fisiológicas no organismo humano (Moll & Davis, 2017). Ela atua como cofator enzimático no metabolismo de ácidos graxos e proteínas e, indiretamente, na síntese de DNA e estruturas neurológicas (Green et al., 2017; Nielsen et al., 2012a). Por conta disso, a deficiência de vitamina B_{12} gera sérios problemas a saúde humana, tais como: mudanças de humor e comportamento, fadiga, depressão e, em casos severos, leva a anemia megaloblástica (Green et al., 2017).

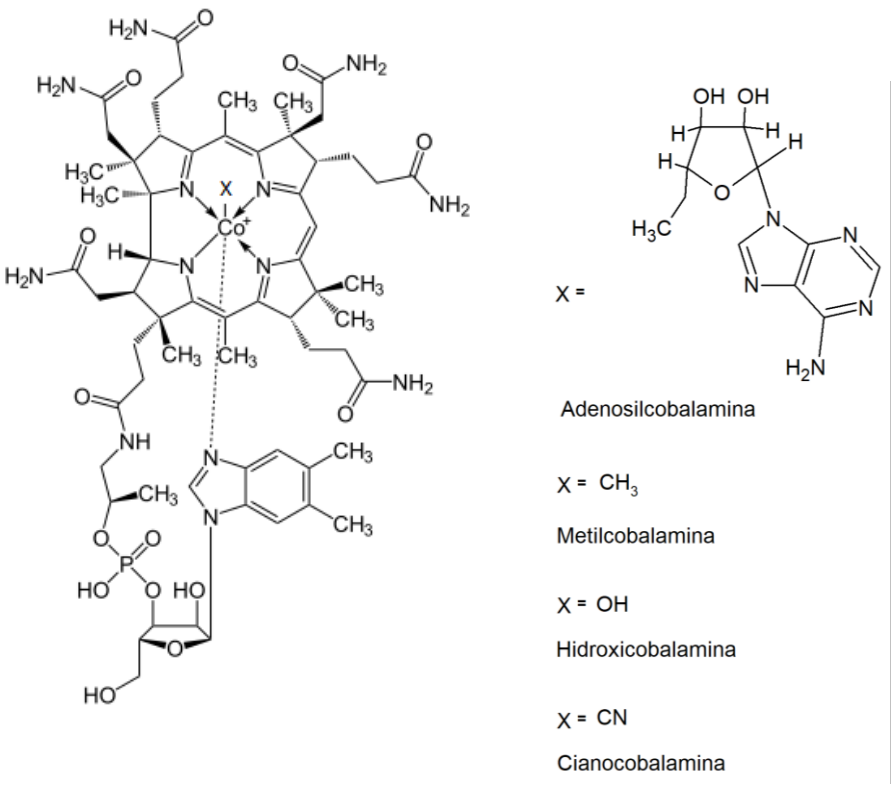

Figura 2. Estrutura química da vitamina B_{12} e seus diferentes configurações biologicamente ativas para os seres humanos.

As bactérias propiônicas ou PAB (*Propionibacterium* sp. e *Acidipropionibacterium* sp.) são os únicos microrganismos seguros para aplicação em alimentos (status GRAS e QPS) capazes de biossintetizar a forma ativa de vitamina B_{12} para os seres humanos (Chamlagain et al., 2015; Xie et al., 2021). Esses microrganismos produzem a vitamina através de uma complexa rota metabólica, extensamente revisada em (Assis, 2019). Recentemente, foi constatado que a bioacessibilidade (quantidade de

micronutriente liberada após digestão) da vitamina B_{12} vinculada a biomassa de *Propionibacterium freudenreichii* é tão alta quanto a bioacessibilidade da vitamina B_{12} cristalina (padrão) quando adicionadas na formulação de pães de trigo (95 % vs 99 %, respectivamente) (Chamlagain et al., 2021).

A absorção desse micronutriente pelo organismo humano ocorre através de uma via complexa e mediada por diferentes proteínas transportadoras de cobalamina, tais como: haptocorrina (HC), fator intrínseco (IF), transcobalamina (TC) e os receptores cubam (Fedosov et al., 2007; Green, 2017; Nielsen et al., 2012). Após desligada da matriz alimentar no estômago, a vitamina B_{12} forma um complexo com a haptocorrina (proteína produzida pelas glândulas salivares), o qual assegura a estabilidade da vitamina B_{12} durante a transição no ambiente gástrico (Nielsen et al., 2012a). No intestino, o complexo HC-Cbl é desfeito pela ação de enzimas proteolíticas e a vitamina B_{12} liberada interage com o fator intrínseco (glicoproteína produzida pelas células parietais do estômago) formando o complexo IF-Cbl, o qual é reconhecido pelos enterócitos intestinais e então absorvido (Green et al., 2017).

As células isoladas de adenocarcinoma humano (Caco-2) tem sido usadas com sucesso em estudos de biocompatibilidade (ex.: ensaios de absorção e toxicidade) de diversos compostos (Kondrashina et al., 2023). Após atingir confluência (3 semanas de crescimento), as células Caco-2 são capazes de diferenciar-se em enterócitos simulando o sistema epitelial do intestino humano *in vitro* (Fedi et al., 2021). Células Caco-2 já foram utilizadas para determinar absorção de novos derivados de vitamina B_{12} (Netsomboon et al., 2016). Porém, ainda não há evidências de aplicação deste modelo celular para deterninar a biocompatibilidade de vitamina B_{12} fornecida através de biomassa microbiana.

3.2. Artigo científico de revisão

O artigo científico de revisão abaixo descreve estratégias de bioprocessos utilizando bactérias propiônicas para produção de metabólitos de interesse em alimentos, incluindo vitamina B_{12}. O artigo foi publicado em janeiro de 2022 no periódico internacional *Food and Bioprocess Technology*, fator de impacto 4,46 e qualis A1 em Ciência de Alimentos.

High cell density culture of dairy *Propionibacterium* sp. and *Acidipropionibacterium* sp.: A review for food industry applications

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Abstract

The dairy bacteria *Propionibacterium sp.* and *Acidipropionibacterium* sp. are versatile and potentially probiotic microorganisms showing outstanding functionalities for the food industry, such as the production of propionic acid and vitamin B_{12} biosynthesis. They are the only food grade microorganisms able to produce vitamin B_{12}. However, the fermentation batch process using these bacteria present some bioprocess limitations due to strong end-product inhibition, cells slow-growing rates, low product titer, yields and productivities, which reduces the bioprocess prospects for industrial applications. The high cell density culture (HCDC) bioprocess system is known as an efficient approach to overcome most of those problems. The main techniques applied to achieve HCDC of dairy *Propionibacterium* are the fed-batch cultivation, cell recycling, perfusion, extractive fermentation, and immobilization. In this review are discussed the techniques available and reported to achieve HCDC of *Propionibacterium* sp. and *Acidipropionibacterium* sp. and it is evaluated the advantages and drawbacks of this system of cultivation in relation to biomass formation, vitamin B_{12} biosynthesis and propionic acid production.

Keywords: Dairy propionic acid bacteria; bioprocess technology; high cell density culture; vitamin B_{12}; propionic acid; probiotics.

Introduction

Propionibacterium sp. is a rod-shaped, gram-positive, facultative anaerobe bacterium, traditionally divided based on its habitat into *classic* dairy-related species and *cutaneous*, skin-related species (Thierry et al., 2011). The single *Propionibacterium* genus was recently taxonomic reclassified into four genera: *Propionibacterium*, *Acidipropionibacterium*, *Cutibacterium*, and *Pseudopropionibacterium* (SCHOLZ; KILIAN, 2016). Dairy *Propionibacterium* sp. and *Acidipropionibacterium* sp. (dairy PAB) comprise the most relevant genera to the food industry due to their role in Swiss cheese ripening, vitamin B_{12} biosynthesis, propionic acid production, as well as their potential probiotic proprieties when added to foods (Chamlagain, 2016; Rabah et al., 2017; Wang et al., 2014;Yang et al., 2018).

Dairy PAB are the only food grade microorganisms presenting the Generally Recognized As Safe (GRAS) standard in the USA, the Qualified Presumption of Safety (QPS) status in the EU, the National Food Safety Standards of the NHS in China, and the Ministerial Ordinance on Milk and Milk products Concerning Compositional Standards in Japan able to produce vitamin B_{12} (Fang et al., 2017).Vitamin B_{12} is essential for human energetic metabolism and DNA replication (Nielsen et al., 2012). Its daily recommended intake is 2.4 µg, and it is important to avoid neurological and physiological disorders, specially anemia (ALLEN, 2008; GREEN *et al.*, 2017). Usually, active vitamin B_{12} is found in appreciable amounts only in foods of animal origin (Bito et al., 2018; Okamoto et al., 2021; Watanabe et al., 2013). However, it is now clear that to mitigate climate change, humans will have to drastically reduce the consumption of animal-origin foods in the near future (POORE; NEMECEK, 2018). Thus, it will be a rise in the demand for alternative vitamin B_{12} sources, which can be obtained by *in situ* fortification of plant-based foods with dairy PAB (CHAMLAGAIN *et al.*, 2018; XIE *et al.*, 2021).

PAB present the natural ability to produce propionic acid through the Wood-Werkman cycle, which is an important property for its bio-based production (Wang & Yang, 2013; Yang et al., 2018). Propionic acid and its calcium, potassium and ammonium salts find several applications in the food industry, as preservative and flavoring agents (Himmi et al., 2000). Additionally, it is a valuable chemical for pharmaceutical, cosmetic, and agricultural applications. Currently, this organic acid is manly produced via petrochemical synthesis ($> 4\ 10^5$ tons per year) due to cost considerations (Jiang et al.,

2015; Piwowarek et al., 2019). However, the growing market for bio-based products, the increase in petroleum prices, and environmental issues, turned its biosynthesis into a desirable option, specially under the biorefinery concept, using renewable feedstocks or industrial wastes such as molasses, residual glycerol from biodiesel, among others, for its bioproduction (Saini et al., 2019; Yang et al., 2018).

Recently, dairy PAB were also being researched for probiotic applications. Probiotics are live microorganisms that, when administrated in adequate amounts, provide health benefits to the host (HILL *et al.*, 2014). Dairy PAB have promising probiotic characteristics such as like immunomodulatory and anti-inflammatory activities, short chain fatty acids production (SCFA), microbiota modulation, and resistance to gastrointestinal conditions (Amadoro et al., 2018; Kouya et al., 2007; Rabah et al., 2018). Their immunomodulatory and anti-inflammatory activities can attenuate Non-Communicable Diseases (NCD) like intestinal bowel diseases (IBD) (PLÉ *et al.*, 2015; UCHIDA; MOGAMI, 2005). These health benefits could potentially prevent the worst effects of SARS-CoV-2 infection as well (Antunes et al., 2020; Singh & Rao, 2021), further stressing the importance to keep a healthy gut microbiota, showing the need to further developments of new functional fermented products or probiotic supplements containing these next-generation therapeutic bacteria (DOUILLARD; VOS, 2019).

Dairy PAB are versatile microorganisms presenting few nutritional requirements, capable to metabolize several carbon sources such as glucose, xylose, molasses, and residual glycerol (Coral et al., 2008; Dishisha et al., 2013; Wang et al., 2014; Yang et al., 2018). However, the growth of these bacteria under batch process systems has some performance limitations due to strong end-product inhibition, slow-growing cells, low product titer, yields and productivities, which reduces the bioprocess outcomes, thus limiting their application at industrial scale (Ahmadi et al., 2017a; Coral et al., 2008; Liang et al., 2012; Ozadali et al., 1996).

On the other hand, high cell density culture (HCDC) is an efficient approach to overcome most of the problems related to batch system, highly improving the bioprocess efficiency (Westman & Franzén, 2015; Yang et al., 2018). In this review are presented the bioprocess techniques available to achieve high cell density cultures of dairy *Propionibacterium* sp. and *Acidipropionibacterium* sp. and it is evaluated their

advantages and drawbacks in relation to biomass formation, vitamin B_{12} synthesis and propionic acid production.

2. Bioprocess parameters

In this section is reviewed key bioprocess parameters that influence the HCDC of *Propionibacterium* sp., with the impacts in the production of vitamin B_{12} and propionic acid, namely: strain selection, growth media, temperature, pH, and aeration conditions. Additionally, a topic on mathematical modeling and statistical optimization for the bioprocess improvement is also presented.

2.1 Selection of Microorganisms

Microorganisms can be isolated from natural sources, purchased from certified collections, or obtained by bio-engineering approaches (random mutagenesis, CRISPR-Cas-mediate genome edition, among others) (Campaniello et al., 2015; Douillard & Vos, 2019). In general, the characteristics required for a competitive bioprocess are high efficiency of substrate conversion into desirable products, low susceptibility towards by-products formation (especially those causing growth arrest), microbial physiologic stability, minimal nutritional requirements, growth in low-cost media culture, and desirable production of extracellular products (Hedayati et al., 2020; Schmidell et al., 2001; Wang & Yang, 2013).

The *Propionibacterium* and *Acidipropionibacterium* genera are comprised of the following species: *P. freudenreichii, P. australiense, P. cyclohexanicum, P. acidifaciens, A. acidipropionici, A. jensenii, A. thoenii, A. microaerophilum, A. damnosum,* and *A. olivae* (SCHOLZ; KILIAN, 2016). Each one of these bacterium present different functionalities in relation to vitamin B_{12} synthesis and propionic acid production, as well as probiotic characteristics. For instance, *P. freudenreichii* and *A. acidipropionici* have the natural capacity to produce high amounts of vitamin B_{12} (0.2 to 1 $mg.g^{-1}$ biomass) and propionic acid (> 50 $g.L^{-1}$), respectively (Martens et al., 2002; Miyano et al., 2000; Wang & Yang, 2013; Yang et al., 2018).

In relation to probiotic applications, attributes such as survival towards gastrointestinal environment, absence of genes of virulence, suspensibility towards antibiotics, adhesion to epithelial cells, and immunomodulation and others health effects are strain-dependent within PAB. These characteristics were extensively reviewed elsewhere (RABAH; CARMO; JAN, 2017). Thus, PAB strains must be screened and certified before any probiotic claim (HILL *et al.*, 2014). Amadoro et al. (2018) reported that *P. freudenreichii* S-1-P stimulated anti-inflammatory response of human peripheral blood mononuclear cells (PBMC). Plé et al. (2015) demonstrated that immunomodulation and anti-inflammatory activity of *P. freudenreichii* CIRM BIA 129 attenuate 2,4,6-Trinitrobenzenesulfonic acid (TNBS) induced colitis in animal models. Additionally, *P. freudenreichii* CIRM BIA 129 cultured in a hyperosmotic environment (> 1,500 $mmol.kg^{-1}$) showed great viability keeping around 70 % of viable cells after bile salts stress, being a promising probiotic strain (HUANG *et al.*, 2016). Omics techniques such as proteomic and genomic, can provide helpful data for screening and selection of new functional PAB strains (DOUILLARD; VOS, 2019). Overall, *P. freudenreichii* and *A. acidipropionici* are the most relevant species to the food industry, thus they will be mentioned as dairy propionic acid bacteria (dairy PAB).

2.2 Growth media composition

Dairy PAB growth media are usually composed by a carbon source (20-50 $g.L^{-1}$), nitrogen source (5-15 $g.L^{-1}$), few micronutrients (e.g. Mg^{2+}, Mn^{2+}, PO_4^{3-}) (1-1,000 $mg.L^{-1}$) and purged with nitrogen before the inoculation process to provide anaerobioses (Ahmadi et al., 2017b; Goswami & Srivastava, 2000; Liu et al., 2011; Martínez-Campos & de la Torre, 2002). In addition, cobalt ions and 5,6-dimethylbenzimidazole (DMBI), which are vitamin B_{12} precursors, are of fundamental importance for active vitamin B_{12} biosynthesis (Assis et al., 2020; Deptula et al., 2015; Hugenschmidt et al., 2011; Vandamme & Revuelta, 2016). For *in situ* fortification of food products the addition of DMBI is undesirable. Thus, in these cases, reduced flavin mononucleotide (FMNH2), a food-grade substrate, is added because dairy PAB can produce active B_{12} from this compound (Chamlagain et al., 2016; Deptula et al., 2015).

PAB can metabolize several carbon sources, such as: sugars (sucrose, glucose, fructose, lactose, galactose, and xylose), molasses (from various sources such as sugarcane and soybeans), organic acids (lactic and glucuronic acids), fatty acids (linolenic, oleic, and palmitic), and other organic compounds such as glycerol (Coral et al., 2008; Goswami & Srivastava, 2000; Hedayati et al., 2020; Wang et al., 2014; Yang et al., 2018). Energy (ATP) and reduced co-factors are initially obtained in pyruvate production throughout glycolysis or the pentose phosphate pathway. Pyruvate is then oxidized to acetic acid and CO_2 or reduced in the Wood-Werkman Cycle (Figure 1) into propionic acid, which are the main dairy PAB fermentation products (Thierry et al., 2011). The metabolic flow between pyruvate oxidation and reduction pathways is essential to maintain the intracellular redox balance (NAD^+/NADH ratio) (Wang & Yang, 2013).

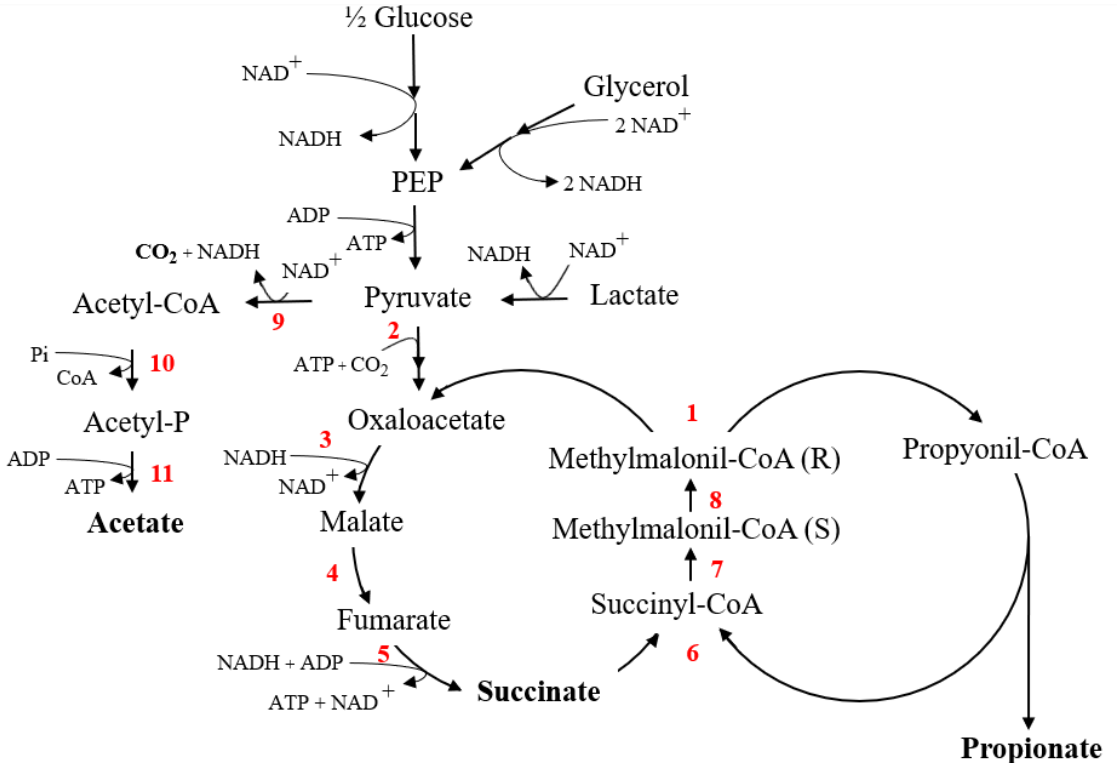

Figure 1. Metabolism of *Propionibacterium* sp. using different carbon sources via the Wood-Werkman cycle. Enzymes involved in the propionate pathway: (1) Methylmalonyl-CoA: Oxaloacetate carboxyltransferase; (2) Pyruvate carboxylase; (3) Malate dehydrogenase; (4) Fumarate hydratase; (5) Succinate dehydrogenase; (6) Propionyl-CoA: Succinate CoA transferase; (7) Methylmalonyl-CoA mutase and (8) Methylmalonyl-CoA epimerase. Enzymes involved in the acetate and

CO_2 pathway: (9) Pyruvate dehydrogenase; (10) Phosphate acetyltransferase; (11) Acetate kinase. PEP: Phosphoenolpyruvate (Piveteau, 1999; Wang & Yang, 2013; Zhang et al., 2015).

Glucose and complex carbon sources such as molasses and whey permeate are the best choices in order to obtain high dairy PAB cell density (Boyaval & Corre, 1987; Coral et al., 2008; Feng et al., 2011; Liu et al., 2016; Ozadali et al., 1996). The carbon source also exerts influence over the propionate/acetate ratio (P/A ratio) and overall fermentation performance. For example, lactate and glucose fermentation by *P. freudenreichii* produces propionic and acetic acids usually in a P/A ratio of 2:1, whilst propionic acid theorical yields from glucose is approximately 0.55 $g.g^{-1}$ (Wang & Yang, 2013). On the other hand, glycerol fermentation favors the formation of reduced compounds and propionic acid is the unique product (achieving theorical yields of 0.80 $g.g^{-1}$), with only traces of acetic acid (P/A ratios > 30:1) been produced (Coral et al., 2008; Himmi et al., 2000; Wang & Yang, 2013).

Cell growth rate and propionic acid productivity (0.01 and 0.20 $g.(L.h)^{-1}$, respectively) are lower using glycerol as carbon source compared to glucose or lactate, caused by cell redox imbalance (Coral et al., 2008; Wang et al., 2014; Wang & Yang, 2013; Zhang & Yang, 2009). However, these parameters can be improved by glycerol/glucose co-fermentation (Liu et al., 2011; Wang et al., 2014; Wang & Yang, 2013). Additionally, it has been reported that balanced glycerol/yeast extract ratio (3:1) could also increase biomass production (~ 9 $g.L^{-1}$), propionic acid productivity (0.31 $g.(L.h^{-1})$) and yields (0.63 $g.g^{-1}$), and P/A ratio (up to 50:1) in batch process (Dishisha et al., 2015). In relation to vitamin B_{12}, it has been reported that glycerol reduces vitamin B_{12} yields in *P. freudenreichii* cultures compared to glucose. However, glycerol co-fermentation with glucose also improves the vitamin B_{12} yields and productivity (Wang et al., 2014).

Lactate metabolism is characteristic of these microorganisms, which is well explored in cheese ripening (Thierry et al., 2005). This carbon source stimulates the production of volatile compounds, organic acids and CO_2, desirable for ripening of Swiss-type cheeses, but the process results in less biomass accumulation since lower energy is recovered from this carbon source (Boyaval & Corre, 1987; Piveteau, 1999; Thierry et al., 2005). Dairy PAB show preference for L-lactate isomer instead of D-lactate and, in

relation to propionic acid production, it can provide higher productivity compared to glycerol (CORAL *et al.*, 2008; CROW, 1986). Additionally, small pH culture variations (less than 0.5 pH units) are observed in lactate fermentation due to almost equimolar acid production, which reduces the need for using alkalis in pH-controlled bioprocess (CORAL *et al.*, 2008; LEWIS; YANG, 1992).

The nitrogen source also affects the growth and productivity, thus affecting a cost-effective bioprocess development. Although dairy PAB can synthesize all amino acids needed from inorganic sources such as $(NH_4)_2SO_4$, organic nitrogen sources like amino acids, protein hydrolysates, yeast extract and corn steep liquor are better growth promoters (Ahmadi et al., 2017b; Thierry et al., 2011). Corn Step Liquor (CSL), a byproduct from starch processing, has been widely used in several PAB bioprocess, due to its low-cost and balanced nutritional content (amino acids, minerals, and vitamins) (Nakano et al., 1996; Quesada-Chanto et al., 1998; Wang & Yang, 2013; Yang et al., 2018). Recently, this byproduct has been proved to be an excellent nitrogen source for PAB biomass production. However, concerning the propionic acid biosynthesis, yeast extract was considered a better option (Ahmadi et al., 2017b).

2.3 Temperature

The culture temperature influences the growth rate and metabolites production of dairy PAB, which can grow in a range of temperatures from 4.5 °C to 40 °C (PIWOWAREK *et al.*, 2019). The growth at low temperatures is very slow, but it is desirable in the Swiss cheese production (Hofherr et al., 1983). The highest cell growth rate and products formation rates are obtained in temperatures around 30 °C (Colomban et al., 1993; Coral et al., 2008; Gorret et al., 2001).

Seshadri and Mukhopadhyay (1993) reported that specific growth rate of *A. acidipropionici* ATCC25562 increased with temperature, from 0.05 h^{-1} at 26 °C up to 0.1 h^{-1} at 34 and 37 °C. Propionic acid productivity did not increase above 30 °C and, at 37 °C, the strain increased the formation of acetic acid as byproduct reducing P/A ratio (SESHADRI; MUKHOPADHYAY, 1993). Similarly, Farhadi et al. (2013) observed the highest propionic acid production at 30 °C in a beverage fermented by *P. freudenreichii* DSM 20270 and *L. acidophilus* LA5. Piwowarek et al. (2019) reported a small increase

in propionic acid production - from 4.70 $g.L^{-1}$ to 5.13 $g.L^{-1}$ - when increasing the growth temperature of *P. freudenreichii* T82 from 30 °C to 37 °C, respectively.

Recently, Hedayati et al. (2020) reported that optimum temperature for vitamin B_{12} biosynthesis by *P. freudenreichii* PTCC1674 was 38.3 °C, under which the increase of biomass production favored vitamin B_{12} accumulation as well (HEDAYATI; HOSSEINI; NAJAFPOUR, 2020). These data indicates that optimal temperature for biomass/vitamin B_{12} production mighty be different from the optimal temperature for propionic acid production by PAB (COLOMBAN; ROGER; BOYAVAL, 1993; CORAL *et al.*, 2008; HEDAYATI; HOSSEINI; NAJAFPOUR, 2020; SESHADRI; MUKHOPADHYAY, 1993).

2.4 pH

In relation to pH, dairy PAB cultures under neutral conditions, pH controlled between 6 and 7, show high cell growth rate (up to 0.12 h^{-1}). Above or below this range, the cell growth rate tends to decrease (as low as 0.06 h^{-1} under pH 5). Also, the lag phase is extended outside neutral conditions (Marshall & Odame-Darkwah, 1995; Seshadri & Mukhopadhyay, 1993; Wang et al., 2012b; Zhuge et al., 2014). Gorret et al. (2001) reported that optimal pH range for *A. acidipropionici* DSM 4900 biomass and EPS production was within pH 6.5 to 7, based on a Response Surface Methodology (RSM). Under acid conditions (pH 5 and 5.5) biosynthesis of propionic acid was favored compared to acetic acid, which increased P/A ratio in a batch process using *A. acidipropionici* ATCC 25562 (SESHADRI; MUKHOPADHYAY, 1993). In uncontrolled pH cultures, *P. freudenreichii* T82 showed drastically reduction in its growth rate and products yields when the pH fell below 5 (PIWOWAREK *et al.*, 2019).

Based on these observations, recently reports have suggested that controlling the culture pH at different values (for instance, pH 6.5 for the initial 48 h and then shifting to pH 5.5 or 6) is an effective strategy to improve propionic acid yields (ZHUGE *et al.*, 2014). In the first stage, pH is set for optimum microbial growth; then, in the second stage pH is reduced to direct the cell metabolic pathway towards propionic acid formation instead of biomass and byproducts formation (Feng et al., 2010b). Using this pH-shift control strategy Feng et al. (2010b) were able to double propionic acid productivity to as

high as 0.18 g.(L.h)$^{-1}$, and the P/A ratio up to 4:1 compared to cultures of *P. freudenreichii* CCTCC M207015 in which the pH was kept constant at 6.5.

2.5 Aeration

Dairy PAB are facultative anaerobe microorganisms and some strains can grow under aerobic conditions, with volumetric oxygen transference coefficient ($k_l a$) as high as 61 h^{-1} and up to 50 % of dissolved oxygen concentration (QUESADA-CHANTO *et al.*, 1997). These microorganisms harbor a partial respiratory system with menaquinones, membrane bound enzymes, cytochromes *b* and *c* that enable them to grow in the presence of oxygen, where it is the final electron acceptor (Ye et al., 1999). Although long exposure to oxygen causes decrease in cell growth due to cytochromes and menaquinones synthesis inhibition, aerobic/microaerophilic cultures can be beneficial for biomass production due to higher ATP generation (Furuichi et al. 2006a, b). On the other hand, propionic acid and vitamin B_{12} production are negatively affected under aerobic conditions (QUESADA-CHANTO *et al.*, 1998).

The growth under aerobic conditions changes the metabolisms pattern and acetic acid becomes the main end product and, at $k_l a$ higher than 20 h^{-1}, propionic acid production stops (KOUYA *et al.*, 2007; QUESADA-CHANTO *et al.*, 1997, 1998). The Wood-Werkman cycle (Figure 1) is reversed in presence of oxygen and propionic acid produced under anaerobioses is consumed under aerobic conditions producing pyruvate, which is further oxidized to acetic acid (YE *et al.*, 1999). Based on that, the oscillation of aeration strategy (anaerobic to aerobic and aerobic to anaerobic) along fermentation can reduce the inhibitory effect of propionic acid and enhance biomass (up to 3 folds) (Furuichi et al., 2006a; Miyano et al., 2000; Ye et al., 1996).

In relation to vitamin B_{12}, even growth at low $k_l a$ (10 h^{-1} and 0 % of dissolved oxygen after 1 h of fermentation), showed a reduction of near 30 % of this vitamin biosynthesis compared to anaerobic growth (QUESADA-CHANTO *et al.*, 1998). Dairy PAB follows the anaerobic pathway for vitamin B_{12} biosynthesis and aeration at the beginning of fermentation reduces the activities of key B_{12}-related enzymes (e.g. δ-aminolevulinic acid synthase and δ-aminolevulinic acid dehydratase), consequently,

reducing the vitamin B_{12} yields (MARTENS *et al.*, 2002; QUESADA-CHANTO *et al.*, 1997, 1998).

However, periodic fluctuations from anaerobic to aerobic and conversely, from aerobic to anaerobic along the cultivation improved vitamin B_{12} yields (2-folds) and productivity (1.4-folds) when compared to fully-anaerobic fermentation (YE *et al.*, 1996). Low dissolved oxygen concentrations (from 0.50 to 1 ppm) allows the propionic acid consumption and the oscillation of aeration strategy decreases the negative effect over vitamin B_{12} biosynthesis (MIYANO; YE; SHIMIZU, 2000; YE *et al.*, 1996). Moreover, oxygen is needed to produce the structural lower ligand 5,6-dimethylbenzimidazole (DMBI) when it is not provided in the culture medium (DEPTULA *et al.*, 2015).

2.6. Mathematical modeling

Mathematical modeling is an important tool for improving the performance of complex fermentation systems (Luo et al., 2021). Modeling takes into count the effect of multiple variables and their interactions upon process outputs making it possible to predict systems behavior in otherwise difficult experimental approaches and can be successfully applied to scaling up (El-Naggar et al., 2019). Thus, relevant modeling approaches for improving dairy PAB bioprocess were reviewed in the following subsections.

2.6.1 Statistical design of experiments (DoE)

Data-driven models based on statistical design of experiments (DoE) are frequently applied for optimization of dairy PAB cultures (Assis et al., 2020; Chen et al., 2013; Hedayati et al., 2020). These experimental designs significantly reduce the number of experiments, costs and time needed in the bioprocess development, being flexible and describing complex systems in relatively simple ways (EL-NAGGAR *et al.*, 2019; LUO; KURIAN; OGUNNAIKE, 2021). On the other hand, they do not extrapolate outcomes beyond defined levels and do not explain the mechanisms of biological phenomena (HEDAYATI; HOSSEINI; NAJAFPOUR, 2020; LUO; KURIAN; OGUNNAIKE, 2021).

Overall, Plackett & Burman design and Response Surface Methodology (RSM) (Central composite design, Box-Behnken design, among others) are chosen for screening

significant variables and to predict their optimal range, respectively, aiming to maximize bioprocess yields and/or productivity (Sindhu et al., 2017). Recently, our research group reached important results using this optimization strategy where biomass and vitamin B_{12} produced by *P. freudenreichii* ATCC13673 were increased up to 4 and 2 folds, respectively (ASSIS *et al.*, 2020).

Following the same method, Hedayati et al. (2020) found synergic interaction within DMBI, elemental solution ($CaCl_2.2H_2O$ and $CoCl_2.6H_2O$) and rice bran oil (RBO) upon vitamin B_{12} biosynthesis, whilst DMBI against temperature interaction presented negative effects. After optimization throughout Box-Behnken design (BBD), the authors were able to increase yields of vitamin B_{12} by 14 % compared to previous experiments (HEDAYATI; HOSSEINI; NAJAFPOUR, 2020). In another research, Chen et al. (2013) increased propionic acid production by 23 % solely using a central composite design (CCD), which highlights the effectiveness of this approach in bioprocess optimization.

2.6.2 Mechanistic models

Mechanistic models are another mathematical representation of bioprocess based in the first-principle mechanisms of microbial activity (e.g. cell growth and metabolism flux) and their related mathematical functions instead to exclusively experimental data (LUO; KURIAN; OGUNNAIKE, 2021). This approach is not as versatile as DoE, requiring extensive knowledge about the subject and substantial efforts for modeling. However, after experimental validation, it becomes a powerful tool to estimate parameters (for instance, lag time, cell growth rate, etc.), to predict microbial behavior and to develop predictive control strategies (Hashemi & Roohi, 2019; López et al., 2004; Sindhu et al., 2017; Zhu et al., 2018).

The Baranyi model successfully described the growth pattern of *P. freudenreichii* PTCC 1674 and *P. shermanii* PTCC 1661 in date syrup medium under different sonication amplitudes and exposure time (HASHEMI; ROOHI, 2019). In the same work, the authors proposed a Gaussian function to predict propionic acid production that showed great accuracy (adj-R^2 > 0.99) (HASHEMI; ROOHI, 2019). Goswami & Srivastava (2000) developed a mathematical model to evaluate the best substrate feeding strategy for propionic acid fermentation. The model showed that maximum substrate consumption and growth rate were reached at 35 h to 40 h, then, a continuous nutrient

feeding (0.04 L $.h^{-1}$) was stablished at this fermentation stage increasing propionic acid productivity (0.23 to 0.4 g.($L.h^{-1}$)) (GOSWAMI; SRIVASTAVA, 2000).

Interestingly, Zhu et al. (2018) enhanced vitamin B_{12} content in soy-milk using Lotka Volterra model to describe interactions between *L. reuteri* ZJ03 and *P. shermanii* ZJ01 in co-fermentation. The authors found that proper anaerobic phase (5 days), temperature (30 °C) and pH (7) provided the least antagonistic effects between strains, as well as enhancing vitamin B_{12} yields (up to 2 folds) (Zhu et al., 2018). Under those circumstances, mechanistic models are a great tool to underline the design of a successful bioprocess using dairy PAB. Further, this modeling process requires fewer experiments for parameters estimation and model validation, which is also desirable for time and costs reduction in the development stage (LUO; KURIAN; OGUNNAIKE, 2021).

3. Bioprocess techniques to obtain high cell density cultures of dairy PAB

The main bioprocess techniques applied to achieve high cell density culture (HCDC) of dairy PAB are the fed-batch bioreactor (GOSWAMI; SRIVASTAVA, 2000; OZADALI; GLATZ; GLATZ, 1996), cell recycling (Crespo et al. 1991; Liu et al., 2016; Miyano et al., 2000), perfusion culture (Boyaval & Corre, 1987; Hatanaka et al., 1988; Nakano et al., 1996), extractive fermentation (Jin & Yang, 1998; Wang et al., 2014; Wang et al., 2012b), and cell immobilization bioreactor (Feng et al., 2011; Rickert et al., 1998; Wallenius et al., 2015).

In this section are presented these techniques and their potential for production of biomass, propionic acid, and vitamin B_{12} by dairy *Propionibacterium* fermentations. The bioprocess design of each technique is illustrated in Figure 2. In the Tables 1 and 2, are summarized their characteristics and outcomes obtained in dairy PAB cultures, respectively.

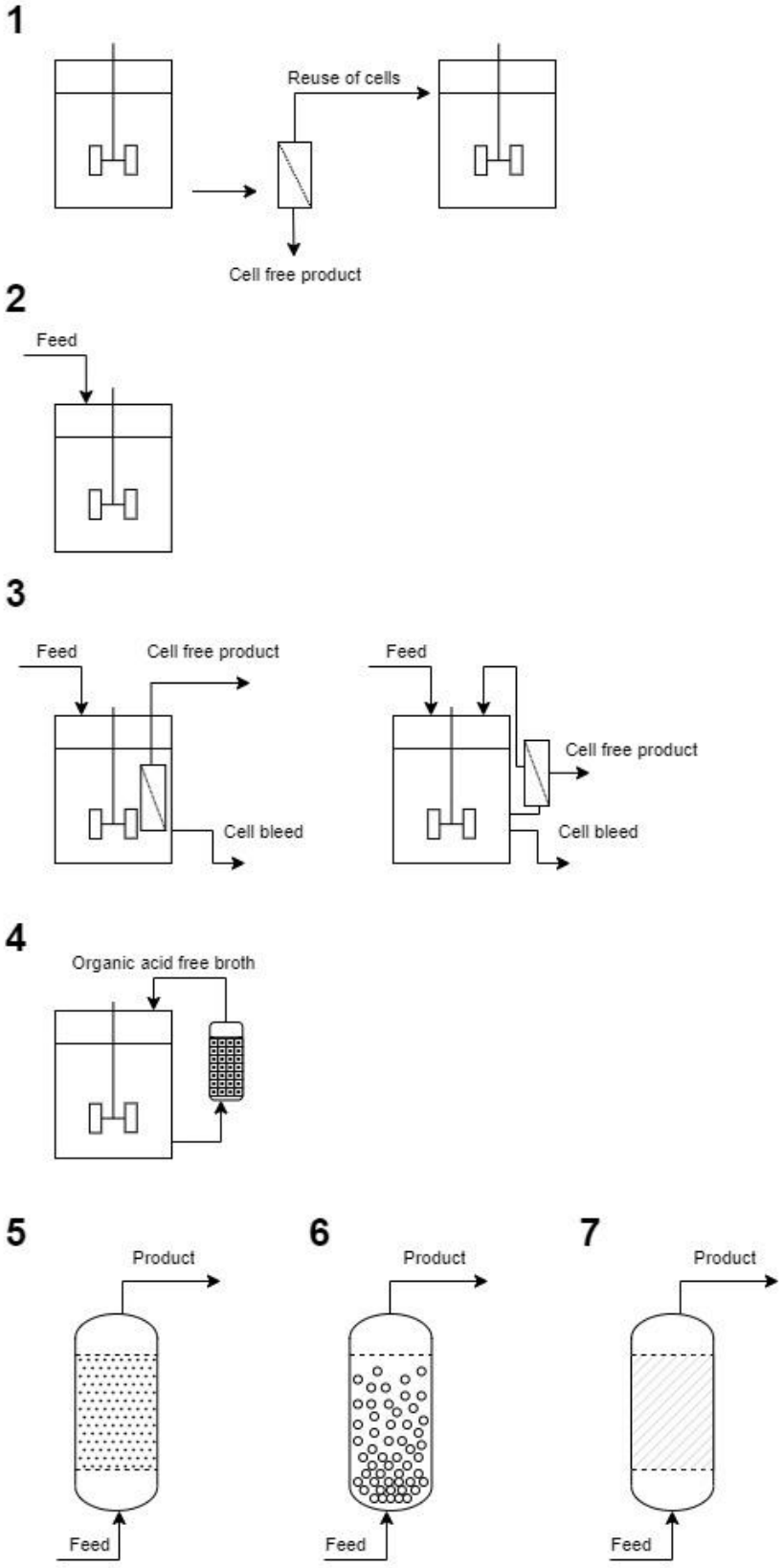

Figure 2. Bioprocess techniques to obtain high cell density cultures of dairy PAB: 1) cell recycling; 2) fed-batch; 3) perfusion culture; 4) extractive fermentation; 5); 6); and 7) cell immobilization (fixed, expanded, and fibrous bed bioreactors, respectively).

3.1 Fed-batch

Fed-batch is a bioprocess technique that consists in substrate addition into the bioreactor throughout constant, intermittent, or exponential feeding (SCHMIDELL *et al.*, 2001). In relation to dairy PAB cultures, common feeding strategies are: constant substrate addition at a pre-established feed rate (often from 0.01 to 0.04 $L.h^{-1}$ initiated after ~ 40 h of growth) or pulses of substrate along bioprocess (Ahmadi et al. 2017a, b; Jiang et al., 2015; Ozadali et al., 1996; Zhu et al., 2010; Zhuge et al., 2014).

Ozadali et al. (1996) achieved high cell density (37 $g.L^{-1}$) of *A. acidipropionici* P9 in a fed-batch with glucose pulses whenever its concentration was close to exhaustion in medium. The authors also reported high propionic acid yields (0.54 $g.g^{-1}$), titer (45 $g.L^{-1}$), and productivity (0.31 $g.(L.h^{-1})$) (OZADALI; GLATZ; GLATZ, 1996). Similar results were obtained in fed-batch cultures using *A. acidipropionici* ATCC 4875 at constant lactose feeding (GOSWAMI; SRIVASTAVA, 2000). On the other hand, Zhu et al. (2010) reported lower *A. acidipropionici* CGMCC 1.2230 cell density (~ 5 $g.L^{-1}$) and propionic acid productivity (0.20 $g.(L.h^{-1})$) in a fed-batch using glycerol as carbon source. However, these authors obtained high yields (0.56 $g.g^{-1}$), titer (44.6 $g.L^{-1}$), and P/A ratio (18:1), which is a characteristic of glycerol fermentation with this bacterium (Zhu et al., 2010).

In relation to vitamin B_{12}, it has been reported that nitrogen sources and other nutrients must be provided in the feed solution to improve biomass yields and to support an efficient HCDC in fed-batch mode operation (Liu et al., 2016; Paik & Glatz, 1994). Biomass production is important for vitamin B_{12} production since it is an intracellular product synthesized during the microbial growth (MARTENS *et al.*, 2002). In addition, availability of vitamin B_{12} precursors such as cobalt ions, DMBI, or $FMNH_2$, are also important for vitamin B_{12} biosynthesis (DEPTULA *et al.*, 2015; HUGENSCHMIDT; SCHWENNINGER; LACROIX, 2011). Therefore, the composition of feeding solution should supply proper nitrogen source as well as precursors for an effective vitamin B_{12} production in fed-batch.

3.2 Cell recycling

3.2.1 Semi-continuous process

In the cell recycling technique, after a batch or a fed-batch phase, microbial cells are harvested by unit operations such as centrifugation, sedimentation, or ultrafiltration, resuspended in fresh medium, and cultured for another cycle, while the previous fermented broth is forwarded to downstream steps (Colomban et al., 1993; Dishisha et al., 2015; Liu et al., 2016; Schmidell et al., 2001). Due to their characteristics (Table 1), cell recycling is considered a potential process for slow-growing microorganisms such as dairy PAB (Quesada-Chanto et al., 1994).

The highest propionic acid productivities obtained in glycerol fermentation under batch process (1 to 1.42 g.(L.h^{-1})) were reported using sequential batches with cell recycling (DISHISHA *et al.*, 2013, 2015). In another research, high propionic acid titer (75 g.L^{-1}) and average productivity (0.32 g.(L.h^{-1})) were obtained in a fed-batch with *A. acidipropionici* ATCC 4875 using a dense inoculum (biomass ~ 14 g.L^{-1}) recycled from a previous HCDC fed-batch (Liu et al., 2016). Colomban et al. (1993). This procedure kept *A. acidipropionici* ATCC 4965 cell concentration around 40 g.L^{-1} by ultrafiltration in a pilot-scale bioreactor (5 m^3) and the authors reported a stable process (15 sequential batches, over 900 h of operation) with high propionic acid productivity (~ 1 g.(L.h^{-1})). That biomass concentration was considered the best choice to improve the bioprocess efficiency, as well as to avoid contamination and clogging issues (COLOMBAN; ROGER; BOYAVAL, 1993).

One of the main advantages of cell recycling is that HCDC can be achieved from the start of the fermentation and be kept relatively constant throughout the experiment (DISHISHA *et al.*, 2013; QUESADA-CHANTO; AFSCHAR; WAGNER, 1994). Additionally, high propionic acid titer and productivities attainable make it a promising technique for propionic acid production in biorefineries (DISHISHA *et al.*, 2013), especially when integrated to the production of other dairy PAB products such as vitamin B_{12} or biomass itself for probiotic or cheese starter applications (COLOMBAN; ROGER; BOYAVAL, 1993; DISHISHA *et al.*, 2013; QUESADA-CHANTO; AFSCHAR; WAGNER, 1994).

In relation to vitamin B_{12}, Miyano et al. (2000) reported a higher B_{12} productivity (0.62 mg.($L.h^{-1}$) in a cell recycling system compared to batch process (0.14 mg.($L.h^{-1}$), although lower specific yields of biomass (0.77 vs 0.98 $mg.g^{-1}$, respectively) were obtained. Quesada-Chanto et al. (1994) developed a bioprocess based in cell recycling for the simultaneous production of propionic acid and vitamin B_{12} by dairy PAB. The authors kept a HCDC (biomass of approximately 75 $g.L^{-1}$) into two connected bioreactors, one under anaerobic conditions, whereas the second was run under microaerophilic conditions, reporting high propionic acid productivity (4.42 g.($L.h^{-1}$) and yields (0.50 $g.g^{-1}$), along with very high vitamin B_{12} productivity (up to 1.50 mg.($L.h^{-1}$) and yields (0.66 $mg.g^{-1}$ biomass) using sugarcane molasses in cultures of *A. acidipropionici* DSM 8250 (QUESADA-CHANTO; AFSCHAR; WAGNER, 1994).

3.2.2 Continuous process (Perfusion)

The perfusion culture system is characterized by continuously feeding fresh medium and fermented broth harvesting whilst microbial cells are kept into the bioreactor by using different cell retention devices such as spin-filters, ultrafiltration modules, among others (Crespo et al., 1991; Nakano et al., 1993; Quesada-Chanto et al., 1994). The removal of inhibitory metabolites and cell retention enable to obtain cell density and productivities 10 times higher than in batch process (CACCIUTTOLO, 2007). Indeed, the best propionic acid productivities (Table 2) ever reported for PAB fermentation were obtained by using continuous process with cell retention by ultrafiltration (BOYAVAL; CORRE, 1987; CRESPO *et al.*, 1991).

Improved results over other techniques have also been reported for biomass production in perfusion cultures (Table 2). The highest biomass obtained for dairy PAB fermentation (over 200 $g.L^{-1}$) was reached in a perfusion process using hollow-fiber module as a cell retention device (HATANAKA *et al.*, 1988). Additionally, this HCDC also produced high vitamin B_{12} titer (52 $mg.L^{-1}$) showing average specific yields of 0.23 $mg.g^{-1}$ of biomass (HATANAKA *et al.*, 1988).

Nakano et al. (1993), using rotative ceramic membranes as the cell retention device reported cell densities around 50 $g.L^{-1}$ in a continuous culture of *P. freudenreichii* ATCC 8262. When the same bioprocess was coupled to a propionic acid removal system,

biomass production increased to 150 $g.L^{-1}$ (NAKANO; KATAOKA; MATSUMURA, 1996). The combined systems enabled the recirculation of fermented broth (propionic acid free) and residual nutrients were efficiently consumed, reducing fresh medium feeding, equivalent to 30 % less glucose being supplied, compared to traditional perfusion (NAKANO; KATAOKA; MATSUMURA, 1996).

The pore size of membrane devices is another variable that influences yields and productivity due its role in cell retention into the bioreactor. Goswami and Srivastava (2001) reported that the higher cell retention using 5 µm spin filter compared to 10 µm spin filter improved propionic acid yields (0.40 vs 0.35 $g.g^{-1}$) and productivities (0.90 vs 0.53 $g.(L.h^{-1})$. In addition, the rotation of devices such as spin filters and rotative ceramic membranes enhances filtration process because it reduces the cake layer formation upon membrane surface, minimizing fouling and clogging issues (GOSWAMI; SRIVASTAVA, 2001; NAKANO; MATSUMURA; KATAOKA, 1993).

High cell density cultures over 100 $g.L^{-1}$ can be obtained using ultrafiltration modules, but it may turn propionic acid fermentation unstable and hinder the operation due to clogging, viscosity increase, and foam formation (BLANC; GOMA, 1989; BOYAVAL; CORRE, 1987; CRESPO *et al.*, 1991). In these cases, a cell bleed rate might be stablished to avoid biomass to reach critical levels and to ensure a stable continuous process (BLANC; GOMA, 1989; CRESPO *et al.*, 1991). Furthermore, the harvested biomass can also be used as probiotics, cheese starter, or vitamin B_{12} source (COLOMBAN; ROGER; BOYAVAL, 1993; DISHISHA *et al.*, 2013; QUESADA-CHANTO; AFSCHAR; WAGNER, 1994).

Currently, the development of new technologies like alternating tangential flow filtration (ATF) as cell retention device, is promising in order to overcome most of those problems and are currently being used in the pharmaceutical industry (Bielser et al., 2018; Zydney, 2021). In an ATF device, the filtration flow is periodically alternated (back and forth) by a diaphragm pump, which continuously harvest the broth while pushing cells back to the bioreactor, thus removing the microbial layer over the membrane surface (KELLY *et al.*, 2014). Therefore, ATF filtration reduces membrane fouling, improves cell viability, medium consumption, cell density and productivity compared to other cell retention devices previously cited (BIELSER *et al.*, 2018; KELLY *et al.*, 2014; ZYDNEY, 2021).

However, at present, there are no reports on perfusion cultures for dairy PAB in ATF systems. This opens the way to further research with these potential probiotic bacteria, especially for biomass and vitamin B_{12} production.

3.3 Extractive fermentation

Propionic acid inhibitory effects over cell cultures begin at concentration range of 5 to 10 $g.L^{-1}$, the critical value being around 30 $g.L^{-1}$, when cell growth is arrested (Suwannakham & Yang, 2005; Wang et al., 2012b), and propionic acid formation is also disrupted due to inhibition of key enzymes propionyl CoA transferase, and oxaloacetate carboxyltransferase (Figure 1) (SUWANNAKHAM; YANG, 2005). This inhibition suppress the propionic acid pathway and increases byproducts formation such as acetic and succinic acids (SUWANNAKHAM; YANG, 2005). Hence, accumulation of propionic acid along fermentation reduces its own yield and productivity (Gu et al., 1998; Suwannakham & Yang, 2005).

It has been suggested that propionic acid concentration should be kept at low level into the bioreactor (< 10 $g.L^{-1}$) in order to achieve high cell growth and products formation rate (Gu et al., 1998; Jin & Yang, 1998; Wang et al., 2012b). To do so, bioreactors can be coupled to activated charcoal-packed columns, ion-exchange columns, system of solvent driven extraction, or electrodialysis system, which enable the selective removal of organic acids from the broth in a process called extractive fermentation or *in situ* product removal (ISPR) (Solichien et al., 1995; Wang et al., 2012b; Zhang et al., 1993).

High propionic acid productivity (~ 1 $g.(L.h^{-1})$ and titer (75 $g.L^{-1}$) were reported by Jin et al. (1998) using solvent driven extraction in a fed-batch fermentations of *A. acidipropionici* ATCC 4875. The authors showed that solvent toxicity could be removed if the extractor were contained in hollow-fiber membranes to reduce contact with microbial cells (JIN; YANG, 1998). Even higher propionic acid titer (91 $g.L^{-1}$) with high yields (0.75 $g.g^{-1}$) but lower productivity (0.36 $g.(L.h^{-1})$ were reported for the fed-batch process of *P. freudenreichii* CICC 10019 using expanded bed adsorption bioreactor (EBAB) for propionic acid removal. In contrast, vitamin B_{12} yields were reduced by approximately 50 % (from 0.72 to 0.37 $mg.g^{-1}$ substrate) compared to conventional fed-batch (Wang et al., 2014). In another research, vitamin B_{12} yields were also reduced in

EBAB fed-batch compared to the batch process (0.95 to 0.37 $mg.g^{-1}$ substrate) using the same strain. However, propionic acid yields were increased (from 0.56 $g.g^{-1}$ to 0.75 $g.g^{-1}$) indicating a metabolic shift into propionic acid biosynthesis in these systems (Wang et al., 2020).

The literature data indicates that extractive fermentation keeping propionic acid at low concentrations (< 10 $g.L^{-1}$) is a better strategy for propionic acid production than for vitamin B_{12} or dairy PAB biomass (Gu et al., 1998; Wang et al., 2020; Wang et al., 2012b; Zhang et al., 1993). However, the control of propionic acid at two levels (i.e. low at first stage and high at late stages of fermentation) along with DMBI addition strategy provided one of the highest vitamin B_{12} titer (59.5 $mg.L^{-1}$), yields (0.98 $mg.g^{-1}$ substrate) and productivity (0.59 $mg.(L.h^{-1})$) ever reported for dairy PAB cultures (Table 2) (Wang et al., 2015). It was suggested that, at late stage of fermentation, inhibitory effects of propionic acid over its own metabolic pathway favors the nutrient shift to vitamin B_{12} biosynthesis (Wang et al., 2015). Thus, extractive fermentation can be effective for vitamin B_{12} production as well, but the process operation must be optimized toward vitamin B_{12} biosynthesis instead of propionic acid formation.

3.4 Cell Immobilized bioreactors

Immobilization technique consists in confining microbial cells into physical structures called support materials such as natural and synthetic polymers, glass beads, *lentikats*, among others, usually through support binding or entrapment methods (Schmidell et al., 2001; Zhu, 2007). After the immobilization, confined cells can be used as biocatalysts in multiple fermentation process (Dishisha et al., 2012; Feng et al., 2011; Rickert et al., 1998).

3.4.1 Fibrous bed bioreactor immobilization

Column bioreactors, consisting of fixed, expanded and fibrous bed bioreactors, are often chosen for fermentation using immobilized cells (SCHMIDELL *et al.*, 2001). In particular, fibrous bed bioreactors (FBB) gained attention because they present less diffusional limitations and pressure issues, maintenance of high active cell density under

long-term operation, reduction of downtime, system stability, and easy immobilization techniques (non-specific adsorption and entrapment) (Feng et al., 2010a; Suwannakham & Yang, 2005; Zhang & Yang, 2009; Zhu, 2007).

In this process, a microbial cell suspension is circulated throughout the column packed with spiral wound fibrous material that can be cotton, terry cloth, bagasse, among others, to enable cells to adhere onto support surface or to get entrapped into void spaces, where they will grow during the bioprocess reaching concentrations of up to 40 $g.L^{-1}$ (Yang, 1996). The major drawbacks observed in this technique are the presence of free-cells into broth (~ 20 %) and additional material costs (Feng et al., 2010a; Yang, 1996; Zhang & Yang, 2009).

The FBB system is self-renewing in the sense that excess of aging or dead cells are continually desorbed whilst new ones are allowed to grow maintaining fermentation for long periods, from months to a year, without clogging or pressure issues (Yang, 1996). Furthermore, cells immobilized into FBB have shown the capacity to modulate their membrane composition, morphological aspects and, more important, the activity of key metabolic enzymes (SUWANNAKHAM; YANG, 2005). This adaptative process strengthens propionic acid tolerance and cell viability, thus high propionic acid titer and less byproducts formation can be achieved by the adapted strains (Feng et al., 2010a; Suwannakham & Yang, 2005; Zhang & Yang, 2009).

One of the highest propionic acid titers ever reported (106 $g.L^{-1}$) was obtained in a long-term fed-batch operation (~ 3,000 h) using *A. acidipropionici* 4875 ACK-Tet immobilized in FBB. However, the acid productivity was low (< 0.04 $g.(L.\ h^{-1})$ due propionic acid accumulation (Zhang & Yang, 2009). Recently, a high trehalose producer mutant strain was able to increase propionic acid titer (135 $g.L^{-1}$) with increased productivity (0.61 $g.(L.h^{-1})$ and yields (0.67 $g.g^{-1}$ lactose) in a fed-batch FBB system (JIANG *et al.*, 2015). The authors suggested that higher trehalose production could enhance microbial tolerance over that propionic acid concentration (JIANG *et al.*, 2015).

Feng et al. (2011) developed a cleaner, effective, and economical bioprocess to produce propionic acid by coupling the use of hydrolyzed cells and molasses as low-cost substrates to fed-batch fermentation with *P. freudenreichii* immobilized in plant-fibrous bed bioreactor. Despite low productivities (0.26 $g.(L.h^{-1})$, they obtained high propionic

acid titer (~ 80 $g.L^{-1}$) and purity (77 %), otherwise unfeasible in batch process, demonstrating the potential of the technique (FENG *et al.*, 2011). In continuous fermentation at high dilution rates (0.1-0.3 h^{-1}) better propionic acid productivities were achieved (up to 1 $g.(L.h^{-1})$, but generating a diluted effluent (less than 15 $g.L^{-1}$ of propionic acid) (DISHISHA; ALVAREZ; HATTI-KAUL, 2012; LEWIS; YANG, 1992).

3.4.2 Alginate beads immobilization

One of the most used immobilization technique is the cell entrapment in Ca-alginate supports (SCHMIDELL *et al.*, 2001). In this process, a microbial suspension is mixed with alginate solution (2-4 % mass fraction) and then dripped into a $CaCl_2$ solution (2-4 % mass fraction) to create a bead-shaped rigid complex that confines the microorganisms (Gardner & Champagne, 2005; Rickert et al., 1998; Xu et al., 2007). It simplifies cells recovery and reuse in repeated bioprocess, protects cells from propionic acid inhibitory effects and improves propionic acid productivity (~ 1 $g.(L.h^{-1})$ in continuous process) (PAIK; GLATZ, 1994). In a consecutive batch, Rickert et al. (1998) reported propionic acid productivity up to 2 $g.(L.h^{-1})$, attributing this good result to high initial substrate level (glucose 75 $g.L^{-1}$) and cell density of *A. thoenii* P20 immobilized in alginate beads.

However, diffusional limitation, contamination, and, in particular, beads stability are the biggest problems to be overcome in alginate immobilization (Duarte et al., 2013). In addition, fermentations of dairy PAB immobilized in alginate beads have shown reduced vitamin B_{12} biosynthesis up to 50 % compared to free cells cultures (Czaczyk et al., 1997; Gardner & Champagne, 2005; Yongsmith et al., 1982). This is attributed to the entrapment of cobalt into the alginate matrix, reducing its availability, thus affecting vitamin B_{12} biosynthesis, based on the central atom of the corrinoid ring (CZACZYK; TROJANOWSKA; GRAJEK, 1997; GARDNER; CHAMPAGNE, 2005). Therefore, results gathered in the literature so far suggest that this technique seems to be more suitable for propionic acid production rather than for vitamin B_{12}.

3.4.3 *Others support materials*

Wallenius et al. (2015) developed an innovative xylan hydrogel matrix that supports high cell density, with estimated concentrations of 99 $g.L^{-1}$ into the column bioreactor (~ 74 g of support material), providing less mass transfer problems. The authors achieved high propionic acid productivity (0.88 $g.(L.h^{-1})$) and yields (0.58 $g.g^{-1}$) in continuous fermentations with *A. acidipropionici* NRRL B-3569 at high dilution rate over a month of bioreactor operation. However, significant damage to the beads was observed after that period (WALLENIUS *et al.*, 2015). Dishisha et al. (2012) observed that immobilization of *A. acidipropionici* DSMZ 4900 in Luffa (vegetal matrix) and Poraver beads (porous glass) was not effective, but modification of supports structures by attaching a cationic polymer such as polyethylenimine improved the immobilization performance (DISHISHA; ALVAREZ; HATTI-KAUL, 2012).

Exopolysaccharide (EPS) producing strains have shown exceptional immobilization performance without any support modification requirements (Belgrano et al., 2018). Olguin et al. (2019) induced EPS production and biofilm formation using stress factors such as sodium chloride and citric acid to immobilize *A. acidipropionici* DSMZ 4900 in Poraver and AnoxKaldnes, a plastic support. Biofilms immobilized in Poraver material provided better propionic acid productivity (0.15 - 0.78 $g.(L.h^{-1})$) in repeated batch cycles (OLGUIN *et al.*, 2019). Therefore, the choice of adequate support material, immobilization technique, as well as microbial strain, growth condition and bioreactor design must be taken into count to achieve the highest fermentation performance with immobilized cells (BELGRANO *et al.*, 2018; DISHISHA; ALVAREZ; HATTI-KAUL, 2012; PAIK; GLATZ, 1994; RICKERT; GLATZ; GLATZ, 1998; WALLENIUS *et al.*, 2015).

Table 1. Characteristics of bioprocess techniques applied to obtain high cell density cultures of dairy PAB cultures.

Bioprocess technique	Advantages	Disadvantages	References
Fed-batch	Relatively simple; avoids substrate inhibition; modulates cell growth rate and metabolic activity.	End-product inhibition due to organic acids accumulation.	(Feng et al., 2010a; Goswami & Srivastava, 2000; Zhu et al., 2010; Zhuge et al., 2014).
Cell recycling	Reduces microbial lag phase; increases cell density; decreases end-product inhibition.	Operational complexity; extra costs with membranes; mechanical stress; clogging of membranes and contamination issues.	(Colomban et al., 1993; Dishisha et al., 2015, 2013; Liu et al., 2016; Miyano et al., 2000; Quesada-Chanto et al., 1994; Westman & Franzén, 2015).
Perfusion	High yields and productivities (up to 10 folds higher than batch); requirement for small equipments; and products homogeneity.	Operational complexity; higher material costs (medium, membranes, harvest tanks, etc.); clogging of membranes and contamination issues.	(Boyaval & Corre, 1987; Cacciuttolo, 2007; Crespo et al., 1991; Croughan, 2015; Pollock et al., 2013).
Extractive fermentation	Reduced end-product inhibitions; increased cell viability and reduced alkali consumption during pH control.	Operational complexity; extra costs; limited extractive capacity and toxicity of resins and solvents.	(Jin & Yang, 1998; Ozadali et al., 1996; Solichien et al., 1995; Wang et al., 2014; Wang et al., 2012a).
Immobilization	Increased cell viability (up to 10^{10} CFU.g^{-1} support); reduced end-product inhibition and lag phase.	Operational complexity; mechanical stability; diffusional limitation; contamination and difficult to scale-up.	(FENG *et al.*, 2011; GARDNER; CHAMPAGNE, 2005; GU; GLATZ; GLATZ, 1998; PAIK; GLATZ, 1994; SUWANNAKHAM; YANG, 2005; WALLENIUS *et al.*, 2015).

Table 2. Comparison of outcomes obtained by dairy *Propionibacterium* sp. cultured under different bioprocess techniques.

Bioprocess technique	Bioprocess parameter								References
	Strain	Temperature (°C)	pH	X ($g.L^{-1}$)	Y_p ($g.g^{-1}$ substrate)	Y_{B12} ($mg.\ g^{-1}$ substrate)	Q_p ($g.(L.h^{-1})$)	Q_{B12} ($mg.(L.h^{-1})$)	
Batch	*A. acidipropionici* ATCC 4965	30	7.0	1.8			0.07		(BOYAVAL; CORRE, 1987)
Batch	*P. freudenreichii* IFO 12424	30	6.7	6.9		0.98*		0.14	(MIYANO; YE; SHIMIZU, 2000)
Batch	*A. acidipropionici* ATCC 4875	30	6.5	14	0.44		0.25		(Goswami and Srivastava 2001)
Batch	*P. freudenreichii* CICC 10019	30	7.0	7.9	0.44		0.20		(Wang et al., 2012b)
Batch	*A. acidipropionici* ATCC 4875	32	6.5		0.55		0.03		(Zhang & Yang, 2009)
Fed-batch	*A. acidipropionici* ATCC 4875	30	6.5	20			0.40		(Goswami and Srivastava 2000)
Fed-batch	*A. acidipropionici* ATCC 4875	30	6.0	24	0.38		0.20		(Liu et al., 2016)
Fed-batch	*A. acidipropionici* CDBB-1049	30	7.0	10	0.48				(MARTÍNEZ-CAMPOS; DE LA TORRE, 2002)
Fed-batch	*P. freudenreichii* CICC 10019	30	7.0		0.71	0.72	0.36	0.36	(Wang et al., 2014)
Fed-batch	*P. freudenreichii* CICC 10019	30	7.0	6	0.35	0.61	0.13	0.23	(Wang et al., 2012a)
Cell recycling	*A. acidipropionici* ATCC 4965	30	7.0	100	0.17		14.3		(BOYAVAL; CORRE, 1987)

Cell recycling	*A. acidipropionici* DSM 8250	37	7.0	75	0.5	0.66	4.42	1	(QUESADA-CHANTO; AFSCHAR; WAGNER, 1994)
Cell recycling	*P. freudenreichii* IFO 12424	30	6.7	35		0.77*		0.62	(MIYANO; YE; SHIMIZU, 2000)
Cell recycling	*A. acidipropionici* ATCC 25562	37	6.0	137	0.56		10.3		(CRESPO *et al.*, 1991)
Cell recycling	*P. shermanii* PZ-3	30	6.5	227		0.23*		0.8	(HATANAKA *et al.*, 1988)
Extractive fermentation	*P. freudenreichii* CICC 10019	30	7.0	6.8	0.78		0.45		(Wang et al., 2012b)
Extractive fermentation	P. shermanii PZ3	30	6.6	8.8			0.39		(Zhang et al. 1993)
Extractive fermentation	*P. freudenreichii* CICC 10019	30	7.0	6.8	0.66	0.54	0.33	0.27	(Wang et al., 2012a)
Extractive fermentation	*P. freudenreichii* CICC 10019	30	7.0		0.75	0.37	0.35	0.36	(Wang et al., 2014)
Extractive fermentation	*A. acidipropionici* ATCC 4875	30	7.1		0.66		1		(JIN; YANG, 1998)
Immobilization	*P. freudenreichii* DSM4902	32	6.5		0.58		0.48		(Wang and Yang 2013)
Immobilization	*A. acidipropionici* ATCC 4875 ACK-Tet	32	7.0	30-60	0.56		0.03		(Zhang and Yang 2009)
Immobilization	*A. acidipropionici* P200910	32	7.0		0.52		0.96		(PAIK; GLATZ, 1994)
Immobilization	*A. acidipropionici* NRRL B-3569	32	7.0	99	0.58		0.88		(WALLENIUS *et al.*, 2015)
Immobilization	*A. acidipropionici* CGMCC 1.2230	37	6.0		0.43		0.71		(Zhu et al., 2012)

X= biomass; Yp= propionic acid yield; Y_{B12}= vitamin B_{12} yield; Q_p= propionic acid productivity; Q_{B12}= vitamin B_{12} productivity. *mg. g biomass.

4. Conclusion

Overall, all techniques reviewed proved to be effective in improving the bioprocess performances of *Propionibacterium* sp. and *Acidipropionibacterium* sp.. However, the implementation of these techniques increases, at least in some level, the bioprocess operational complexity and material costs. Therefore, the HCDC advantages and disadvantages should be taken into count before choosing a particular HCDC technique for dairy PAB fermentations. In special, fed batch using immobilized cells in fibrous bed bioreactors (FBB) appears to be a very promising technique for propionic acid production based on its relative simplicity and the possibility to produce high yields of this organic acid. On the other hand, the highest biomass production and vitamin B_{12} biosynthesis are obtained in cell recycling systems but, unfortunately, the very promising ATF system, already in use for some pharmaceutical applications, have not been tested for dairy PAB production, which opens the window for new research in the field. Despite its high operational complexity and costs, cell recycling could be explored to produce these outstanding microorganisms, especially for value-added applications, such as probiotic supplements and vitamin B_{12} fortification of plant-based products. Other applications are still to be explored with the increasing interest for vitamin B_{12}/probiotic-rich foods and beverages.

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Conflict of Interest

The authors of this work declare no conflicts of interest.

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4. Artigos científicos experimentais

4.1. Otimização da produção de vitamina B_{12} por P. shermanii ATCC 13673 em biorreatores STR

O processo de otimização da produção de vitamina B_{12} por *P. shermanii* ATCC 13673 em biorreatores STR de 2 L utilizando resíduo agroindustrial de soja como substrato foi descrito na forma de artigo científico (a ser submetido). Segue a apresentação do mesmo.

5. Discussão geral

Mudanças no padrão de produção e consumo são essenciais para cumprir os objetivos do desenvolvimento sustentável, assim como, os acordos internacionais sobre as mudanças climáticas. Órgãos científicos endossam a necessidade de adoção de dietas saudáveis com teor reduzido de alimentos derivados de animais, sobretudo carnes vermelhas, e açúcares refinados, ao longo que se aumente a participação de alimentos vegetais, como leguminosas, frutas e nozes na dieta mundial (IPCC, 2022; Willett et al., 2019). A conscientização da população sobre os desafios de nossa era, o Antropoceno: a era dos seres humanos, catalisam a busca por alternativas sustentáveis em todos setores, incluindo o de alimentos. Nesse contexto, a adoção de dietas alternativas, assim como, o setor de proteínas vegetais alternativas vem aumentando a sua participação no mercado de alimentos anualmente (Biscarra-Bellio et al., 2023).

A ingestão de vitamina B_{12} em dietas vegetais alternativas pode ser desafiadora, devido a inabilidade natural das plantas em produzir este micronutriente (Niklewicz et al., 2022). Porém, através de estratégias de bioprocessos pode-se suprir esta demanda nutricional de forma potencialmente eficaz, conforme observado ao longo deste estudo. A otimização das condições de bioprocessos possibilitou a obtenção de altos rendimentos de vitamina B_{12} (acima de 800 µg/g biomassa seca), mais de 300 vezes a recomendação diária de vitamina B_{12} (2.4 µg/dia). Por ser uma linhagem GRAS (*Generally Reconized As Safe*), a biomassa de *P. shermanii* ATCC 13673 pode ser adicionada diretamente aos alimentos vegetais em estratégias de fortificação de alimentos. Esse fato pode reduzir os custos da purificação do micronutriente e torná-lo acessível para fortificar diversos alimentos de consumo diário da população.

Além disso, a linhagem *P. shermanii* ATCC 13673 foi cultivada em resíduo agroindustrial de soja. Esse fato é de suma importância para o desenvolvimento de processos produtivos dentro do conceito de economia circular, onde resíduos das atividades industriais devem ser reintegrados em novos ciclos produtivos, gerando produtos de valor agregado ou bioenergia (Ellen MacArthur Foundation, 2024). Mais de 50.000 m^3 de LAPRS (Liquid Acid Protein Residue of Soybean) são gerados por uma única empresa de processamento do grão de soja para obtenção de proteína isolada. Por conta da alta carga orgânica, o resíduo exige um oneroso tratamento de efluentes. Porém, os dados obtidos neste estudo demonstram o seu potencial para ser utilizado como substrato de *P. shermanii* ATCC 13673 para produção de vitamina B_{12}.

Vale pontuar que pré-tratamentos enzimáticos foram aplicados no substrato, sobretudo para quebra de açúcares complexos da matriz vegetal (ex.: estaquiose e rafinose). A investigação de novas linhagens naturalmente equipadas com arsenal enzimático para degradar substratos de soja, tais como: linhagens do gênero *Acidipropionibacterium* (anteriormente *Propionibacterium acidipropionicii*) (Yang et al., 2018). Ou ainda, o desenvolvimento de co-culturas de microrganismos e cultivos de alta densidade celular (Assis et al., 2022; Tangyu et al., 2022). Podem ser alternativas para aumentar a eficiência deste bioprocesso baseado na utilização de resíduos agroindustriais como substrato, sem a necessidade de utilização de insumos tecnológicos externos.

O estudo de caracterização da biomassa de *P. shermanii* ATCC 13673 seca por atomização revelou boa retenção de vitamina B_{12} (em torno de 18 µg/g biomassa seca). Porém, a análise em sistema ICP-MS também revelou a presença de resíduos de cobalto (~ 3 µg/g biomassa seca), o qual pode ser tóxico em altas concentrações. Apesar da baixa concentração de cobalto nas amostras, a investigação da biocompatibilidade de produtos fortificados com a biomassa de *P. shermanii* ATCC 13673 produzida nas condições deste estudo se fez necessária para aferir, tanto aspectos de funcionalidade (ex.: liberação de vitamina B_{12} da matriz microbiana), quanto aspectos de segurança (ex.: toxicidade relacionada aos traços de cobalto).

A análise de citotoxicidade e integridade das monocamadas de células Caco-2 (modelo celular do epitélio intestinal humano) demonstrou que amostras de alimentos fortificadas com biomassa da linhagem não exerceram toxicidade aguda sobre o modelo celular após digestão simulada (INFOGEST 2.0). Os dados gerados são um indicativo inicial importante da segurança do consumo de alimentos fortificados com a biomassa de *P. shermanii* ATCC 13673 produzida neste estudo. Estes dados corroboram com os relatórios de agências internacionais de saúde, as quais indicam níveis toleráveis de cobalto em torno de 700 µg/dia para um adulto de 70 kg (EFSA, 2012). Assim como, estudos da influência da suplementação de sais de cobalto em voluntários humanos (1000 µg/dia) (Tvermoes et al., 2014). Tais valores estão muito acima da concentração de cobalto presentes na biomassa de *P. shermanii* ATCC 13673 produzida neste estudo. E os ensaios em modelo celular intestinal confirmaram a sua irrelevância para gerar efeito tóxico *in vitro*.

O ensaio de absorção epitelial da vitamina B_{12} e estimativa da sua biodisponibilidade in vitro era um dos objetivos nos experimentos de biocompatibilidade com células Caco-2. Porém, o tratamento térmico necessário para inativação de enzimas digestivas também degradou a

vitamina B_{12} presente nas amostras, provavelmente devido a sensibilidade térmica da HOCbl agora livre nas amostras digeridas. Consequentemente, a análise de biodisponibilidade da vitamina B_{12} *in vitro* em células Caco-2 foi inviabilizada.

Alternativamente, a conversão da OHCbl para CNCbl poderia solucionar este entrave tecnológico, aumentando a estabilidade térmica da vitamina B_{12} frente ao processo de inativação térmica das enzimas digestivas após a digestão simulada das amostras fortificadas. Ainda assim, os resultados não refletiriam a biodisponibilidade da HOCbl, forma de vitamina B_{12} majoritária na biomassa *P. shermanii ATCC 13673* seca. Outra alternativa seria o co-cultivo das células Caco-2 com a linhagem HT29-MTX. Essa linhagem celular é conhecida pela sua produção de uma camada protetora de muco, a qual poderia proteger as células Caco-2 do efeito citotóxico das amostras de digestão simulada apenas diluídas em tampão (Guri et al., 2014). Entretanto, o período de doutorado sanduíche no exterior chegou ao seu final. Além disso, o processo de otimização do ensaio de biodisponibilidade necessitaria de uma quantidade considerável de tempo, tendo em vista que as células Caco-2 possuem crescimento lento (> 21 dias para diferenciação em enterócitos).

Ainda assim, o estudo de liberação da vitamina B_{12} (bioacessibilidade) após a digestão simulada *in vitro* (INFOGEST 2.0) revelou que o micronutriente fornecido pela biomassa de *P. shermanii* 13673 possuí bioacessibilidade tão alta quanto a vitamina B_{12} cristalina (encontrada em suplementos farmacêuticos). Esses resultados se aplicam biomassa microbiana adicionada em produtos de panificação e vão de encontro aos relatos Chamlagain et al. (2021) utilizando biomassa de *P. freudenreichii* DSM 20271 em pães fortificados. Vale ressaltar que, ensaios de biocompatibilidade e bioacessibilidade *in vitro* comumente possuem boa correlação com estudos *in vivo* e geram informações úteis sobre funcionalidade e os potenciais efeitos das substâncias teste ao organismo humano (Fedi et al., 2021; Kondrashina et al., 2023; Verhoeckx et al., 2015).

6. Conclusão geral e perspectivas futuras

A linhagem propiônica *P. shermanii* ATCC 13673 foi capaz utilizar o resíduo agroindustrial de soja como substrato eficazmente para produção de vitamina B_{12}. Após a otimização do bioprocesso em reatores STR 2 L, quantidades apreciáveis do micronutriente foram obtidas. A disponibilidade de açúcares simples foi uma das variáveis mais significativa sobre o rendimento do bioprocesso desenvolvido neste estudo. A busca por linhagens naturalmente adaptadas a substratos vegetais de soja e/ou desenvolvimento de estratégias de co-cultivos e cultivos de alta densidade celular podem conduzir a rendimentos de vitamina B_{12} ainda maiores. Este estudo foi pioneiro na demonstração de biocompatibilidade da vitamina B_{12} de biomassa microbiana sobre modelo *in vitro* do epitélio intestinal humano (células Caco-2). Os resultados obtidos foram promissores e indicaram que: alimentos vegetais fortificados com a biomassa da linhagem *P. shermanii* ATCC 13673 cultivada em resíduo agroindustrial de soja podem suprir a demanda nutricional por vitamina B_{12} em dietas alternativas de forma segura e eficaz. Esses dados são um ponto de partida importante e contribuem para enriquecer a discussão acerca deste tema inovador.

Ainda assim, estudos complementares são necessários para afirmar segurança no longo prazo (ex.: ensaios de toxicidade crônica) e estimar a biodisponibilidade do micronutriente *in vivo*. Adicionalmente, também se faz necessário avaliar a influência do uso da biomassa microbiana sobre características tecnológicas, sensoriais e de armazenamento dos produtos alimentícios. No mesmo sentido, podem ser desenvolvidas estratégias de recuperação do ácido propiônico produzido pela linhagem para investigar as suas propriedades antifúngicas naturais nos produtos de panificação fortificados com a biomassa microbiana. Além disso, a investigação de diferentes processos de secagem da biomassa e a fortificação alimentos vegetais conservados sob refrigeração (ex.: sucos, análogos vegetais de produtos lácteos *etc*.) podem resultar em maiores percentuais de recuperação da vitamina B_{12} e viabilidade celular por longos períodos, respectivamente. Tendo em vista que as linhagens propiônicas possuem atributos probióticos emergentes, elas poderão ser utilizadas para o desenvolvido de alimentos vegetais com propriedades probióticas, ricos em vitamina B_{12} e com ação antifúngica natural. As quais são perspectivas para o desenvolvimento de trabalhos futuros.

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