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Tese

miRNAs: Expressão heteróloga e sua aplicação como componente de novas tecnologias na aquicultura

Nyelson da Silva Nonato

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“A tarefa não é tanto ver aquilo que ninguém viu, mas pensar o que ninguém ainda pensou sobre aquilo que todo mundo vê.” (Arthur Schopenhauer)

Resumo

NONATO, Nyelson da Silva. **miRNAs: Expressão heteróloga e sua aplicação como componente de novas tecnologias na aquicultura**. 2025. XXf. Tese (Doutorado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

A aquicultura enfrenta desafios críticos, como estresses ambientais e doenças, que impactam a produtividade. Esta tese propõe uma solução inovadora baseada na produção heteróloga de microRNAs (miRNAs) em bactérias e seu uso como suplemento alimentar para teleósteos, combinando uma revisão sistemática do estado da arte com o desenvolvimento de uma patente. Inicialmente, conduziu-se uma análise abrangente de plataformas bacterianas para expressão de miRNAs, identificando 832 estudos em três bases de dados, dos quais 15 foram selecionados. Os resultados demonstraram que *Escherichia coli*, *Salmonella typhimurium* e *Rhodovulum sulfidophilum* são as principais espécies utilizadas, com 78 miRNAs produzidos heterologamente – 75 em *E. coli* (como miR-21 e miR-155), 1 em *R. sulfidophilum* (miR-29b) e 2 em *S. typhimurium* (mi-INHA e miRNA CCL22). *R. sulfidophilum* destacou-se por sua capacidade de secretar ácidos nucleicos, ausência de RNases e cultivo em água salgada, enquanto *E. coli* manteve-se como modelo preferencial para produção em larga escala. Plasmídeos com promotor lpp e sítios de clonagem múltipla mostraram-se os mais eficientes para expressão de miRNAs, apontando para a importância do design de vetores na otimização da produção. Com base nesses achados, desenvolveu-se uma patente de suplemento alimentar contendo miRNAs e antagomiRs sintéticos, destinado a aumentar a tolerância de peixes a baixas temperaturas. O método envolve: (1) design de construções de DNA recombinante com sequências específicas de miRNAs, (2) transformação em *E. coli* para produção em biorreatores, (3) inativação térmica da biomassa bacteriana e (4) encapsulação em ração com adesivo graxo, garantindo estabilidade e absorção intestinal. A formulação atua modulando vias antioxidantes e metabólicas (como a via da glicose), reduzindo os efeitos do estresse térmico. Diferencia-se de abordagens convencionais por ser não transgênica, de administração oral e sazonal, o que facilita sua adoção comercial. Os resultados indicam que a combinação de produção bacteriana e aplicação nutracêutica de miRNAs oferece uma solução viável para desafios da aquicultura. Conclui-se que a plataforma desenvolvida não apenas avança o conhecimento sobre expressão heteróloga de miRNAs em procariotos, mas também estabelece um novo paradigma para intervenções baseadas em RNA na produção animal, com potencial adaptação a outros estresses ambientais ou espécies de interesse econômico.

Palavras-chave: RNAs não codificantes; RNA bioengenheirado; Sistemas de expressão de miRNA; Aquicultura; Nutrigenômica.

ABSTRACT

NONATO, Nyelson da Silva. **miRNAs: Heterologous expression and its application as a component of new technologies in aquaculture**. 2025. XXf. Thesis (Doctorate) – Graduate Program in Biotechnology, Federal University of Pelotas, Pelotas.

Aquaculture faces critical challenges, such as environmental stressors and diseases, which impact productivity. This thesis proposes an innovative solution based on the heterologous production of microRNAs (miRNAs) in bacteria and their use as a dietary supplement for teleosts, combining a systematic review of the state of the art with the development of a patent. Initially, a comprehensive analysis of bacterial platforms for miRNA expression was conducted, identifying 832 studies across three databases, of which 15 were selected. The results demonstrated that *Escherichia coli*, *Salmonella typhimurium*, and *Rhodovulum sulfidophilum* are the main species used, with 78 miRNAs produced heterologously—75 in *E. coli* (e.g., *miR-21* and *miR-155*), 1 in *R. sulfidophilum* (*miR-29b*), and 2 in *S. typhimurium* (mi-INHA and miRNA CCL22). *R. sulfidophilum* stood out for its ability to secrete nucleic acids, absence of RNases, and cultivation in saltwater, while *E. coli* remained the preferred model for large-scale production. Plasmids with the *lpp* promoter and multiple cloning sites proved to be the most efficient for miRNA expression, highlighting the importance of vector design in production optimization. Based on these findings, a patent was developed for a dietary supplement containing synthetic miRNAs and antagomiRs, aimed at enhancing fish tolerance to low temperatures. The method involves: 1) Designing recombinant DNA constructs with specific miRNA sequences; 2) Transformation into *E. coli* for production in bioreactors; 3) Thermal inactivation of bacterial biomass; 4) Encapsulation in feed with a fatty adhesive, ensuring stability and intestinal absorption. The formulation works by modulating antioxidant and metabolic pathways (e.g., the glucose pathway), reducing the effects of thermal stress. It differs from conventional approaches by being non-transgenic, orally administered, and seasonal, facilitating commercial adoption. The results indicate that combining bacterial production and nutraceutical application of miRNAs offers a viable solution to aquaculture challenges. In conclusion, the developed platform not only advances knowledge about heterologous miRNA expression in prokaryotes but also establishes a new paradigm for RNA-based interventions in animal production, with potential adaptation to other environmental stressors or economically relevant species.

Keywords: Non-coding RNAs; Bioengineered RNA; miRNA expression systems; Aquaculture; Nutrigenomics.

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miRNA – MicroRNA (pequeno RNA não codificante regulatório)

RNases – Ribonucleases (enzimas que degradam RNA)

AntagomiRs – Antagomirs (oligonucleotídeos sintéticos que inibem miRNAs específicos)

DNA – Ácido desoxirribonucleico

RNA – Ácido ribonucleico

exog-miRNAs – MicroRNAs exógenos (introduzidos artificialmente no organismo)

RT-qPCR – Quantitative Reverse Transcription Polymerase Chain Reaction (PCR quantitativo com transcrição reversa)

dsRNA – Double-stranded RNA (RNA de fita dupla)

RNAi – RNA interference (interferência por RNA)

pré-miRNAs – precursores de miRNAs (transcritos intermediários na biogênese de miRNAs)

pH – Potencial Hidrogeniônico (medida de acidez/alcalinidade)

Sumário

1 INTRODUÇÃO GERAL

Desde os tempos da antiga China, a produção de organismos aquáticos é documentada, sendo os primeiros relatos de cultivos rudimentares de macroalgas. Não demorou muito para a proteína animal aquática ser domesticada, e os peixes VALENTI, W. C. et al., 2021). De acordo com o relatório mais recente da FAO, The State of World Fisheries and Aquaculture 2024, a aquicultura foi responsável por 51% da produção global de animais aquáticos em 2022, superando pela primeira vez a pesca extrativa (FAO 2024)., demonstrando assim a importância deste segmento na alimentação do *Homo sapiens sapiens* do antropoceno (VALENTI, W. C. et al., 2021). Na piscicultura brasileira percebe-se o desenvolvimento exponencial com crescimento de 4,9 % em 2019, em um total de 799.560 toneladas de peixe no ano (CYRINO, J. E. P. et al., 2019).

Dentre as espécies de peixes produzidas, podemos destacar as de água doce: a tilápia (*Oreochromis niloticus*); o tambaqui (*Colossoma macropomum*), e seus híbridos como o híbrido de tambacu (*Piaractus mesopotamicus* × *C. macropomum*) e o híbrido de tambatinga (*C. macropomum* × *P. brachypomus*); pacu (*Piaractus mesopotamicus*); a carpa (*Cyprinus carpio*); o pirarucu (*Arapaima gigas*) e o bagre (*Ictalurus punctatus*). Na cultura dos peixes marinhos podemos destacar a tainha (*Mugil liza*); o linguado (*Paralichthys orbignyanus*); o robalo (*Centropomus parallelus* a *C. undecimalis*); o bijupirás (*Rachycentron canadum*); o peixe vermelho (*Lutjanus spp.*); a garoupa (*Epinephelus marginatus*) e a sardinha (*Sardinella brasiliensis*), correspondendo a 7,3 milhões toneladas, cerca de 23% do que foi produzido pela maricultura mundial em 2018 (LISBOA, V. et al., 2020).

A tilápia (*O. niloticus*) representa cerca de 60,6% da produção de peixes produzidos no Brasil, cerca de 486.155 toneladas no ano de 2020, segundo dados do “Anuário Peixe BR da Piscicultura”. Este é um peixe de rápida taxa de crescimento e engorda, altos níveis de fecundidade e adaptação, apresentando uma carne branca, com baixos teores de gordura e um alto valor nutricional agregado (BHUJEL, R. C., 2000; BOSCOLO, W. R. et al., 2008). O principal produtor da região Sul do país é o estado do Paraná, na região Sudeste, os estados de São Paulo e Minas Gerais, e na região Nordeste, o Ceará, Bahia e Pernambuco, havendo nestes estados grandes celeiros de produção (BARROSO, R. M. et al., 2019; VALENTI, W. C. et al., 2021).

No Brasil, até meados da década de 90, os cultivos de tilápia se davam em viveiros escavados no chão, sistemas que representam por volta de 14,36 ± 5,3% dos custos iniciais para construção, sem falar dos tratamentos de fertilização, calagem, aeradores, dentre outros (DE OLIVEIRA, E. G. et al., 2007; CASTILHO-BARROS, L. et al., 2020). A partir do ano de 2000, surgem os cultivos em tanques-rede, sistemas flutuantes que requerem um menor investimento por quilo de animal produzido, instalados principalmente em locais com vastas vazões hídricas, como reservatórios públicos, represas, lagos e rios, sendo estes produtivos na criação de peixes em altas densidades (FURLANETO, F. P. B. et al., 2010; SCORVO FILHO, J. D. et al., 2010). Em termos produtivos, a conversão de alimentação melhorada é 22,2% menor em tanques de terra em relação aos tanques-rede (VALENTI, W. C. et al., 2021).

Estudos avaliam que em sistemas de cultivo em tanques-rede é possível a redução da oferta de ração sem afetar a produção do pescado, visto seus hábitos alimentares omnívoros e planctívoros, reduzindo drasticamente os gastos produtivos (DE MORAES, C. R. F., et al., 2020). Mesmo com todo desenvolvimento tecnológico datado desde 2000 a.C., ainda existem gargalos produtivos a serem estudados e novas tecnologias a serem geradas neste sistema de produção. Fatores ambientais como salinidade e temperatura, ligadas aos períodos sazonais de cada região, são atualmente um dos maiores fatores que limitam a produção na aquicultura, principalmente de espécies que estão fora de sua faixa de tolerância ideal (REBOUÇAS, P. M. et al., 2020). A tilápia é um animal pecilotérmico, oriundo de regiões tropicais e subtropicais de águas quentes (26°C e 28°C), e sua reprodução está diretamente ligada à temperatura (±24°C). Quando abaixo de 20°C, o animal passa a um estágio de estresse, já demonstrando redução da movimentação e de hábitos alimentares, e indo à óbito em temperaturas entre 11 e 17°C, acarretando maiores custos e perdas de produção em períodos mais frios (inverno) em regiões mais distantes a linha do equador (NICO, L.G., 2019; REBOUÇAS, P. M. et al., 2020).

A alimentação fornecida é significativamente importante no cultivo de tilápias na busca por reduzir esses efeitos metabólicos indesejáveis. A alimentação é responsável por grande parte dos gastos da produção, mantendo assim suas taxas corporais em condições menos estressantes, favorecendo seu desenvolvimento

(XIONG, Y. et al., 2014; XIE, D. et al., 2017). Nutricionalmente falando, as tilápias necessitam de taxas específicas de lipídeos (10-15%), proteínas (>40%), assim como carboidratos, sais minerais e vitaminas (CHOU, B.S.; SHIAU, S.Y. ,1996; NICO, L. G., 2019).

Com o avanço da ciência, mais pesquisas têm buscado melhorar o desenvolvimento comercial de *O. niloticus*. Artigos científicos já demonstraram que a otimização da relação ácido linoleico (18:2) e ácido linolênico (18:3) na dieta melhora o perfil de ácidos graxos nos peixes (PAULINO et al., 2018); que probióticos e prebióticos específicos podem conferir melhorias substanciais no crescimento, engorda e respostas imunológicas (ZHOU, X. et al., 2010; XIA, Y. et al., 2020; POOLSAWAT, L. et al., 2021); que é possível a produção de vacinas comestíveis (PLANT, K. P.; LAPATRA, S. E., 2011; ISMAIL, M.S. et al., 2016; HOARE, R. et al., 2021); e que a adição de biomassa de microalgas favorece o desenvolvimento quando comparado à dietas convencionais baseadas em rações comerciais (SARKER, P. K. et al., 2018; BARRAZA-GUARDADO, R. H. et al., 2020; ALAGAWANY, M. et al., 2021). Especificando este último item, a spirulina (*Arthrospira platensis*) é uma das algas mais testadas e utilizadas como suplemento alimentar contra distúrbios fisiológicos, conferindo proteção contra danos oxidativos e efeitos imunoestimulantes (TAKEUCHI, T. et al., 2002; RAGAP, H. M. et al., 2012; ABDELKHALEK, N. K. M et al., 2017; SIRINGI, J. O., et al., 2021).

Além dos contribuintes oriundos da alimentação, os processos fisiológicos e metabólicos podem ser controlados por mecanismos endógenos, categorizados como mecanismos epigenéticos, dentre os quais, destacam-se os microRNAs (miRNAs), moléculas de ácido ribonucleico (RNA) medindo 18-22 nucleotídeos não codificantes com atividade de regulação pós-transcricional da expressão gênica (CAMPOS, V. F. et al., 2011; BLÖDORN, E. B. et al., 2021; RAZA, S. H. A. et al., 2022). Por seu caráter modulador de fenótipos fisiológicos e natureza pleiotrópica, essas moléculas estão sendo estudadas para diferentes aplicações e dentre diferentes espécies e reinos (LI, D. et al., 2021; DA SILVA, L. S. et al., 2021; YU, Y. et al., 2021). Mesmo com todo esse conhecimento, muito ainda tem de ser descoberto, como por exemplo a utilização de miRNAs exógenos (exog-miRNAs) por via intravenosa em terapias moleculares (RUPAIMOOLE, R.; SLACK, F. J., 2017; GANDHI, G. et al., 2021; SAQUIB, M. et al., 2021) ou por via oral através da

alimentação (KHURANA, P., et al., 2021; DEL POZO-ACEBO, L. et al., 2021). Tal técnica tem sido relatada por possuir potencial de aplicação, principalmente no contexto produtivo da aquicultura (ZHOU et al., 2021).

Cui e colaboradores (2021) demonstraram que, o fornecimento de bactérias que superexpressam miR-34 através da alimentação, promoveu uma resposta imune antiviral em camarões contra a síndrome da mancha branca. Ao mesmo tempo, o miR-34 antiviral exibiu atividade antitumoral em camundongos alimentados com camarão que superexpressam miR-34 e se alimentavam com as bactérias produtoras de miR-34. Tal estudo demonstrou que os miRNAs disponibilizados na dieta foram capazes de controlar simultaneamente a doença viral do camarão e a tumorigênese humana, sendo esta uma estratégia eficaz e econômica (CUI; WAN; ZHANG, 2021).

No contexto do enfrentamento a baixas temperaturas, a literatura relata a atuação de vários miRNAs que trabalham da manutenção fisiológica e metabólica de peixes teleósteos (BIZUAYEHU, T. T.; BABIAK, I., 2014; WOLDEMARIAM, N. T. et al., 2019; BLÖDORN, E. B. et al., 2021; RAZA, S. H. A. et al., 2022). Através da revisão sistemática da literatura e projeções *in sílico* produzidas por Blödorn e colaboradores (2021) foi possível identificar que o miR-9-3p, miR-135c, miR-9-5p, miR-30b, miR-122 e miR-92a-3p são moléculas potenciais para regulação da adaptação de peixes teleósteos ao frio (BLÖDORN, E. B. et al., 2021). Desse modo, o desenvolvimento de suplementos alimentares baseados na tecnologia de miRNAs é de grande interesse comercial, principalmente na busca da tolerância de peixes de água doce ao frio, conferindo uma melhor produção do pescado em condições antes desfavoráveis.

2 REVISÃO BIBLIOGRÁFICA

2.1 miRNAs como moduladores metabólicos por via oral

A pesquisa de base sempre foi o impulso para inovação, gerando inúmeras descobertas nas diversas áreas do conhecimento, entre estes temos os microRNAs ou miRNAs, um grupo de moléculas de RNA não codificantes contendo de 22~25 nucleotídeos compondo sua estrutura. Descoberto em 1993 estas moléculas

revolucionaram a compreensão acerca dos processos pós-transcricionais. Inicialmente foi identificado o *lin-4* em um nematódea (*Caenorhabditis elegans*), regulando seu desenvolvimento larval (LEE et al., 1993). Anos depois foram sendo encontrados vários outros, em diferentes espécies e responsáveis por diferentes processos, cada um regulando negativamente ou positivamente a expressão gênica, degradando ou bloqueando a tradução de RNAs mensageiros (mRNAs; CAMPOS et al., 2011).

A biogênese dos miRNAs se inicia no núcleo (Fig. 1), sendo o RNA primário (pri-miRNA) transcrito pela Polimerase II ou III e pré-processado pela endonuclease Drosha RNase III para formação dos miRNAs precursores (pré-miRNAs), quando os pré-miRNAs são transportados ao citoplasma passam por um processo de maturação pela RNase III e Dicer, resultando em uma fita dupla de miRNA nos metazoários (LI, Z.; XU, R.; LI, N., 2018). As plantas diferem dos metazoários no local de maturação dos pré-miRNA, que acontece ainda no núcleo, a enzima DCL1 tem sido proposta como responsável pelos cortes realizados durante a maturação dos miRNA, até a formação da dupla fita. Outra diferença é a metilação obrigatória que ocorre no final da maturação, onde a enzima RNA metil transferase, chamada Hua Enhancer (HEN) 1, metila os duplexes de miRNA/miRNA em suas extremidades 3 ' (2'-O-metilados 3′-terminal; LI, Z.; XU, R.; LI, N., 2018). Por final sendo transportado pelo complexo proteico Ran-GTP em animais, e a carioferina HASTY, o ortólogo vegetal da Exportin-5 (BARTEL, D. P., 2004).

A regulação da expressão pós-transcricional só ocorre com o complexo RISC, complexo de silenciamento induzido por RNA, ele se associa ao microRNA fita dupla o separando, uma das fitas é associada e a outra degradada ou anexada a outra RISC, só assim teremos um miRNA maduro (FERNANDES-SILVA et al., 2012; SILVA et al., 2018), não diferindo entre as plantas e os metazoários (BARTEL, D. P., 2004).

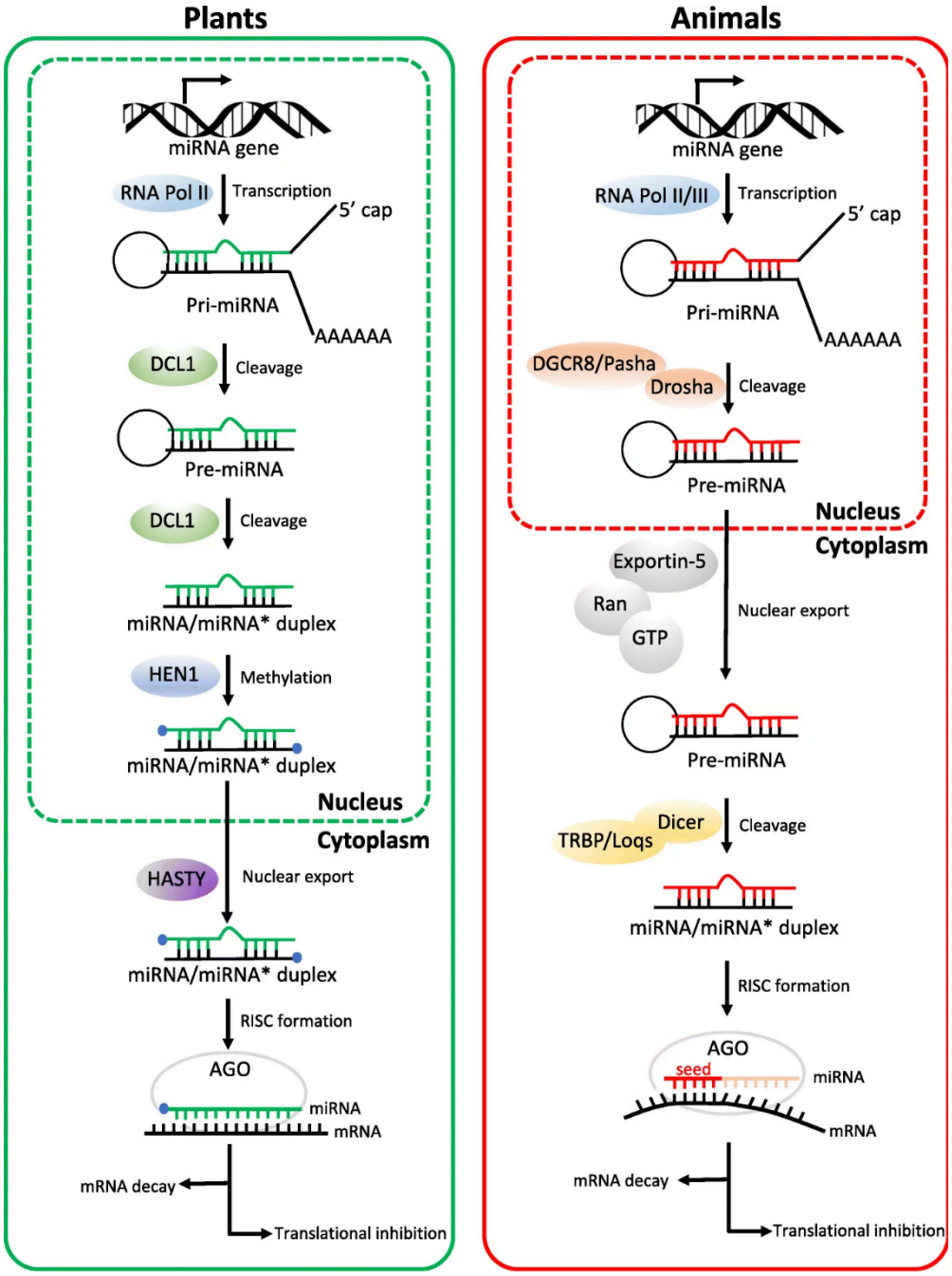

Figura 1 – Biogênese e processo de maturação de miRNA em plantas e animais. Fonte: LI, Z.; XU, R.; LI, N., 2018.

O complexo RISC também funciona no silenciamento pós trancricional com outro grupo de moléculas de RNA, os pequenos RNAs interferentes (siRNAs) que medeiam o RNA interferente (RNAi), e isso é uma das características que os assemelham aos miRNA. A partir de uma fita de RNA dupla (dsRNA) a enzima DICER faz clivagens consecutivas, processando a molécula e gerando vários siRNAs com aproximadamente 22-19 nucleotídeos, o siRNA se acopla ao complexo

RISC, ficando a fita antisense acoplada para assim o complexo atuar na clivagem do mRNA em sua área complementar a do siRNAs (BARTEL, D. P., 2004; ABO-AL-ELA, H. G., 2021).

Para que a regulação ocorra efetivamente é necessária uma complementaridade, seja ela total ou parcial, entre o miRNA maduro e o mRNA, em animais miRNAs têm alvos com complementaridade imperfeita (LI, Z.; XU, R.; LI, N., 2018), a maioria das interações ocorre com a região 3′UTR dos mRNAs alvo, bloqueando a expressão. Outros alvos de interação já foram detectados, por exemplo, as sequências das regiões 5'UTR também podem mediar a interação mRNA – miRNA (BROUGHTON et al., 2016). Em plantas, miRNAs têm complementaridade quase perfeita com seus mRNAs alvo (LI, Z.; XU, R.; LI, N., 2018). Em estudos utilizando espécies vegetais de Arabidopsis puderam observar que os miRNAs vegetais não agem apenas clivando o alvo, também poderiam induzir a repressão traducional a partir do seu acúmulo na planta. Contudo, ainda não se tem conhecimento como esta repressão é regulada (GANDIKOTA et al., 2007; LI, Z.; XU, R.; LI, N., 2018). Ainda não está completamente elucidado como ocorre a regulação endógena mediada por microRNAs de origem vegetal ingeridos por meio da alimentação, embora evidências apontem que esses miRNAs podem exercer algum nível de influência sobre a expressão gênica em organismos consumidores. Com os avanços nas metodologias de detecção e análise funcional de RNAs não codificantes, espera-se que em breve seja possível compreender com maior precisão se essa interação cruzada realmente ocorre, e de que forma ela se estabelece no contexto interespécies (LI, Z.; XU, R.; LI, N., 2018).

Todas essas características abrem o leque de interação e aplicação dos miRNAs, atuando dentro ou fora da célula como os hormônios. Eles podem ser produzidos endogenamente ou incorporados/absorvidos por via intravenosa e alimentar. Inúmeras são as aplicações projetadas, como possíveis terapias para saúde humana e soluções para aquicultura (MORI et al., 2019; CUI, Y.; WAN, H.; ZHANG, X., 2021). Com o avanço no desenvolvimento das técnicas, escolhendo melhor os controles (positivo e negativo) e protocolos de análise, evitando ruídos de sinais de fundo durante RT-qPCR ou artefatos de procedimento de sequenciamento, em breve entenderemos se há, e como funciona essa interação cruzada (LI, Z.; XU, R.; LI, N., 2018).

A comunicação extracelular, célula a célula, se dá por miRNAs circulantes (circRNAs) que são sintetizados intracelularmente e secretados nos fluídos extracelulares, como sangue, leite, fluido seminal e suor, atuando endocrinamente nos diferentes tecidos (O'BRIEN et al., 2018). Outro modo de entrega dos miRNAs aos tecidos e células alvo é por via alimentar. A interação com o intestino tem sido algo bem pesquisado, visto as características particulares deste órgão, como seu pH, diversidade microbiana e conexões com o sistema nervoso (O'BRIEN et al., 2018). Para manter a atividade e aumentar sua estabilidade estas moléculas são agregadas em exossomos e vesículas, ou associadas a proteínas, como lipoproteína de alta densidade (HDL), só assim vão conseguir cumprir suas funções extracelulares. Estando protegidos por uma vesícula são captados através da membrana celular por endocitose, por exemplo, e os miRNA extracelulares sem revestimento são captados por receptores específicos na célula.Mesmo com essas observações os mecanismos de captação ainda não são bem compreendidos (O'BRIEN et al., 2018; PENG; WANG, 2018). Foram observados alguns mecanismos de captação intestinal de miRNA’s e dsRNAs, entre eles por transportadores do tipo SID (Proteína defeituosa de interferência de RNA sistêmica), sendo os mais estudados SID1, SID2 e SID5, assim como células imunes da barreira intestinal por exemplo (LI, Z.; XU, R.; LI, N., 2018; Cui, Y. et al., 2021).

Estudos recentes (CHEN et al. 2021) conseguiram mapear o caminho que os miRNA dietéticos percorrem (Fig. 2). Após todo processo de mastigação e deglutição do alimento os miRNA presentes são liberados por digestão mecânica e absorvidos pelos transportadores SIDT1, expressos em células PIT do epitélio estomacal. Para uma eficiente entrega dessas moléculas a estabilidade é algo extremamente importante, o ambiente ácido contribui para não degradação a partir de RNases presentes. Exossomos medeiam a absorção de miRNA, assim como conferem uma proteção extra contra sua degradação. Após captação pelas células PIT os miRNA são secretados para o sistema circulatório como entidades funcionais em exossomos e vão se encaminhando aos seus alvos (CHEN et al. 2021).

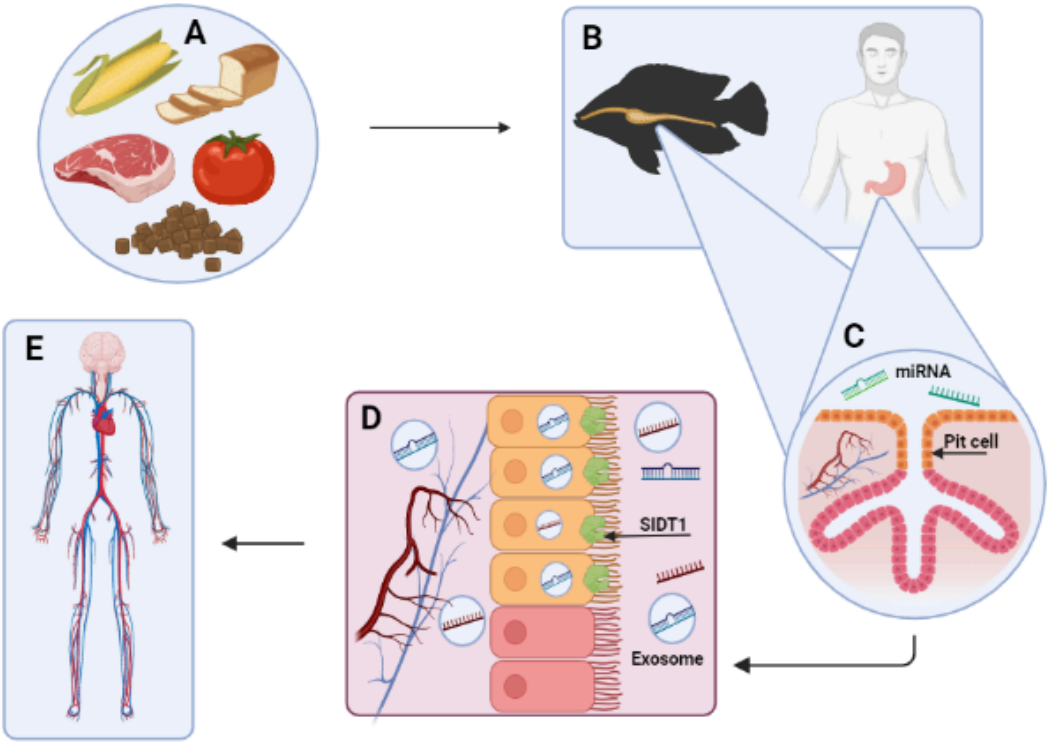

Figura 2 – Representação Semiesquemática do caminho que os miRNA dietéticos percorrem. A) Alimentos como principais fontes de miRNAs e dsRNAs; B) Após o processo de mastigação e deglutição o alimento segue para o estômago dos animais, onde processos digestivos ocorrem; C) miRNAs e dsRNAs encapsulados em exossomos ou agregados proteicos são liberados no lúmen do estomago; D) Receptores SIDT1 presentes na membrana de células PIT captam os miRNAs e dsRNAs, sendo posteriormente encapsulados em exossomos intracelularmente e secretados no tecido; E) Exossomos secretados pelas células PIT entram no sistema circulatório e são entregues as células e tecidos de destino. Imagem criada em BioRender.com.

Buscando testar a aplicação dos miRNA através da alimentação na regulação de doenças Cui, Y. et al. (2021) desenvolveram um camarão modificado geneticamente para expressar o mir-34, um miRNA com potencial antiviral. Além de expressar essa molécula esses camarões foram alimentados com bactérias, também modificadas, que expressavam esse miRNA. Quando expostos ao vírus da mancha branca (WSSV) os animais conseguiram resistir a infecção. Por ser uma molécula altamente versátil, tem uma estrutura que pode se acoplar e direcionar vários genes-alvos. Essas características permitiram realizar a testagem do mir-34 contra doenças metabólicas, mais precisamente a tumorigênese humana. Células doentes foram cultivadas e inoculadas em camundongos. Os camarões que super expressam e se alimentam do miRNA, foram preparados e oferecidos na alimentação dos

camundongos. Foi observado que o mir-34 controlou simultaneamente a doença viral do camarão anteriormente e agora a tumorigênese de células humanas (Cui, Y. et al., 2021).

Gismondi et al. (2021) estudaram a interação do miR171vr, uma isoforma do miR171 de plantas, em células de rim embrionário humano 293 (HEK293) e confirmaram as projeções *in silico*, onde houve a interação. Células tratadas com miR171vr mostraram uma diminuição significativa dos níveis de mRNA e proteína de GNA12. Os fatores de sinalização a jusante do GNA12, incluindo o mTOR, foram regulados para cima após a exposição ao miRNA da planta. Através de testes, o miRNA da planta sintética permaneceu intacto após exposição a pH ácido e neutro ou alta temperatura, enquanto parecia muito sensível ao pH básico e à atividade da RNase A. Esses dados suportam a hipótese de que o miR171vr pode ser realmente absorvido pela dieta. O miR171vr tem potencial de ajudar no tratamento de distúrbios em que o GNA12 é superexpresso (ou seja, câncer oral, adenocarcinoma de mama e próstata) ou mTOR quinase é regulado para baixo (por exemplo, obesidade, diabetes tipo 2, neurodegeneração), sendo introduzido pela dieta ou como suplemento em terapias gênicas.

Estudos *in vivo* utilizando os RNA's não codificantes na regulação metabólica são extremantes potenciais para o desenvolvimento de tecnologias para saúde humana, ainda mais quando tratados no contexto de sua implementação alimentar, método de aquisição, disseminação e atuação pelo corpo, células e tecidos.

3 HIPÓTESE E OBJETIVOS

3.1 Hipótese

A a identificação de plataformas bacterianas, bem como a otimização de vetores de expressão é essencial para uma produção efetiva de miRNAs heterólogos, aplicado ao desenvolvimento de tecnologias nutracêuticas para peixes.

3.2 Objetivo Geral

O presente estudo tem como objetivo a otimização da produção de miRNAs heterólogos em plataformas bacterianas, como suplemento nutracêutico para peixes.

3.3 Objetivos Específicos

- Identificar as melhores plataformas bacterianas para expressão heteróloga de miRNAs;
- Identificar os elementos essenciais necessários em uma construção para aumentar a expressão de miRNA;
- Revisar qual o plasmídeo comercial que tem sido mais utilizado para expressar miRNA;
- Otimizar vetores de expressão e construir uma molécula de DNA sintético contendo sequência nucleotídica para produção eficiente de miRNAs;
- Patentear as sequências sintéticas desenhadas, assim como a formulação do suplemento alimentar acrescido com cepa recombinante expressando miRNAs de interesse;
- Revisar artigos científicos e desenvolver um artigo de revisão bibliográfica sobre a expressão heteróloga de miRNAs em bactérias.

4 CAPÍTULOS

4.1 Artigo 1

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miRNA heterologous production in bacteria: A systematic review focusing on the choice of plasmid features and bacterial/prokaryotic microfactory

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Abstract

Bacteria, the primary microorganisms used for industrial molecule production, do not naturally generate miRNAs. This study aims to systematically review current literature on miRNA expression systems in bacteria and address three key questions: (1) Which microorganism is most efficient for heterologous miRNA production? (2) What essential elements should be included in a plasmid construction to optimize miRNA expression? (3) Which commercial plasmid is most used for miRNA expression? Initially, 832 studies were identified across three databases, with fifteen included in this review. Three species—*Escherichia coli*, *Salmonella typhimurium*, and *Rhodovulum sulfidophilum*—were found as host organisms for recombinant miRNA expression. A total of 78 miRNAs were identified, out of which 75 were produced in

E. coli, one in *R. sulfidophilum* (miR-29b), and two in *S. typhimurium* (mi-INHA and miRNA CCL22). Among gram-negative bacteria, *R. sulfidophilum* emerged as an efficient platform for heterologous production, thanks to features like nucleic acid secretion, RNase non-secretion, and seawater cultivation capability. However, *E. coli* remains the widely recognized model for large-scale miRNA production in biotechnology market. Regarding plasmids for miRNA expression in bacteria, those with an lpp promoter and multiple cloning sites appear to be the most suitable due to their robust expression cassette. The reengineering of recombinant constructs holds potential, as improvements in construct characteristics maximize the expression of desired molecules. The utilization of recombinant bacteria as platforms for producing new molecules is a widely used approach, with a focus on miRNAs expression for therapeutic contexts.

Keywords: Non-coding RNAs; Genetic engineering; Bioengineering; Bioengineered RNA; miRNA expression systems; Therapeutics;

1. Introduction

microRNAs (miRNAs or miRs) are small non-coding RNA molecules, composed of around 18 to 25 nucleotides, with activity in post-transcriptional regulation of gene expression through base pairing with complementary sequences of messenger RNA (mRNA), resulting in mRNA degradation or inhibition of translation (Campos et al., 2011; Blödorn et al., 2021; Raza et al., 2022). Due to their modulator character of physiological phenotypes and pleiotropic nature, these molecules were studied for different applications, among different species and kingdoms (Li et al., 2021; Da Silva et al., 2021; Yu et al., 2021). Nevertheless, much remains to be explored, particularly regarding the deregulation of miRNAs and their association with cancer (Volinia et al., 2006; Stabile et al., 2023), as well as their role in modulating immune system responses (Zhou et al., 2012; Rose et al., 2021), both of which serve as notable examples.

The natural expression of miRNAs is observed in viruses, fungi, algae, plants and animals, which have a whole physiological structure that facilitates the encoding, transport and maturation of miRNAs through the RNase III Drosha molecular system in the nucleus and DICER in the cytoplasm (Lee et al., 2010; Cuperus et al., 2011; Cock et al., 2017). Bacteria lack true miRNAs, even though there is the production of

small non-coding RNAs (sRNAs) which have the same miRNA behavior, circulating in extracellular vesicles (Wassarman et al., 1999; Choi et al., 2017). Bacterial production of sRNAs is linked to the synthesis of ribonucleoproteins that control cellular functions, which changes the stability and efficiency of mRNA translation, through the formation of sRNA/mRNA duplexes (Driessen and Nouwen, 2008; Furuse et al., 2014). Even though it is not inherent to their biology, the production of miRNAs in bacterial cells has been an object of recent studies. This approach to express heterologous miRNA in bacteria may find wide biotechnological applications.

Bacteria are often used as host factories for the expression of various molecules of industrial interest. Some examples of molecules that can be expressed in bacteria include insulin (Allen et al., 2019; Alyas et al., 2021), growth factor (Tian et al., 2011), interferons (Bis et al., 2014; Alrseetmiwe et al., 2020), pigments (Lim et al., 2015; Linke et al., 2023), and hyaluronic acid (Sheng et al., 2015; Sunguroğlu et al., 2018). The expression of these molecules in bacteria may be advantageous due to their ability to grow rapidly in large quantities, be easily genetically manipulated, and be able to produce complex molecules in a high-yield and low-cost system (Posada et al., 2012; Falak et al., 2022; Ganjave et al., 2022). To express miRNA in bacteria, an appropriate expression system is needed. There are several types of miRNA expression systems available, including expression of miRNA from a plasmid (Yi et al., 2020), the insertion of miRNA into an endogenous bacterial gene (Pereira et al., 2017), and miRNA expression from a phage (Liu and Berkhout, 2011; Hashiro et al., 2019).

The ease of manipulation and amplification make a plasmid-based miRNA expression system a widely favored method. However, it is important to consider the essential characteristics that must be contained in an expression plasmid (Keasling, 1999; Choi et al., 2010; Terol et al., 2021). Expression plasmids can be customized to meet the specific expression needs of the molecule of interest. For example, they can be designed to produce proteins under different growth conditions, including different temperatures, pH and culture media, as well as with different post-translational modifications, such as glycosylation (Brooks, 2004; Kightlinger et al., 2020).

In this systematic literature review, we examine some miRNA expression systems in bacteria and provide answers to 3 distinct questions: (1) Which microorganism is the most effective for heterologous miRNA production? (2) What are the essential elements required in a construct to enhance miRNA expression? (3) What is the commercial plasmid that has been most used to express miRNA? In view of the economic importance of producing heterologous molecules through expression systems in bacteria and the trend towards large-scale production of other molecules of commercial interest, this review is important not only to support and direct new studies in the area, but also to investigate more efficient expression systems to produce miRNAs of technological interest.

2. Materials and methods

2.1. Review question and search

To find plasmids used for expression of miRNAs in bacteria, a systematic review of the scientific literature was carried out in three different databases: PubMed, Web of Science and Scopus. Scientific articles up to June 2024 were selected with the following keywords and terms: “microRNA” or “miRNA” combined with “heterologous expression” or “recombinant RNA” or “bioengineered microRNA” or “recombinant DNA” or “expression system” or plasmid and “bacteria”. The complete structure of the search strategy is described in Table 1. Only peer-reviewed articles written in English were selected.

2.2. Screening and selection

Next, all the results from the three databases were gathered in text format (.txt) and added to a single folder in Excel to start the integrity analysis of the assembled library, as well as to remove duplicates. Subsequently, three independent reviewers examined all titles and abstracts, and a fourth reviewer contributed if there was any disparity in the inclusion or not of the articles. To be part of the sample, the study should meet the following inclusion criteria: (1) original research, (2) use of bacteria, (3) use of constructs to express miRNAs, (4) expression of recombinant molecules, and (5) open access results. Studies that did not meet the inclusion criteria as review articles, abstracts or presentations of scientific events, books or book chapters,

papers in languages other than English, research with eukaryotes, CRISPR/Cas, archaea or viruses were excluded.

2.3. Data collection

Lastly, data were extracted from all publications that met the inclusion criteria. The full text and supplemental sections of each study were scrutinized by two independent reviewers. The data collected include the author's name, year, keywords, species of bacteria used, plasmid used for expression, how the miRNA was expressed and for what purpose, characteristics of the plasmid and possible methodological descriptions. In addition, there was a manual investigation in the reference sections, in search of possible additions, as some articles that met the inclusion criteria did not appear in the searches using the keywords in the databases, completing the final library.

3. Results and discussion

3.1. Study selection and data collection

A total of 832 studies were initially identified in three databases using the search terms (Table 1), of which 414 were in PubMed, 266 in Scopus and 152 in Web of Science (Fig. 1). Nine studies were manually added to the pool after being identified in a reference list. The discrepancy between the number of studies in the different databases is mainly due to the different indexing rules of each database, for example the terms MeSH (Medical Subject Headings), these parameters being considered a reflection of the quality of journals by many researchers. Studies in the health and medical areas were the first to enter this quality dispute, mainly in private databases, where they often concentrate a greater number of indexed texts, as is the case of the PubMed platform (Mao and Lu, 2017).

However, other factors may contribute to it, such as the lack of permission to use all features of a thesaurus by Scopus (Bramer et al., 2017). Another possible explanation for the reduced number of studies found in Web of Science and Scopus is the subscription cost, reducing the power of choice of institutions to carry out publications (Pranckutė, 2021). After assembling the library, 21 duplicates were removed, and 796 studies were excluded by screening titles and abstracts. Of the

total number of exclusions, 59 studies aimed the expression of miRNA/RNAi in eukaryotic cells, 240 studies using machinery from archaea or viruses, 33 studies using CRISPR/Cas, 413 studies on genetic functioning and molecular pathways in pro/eukaryotes and 51 articles reviews, abstracts or presentations of scientific events, books, or book chapters.

Subsequently, 24 studies were selected for the next step, the full-text evaluation. From the last selection, nine articles were removed from the sample for not expressing miRNA in bacteria, resulting in a final library comprising fifteen studies: Yoon et al. (2011); Li et al. (2014); Li et al. (2015); Wang et al. (2015); Chen et al. (2015); Pereira et al. (2016); Ho et al. (2018); Petrek et al. (2019); Pereira et al. (2020); Yi et al. (2020); Cui et al. (2021); Li et al. (2021); Yoon et al. (2021); Chen et al. (2023) and Traber et al. (2024).

Among all the selected studies, three different species were identified, including *Escherichia coli*, *Salmonella typhimurium* and *Rhodovulum sulfidophilum*. Furthermore, a total of 78 miRNAs (Table 2) were expressed. Most of the applications of the miRNAs produced were for the treatment of cancer. Several expression systems were used, each one with its particularities towards achieving improved expression of the desired miRNA. The selected studies were published within the timeframe spanning from 2011 to 2024. A complete overview of all included studies is available in Table 2.

3.2. Summary of selected articles

Furthermore, to seek mechanisms to overcome atopic dermatitis, Yoon et al. (2011) described in their study the therapeutic use of recombinant *S. typhimurium* carrying miRNA CCL22 in a mouse skin model. Using an RNAi designer program, two single-stranded DNA oligonucleotides encoding the pre-miRNA target of the regulatory chemokine CCL22 were designed. After oligonucleotide annealing, cloning was performed in the pcDNA™ 6.2-GW/EmGFP-miR expression vector. The study showed that the new plasmid reduced the gene expression of the regulatory chemokine CCL22 *in vitro* and *in vivo* in models of atopic dermatitis in mice and induced therapeutic effects with Th-1 immune responses. It has been shown that topical application of the recombinant bacteria resulted in a significant improvement in skin inflammation, reduced infiltration of inflammatory cells and decreased

expression of pro-inflammatory cytokines, as well as an overall improvement in skin barrier function, demonstrating potential for the development of new therapies for inflammatory skin diseases.

Li et al. (2014) describes a method for the rapid production of a pre-miRNA molecule, specifically pre-miR-27b. It was expressed in *E. coli* bacteria using recombinant RNA technology, and the pre-miR produced were applied in cell cultures of human carcinoma. Purified tRNA/mir-27b agents were found to be processed to mature miR-27b in human carcinoma LS-180 cells, which consequently reduced CYP3A4 protein expression and resulted in lower midazolam 19-hydroxylase activity. Consequently, it influenced the ability of cellular metabolism of drugs. The work brings new clues for the development of natural pre-miRNA agents for functional studies in drug metabolism.

Li et al. (2015), on the other hand, describe the efficient production of intracellular chimeric pre-miR-1291 in *E. coli*, as well as its effectiveness in reducing their target gene expression. The DNA fragments corresponding to the miRNA were cloned into the pBSMrnaSeph vector, a recombinant RNA technology using a methionyl transfer RNA (tRNA) scaffold. Freshly transformed *E. coli* HST08 cells were seeded on an LB broth agar plate containing antibiotics for selection, followed by culture at 37 °C. For large-scale production of RNA, transformed cells were cultured in 1 L of medium. Total RNAs were isolated from bacteria using the phenol extraction method and quantified with a spectrophotometer. To examine the expression of recombinant ncRNA produced, the extract was subjected to polyacrylamide gel electrophoresis, demonstrating optimal expression of the recombinant tRNA/mir −1291.

The publication by Wang et al. (2015) describes a novel approach for high-yield biosynthesis in *E. coli*, utilizing a chimeric tRNA/miR-34a that can be employed as a prodrug in cancer therapy. To achieve this, the vector pBSMrnaSeph was constructed for the expression of tRNA/miR-34a, and the tRNA/miR-34a expression plasmids were confirmed through sequencing analysis. The expression of recombinant ncRNAs was carried out in *E. coli*. Total RNAs were isolated and analyzed via urea-PAGE to evaluate the expression of recombinant ncRNAs. The study reported that the chimeric tRNA/miR-34a could be efficiently biosynthesized on

a large scale in a common strain of *E. coli* and rapidly purified with a high degree of homogeneity. Additionally, the chimeric tRNA/miR-34a demonstrated remarkable cellular stability. Deep sequencing and qRT-PCR analyses revealed that the tRNA-carried pre-miR-34a was accurately processed into mature miR-34a within human carcinoma cells. Furthermore, tRNA/miR-34a inhibited the proliferation of various types of human carcinoma cells in a dose-dependent manner. It also significantly suppressed the growth of A549 non-small cell lung cancer and HepG2 hepatocarcinoma xenograft tumors in mice. Finally, the recombinant tRNA/miR-34a had minimal or no effect on blood chemistry and interleukin-6 levels in mouse models, suggesting that the recombinant RNAs were well-tolerated.

In the study by Chen et al. (2015), an advanced method for the creation of high-yield chimeric RNAs was developed, using various types of small functional RNAs with broad applications. A series of plasmids were created using different amplicons along with the pBSMrnaSeph vector, and the chimeric RNAs were expressed in *E. coli*. The analysis of total RNAs revealed that the levels of recombinant pre-miRNA chimeras expressed in *E. coli* varied significantly, even using the same tRNA scaffold. Additionally, many of the target RNAs were not expressed or showed minimal expression. A549 cells were transfected with OnRS/miR-124 and tRNA/mir-34a (OnRS) purified by FPLC. It was observed that mature miR-124 could be selectively produced from OnRS/miR-124 in human cells. Through deep sequencing and selective RT-qPCR analysis with stem-loop, it was determined that the levels of mature miR-124 were approximately 1000 times higher in A549 cells from day 1 to day 4 after transfection with OnRS/miR-124. Moreover, the increase in miR-124 in A549 cells treated with OnRS/miR-124 resulted in a 60 % reduction in STAT3 protein levels, and a 1 to 2-fold increase in early and late apoptosis rates, as well as necrosis. The researchers highlight that this approach is robust and versatile, with extensive potential applications in the engineering of chimeric non-coding RNAs, which could be used as research tools both *in vitro* and *in vivo*, and developed as diagnostic and therapeutic agents.

Pereira et al. (2016) describe a method to produce human pre-miR-29b using a mutant strain of *R. sulfidophilum* that has been engineered to secrete pre-miR-29b directly into the culture medium. They show that this mutant strain can produce

pre-miR-29b in significant amounts in a relatively short time, and that the secreted pre-miR-29b is biologically active. Finally, the authors also discuss some of the potential applications of large-scale production of pre-miR-29b, including a possible therapeutic treatment for diseases, such as cancer. They suggest that the direct secretion of produced pre-miR-29b into the extracellular environment may have advantages over traditional production methods such as recombinant pre-miR-29b production in *E. coli*.

Ho et al. (2018) highlighted a new ncRNA bioengineering technology by introducing a more stable carrier known as nCAR, which includes a derivative of pre-miR-34a. This study investigated the effects of nCARs on miRNA profiles in human lung carcinoma cell lines. The pre-miR-34a G138U/139ΔG derivative was selected and fused with a tRNAMet to form the nCAR, designed as a carrier for RNAi agents. This nCAR facilitated the assembly of targeted RNAi agents, where small RNAs (sRNAs) such as miRNAs, siRNAs, and aptamers replaced the miR-34a sequences or were directly added to designated locations. The resultant constructs were assembled into the pBSTNAV vector. After transformation and fermentation, the expression of the target BERAs was evaluated by urea-PAGE analysis of total RNAs isolated from *E. coli*. They found successful expression of 33 BERAs at a remarkably high level (40 %–80 % of total RNAs), representing an 80 % success rate (33 out of 42 target ncRNAs). Additionally, the release of target miRNAs in human cells was found to be Dicer-dependent in the case of miR-34a-5p and Dicer-independent in miR-124a-3p, with subsequent changes in miRNome and transcriptome profiles. Furthermore, the nCAR assembled with miR-34a-5p and miR-124-3p effectively suppressed cell proliferation in human lung carcinoma by modulating the expression of target genes such as cMET and CDK6 for miR-34a-5p, and STAT3 and ABCC4 for miR-124-3p. This ncRNA bioengineering platform, utilizing nCAR for the production of various ncRNA molecules, offers promising potential as a new therapeutic strategy against cancer.

Petrek et al. (2019) investigated the production of a single long non-coding RNA molecule carrying multiple small RNAs, known as CO-BERAs, evaluating its antiproliferative effect on human non-small cell lung cancer cell lines. The CO-BERA sequences were generated by replacing the miR-34a duplexes with specific miRNA

or siRNA sequences (miR34a, miR-124, let-7c, anti-miR-21, or NRF2-siRNA), and then each of the inserts was ligated into the pBSTNAV vector. The resulting plasmids were transformed into *E. coli*, and the expression of all target CO-BERAs was confirmed by sequencing analysis. The CO-BERAs were successfully expressed in *E. coli* at levels exceeding 40 % of the total bacterial RNA. Additionally, the recombinant CO-BERAs were purified to high homogeneity by protein liquid chromatography. To assess antiproliferative activity, cells were seeded at densities of 3000 or 5000 cells per well in 96-well plates overnight, and then transfected with 15 nM of ncRNA or control tRNA using Lipofectamine 3000. Cell viability was determined by MTT assay 72 h post-transfection. The CO-BERAs demonstrated significant antiproliferative activity against various human non-small cell lung cancer cell lines. Specifically, htRNALeu/miR-34a/miR-124 and htRNALeu/let-7c/miR-124 consistently exhibited the highest degrees of antiproliferative activity against all non-small cell lung cancer (NSCLC) cell lines analyzed. The article also highlights the importance of this technology, emphasizing its potential to develop new experimental, diagnostic, and therapeutic tools based on miRNA.

Pereira et al. (2020) describe the development and evaluation of a polymeric delivery system (nanocarriers) for the targeted delivery of recombinant precursor miRNA (pre-miR-29b), with potential implications in the treatment of neurological disorders. After the biosynthesis by *R. sulfidophilum*, the recombinant pre-miR-29b was recovered from the total RNA content and purified by affinity chromatography, with later confirmation of expression by polyacrylamide electrophoresis. The authors designed a nanocarrier consisting of a chitosan/polyethyleneimine polyplex modified with lactoferrin and stearic acid to enhance its brain targeting ability. The nanocarrier was loaded with pre-miR-29b, a miRNA with reported neuroprotective and neuroregenerative effects. The nanocarrier was characterized for its size, payload, and morphology, and evaluated for its ability to deliver pre-miR-29b to human neural cells and mouse brain tissue. Results showed that the nanocarrier efficiently delivered pre-miR-29b to both neural cells and brain tissue, resulting in the upregulation of miR-29b and its downstream targets. *In vitro* analysis demonstrated that the pre-miR-29b-CS/PEI-SA-Lf system delivered pre-miR-29b to neuronal cells after 1 h of transfection. The transport experiment across the blood-brain barrier (BBB) showed that CS-SA-Lf delivered 65 % recombinant pre-miR-29b over a 4 h

period. The study suggests that this nanocarrier system could be a promising approach for treating neurodegenerative diseases by selectively delivering miRNAs to the brain.

In the study conducted by Yi et al. (2020), the fully humanized hBERA/miR-328-3p molecule was developed, and the role of miR-328-3p in regulating the uptake of essential nutrients for cellular metabolism was explored. The plasmid for recombinant expression of hBERA/miR-328 was constructed using htRNASer/pre-miR-34a, where the miR-34a duplexes were substituted with miR-328 target sequences. Following the overexpression of the recombinant ncRNA in *E. coli*, the total bacterial RNA was extracted and subjected to urea-PAGE analysis, demonstrating successful heterogeneous and high-level expression of the molecule. To evaluate the levels of mature miR-328-3p in human osteosarcoma (OS) 143B and MG63 cells after 48 h of treatment with recombinant ncRNA, an RT-qPCR assay was performed. The data revealed significantly higher levels of miR-328-3p. It was also confirmed that miR-328-3p specifically processes to inhibit the growth of human OS cells (143B and MG63). Additionally, it was observed that miR-328-3p can regulate the expression of LAT1 and GLUT1 transporters through their 3' UTRs, thereby modulating the mTOR pathway. Finally, the combination of miR-328-3p with chemotherapy (cisplatin or doxorubicin) showed a synergistic inhibition of OS cell proliferation. The article highlights the development of ncRNA biological agents and demonstrates a novel role of miR-328 in regulating xenobiotic/nutrient transport and the underlying homeostasis in cancer metabolism.

Cui et al. (2021) explored the potential use of miRNAs through feeding as a therapeutic strategy to control viral diseases in animals, inhibiting white spot syndrome virus (WSSV) replication in shrimp, as well as controlling the growth of human tumor cells in an animal model. To express the mature miRNA in bacteria, the miR-34 from the shrimp *Marsupenaeus japonicus* was cloned into a commercial plasmid. The construct was then transformed into *E. coli* HT115 (DE3) cells. To test the control of viral infection using miR-34, shrimps were initially infected intramuscularly with the WSSV virus (105 copies/mL), then the crustaceans were fed a mixed feed containing recombinant bacteria expressing miR-34. Suppression of WSSV infection was observed through a significant reduction in WSSV copy number

and mortality of infected shrimp. By offering shrimp fed with *E. coli* that was overexpressing miR-34 in the mice diet, it was observed the direct influence of miR-34 on the growth and metastasis of breast cancer cells, arresting them in the G0/G1 phase, thus, suppressing the progression of cancer. Furthermore, the article discusses the potential advantages of using food miRNAs as a therapeutic strategy, as well as discussing the challenges of this approach.

Li et al. (2021) developed a method for *in vivo* fermentation to produce humanized non-coding RNAs (ncRNAs) carrying payload miRNAs for targeted anticancer therapy. Competent *E. coli* HST08 cells were transformed with target plasmids and confirmed by DNA sequencing. Electrophoretic analysis of total bacterial RNAs revealed that, except htRNATyr/pre-miR-34a, all other target ncRNAs studied were efficiently accumulated in *E. coli*, representing around 40 % of the total bacterial RNA content. To define the antitumor efficacy of miR-34a-5p and miR-124-3p, oncolytic miRNAs were applied in xenograft models of orthotropic osteosarcoma and spontaneous metastasis in mice. The results showed that hBERA/miRNAs are selectively processed to mature miRNAs intracellularly, as well as that they modulate target gene expression and cellular processes in human carcinoma cells *in vitro*. Furthermore, hBERA/miR-34a-5p and miR-124-3p were effective in reducing tumor growth and lung metastasis in *in vivo* models (mouse). The authors suggest that large-scale production of humanized ncRNAs carrying payload miRNAs through *in vivo* fermentation processes has the potential to become a new platform for targeted anticancer therapy.

The article by Yoon et al., 2021 evaluated B16F10 cells infected with the BRD509 strain of *S. typhimurium* expressing miRNA for the regulation of the INHA gene (alpha subunit of inhibin) in association with radiation treatment in melanoma. Initially, *S. typhimurium* SF586 was transformed to contain vectors encoding miRNA against INHA and a scrambled miRNA, generating the SF509 strain. The plasmid isolated from the Salmonella SF509 strain was used to construct the BRD509 strain, which already contained the INHA sequence. To construct plasmids expressing INHA siRNAs, the modified RNAi expression vector miR BLOCK-iT Pol II (Invitrogen, Carlsbad, CA, USA) was used. The tests conducted confirmed that the recombinant Salmonella drives effective cell death, and when used in conjunction with

radiotherapy, maximizes the outcome. The cytotoxic effect of the miRNA INHA, as well as the production of ROS, contributes to the reduction of cancer cells.

In 2023, Chen et al. expressed three recombinant miRNAs in *E. coli* to investigate their potential as antimetabolites for the inhibition of human NSCLC cells. Each plasmid was constructed by inserting the target miRNA coding sequence (miR-22-3p, miR-9-5p, miR-218-5p) into an htRNAleu/pre-miR-34a carrier. The resulting sequences were then inserted into the pBSMrna vector. The individual plasmids were confirmed by DNA sequencing analysis. The recombinant miRNAs were expressed in the *E. coli*, and the total bacterial RNAs were isolated by phenol extraction and visualized by urea-PAGE. Subsequently, the recombinant miRNAs were purified using fast protein liquid chromatography (FPLC) with anion exchange. The purity of the miRNAs was validated by urea-PAGE and quantified by high-performance liquid chromatography (HPLC). Human NSCLC cells were transfected with these recombinant hBERAs using Lipofectamine 3000. Selective RT-qPCR analyses were conducted to verify the targeted release of the miRNAs from the hBERAs in human NSCLC cells. A significant increase in miR-22-3p, miR-9-5p, and miR-218-5p was observed in cells treated with the corresponding hBERA/miRNA. Additionally, it was demonstrated that recombinant miR-22-3p, miR-9-5p, and miR-218-5p regulate key folate metabolic enzymes, inhibiting their metabolism and altering the amino acid metabolome in NSCLC cells. The authors highlight the relevance of the antifolate action of these oncolytic miRNAs, emphasizing the importance of folate metabolism as a crucial pathway in NSCLC cells, and providing perspectives for the development of new therapies against NSCLC.

Recently, in the study by Traber et al. (2024), an innovative RNA bioengineering (BioRNA) platform technology, also known as bioengineered or biologic RNA agents (BERAs), was developed to enable *in vivo* production of BioRNA/miRNA agents through fermentation using stable glycyl and leucyl htRNA/hsa-premiR-34a carriers. The BioRNAGly/miRNAs and BioRNALeu/miRNAs were cloned into the pBSKrna vector and verified by DNA sequencing. Subsequently, these plasmids were transformed into competent *E. coli*. The overexpression of 26 BioRNAGly variants and 22 BioRNALeu variants was confirmed by urea-PAGE

analysis, with a 100 % success rate, each representing >40 % of the total RNA based on band density. The BioRNAs were purified by FPLC with a purity exceeding 97 %. After synthesizing these BioRNA/miRNAs in the bacteria and subsequent purification, their antiproliferative effects and ability to regulate MRP1 and VDAC1 proteins in NSCLC cells were evaluated. In NSCLC cell viability assays, both BioRNAGly and BioRNALeu at 15 nM showed variable reductions in cell viability, with comparable results. Additionally, miR-7-5p demonstrated similar antiproliferative activities in both BioRNAGly and BioRNALeu carriers, evaluated in the A549 and H1975 NSCLC cell lines. To investigate the selective processing of miR-7-5p (BioRNAGly/miR-7-5p or BioRNALeu/miR-7-5p) in NSCLC cells, selective RT-qPCR was used, showing significantly higher levels of mature miR-7-5p with BioRNAGly/miR-7-5p compared to BioRNALeu/miR-7-5p. The Western blot analysis revealed that BioRNAGly/miR-7-5p downregulates the expression of several proteins in NSCLC cells, decreasing EGFR by 68 % and 76 %, MRP1 by 73 % and 75 %, and VDAC1 by 72 % and 82 % in the A549 and H1975 cell lines, respectively. This article addresses the applications of RNA biotechnology using tRNA/pre-miRNA carriers as a promising strategy for the treatment of NSCLC.

3.3. Purple marine bacteria as an efficient platform for the heterologous production of miRNAs

Three species of bacteria have been employed for miRNA expression, all three belonging to the Gram-negative group. These species are: *E. coli*, *S. typhimurium* and *R. sulfidophilum*, as shown in Table 2.

E. coli is a well-known bacterium in the scientific community due to its importance in molecular biology research and biotechnology applications. The existence, maintenance, and propagation of *E. coli* strains of biotechnological and commercial interest are crucial for the advancement of the biotechnological industry. Out of the fifteen studies analyzed, eleven used *E. coli* bacteria to express ncRNA molecules. Among the existing strains, we found that HST08, TOP10, DH5aα, BL21(DE3), and HT115 (DE3) were used for the production of miRNAs.

To the best of our knowledge, the HST08 strain was initially used for miRNA production through heterologous expression (Li et al., 2014). Furthermore, since

Wang et al. (2015) found higher levels of recombinant ncRNAs in HST08 compared to other strains such as BL21, DH5α, and TOP10, subsequent research has focused on using this strain, leading to the production of a variety of miRNAs (Li et al., 2015; Chen et al., 2015; Ho et al., 2018; Petrek et al., 2019; Yi et al., 2020; Li et al., 2021; Chen et al., 2023; Traber et al., 2024). The mechanisms underlying this improvement are still unknown; however, the enhanced expression of recombinant ncRNA in the HST08 strain could be related to the lack of gene clusters in HST08 cells for digesting methylated DNA or a reduced capacity to polyadenylate ncRNA for degradation (Wang et al., 2015). Therefore, the HST08 strain, with its smaller genome size, could facilitate mutation screening, making it a more efficient host for producing recombinant proteins and various types of miRNAs.

The DH10β strain (also known as TOP10) was used in two studies for miRNAs production by Wang et al. (2015) and Ho et al. (2018). As mentioned, the TOP10 strain is considered inferior to HST08 (Wang et al., 2015); however, Ho et al. (2018), through a modification with tRNA/pre-miR-34a-G138U/139ΔG, did not find such differences between the two strains. The DH10β strain belongs to the K-12 type, significantly differing from the B strain family. This strain is often used because it allows stable replication and a high number of plasmid copies, making it suitable for plasmid production (Taylor et al., 2017). It is known that DH10β requires leucine for growth due to the loss of the leuLABCD operon. DH10β also contains two alleles (relA1 and spoT1) that cause sensitivity to nutrient changes and slower growth rates compared to wild-type *E. coli* (Durfee et al., 2008). On the other hand, the DH5α strain was also used to produce miRNA (Wang et al., 2015), where recombinant miRNA production was not detectable in the gel after electrophoresis.

Furthermore, Wang et al. (2015) used another strain known as BL21(DE3). This strain is commonly used for producing recombinant proteins, whose main characteristic is to carry the T7 RNA polymerase gene, originating from a bacteriophage, and is under the control of the lacUV5 promoter. This strategy enables efficient expression of recombinant proteins using the T7 expression system (Jeong et al., 2015). IPTG (Isopropyl β-D-1-thiogalactopyranoside), a structural analog of lactose, is necessary in this case to induce the maximum expression of the polymerase and thus express the cloned recombinant gene downstream of the

promoter. Another interesting feature of the BL21(DE3) strain is the absence of outer membrane proteases, which degrade proteins. Based on this feature, BL21(DE3) is ideal for efficiently expressing sensitive molecules. Due to the absence of proteases, what is expressed is not degraded, contributing to the processing of the molecules produced (Jeong et al., 2015).

Cui et al. (2021) used the HT115(DE3) strain, which is noteworthy not only for carrying the T7 RNA polymerase gene under the control of the lac promoter, as BL21(DE3) competent cells do, but also for its capability to produce small RNAs. This remarkable feature renders the HT115(DE3) strain particularly useful for experiments involving double-stranded RNA (dsRNA; Papić et al., 2018).

S. typhimurium is a facultative anaerobic bacterium that can grow under both aerobic and anaerobic conditions, used in laboratory research as a model organism to study bacterial pathogenesis and host-pathogen interactions. In the laboratory, it is commonly grown using a variety of media, including nutrient agar, tryptic soybean agar, and broth at temperatures ranging from 37 to 42 °C (Hapfelmeier and Hardt, 2005; Taskila et al., 2012). The two main strains of *S. typhimurium* used in biotechnology are LT2 and SL1344. These strains have been extensively studied and their genomes sequenced (Hoiseth and Stocker, 1981; Bullas and Ryu, 1983; Tsai et al., 1989; Qi et al., 1996). The LT2 strain is commonly used as a reference strain for studying the biology and pathogenesis of *S. typhimurium*, while SL1344 has been used as a model for studying the interaction between Salmonella and host cells. Two of the studies scrutinized in this review utilized strains of *S. typhimurium* for the expression of miRNAs. Both studies were conducted by Yoon and collaborators, and yet span distinct timeframes for experiment execution, article composition and article writing (Yoon et al., 2011; Yoon et al., 2021), as well as different strains: SF586 and BRD509. The SF586 strain has been shown to induce a robust immune response in animal models, making it a promising candidate for the development of new vaccines, having a well-characterized genome is known to possess several virulence factors, including secretion systems type III and fimbriae. SF586 was engineered to express T7 RNA polymerase, which allows efficient expression of genes under the control of the T7 promoter (Bullas and Ryu, 1983; Bang et al., 2000). The strain BRD509, derived from *S. typhimurium* SL1344. It is characterized by having an aroA

deletion and was introduced into *S. typhimurium* SL1344 by transduction with a P22 lysate prepared in C5 aroA554::Tn10 (Hoiseth and Stocker, 1981; Strugnell et al., 1992). Among the characteristics of the BRD509 strain, its ability to survive in the gastrointestinal tract of chickens, its absence of genes related to pathogenicity, its resistance to antibiotics and its ability to reduce the colonization of pathogenic *S. typhimurium* (Xu et al., 1995; Liang et al., 2019). Despite the interesting characteristics for heterologous expression, this species has the disadvantage of high biological risk and biosafety when used in large-scale cultivation, as this bacterium is a common cause of foodborne illness in humans (Moffatt et al., 2016; Galán, 2021).

R. sulfidophilum is a chemolithotrophic bacterium capable of using inorganic sulfur compounds as an energy source. In the laboratory, it is typically grown in a photobioreactor under anoxic conditions with light as an energy source. The medium used for cultivation is often a minimal medium supplemented with thiosulfate, which is a source of sulfur. The culture is typically grown at a temperature between 25 and 30 °C with constant agitation (Azad et al., 2001; Carlozzi and Touloupakis, 2021). Large-scale cultivation of *R. sulfidophilum* can be achieved using a fermentation process in a bioreactor. Parameters for large-scale cultivation such as temperature, pH and agitation rate are optimized to achieve maximum growth and product yield. The use of *R. sulfidophilum* in biotechnology applications such as wastewater treatment and bioenergy production, highlights the importance of understanding the cultivation requirements of this bacterium for laboratory and industrial scale applications (Azad et al., 2001; Cai and Wang, 2013; Srisawat et al., 2022). The main advantage of using *R. sulfidophilum* as a biofactory is its ability to secrete heterologous nucleic acids during cell growth. Additionally, it does not secrete RNases, thus maintaining the integrity and activity of the produced RNA (Kikuchi and Umekage, 2018; Yu et al., 2020). The studies by Pereira et al., 2016, Pereira et al., 2020 used the DSM 1374 strain, also known as the W4 strain (Hansen and Veldkamp, 1973; Hiraishi and Ueda, 1994; Masuda et al., 2013). DSM 1374 has several important characteristics for biotechnological and commercial applications, as a source of bioenergy, carotenoid pigments, having applications in the food, pharmaceutical and aquaculture industries (Maeda et al., 2006; Cai and Wang, 2013; Srisawat et al., 2022).

Bacterial strains have been extensively explored as carriers of heterologous genomes for biotechnological applications, including the production of miRNAs. These strains offer the ability to incorporate and maintain foreign genetic sequences in their genome, enabling the expression of heterologous genes of interest (Shizuya et al., 1992). This feature has proven valuable in the field of miRNA expression, where bacterial strains are used as efficient hosts to produce these biological molecules (Pereira et al., 2016; Cui et al., 2021). Among the strains used, purple marine bacteria have shown promise due to their intrinsic characteristics, such as the ability to perform complex post-translational modifications and tolerance to high cell densities (Azad et al., 2001; Yu et al., 2020). However, other bacterial strains have also been successfully explored for miRNA expression, offering different advantages and flexibility for specific applications as we can see throughout this work. The use of these bacterial strains as carriers of heterologous genomes opens doors for significant advancements in miRNA production and other areas of biotechnology, driving the development of innovative biological therapies and applications.

3.4. Efficient plasmids produce a high number of transformed colonies and high product load

The efficiency of a plasmid can be measured in different ways, depending on the application or purpose. Some common ways of measuring plasmid efficiency are through transformation efficiency analyses, quantification of the expressed molecule of interest and with reporter gene assays.

Transformation efficiency measures the ability of a plasmid to transform bacterial cells. The number of colonies can be used to calculate transformation efficiency, which is expressed as the number of transformants per microgram of plasmid DNA. Using this technique Pereira et al. (2016) analyzed the incubation time of bacteria with DNA, the plasmid DNA used in the transformation mixture, the incubation temperature and the growth. After the experiment, it was observed that the optimal conditions for the transformation of *R. sulfidophilum* are the use of 0.025 µg of plasmid DNA, with a thermal shock of 2 min at 35 °C in cells in the middle

phase of culture (OD = 0.4). So, a high transformation efficiency was observed with 1.9×10^4 transformers/μg plasmid.

If the plasmid is designed for the expression of molecules its efficiency can be measured by determining the amount of the molecule produced. This can be done using techniques such as Western blotting, ELISA, mass spectrometry, urea-PAGE or qPCR.

The urea-polyacrylamide gel electrophoresis (urea-PAGE) has been used to assess the expression of recombinant ncRNAs. Initially, total RNAs are isolated using the Tris-HCl saturated phenol extraction method and then quantified with a spectrophotometer. Subsequently, total RNAs are analyzed using denaturing urea-polyacrylamide gel electrophoresis. Typically, 0.1–1 μg of total RNA per lane is loaded for urea-PAGE analysis. To visualize under a UV detector using the Imaging System, urea-PAGE gels are incubated with ethidium bromide (0.5 μg/mL) for 5 min on a shaker. Afterwards, images are analyzed and used to estimate the relative levels of recombinant ncRNAs present in the total RNAs (Li et al., 2014; Li et al., 2015; Wang et al., 2015; Chen et al., 2015; Ho et al., 2018; Petrek et al., 2019; Yi et al., 2020; Li et al., 2021; Chen et al., 2023; Traber et al., 2024).

Thus, urea-PAGE has been used to verify the production of a single miRNA, as, for example, Li et al. (2014) achieved good purity (0.85 %; based on gel electrophoresis) and a reasonable yield, approximately 2 % of recombinant ncRNA/total RNA or 2–3 mg of ncRNAs from 1 L of bacterial culture for tRNA/mir-1291–123 nt and tRNA/MSA. Li et al. (2015) showed that the maximum production of pre-miR-1291 was 2–3 mg/L of bacterial culture. Meanwhile, Wang et al. (2015) demonstrated consistent and efficient expression of tRNA/mir-34a biological agents, and the highest accumulation levels were found 9–14 h post-transformation. On the other hand, Yi et al. (2020) confirmed the success of high-level heterologous expression of the target molecule hBERA/miR-328 by the appearance of a new and strong band of the expected size in transformed bacteria. Moreover, urea-PAGE has also been used to verify the production of a large number of miRNAs as reported by Chen et al. (2015), Ho et al. (2018), Petrek et al. (2019), Li et al. (2021), Chen et al. (2023), and Traber et al. (2024). The total number of miRNAs found can be seen in Table 2.

The amount of pre-miR-29b produced by Pereira et al. (2016) was measured through reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR). The calibration curve to determine the pre-miR-29b concentration was constructed by serial dilutions of the synthetic pre-miR-29b sample. A total yield of 358 µg/L of pre-miR-29b was observed inside the cells during 32 h cultivation, and the maximum extracellular production achieved was approximately 182 µg/L of culture.

If the plasmid contains a reporter gene, its efficiency can be measured by quantifying the expression of the reporter gene in cells transfected with the plasmid. This can be done by using techniques such as flow cytometry, luminescence assays or colorimetric assays.

Yoon et al. (2011) observed through fluorescence microscopy that after 48 h of transfection, GFP expression was occurring, followed by miRNA expression in the cell. To assess miRCCL22 expression in bacteria, green fluorescent proteins were also observed in ST-miRCCL22 and ST-miRCV by Western blot analysis. GFP was observed in ST-miRCCL22 and ST-miRCV but was not observed in the control group.

3.5. Good miRNA expression plasmids contain multiple cloning sites and a lpp promoter

Plasmids are circular double-stranded DNA molecules found naturally in many bacteria and some species of fungi. They carry non-essential genes that provide a selective advantage to the host organism, such as resistance to antibiotics or the ability to degrade unusual compounds. Moreover, they can be easily manipulated in the laboratory, making them useful tools for molecular biology in the expression of genes and proteins of interest in host cells, and genetic engineering, as vectors for the transfer of genetic material between different organisms (Mentel et al., 2006; Monzani et al., 2022).

There are several types of plasmids, which can be classified based on their origin of replication, size, number of copies, selection markers and gene expression systems (Couturier et al., 1988). Cloning vectors are designed to take fragments of

foreign DNA and replicate them in host cells, usually having sites for easy insertion of DNA molecules. Expression vectors are designed to express the cloned gene of interest, as recombinant proteins. It contains regulatory elements, which allow the controlled expression of the gene of interest (Carnes and Williams, 2007).

The design of an effective miRNA expression plasmid involves the inclusion of key components that facilitate efficient gene expression (Tortora et al., 2016). These components include: The origin of replication, promoters, multiple cloning site (MCS), selection marker, and enhancers. The origin of replication (ori or rep) is a sequence that allows the plasmid to replicate independently within the host cell. Promoters are DNA sequences located upstream of the gene sequence that guide RNA polymerase to initiate transcription. The MCS or polylinker is represented by some elements that allow the insertion of the gene of interest into the plasmid. Additionally, this versatile feature allows for easy manipulation and customization of the plasmid. The selection marker component enables the selection of cells that contain the inserted plasmid after transformation, as they contain antibiotic resistance or fluorescence genes. Enhancers are DNA sequences that increase the transcriptional activity of a promoter, thereby enhancing the expression of the gene of interest. Regulatory elements can be arranged in various combinations and configurations to generate plasmids optimized for specific gene expression applications (Xu et al., 2001).

Analyzing the studies selected for this review, we can observe a variety of plasmids used for the expression of miRNAs, either in its pre-miRNA or mature form. Thus, for the production of miRNAs, plasmids such as pBSMrnaSeph, pBSTNAV, LITMUS 38i, and pBSKrna have been used in the bacteria *E. coli*. In the bacteria *S. typhimurium*, plasmids such as miRNACCL22 and pcDNA6 were used, and in *R. sulfidophilum*, it was pBHSR1-RM, as shown in Table 2.

Yoon et al. (2011), using the MultiSite Gateway™ recombination system, cloned the gene for the pre-miRNA target of the regulatory chemokine CCL22 in the expression vector pcDNA™ 6.2-GW/EmGFP-miR, resulting on the plasmid named miRCCL22. Although the pcDNA™ 6.2-GW/EmGFP-miR vector is recommended for use in eukaryotes, it was possible to observe that this expression system works in *E. coli* and *S. typhimurium*, two prokaryotes (Goussard et al., 2004). Thus, this is due to the presence of a eukaryotic DNA fragment flanking the EmGFP-miR region of the

vector, the cytomegalovirus (CMV) promoter, and pUC origin for selection and maintenance in *E. coli* (Yoon et al., 2011). Species of facultative intracellular pathogens, such as those used by Yoon et al., 2011, can live and reproduce both inside and outside host cells, already possessing a machinery for interaction with eukaryotic cells (Goussard et al., 2004; Fajac et al., 2004).

Ho et al. (2018), Petrek et al. (2019), and Li et al. (2021) used the pBSTNAV plasmid for the production of a large number of miRNAs through the BERAMet, CO-BERALeu/ser, and hBERASer/Leu/Lys/Cys/Gln/Tyr platforms, respectively. Regarding the studies by Ho et al. (2018), for plasmid expression in bacteria, the ncRNAs of interest were cloned into the target vector, where the miR-34a duplex was replaced by the target RNAs (e.g., miRNA, siRNA, or antisense RNA, RNA aptamers, etc.) of interest, with the insertion of the target ncRNA sequences achieved by PCR amplification using primers. Petrek et al. (2019) created the CO-BERA by replacing the miR-34a duplexes with specific miRNA or siRNA sequences (miR-34a, miR-124, let-7c, anti-miR-21, or NRF2-siRNA), which were then inserted into the vector. Li et al. (2021) constructed ncRNA expression plasmids by inserting human tRNASer/Leu/Lys/Cys/Gln/Tyr sequences and miRNAs into the pBSTNAV vector. These were then expressed in bacteria, resulting in the successful expression of various types of miRNAs with different htRNAs (htRNASer/Leu/Lys/Cys/Gln). The pBSTNAV plasmid is driven by a strong lipoprotein (lpp) gene promoter, terminated with a transcription terminator from the ribosomal RNA operon rrnC (rrnC), and selected with the ampicillin resistance (AmpR) gene (Meinnel et al., 1988a, Meinnel et al., 1988b; Ponchon et al., 2009; Tu et al., 2021).

Currently, the use of the pBSKrna vector has been reported by Traber et al. (2024). The BioRNALeu and BioRNAGly inserts were produced by PCR amplification using the respective BioRNALeu/miRNA and BioRNAGly/miRNA plasmids as templates and specific primers for htRNALeu and htRNAGly, and were cloned into the linearized pBSKrna vector. As a result, the formed plasmids were named pBSK/BioRNA. After fermentation in the bacteria, the researchers produced several types of miRNAs (22 BioRNALeu and 26 BioRNAGly). Like the pBSMrnaSeph and pBSTNAV vectors, the pBSKrna vector is composed of a lipoprotein (lpp) promoter

and a terminator from the ribosomal RNA operon rrnC (rrnC). However, the gene encoding antibiotic resistance and selection is β-lactamase (bla) (Traber et al., 2024).

Pereira et al. (2016) cloned DNA fragments corresponding to pre-miR-29b into the vector pBHSR1-RM, resulting in the plasmid pBHSR1-RM-pre-miR-29b. Later, in 2020, Pereira et al. conducted a study aiming to express the pre-miRNA. For this purpose, they replicated the sequence related to human pre-miR-29b. The amplicons were cloned into the plasmid pBHSR1-RM, which was subsequently used for transformation of *R. sulfidophilum*. The pBHSR1-RM vector contains the rrn promoter region, the streptavidin RNA aptamer sequence with ribozyme sequences flanking the pre-miRNA target, and the puf terminator region and kanamycin resistance region. The mutant rrn promoter may be favorable for the extracellular production of aptamer RNA, in principle the −35 mutation may increase the affinity of the RNA polymerase σ factor (Suzuki et al., 2011). Moreover, to improve RNA expression in *R. sulfidophilum*, the plasmid pBHR1 was modified. The modifications to the plasmid include the insertion of an enhanced promoter sequence (promoter region pCF-rpAM1) and mRNA stabilization elements (structural gene of the RNA aptamer phSR1) (Suzuki et al., 2011).

Seeking for the expression of mature miRNAs in *E. coli*, Cui et al. (2021) cloned the sequence corresponding to the mature miR-34 of a crustacean in the plasmid LITMUS 38i. Designed to perform efficient transcription of double-stranded RNA, the LITMUS 38i plasmid contains the pUC19 origin of replication, an ampicillin selectable marker, an M13 origin of replication, and T7 promoters flanking the multiple cloning sites. The plasmid lacks the MultiSite Gateway™ Recombination System. Because it has a lacZα gene, the expression of the heterologous molecule was induced by IPTG.

For the construction of expression plasmid sequences, Yoon et al. (2021) designed *in silico* two single-stranded DNA oligonucleotides complementary to pre-miRNAs targeting the gene encoding inhibin alpha subunit (INHA). To increase the efficiency of miRNA expression in melanoma cancer cells, two single-stranded oligos were annealed and cloned into a pcDNATM6.2-GW/miR vector, in which the CMV promoter was replaced by the Salmonella-operated T7 promoter. After those modifications, the plasmid was named pcDNA6-emi-INHA.

The success in the performance of heterologous expression of miRNAs in bacteria is due to a wide variety of factors, where the interaction between vector properties/sequences, along with the bacterial environment in which they are expressed, plays a significant role. Thus, the properties or sequences of a plasmid—replication origin, promoters, MCS, selection marker, and enhancers—can be major factors; additionally, we believe that the vector promoter may have greater relevance for miRNAs overexpression. Namely, the promoter is the key component of an expression system due to its role in the overall strength of the expression of the miRNA of interest. In this context, the vector's performance in inducing miRNA expression would be enhanced with a promoter that favors overexpression (Kaur et al., 2018).

Among the vectors examined in this review, four different promoters have been identified for miRNA production. Although a comparative study of various plasmids with different promoters to evaluate miRNA production performance was not conducted, it can be stated that vectors utilizing CMV, T7, lpp, and rrn promoters have shown potential for successful miRNA production. Specifically, the lpp promoter has stood out for its high-level expression, making it particularly favored for producing a wide range of miRNAs (Ho et al., 2018; Petrek et al., 2019; Li et al., 2021; Traber et al., 2024).

On the other hand, to enhance the yield in miRNAs production, the platform of tRNA fused with pre-miR-34a (BERAs or BioRNAs) has been modified. These modifications include strategies such as altering bases or adding specific sequences such as tRNALeu, Gly, Ser, etc., optimal non-coding RNA scaffold (OnRS), modification at G138U/139ΔG, Sephadex aptamer (MSA), and combinatorial BERAs (CO-BERAs). Consequently, significant improvements have been achieved, resulting in consistent high-level expression of chimeric miRNAs (Chen et al., 2015; Ho et al., 2018; Petrek et al., 2019; Traber et al., 2024).

4. Conclusion

Upon the completion of all the research in the present systematic review, our standpoint is that the most efficient microorganism for the heterologous production of

miRNAs of commercial interest seems to be the marine bacterium *R. sulfidophilum* (DSM 1374). It can be chosen due to its characteristics such as secretion of heterologous nucleic acids and the non-secretion of RNases during its cultivation (Suzuki et al., 2009). In addition, it can be cited the simple culture medium, needing for basic inorganic nutrients only; the capacity to perform facultative photosynthesis; and to be allowed for being cultivated in salt water (Suzuki et al., 2010; Higuchi-Takeuchi and Numata, 2019). Compared to conventional microorganisms (*E. coli*, *S. typhimurium*, etc.) which produce heterologous proteins, *R. sulfidophilum* is not pathogenic, and from its cultivation characteristics it opens a range of possibilities for the application of its biomass after - miRNA expression in biorefineries, aquaculture and bioenergy (Higuchi-Takeuchi and Numata, 2019).

Also, another interesting bacterium as a potential microorganism for miRNAs production that we can mention is *E. coli.* Even being a pathogenic strain in its wild strain (Kaper et al., 2004; Mayer et al., 2023), most *E. coli* strains are harmless, and highly used in laboratory studies and biotechnology companies, with large-scale production (Nuge et al., 2023). In addition, this species has its DNA fully mapped. Even with new strains mutating naturally from the interaction of genes with each other and with the environment, periodic monitoring is carried out to get a sense of these modifications (Rousset et al., 2021).

Among the expression plasmids analyzed in this review, we believe that the most suitable ones for expressing miRNAs in *E. coli* bacteria are those containing multiple cloning sites for the insertion of heterologous genes and an lpp promoter. This choice is due to their robust expression cassette, capable of producing a wide variety of different types of miRNAs. However, when the chosen is *R. sulfidophilum*, the best plasmid seems to be the pBHSR1-RM, is a plasmid derived from the pBHR1 plasmid that was modified to improve the expression of RNAs in *R. sulfidophilum*. The modifications included the insertion of an enhanced promoter sequence (pCF-rpAM1 promoter region) and mRNA stabilization elements (phSR1 RNA aptamer structural gene) into the plasmid (Suzuki et al., 2011).

Plasmids containing the lpp promoter seem to be the best choice according to the works analyzed for the expression of miRNAs in bacteria. The promoter has several characteristics that make it an attractive choice for performing gene

expression, and one of the main is the capacity to transcript cloned genes into messenger RNA from complementary DNA. This feature can be useful for gene expression studies and for producing RNA for applications in biotechnology. In the context of expression of miRNAs in bacteria, some additional resources must be considered in the construction of expression plasmids. Because miRNAs are small, stable molecules, precise cleavage of miRNA precursors (pre-miRNAs) is essential.

Overall, the design of a miRNA expression plasmid is critical for the efficient expression and processing of the miRNA molecule in cells. Advances in plasmid design and genetic engineering techniques have enabled the development of more efficient and specific miRNA expression plasmids, which have greatly contributed to the study of miRNA function and therapeutic applications.

Author contributions statement

NN, LN e VC conceived and planned the systematic review. AW, LN and carried out the data analysis. VC, WB, MR and DB contributed to the interpretation of the results. NN, DB and VC took the lead in writing the manuscript. All authors provided critical feedback and helped shape the data collection and extraction, analysis, and manuscript.

CRediT authorship contribution statement

Nyelson da Silva Nonato: Writing – review & editing, Writing – original draft, Methodology, Conceptualization. Leandro Silva Nunes: Writing – review & editing, Methodology, Formal analysis, Data curation. Amanda Weege da Silveira Martins: Methodology, Formal analysis, Data curation. Danillo Pinhal: Writing – review & editing, Methodology, Formal analysis, Data curation. William Borges Domingues: Writing – review & editing, Writing – original draft, Data curation, Conceptualization. Dionet Keny Bellido-Quispe: Writing – review & editing, Investigation, Formal analysis. Mariana Härter Remião: Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. Vinicius Farias Campos: Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

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References

Allen, D., et al., 2019. Recent advances and near future of insulin production and therapy. **Future Med. Chem.** 11 (13), 1513–1517. https://doi.org/10.4155/fmc-2019-0134.

Alrseetmiwe, et al., 2020. *Escherichia coli* strain bl21: cloning and expression of an optimized interferon alpha 2B (DE3). **J. Life Sci. Appl. Res.** 1 (2), 40–44. https://doi.org/10.59807/jlsar.v1i2.15.

Alyas, J., et al., 2021. Human insulin: history, recent advances, and expression systems for mass production. **Biomed. Res. Ther.** 8 (9), 4540–4561. https://doi.org/10.15419/bmrat.v8i9.692.

Azad, S.A., et al., 2001. Growth and production of biomass of *Rhodovulum sulfidophilum* in sardine processing wastewater. **Lett. Appl. Microbiol.** 33 (4), 264–268. https:// doi.org/10.1046/j.1472-765X.2001.00993.x.

Bang, I.S., et al., 2000. OmpR regulates the stationary-phase acid tolerance response of Salmonella enterica serovar typhimurium. **J. Bacteriol.** 182 (8), 2245–2252. https:// doi.org/10.1128/jb.182.8.2245-2252.2000.

Bis, R.L., et al., 2014. High yield soluble bacterial expression and streamlined purification of recombinant human interferon α-2a. **Protein Expr. Purif.** 99, 138–146. https://doi.org/10.1016/j.pep.2014.04.010.

Bl¨odorn, E.B., et al., 2021. MicroRNA roles and their potential use as selection tool to cold tolerance of domesticated teleostean species: a systematic review. **Aquaculture,** 736747. https://doi.org/10.1016/j.aquaculture.2021.736747.

Bramer, W.M., et al., 2017. Optimal database combinations for literature searches in systematic reviews: a prospective exploratory study. **Syst. Rev.** 6, 1–12. https://doi.org/10.1186/s13643-017-0644-y.

Brooks, S.A., 2004. Appropriate glycosylation of recombinant proteins for human use: implications of choice of expression system. Mol. Biotechnol. 28, 241–255. https://doi.org/10.1385/MB:28:3:241.

Bullas, L.R., Ryu, J.I., 1983. *Salmonella typhimurium* LT2 strains which are r-m+for all three chromosomally located systems of DNA restriction and modification. **J. Bacteriol.** 156 (1), 471–474. https://doi.org/10.1128/jb.156.1.471-474.1983.

Cai, J., Wang, G., 2013. Screening and hydrogen-producing characters of a highly efficient H2-producing mutant of *Rhodovulum sulfidophilum* P5. **Bioresour. Technol.** 142, 18–25. https://doi.org/10.1016/j.biortech.2013.05.009.

Campos, V.F., et al., 2011. MicroRNAs e seu papel no desenvolvimento embrionário. **Ciência Rural** 41, 85–93. https://doi.org/10.1590/S0103-84782011000100014.

Carlozzi, P., Touloupakis, E., 2021. Bioplastic production by feeding the marine *Rhodovulum sulfidophilum* DSM-1374 with four different carbon sources under batch, fed-batch and semi-continuous growth regimes. **New Biotechnol.** 62, 10–17. https://doi.org/10.1016/j.nbt.2020.12.002.

Carnes, A.E., Williams, J.A., 2007. Plasmid DNA manufacturing technology. **Recent Pat. Biotechnol.** 1 (2), 151–166. https://doi.org/10.2174/187220807780809436.

Chen, Q.X., Wang, W.P., Zeng, S., Urayama, S., Yu, A.M., 2015. A general approach to high-yield biosynthesis of chimeric RNAs bearing various types of functional small RNAs for broad applications. **Nucleic Acids Res.** 43 (7), 3857–3869. https://doi.org/10.1093/nar/gkv228.

Chen, Y., Tu, M.J., Han, F., Liu, Z., Batra, N., Lara, P.N., Yu, A.M., 2023. Use of recombinant microRNAs as antimetabolites to inhibit human non-small cell lung cancer. **Acta Pharm. Sin. B** 13 (10), 4273–4290. https://doi.org/10.1016/j.apsb.2023.07.011.

Choi, Y.J., et al., 2010. Novel, versatile, and tightly regulated expression system for *Escherichia coli* strains. **Appl. Environ. Microbiol.** 76 (15), 5058–5066. https://doi.org/10.1128/AEM.00413-10.

Choi, J., et al., 2017. Tiny RNAs and their voyage via extracellular vesicles: secretion of bacterial small RNA and eukaryotic microRNA. **Exp. Biol. Med.** 242 (15), 1475–1481. https://doi.org/10.1177/1535370217723166.

Cock, J.M., et al., 2017. Rapid evolution of microRNA loci in the brown algae. **Genome Biol. Evol.** 9 (3), 740–749. https://doi.org/10.1093/gbe/evx038.

Couturier, M., et al., 1988. Identification and classification of bacterial plasmids. **Microbiol. Rev.** 52 (3), 375–395. https://doi.org/10.1128/mr.52.3.375-395.1988.

Cui, Y., Wan, H., Zhang, X., 2021. miRNA in food simultaneously controls animal viral disease and human tumorigenesis. **Mol. Ther. Nucleic Acids** 23, 995–1006. https://doi.org/10.1016/j.omtn.2021.01.011.

Cuperus, J.T., Fahlgren, N., Carrington, J.C., 2011. Evolution and functional diversification of MIRNA genes. **Plant Cell** 23 (2), 431–442. https://doi.org/10.1105/tpc.110.082784.

Da Silva, L.S., et al., 2021. Capillary electroporation affects the expression of miRNA-122-5p from bull sperm cells. **Gene** 768, 145286. https://doi.org/10.1016/j.gene.2020.145286.

Driessen, A.J.M., Nouwen, N., 2008. Protein translocation across the bacterial cytoplasmic membrane. **Annu. Rev. Biochem.** 77, 643–667. https://doi.org/10.1146/annurev.biochem.77.061606.160747.

Durfee, T., Nelson, R., Baldwin, S., Plunkett III, G., Burland, V., Mau, B., Blattner, F.R., 2008. The complete genome sequence of *Escherichia coli* DH10B: insights into the biology of a laboratory workhorse. **J. Bacteriol.** 190 (7), 2597–2606. https://doi.org/10.1128/JB.01695-07.

Fajac, I., et al., 2004. Recombinant *Escherichia coli* as a gene delivery vector into airway epithelial cells. **J. Control. Release** 97 (2), 371–381. https://doi.org/10.1016/j.jconrel.2004.03.025.

Falak, S., Sajed, M., Rashid, N., 2022. Strategies to enhance soluble production of heterologous proteins in *Escherichia coli.* **Biologia** 77 (3), 893–905. https://doi.org/10.1007/s11756-021-00994-5.

Furuse, Y., et al., 2014. Search for microRNAs expressed by intracellular bacterial pathogens in infected mammalian cells. **PLoS One** 9 (9), e106434. https://doi.org/10.1371/journal.pone.0106434.

Gal´an, J.E., 2021. *Salmonella typhimurium* and inflammation: a pathogen-centric affair. **Nat. Rev. Microbiol.** 19 (11), 716–725. https://doi.org/10.1038/s41579-021-00561- 4.

Ganjave, S.D., et al., 2022. High cell density cultivation of *E. coli in* shake flasks for the production of recombinant proteins. **Biotechnol. Reports** 33, e00694. https://doi.org/10.1016/j.btre.2021.e00694.

Goussard, S., Grillot-Courvalin, C., Courvalin, P., 2004. Eukaryotic promoters can direct protein synthesis in gram-negative bacteria. **Microb. Physiol.** 6 (3–4), 211–218. https://doi.org/10.1159/000077252.

Hansen, T.A., Veldkamp, H., 1973. *Rhodopseudomonas sulfidophila*, nov. spec., a new species of the purple nonsulfur bacteria. **Arch. Mikrobiol.** 92, 45–58. https://doi.org/10.1007/BF00409510.

Hapfelmeier, S., Hardt, W., 2005. A mouse model for *S. Typhimurium*-induced enterocolitis. **Trends Microbiol.** 13 (10), 497–503. https://doi.org/10.1016/j.tim.2005.08.008.

Hashiro, S., Mitsuhashi, M., Yasueda, H., 2019. Overexpression system for recombinant RNA in *Corynebacterium glutamicum* using a strong promoter derived from corynephage BFK20. **J. Biosci. Bioeng.** 128 (3), 255–263. https://doi.org/10.1016/j. jbiosc.2019.03.003.

Higuchi-Takeuchi, M., Numata, K., 2019. Marine purple photosynthetic bacteria as sustainable microbial production hosts. **Front. Bioeng. Biotechnol.** 7, 258. https://doi.org/10.3389/fbioe.2019.00258.

Hiraishi, A., Ueda, Y., 1994. Intrageneric structure of the genus Rhodobacter: transfer of *Rhodobacter sulfidophilus* and related marine species to the genus Rhodovulum

gen. **Nov. Int. J. Syst. Evol. Microbiol.** 44 (1), 15–23. https://doi.org/10.1099/00207713- 44-1-15.

Ho, P.Y., Duan, Z., Batra, N., Jilek, J.L., Tu, M.J., Qiu, J.X., Yu, A.M., 2018. Bioengineered noncoding RNAs selectively change cellular miRNome profiles for cancer therapy. **J. Pharmacol.** Exp. Ther. 365 (3), 494–506. https://doi.org/10.1124/jpet.118.247775.

Hoiseth, S.K., Stocker, B.A.D., 1981. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. **Nature** 291, 238–239. https://doi.org/10.1038/291238a0.

Jeong, H., Kim, H.J., Lee, S.J., 2015. Complete genome sequence of *Escherichia coli* strain BL21. **Genome Announc.** 3 (2). https://doi.org/10.1128/genomea.00134-15 p. e00134–15.

Kaper, J.B., Nataro, J.P., Mobley, H.L.T., 2004. Pathogenic *Escherichia coli*. **Nat. Rev. Microbiol.** 2 (2), 123–140. https://doi.org/10.1038/nrmicro818.

Kaur, J., Kumar, A., Kaur, J., 2018. Strategies for optimization of heterologous protein expression in *E. coli*: roadblocks and reinforcements. **Int. J. Biol. Macromol.** 106, 803–822. https://doi.org/10.1016/j.ijbiomac.2017.08.080.

Keasling, J.D., 1999. Gene-expression tools for the metabolic engineering of bacteria. **Trends Biotechnol.** 17 (11), 452–460. https://doi.org/10.1016/S0167-7799(99) 01376-1.

Kightlinger, W., et al., 2020. Synthetic glycobiology: parts, systems, and applications. **ACS Synth. Biol**. 9 (7), 1534–1562. https://doi.org/10.1021/acssynbio.0c00210.

Kikuchi, Y., Umekage, S., 2018. Extracellular nucleic acids of the marine bacterium *Rhodovulum sulfidophilum* and recombinant RNA production technology using bacteria. **FEMS Microbiol. Lett.** 365 (3). https://doi.org/10.1093/femsle/fnx268 p. fnx268.

Lee, H., et al., 2010. Diverse pathways generate microRNA-like RNAs and Dicer-independent small interfering RNAs in fungi. **Mol. Cell** 38 (6), 803–814. https://doi.org/10.1016/j.molcel.2010.04.005.

Li, M., et al., 2014. Rapid production of novel pre-microRNA agent hsa-mir-27b in *Escherichia coli* using recombinant RNA technology for functional studies in mammalian cells. **Drug Metab. Dispos.** 42 (11), 1791–1795. https://doi.org/10.1124/dmd.114.060145.

Li, M.M., Addepalli, B., Tu, M.J., Chen, Q.X., Wang, W.P., Limbach, P.A., Yu, A.M., 2015. Chimeric microRNA-1291 biosynthesized efficiently in *Escherichia coli* is effective to reduce target gene expression in human carcinoma cells and improve chemosensitivity. **Drug Metab. Dispos.** 43 (7), 1129–1136. https://doi.org/10.1124/dmd.115.064493.

Li, P.C., Tu, M.J., Ho, P.Y., Batra, N., Tran, M.M., Qiu, J.X., Yu, A.M., 2021. *In vivo* fermentation production of humanized noncoding RNAs carrying payload miRNAs for targeted anticancer therapy. **Theranostics** 11 (10), 4858. https://doi.org/10.7150/thno.56596.

Liang, K., et al., 2019. Genetically engineered *Salmonella typhimurium*: recent advances in cancer therapy. **Cancer Lett.** 448, 168–181. https://doi.org/10.1016/j.canlet.2019.01.037.

Lim, C.G., et al., 2015. Development of a recombinant *Escherichia coli* strain for overproduction of the plant pigment anthocyanin. **Appl. Environ. Microbiol.** 81 (18), 6276–6284. https://doi.org/10.1128/AEM.01448-15.

Linke, J.A., Rayat, A., Ward, J.M., 2023. Production of indigo by recombinant bacteria. **Bioresour. Bioprocess.** 10 (1), 20. https://doi.org/10.1186/s40643-023-00626-7.

Liu, Y.P., Berkhout, B., 2011. miRNA cassettes in viral vectors: problems and solutions. **Biochim. Biophys. Acta** 1809 (11–12), 732–745. https://doi.org/10.1016/j.bbagrm.2011.05.014.

Maeda, I., et al., 2006. Colorimetric dimethyl sulfide sensor using *Rhodovulum sulfidophilum* cells based on intrinsic pigment conversion by CrtA. **Appl. Microbiol. Biotechnol.** 70, 397–402. https://doi.org/10.1007/s00253-005-0117-4.

Mao, Y., Lu, Z., 2017. MeSH now: automatic MeSH indexing at PubMed scale via learning to rank. **J. Biomed. Semant.** 8, 1–9. https://doi.org/10.1186/s13326-017-0123-3.

Masuda, S., et al., 2013. Whole-genome sequence of the purple photosynthetic bacterium *Rhodovulum sulfidophilum* strain W4. **Genome Announc.** 1 (4). https://doi.org/ 10.1128/genomea.00577-13 p. e00577–13.

Mayer, C., et al., 2023. Quorum sensing architecture network in *Escherichia coli* virulence and pathogenesis. **FEMS Microbiol. Rev.** https://doi.org/10.1093/femsre/fuad031 p. fuad031.

Meinnel, T., Mechulam, Y., Fayat, G., 1988a. Fast purification of a functional elongator tRNAmet expressed from a synthetic gene *in vivo*. **Nucleic Acids Res**. 16 (16), 8095. https://doi.org/10.1093/nar/16.16.8095.

Meinnel, T., Mechulam, Y., Fayat, G., 1988b. Fast purification of a functional elongator tRNAmet expressed from a synthetic gene *in vivo*. **Nucleic Acids Res.** 16 (16), 8095. https://doi.org/10.1093/nar/16.16.8095.

Mentel, M., et al., 2006. Transfer of genetic material between pathogenic and food-borne yeasts. **Appl. Environ. Microbiol.** 72 (7), 5122–5125. https://doi.org/10.1128/ AEM.00293-06.

Moffatt, C.R.M., et al., 2016. *Salmonella typhimurium* and outbreaks of egg-associated disease in Australia, 2001 to 2011. **Foodborne Pathog.** Dis. 13 (7), 379–385. https:// doi.org/10.1089/fpd.2015.2110.

Monzani, P.S., et al., 2022. Cows as bioreactors for the production of nutritionally and biomedically significant proteins. Recent advances in animal. **Nutr. Metab.** 299–314. https://doi.org/10.1007/978-3-030-85686-1_15.

Nuge, T., et al., 2023. Enhanced large-scale production of recombinant phytase in *E. coli* DH5 α: medium components optimization and thermodynamic studies. **J. Mol. Liq.** 370, 120965. https://doi.org/10.1016/j.molliq.2022.120965.

Papi´c, L., et al., 2018. Double-stranded RNA production and the kinetics of recombinant *Escherichia coli* HT115 in fed-batch culture. **Biotechnol. Reports** 20, e00292. https:// doi.org/10.1016/j.btre.2018.e00292.

Pereira, P., Pedro, A.Q., Tom´as, J., Maia, C.J., Queiroz, J.A., Figueiras, A., Sousa, F., 2016. Advances in time course extracellular production of human pre-miR-29b from *Rhodovulum sulfidophilum.* **Appl. Microbiol. Biotechnol.** 100, 3723–3734. https:// doi.org/10.1007/s00253-016-7350-x.

Pereira, P., et al., 2017. New insights for therapeutic recombinant human miRNAs heterologous production: *Rhodovolum sulfidophilum* vs *Escherichia coli*. **Bioengineered** 8 (5), 670–677. https://doi.org/10.1080/21655979.2017.1284710.

Pereira, P., Barreira, M., Cruz, C., Tom´as, J., Luís, ˆA., Pedro, A.Q., Sousa, F., 2020. Brain- targeted delivery of pre-miR-29b using lactoferrin-stearic acid-modified-chitosan/ polyethyleneimine polyplexes. **Pharmaceuticals** 13 (10), 314. https://doi.org/ 10.3390/ph13100314.

Petrek, H., Batra, N., Ho, P.Y., Tu, M.J., Yu, A.M., 2019. Bioengineering of a single long noncoding RNA molecule that carries multiple small RNAs. Appl. Microbiol. Biotechnol. 103, 6107–6117. https://doi.org/10.1007/s00253-019-09934-5.

Ponchon, L., et al., 2009. A generic protocol for the expression and purification of recombinant RNA in *Escherichia coli* using a tRNA scaffold. **Nat. Protoc.** 4 (6), 947–959. https://doi.org/10.1038/nprot.2009.67.

Posada, J.A., Cardona, C.A., Gonzalez, R., 2012. Analysis of the production process of optically pure D-lactic acid from raw glycerol using engineered *Escherichia coli* strains. **Appl. Biochem. Biotechnol.** 166, 680–699. https://doi.org/10.1007/s12010-011-9458-x.

Pranckut˙e, R., 2021. Web of science (WoS) and Scopus: the titans of bibliographic information in today's academic world. **Publications** 9 (1), 12. https://doi.org/ 10.3390/publications9010012.

Qi, S., Moir, A., O'connor, C.D., 1996. Proteome of *Salmonella typhimurium* SL1344: identification of novel abundant cell envelope proteins and assignment to a two-dimensional reference map. **J. Bacteriol.** 178 (16), 5032–5038. https://doi.org/ 10.1128/jb.178.16.5032-5038.1996.

Raza, S.H.A., et al., 2022. MicroRNAs mediated environmental stress responses and toxicity signs in teleost fish species. **Aquaculture**, 737310. https://doi.org/10.1016/ j.aquaculture.2021.737310.

Rose, S.A., et al., 2021. A microRNA expression and regulatory element activity atlas of the mouse immune system. **Nat. Immunol.** 22 (7), 914–927. https://doi.org/ 10.1038/s41590-021-00944-y.

Rousset, F., et al., 2021. The impact of genetic diversity on gene essentiality within the *Escherichia coli* species. **Nat. Microbiol.** 6 (3), 301–312. https://doi.org/10.1038/ s41564-020-00839-y.

Sheng, J., Ling, P., Wang, F., 2015. Constructing a recombinant hyaluronic acid biosynthesis operon and producing food-grade hyaluronic acid in *Lactococcus lactis*.

J. Ind. Microbiol. Biotechnol. 42 (2), 197–206. https://doi.org/10.1007/s10295-014-1555-8.

Shizuya, H., et al., 1992. Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in *Escherichia coli* using an F-factor-based vector. **Proc. Natl. Acad. Sci.** 89 (18), 8794–8797. https://doi.org/10.1073/pnas.89.18.8794.

Srisawat, P., Higuchi-Takeuchi, M., Numata, K., 2022. Microbial autotrophic biorefineries: perspectives for biopolymer production. **Polym. J**. 54 (10), 1139–1151. https://doi.org/10.1038/s41428-022-00675-3.

Stabile, R., et al., 2023. The deleted in oral cancer (DOC1 aka CDK2AP1) tumor suppressor gene is downregulated in oral squamous cell carcinoma by multiple microRNAs. **Cell Death Dis.** 14 (5), 337. https://doi.org/10.1038/s41419-023-05857-2.

Strugnell, R., et al., 1992. Characterization of a *Salmonella typhimurium* aro vaccine strain expressing the P. 69 antigen of *Bordetella pertussis*. **Infect. Immun.** 60 (10), 3994–4002. https://doi.org/10.1128/iai.60.10.3994-4002.1992.

Sunguroˇglu, C., et al., 2018. Higher titer hyaluronic acid production in recombinant *Lactococcus lactis*. **Prep. Biochem. Biotechnol.** 48 (8), 734–742. https://doi.org/10.1080/10826068.2018.1508036.

Suzuki, H., et al., 2009. Characterization of extracellular DNA production and flocculation of the marine photosynthetic bacterium *Rhodovulum sulfidophilum*. **Appl. Microbiol. Biotechnol.** 84, 349–356. https://doi.org/10.1007/s00253-009-2031-7.

Suzuki, H., et al., 2010. Extracellular production of an RNA aptamer by ribonuclease-free marine bacteria harboring engineered plasmids: a proposal for industrial RNA drug production. **Appl. Environ. Microbiol.** 76 (3), 786–793. https://doi.org/10.1128/ AEM.01971-09.

Suzuki, H., et al., 2011. Artificial RNA aptamer production by the marine bacterium *Rhodovulum sulfidophilum*: improvement of the aptamer yield using a mutated transcriptional promoter. **J. Biosci. Bioeng.** 112 (5), 458–461. https://doi.org/10.1016/j.jbiosc.2011.07.025.

Taskila, S., Tuomola, M., Ojamo, H., 2012. Enrichment cultivation in detection of food- borne Salmonella. **Food Control** 26 (2), 369–377. https://doi.org/10.1016/j.foodcont.2012.01.043.

Taylor, T., Denson, J.P., Esposito, D., 2017. Optimizing expression and solubility of proteins in *E. coli* using modified media and induction parameters. **Heterol. Gene Express.** *E. coli* 65–82. https://doi.org/10.1007/978-1-4939-6887-9_5.

Terol, G.L., et al., 2021. Impact of the expression system on recombinant protein production in *Escherichia coli* BL21. **Front. Microbiol.** 12, 682001. https://doi.org/10.3389/fmicb.2021.682001.

Tian, H., et al., 2011. Lactose induction increases production of recombinant keratinocyte growth factor-2 in *Escherichia coli*. **Int. J. Pept. Res. Ther.** 17, 123–129. https://doi.org/10.1007/s10989-011-9249-9.

Tortora, G.J., Case, C.L., Funke, B.R., 2016. **Microbiologia**-12a Edição (Artmed Editora).

Traber, G.M., Yi, C., Batra, N., Tu, M.J., Yu, A.M., 2024. Novel RNA molecular bioengineering technology efficiently produces functional miRNA agents. RNA 30 (6), 680–694. https://doi.org/10.1261/rna.079904.123.

Tsai, S.P., Hartin, R.J., Ryu, J., 1989. Transformation in restriction-deficient *Salmonella typhimurium* LT2. **Microbiology** 135 (9), 2561–2567. https://doi.org/10.1099/ 00221287-135-9-2561.

Tu, M.J., Wright, H.K., Batra, N., Yu, A.M., 2021. Expression and purification of tRNA/pre-miRNA-based recombinant noncoding RNAs. In: **RNA Scaffolds: Methods and Protocols.** Springer US, New York, NY, pp. 249–265. https://doi.org/10.1007/978-1-0716-1499-0_18.

Volinia, S., et al., 2006. A microRNA expression signature of human solid tumors defines cancer gene targets. **Proc. Natl. Acad. Sci.** 103 (7), 2257–2261. https://doi.org/ 10.1073/pnas.0510565103.

Wang, W.P., Ho, P.Y., Chen, Q.X., Addepalli, B., Limbach, P.A., Li, M.M., Yu, A.M., 2015. Bioengineering novel chimeric microRNA-34a for prodrug cancer therapy: high- yield expression and purification, and structural and functional characterization. **J. Pharmacol. Exp. Ther.** 354 (2), 131–141. https://doi.org/10.1124/jpet.115.225631.

Wassarman, K.M., Zhang, A., Storz, G., 1999. Small RNAs in *Escherichia coli*. **Trends Microbiol.** 7 (1), 37–45. https://doi.org/10.1016/S0966-842X(98)01379-1.

Xu, et al., 1995. Protection against Leishmania major infection in genetically susceptible BALB/c mice by gp63 delivered orally in attenuated *Salmonella typhimurium* (AroA- AroD-). Immunology 85 (1), 1.

Xu, Z., et al., 2001. Optimization of transcriptional regulatory elements for constructing plasmid vectors. **Gene** 272 (1–2), 149–156. https://doi.org/10.1016/S0378-1119 (01)00550-9.

Yi, W., Tu, M.J., Liu, Z., Zhang, C., Batra, N., Yu, A.X., Yu, A.M., 2020. Bioengineered miR-328-3p modulates GLUT1-mediated glucose uptake and metabolism to exert synergistic antiproliferative effects with chemotherapeutics. **Acta Pharm. Sin.** B 10 (1), 159–170. https://doi.org/10.1016/j.apsb.2019.11.001.

Yoon, W.S., Lee, S.S., Chae, Y.S., Park, Y.K., 2011. Therapeutic effects of recombinant *Salmonella typhimurium* harboring CCL22 miRNA on atopic dermatitis-like skin in mice. **Exp. Mol. Med.** 43 (2), 63–70. https://doi.org/10.3858/emm.2011.43.2.008.

Yoon, W., Park, Y., Kim, S., Park, Y., Kim, C.Y., 2021. Combined therapy with microRNA- expressing Salmonella and irradiation in melanoma. **Microorganisms.** 9 (11), 2408. https://doi.org/10.3390/microorganisms9112408.

Yu, A., et al., 2020. Novel approaches for efficient *in vivo* fermentation production of noncoding RNAs. **Appl. Microbiol. Biotechnol.** 104, 1927–1937. https://doi.org/10.1007/s00253-020-10350-3.

Yu, Y., et al., 2021. MicroRNAs: the novel mediators for nutrient-modulating biological functions. **Trends Food Sci. Technol.** 114, 167–175. https://doi.org/10.1016/j.tifs.2021.05.028.

Zhou, Q., et al., 2012. Immune-related microRNAs are abundant in breast milk exosomes. **Int. J. Biol. Sci.** 8 (1), 118. https://doi.org/10.7150/ijbs.8.118.

Figures and Tables

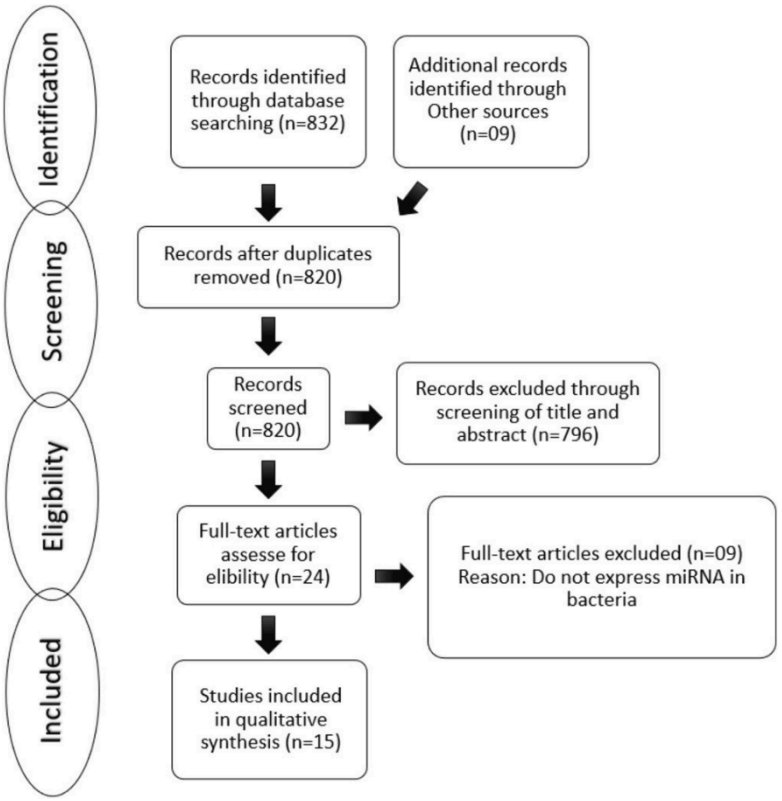

Figura 3 - Representative research flowchart.

Table 1
Search terms.

PubMed, Web of Science e Scopus	#1 ("microRNA" OR "miRNA") #2 ("heterologous expression" OR "recombinant RNA" OR "bioengineered microRNA" OR "recombinant DNA" OR "expression system" OR plasmid) #3 (bacteria)
[a]Search combination:	#1 AND #2 AND #3

Table 2
Overview of studies included in the systematic review.

Author	Year	Species	Plasmid used	Expressed miRNA	Target
Yoon et al.	2011	*S. typhimurium*	miRNACCL22	miRNA CCL22	Chemokine (C—C motif) ligand 22 (CCL22)
Li et al.	2014	*E. coli*	pBSMrnaSeph/tRNA/miR-27b	miR-27b and hsa-mir-27b	Cytochrome P450 3A4 (CYP3A4) in human carcinoma cells LS-180
Li et al.	2015	*E. coli*	pBSMrnaSeph/tRNA/pre-miR-1291	pre-miR-1291 and tRNA/miR-1291	ABCC1, FOXA2, and MeCP2
Wang et al.	2015	*E. coli*	pBSMrnaSeph/tRNA/pre-miR-34a	tRNA/miR-34a	CDK6, MET and SIRT1
Chen et al.	2015	*E. coli*	pBSMrnaSeph/tRNA/miRNA pBSMrnaSeph/OnRS/miRNA	tRNA/Let-7a, tRNA/mir-1291, tRNA/mir-221, tRNA/mir-155, tRNA/mir-125-2, tRNA/mir-125-1, tRNA/mir-124-2, tRNA/mir-124-1, tRNA/mir-122, tRNA/mir-34a, tRNA/mir-27b and OnRS/miR-124	OnRS/miR-124: STAT3
Pereira et al.	2016	*R. sulfidophilum*	pBHSR1-RM-pre-miR-29b	pre-miR-29b	Regulatory pathways in neurodegenerative diseases and exhibits tumor suppressor and immunomodulatory properties
Ho et al.	2018	*E. coli*	pBSTNAV/BERA^{Met}	33 ncRNAs were successfully expressed (miR- 27a-3p, miR-27b-3p, miR-892b-3p, miR-451a-5p, ApoB-siRNA, anti-miR-126-3p, ICAM1-siRNA, miR-298-5p, miR-519c-5p, miR-122-5p, Nrf2-siRNA, miR-126-3p, miR-144-5p, ARV7-siRNA, GFP-siRNA, let-7c-5p, miR-127-3p, miR-34a-5p, miR-124-3p, miR-328-3p, anti-miR-451b-5p, MAS, anti-miR-21- 5p-3, miR-33a-5p, anti-miR-21- 5p-2, anti-miR-122-5p, scrm-5, scrm-4, scrm-3, anti-miR-21-5p, miR-34a/TPA3' + TPA5', miR-888/pegapta nib3' and miR-34a/EpCAM) 2 miRNAs were purified (nCARMet/miR-34a-5p and nCARMet/miR-124-3p)	miR-34a-5p: cMET and CDK6 miR-124-3p: STAT3 and ABCC4
Petrek et al.	2019	*E. coli*	pBSTNAV/CO-BERA$^{Leu/ser}$	htRNA^{Leu}/miR-34a/miR-124, htRNA^{Leu}/miR-124/miR-34a, htRNA^{Leu}/let-7c/miR-124, htRNA^{Leu}/let-7c/miR-34a, htRNA^{Ser}/miR-124/miR-34a, htRNA^{Ser}/let-7c/miR-124, htRNA^{Ser}/let-7c/miR-34a, htRNA^{Leu}/miR-34a/anti-miR-21, htRNA^{Leu}/NRF2-siRNA/miR-34a and htRNA^{Ser}/NRF2-siRNA/miR-34a	Anti-proliferative against several human NSCLC cell lines
Pereira et al.	2020	*R. sulfidophilum*	pBHSR1-RM-pre-miR-29b	pre-miR-29b	BACE1
Yi et al.	2020	*E. coli*	Unspecified vector/hBERA^{Ser}	hBERA^{Ser}/miR-328-3p	SLC7A5, LAT1, SLC2A1 and GLUT1
Cui et al.	2021	*E. coli*	LITMUS 38i	shrimp miR-34	Viral genes in shrimp (wsv330 and wsv359) and carcinogenic genes (CCND1, CDK6, CCNE2, E2F3, FOSL1 and MET)
Li et al.	2021	*E. coli*	pBSTNAV/hBERA$^{Ser/Leu/Lys/Cys/Gln/Tyr}$	htRNA$^{Ser/Leu/Lys/Cys/Gln}$/miR-34a-5p, 20 ncRNAs with htRNA^{Ser}/hsa-pre-miR-34a (miR-124-3p, let-7c-5p, NRF2-siRNA, miR-200b-3p, miR-148-3p, miR-22-3p, miR-6775-3p, miR-133a-3p, miR-122-5p, miR-328-3p, miR-7-1-5p, miR-92a-3p, miR-126-3p, miR-132-3p, miR-137-3p, miR-140-5p, miR-141-3p, miR-142-3p, miR-194-3p and miR-205-5p) and 9 ncRNAs with htRNA^{Leu}/hsa-pre-miR-34a (BCL2-siRNA, NRF2-siRNA, AGR2-siRNA, miR-124-3p, let-7c-5p, miR-328-3p, miR-200b-3p, miR-22-3p and miR-148-3p)	miR-34a (BCL2, SIRT1, and c-MET) and miR-124 (VAMP3, MCT1, and STAT3)
Yoon et al.	2021	*S. typhimurium*	pcDNA6-emi-INHA	mi-INHA	Gene INHA (inhibin alpha subunit)
Chen et al.	2023	*E. coli*	pBSMrna/hBERA^{Leu}	hBERA^{Leu} (miR-22-3p, miR-9-5p and miR-218-5p)	Metabolic folate enzymes (SHMT1, MTHFR, MTHFD2 and MTHFD1L) Alteration in the transfer of one carbon from serine to the folate cycle, glucose uptake and regulate serine biosynthesis
Traber et al.	2024	*E. coli*	pBSKrna/BioRNA$^{Leu/Gly}$	22 BioRNA^{Leu} and 26 BioRNA^{Gly} (miR-143-3p, miR-206-3p, miR-375-3p, miR-497-5p, miR-125b-5p, let-7a-5p, let-7b-5p, let-7c-5p, let-7d-5p, let-7e-5p, let-7f-5p, let-7 g-5p, miR-101-3p, miR-195-5p, miR-370-3p, miR-495-3p, miR-519a-5p, miR-29a-3p, miR-99-5p, miR-100-5p, miR-146a-5p, miR-146b-5p, miR-126b-3p, miR-133a-3p, miR-200b-3p and miR-7-5p)	Except for miR-125b-5p, all the BioRNAs inhibit the proliferation of human NSCLC cells miR-7-5p: EGFR, MRP1 and VDAC1

4.2 Patente de Invenção 1

Pedido nacional de Invenção, Modelo de Utilidade, Certificado de Adição de Invenção e entrada na fase nacional do PCT

Número do Processo: BR 10 2023 012168 3

Figura 4 - Principais informações referentes ao depósito da patente.

Título: “Suplemento alimentar contendo miRNAs para o aumento a tolerância de peixes em baixas temperaturas”

Resumo

A presente invenção abrange as áreas da biotecnologia animal, nutrigenômica e piscicultura. Visa apresentar o processo de produção de um suplemento alimentar contendo miRNAs e antagomiR artificiais capazes de modular as defesas antioxidantes e o nível de glicose, contribuindo para uma melhor tolerância de teleósteos ao frio, logo redução com as percas na produção. O método inclui o desenho e confecção de uma construção de DNA sintético recombinante contendo sequência suficiente e necessária para a síntese das moléculas maduras, seguido da transformação de cepa procariota para produção em pequena, média ou larga escala dos miRNA e antagomiR de interesses. Após a inativação térmica da biomassa microbiana, é realizado o processo de mistura da ração com um adesivo graxo para peixes, finalizando o processo com a secagem do material, para posterior armazenamento adequado, comercialização e utilização na piscicultura. A aplicação de um suplemento alimentar contendo miRNAs artificiais caracteriza-se por apresentar uma solução biotecnológica frente a problemática da produção de peixes em períodos e regiões geográficas de baixa temperatura, diferenciando-se de outros métodos por ser de administração oral e sazonal, característica que corrobora sua produção e aplicação comercial.

Palavras-chave associadas: miRNA; ração; Teleósteo; *O. niloticus*; sobrevivência ao frio;

Busca de anterioridade

Tabela 3 - Apresentação do número de patentes observados em diversos bancos mundiais proteção industrial, a partir de diferentes termos de busca.

Termos de buscas		Resultados da busca					
		INPI	WIPO	USPTO	Google Patents	Espacenet	Portal Capes
1 termo	Suplemento alimentar	146	342	152.712	14.691	230.917	1.273
	Ração	912	21.306	148.247	24.026	301.542	20.549
	miRNA	24	1.286	45.781	248.248	38.868	152.227
	Expressão Heteróloga	32	6.280	200.845	7.645	147.726	33
2 termos	Suplemento alimentar; miRNA	0	119	4.896	206	2.456	0
	Ração; peixe	6	2.282	10.221	2.311	10.624	788
	miRNA; frio	0	116	26.908	8.153	17.926	4
	Expressão Heteróloga; miRNA	0	183	16.177	807	10.880	0
3 termos	Suplemento alimentar; miRNA; *E. coli*	0	92	65	146	1.342	0
	Ração; peixe; miRNA	0	16	530	61	292	0
	miRNA; frio; Tilápia	0	0	0	7	106	0
	Expressão Heteróloga; miRNA; *E. coli*	0	140	33	469	5.878	0

Campo da invenção

A presente invenção refere-se a um suplemento alimentar contendo miRNAs artificiais. Particularmente ao método de produção do suplemento, envolvendo a expressão de miRNAs artificiais de interesse em sistema procariótico e o processo de preparo da ração suplementada com os miRNAs.

A ideia propõe uma solução biotecnológica frente a redução da produção de peixes em períodos e regiões geográficas de baixa temperatura, que acarreta maiores investimentos em alimentação, vacinação, aumento da mortalidade e redução do tamanho e taxa de crescimento dos peixes. Logo, a invenção de um suplemento alimentar apresentado aqui trata de um produto inovador e com

potencial de transferência para empresas de piscicultura e relacionadas a soluções em agropecuária.

Fundamentos da invenção

Da importância da aquicultura

A aquicultura é uma das atividades mais antigas e importantes, sendo a produção de organismos aquáticos documentada desde o tempo imperial chinês, gerando proteína (animal, alga) para consumo alimentar, além de outros produtos de valor agregado, como substâncias emulsificantes e couro. Com o aumento das demandas de proteína animal para consumo, o cultivo de peixes foi sendo modernizado e ampliado, além do cultivo em tanques de terra, a utilização de tanques-rede demonstraram redução da oferta de ração sem afetar a produção do pescado. Segundo a FAO (Food and Agriculture Organization) a aquicultura é responsável por 52% do pescado consumido, demonstrando assim a importância deste segmento na alimentação humana. Observando os dados dos últimos três anos podemos constatar o importante crescimento da piscicultura brasileira, sendo em 2019 produzido um total de 799.560 toneladas de peixe (↑4,9%), em 2020 cerca de 802.930 toneladas (↑5.93%) e em 2021 atingiu 841.005 toneladas, com receita de cerca de R$8 bilhões e crescimento de 4,7%.

Dentre as espécies de peixes produzidas destaca-se a Tilápia-do-nilo (*Oreochromis niloticus*), peixe de água doce que representa cerca de 60,6% da produção de peixes produzidos no Brasil, cerca de 486.155 toneladas no ano de 2020, segundo dados do “Anuário Peixe BR da Piscicultura”, demonstrando assim a importância deste setor produtivo. Nos primeiros nove meses de 2021, as exportações da piscicultura brasileira alcançaram aproximadamente US $12,8 milhões, representando um aumento de 48,3% em relação ao mesmo período do ano anterior. Os principais países importadores são os Estados Unidos, China, Colômbia, Peru e Chile. O México é o segundo maior mercado internacional de produtos de tilápia, e as 228.000 toneladas equivalentes de peso vivo de sua importação de tilápia em 2018 foi superior à sua produção doméstica. No Brasil o principal produtor da região Sul do país é o estado do Paraná, na região Sudeste, os estados de São Paulo e Minas Gerais, e na região Nordeste, o Ceará, Bahia e Pernambuco, havendo nestes estados grandes celeiros de produção.

Dos fatores ambientais atrelados à produtividade

Fatores ambientais como salinidade e temperatura, ligadas aos períodos sazonais de cada região, são atualmente grandes limitantes da piscicultura, principalmente de espécies que estão fora de sua faixa de tolerância ideal. Os animais ficam mais susceptíveis a infecções microbianas, enfraquecimento do sistema imunológico e pressão homeostática, levando ao aumento da mortalidade ou condições subletais.

A tilápia é considerada um animal pecilotérmico, oriundo de regiões tropicais e subtropicais de águas quentes (26°C e 28°C) e sua reprodução está diretamente ligada à temperatura (±24°C). Quando abaixo de 20°C o animal passa a um estágio de estresse, já demonstrando redução da movimentação e de hábitos alimentares e indo à óbito em temperaturas entre 11 e 17°C, acarretando maiores custos e perdas de produção em períodos mais frios (inverno), em regiões mais distantes da linha do equador. Após o cultivo de tilápias em diferentes períodos (verão/inverno) foi observado uma redução de ±20% no peso médio dos animais durante o inverno, enquanto os peixes cultivados no verão apresentam melhores desempenhos (média de 460,40 g).

Mudanças bruscas de temperatura geram várias respostas oxidativas, frente a adaptação celular a nova condição os organismos desenvolveram mecanismos de defesa antioxidantes. Para se adaptar à nova condição ambiental de frio e manter as funções fisiológicas, os peixes aumentam os níveis de insaturação de ácidos graxos dos fosfolipídios que compõem suas membranas celulares, adaptação homeoviscosa. Períodos sazonais requerem todo um tratamento mais acurado no cultivo de peixes, seja com o manejo alimentar ou fornecimento de antibióticos para prevenção e tratamento de patologias associadas. Tratando-se de adaptações sazonais em regiões subtropicais, o inverno (baixa da temperatura) é responsável por grandes perdas econômicas na região sul do Brasil, assim como em países que apresentam um inverno mais rigoroso, regiões de clima temperado e subtropical, logo, é de grande importância a utilização e desenvolvimento de técnicas e produtos eficazes na adaptação e manutenção da produtividade de organismos oriundos da aquicultura em períodos frios.

Da suplementação alimentar

A qualidade da alimentação fornecida é significativamente importante no cultivo de qualquer organismo, favorecendo a manutenção do seu desenvolvimento (crescimento, reprodução), e reduzindo efeitos metabólicos indesejáveis e infecções. A alimentação é responsável por grande parte dos gastos da produção em aquicultura, sendo fornecida em quantidades e concentrações pré-estabelecidas, ela mantém as taxas corporais em condições menos estressantes e contribui diretamente no incremento muscular da carcaça dos animais. Nutricionalmente falando, as tilápias necessitam de taxas específicas de lipídeos (10-15%), proteínas (>40%), assim como carboidratos, sais minerais e vitaminas.

Durante adaptações a condições de baixas temperaturas as tilápias requerem a adição de suplementos a ração, como vitaminas (C e E), aminoácidos, probióticos e prebióticos específicos, conferindo melhorias substanciais no crescimento, engorda e respostas imunológicas. Além destes a otimização da relação ácido linoleico (18:2) e ácido linolênico (18:3) na dieta melhora o perfil de ácidos graxos dos peixes. Em uma temperatura fria abaixo do ideal, a Tilápia-do-nilo requer maiores proporções de ácidos graxos poliinsaturados (PUFA), fosfolipídios (PL),

colesterol e l-carnitina podem melhorar a tolerância térmica dos peixes, melhorando sua digestibilidade e desenvolvimento em condições adversas.

A adição de biomassa de microalgas ou fungos favorece o desenvolvimento quando comparado a dietas convencionais baseadas em rações comerciais. A spirulina (*Arthrospira platensis*) é uma das algas mais testadas e utilizadas como suplemento alimentar contra distúrbios fisiológicos, conferindo proteção contra danos oxidativos e efeitos imunoestimulantes. Por ser um bom produtor de ômegas o fungo *Aurantiochytrium* sp. também já foi testado como suplemento alimentar, resultando num melhor crescimento dos animais, uma ótima eficiência alimentar, além de uma composição lipídica muscular de ácidos graxos poliinsaturados (n-3/n-6) satisfatória para comercialização.

Assim, faz-se necessário a busca por novas moléculas e produtos que contribuam para o desenvolvimento de animais em sistemas de aquicultura, principalmente em condições adversas, como períodos sazonais de inverno. Com os avanços obtidos no campo da nutrição animal está sendo possível driblar alguns gargalos produtivos, mas ainda existe necessidade e espaço para inovação e desenvolvimento de tecnologias no campo da produção animal.

Dos microRNAs na alimentação

Além dos contribuintes oriundos da alimentação, os processos fisiológicos e metabólicos podem ser controlados por mecanismos endógenos, categorizados como mecanismos epigenéticos, dentre os quais, destacam-se os microRNAs (miRNAs), que são moléculas de ácido ribonucleico (RNA) medindo 18-22 nucleotídeos não codificantes com atividade de regulação pós-transcricional da expressão gênica. Por seu caráter modulador de fenótipos fisiológicos e natureza pleiotrópica, essas moléculas estão sendo estudadas para diferentes aplicações e dentre diferentes espécies e reinos.

Naturalmente fazemos ingestão diária de vários microRNAs através da alimentação, seja através de frutas, grãos, carne e chás. Estudos recentes conseguiram mapear o caminho que os miRNA dietéticos percorrem. Após todo processo de mastigação e deglutição do alimento os miRNAs presentes são liberados por digestão mecânica e absorvidos pelos transportadores SIDT1, expressos em células PIT do epitélio estomacal. Exossomos estão envolvidos em diferentes passos do processamento, degradação e a absorção de miRNAs, assim como conferem uma proteção extra contra sua degradação. Após a captação pelas células PIT os miRNAs são secretados para o sistema circulatório como entidades funcionais em exossomos e vão se encaminhando aos seus alvos.

microRNAs são capazes de modular genes e vias moleculares. Estudos demonstraram que a superexpressão de miR-34 através da alimentação promoveu uma resposta imune antiviral em camarões contra a síndrome da mancha branca. Ao mesmo tempo, o miR-34 antiviral exibiu atividade antitumoral em camundongos alimentados com camarão que superexpressam miR-34. Tal estudo demonstrou que

os miRNAs disponibilizados na dieta foram capazes de controlar simultaneamente a doença viral do camarão e a tumorigênese humana, sendo esta uma estratégia eficaz e econômica.

Objetivos da invenção

Atualmente existe uma demanda por um suplemento alimentar que promova o melhoramento da tilapicultura de forma barata e segura em períodos e regiões geográficas de baixas temperaturas. Assim, se faz necessário o desenvolvimento de tecnologias que se baseiam em uma via natural de introdução de miRNAs, a alimentação. Assim, a invenção aqui descrita tem como objetivo o desenvolvimento de um suplemento alimentar contendo miRNAs artificiais que permitam uma redução dos efeitos oxidativos do metabolismo, da desregulação da homeostase da glicose, contribuindo para uma melhor tolerância ao frio em peixes e, consequentemente, maiores taxas de sobrevivência durante o estresse causado por este fator.

Descrição detalhada da invenção

A presente invenção refere-se ao método de produção de um suplemento alimentar contendo miRNAs artificiais para o aumento da tolerância de peixes em baixas temperaturas. O miRNAs artificiais foram desenhados baseando-se na sequência de interesse, mRNA alvo, utilizando o programa AmiRNA Designer.

Preferentemente, o método pode ser realizado obedecendo o seguinte protocolo: inicialmente deve ser realizado a expressão das moléculas sintéticas correspondentes aos miR-122, miR-30b, miR-135c, miR-29b e miR-9-3p maduros no citoplasma do sistema procariótico de *Escherichia coli*. Deve ser usado um plasmídeo comercial com sítios múltiplos de clonagem para a inserção das sequências dos microRNAs artificiais de interesse posteriormente. As sequências do DNA sintético para expressão de cada molécula madura de fita dupla com sítios de restrição podem ser encontradas sob as seguintes identificações: SEQ ID No.1, SEQ ID No.2, SEQ ID No.3, SEQ ID No.4, SEQ ID No.5, SEQ ID No.6, SEQ ID No.7, SEQ ID No.8, SEQ ID No.9, SEQ ID No.10, SEQ ID No.11, SEQ ID No.12, SEQ ID No.13, SEQ ID No.14, SEQ ID No.15, SEQ ID No.16, SEQ ID No.17, SEQ ID No.18, SEQ ID No.19 e SEQ ID No.20. Separadamente cada sequência de DNA sintético deve ser inserida ao respectivo plasmídeo a ser utilizado em um eppendorf, sendo o plasmídeo do vetor LITMUS 38i tratado a 35-38°C por 55-65 minutos com endonucleases, após a digestão dos sítios de restrição as moléculas devem ser unidas utilizando a enzima de ligação T4 DNA Ligase. A inserção dos plasmídeos nas células deve ser realizada através de choque de temperatura, as bactérias recombinantes devem ser selecionadas utilizando 80-110 µg.mL-1 de ampicilina no meio de cultivo LB (Luria Bertani) a 36-37°C durante 12h.

O cultivo das células recombinantes de *Escherichia coli* pode ser realizado em biorreatores convencionais, do tipo tanque agitado e aerado, e em biorreatores não convencionais pneumáticos, tipo coluna de bolhas e tipo airlift. As células podem ser

cultivadas utilizando diferentes modos de operação, dentre eles o modo descontínuo (batch), o qual tem sido utilizado amplamente na indústria.

Subsequente, após o cultivo das bactérias que expressam as moléculas de interesse, deve ser realizada a separação do meio de cultivo, a filtragem das células pode ser realizada utilizando uma membrana 0,22 µm. Para seguir com a mistura na ração as células devem ser inativadas em banho-maria a 55-65°C pelo período de tempo compreendendo de 20 min a 30 min, seguido da filtragem e secagem em estufa de circulação forçada na faixa de temperatura de 55°C a 65ºC por 50-60 min.

Posteriormente, em um misturador industrial deve ser colocado as células de *E. coli* secas, uma ração comercial devidamente balanceada para peixes, assim como um óleo para facilitar a incorporação dos componentes. O material deve ser misturado entre 15 e 30 minutos de forma homogênea. Após a mistura dos ingredientes o suplemento deve ser seco em estufa de circulação forçada na faixa de 55 a 65°C por 45-75 h. O suplemento deve ser armazenado em recipiente estéril, selado e longe da luz até o uso para preservar as características alimentares do produto.

5 CONCLUSÃO GERAL

Esta tese demonstrou, por meio de uma revisão sistemática e do desenvolvimento de uma patente, o potencial da produção heteróloga de miRNAs em bactérias como ferramenta biotecnológica para a aquicultura. A análise crítica da literatura identificou *Rhodovulum sulfidophilum* como o microrganismo mais promissor para expressão de miRNAs, devido a suas características únicas: secreção de ácidos nucleicos, ausência de RNases, cultivo em água salgada e não patogenicidade. Esses atributos a tornam ideal para aplicações em biorrefinarias e aquicultura, com vantagens sobre modelos convencionais como *Escherichia coli* – esta, por sua vez, mantém relevância para produção em larga escala, dada sua versatilidade e infraestrutura industrial consolidada.

Quanto aos vetores de expressão, os resultados destacaram a superioridade de plasmídeos com promotor lpp e sítios de clonagem múltipla em *E. coli*, enquanto para *R. sulfidophilum* o plasmídeo pBHSR1-RM (modificado com promotor pCF-rpAM1 e elementos de estabilização de RNA) mostrou-se mais eficiente. Esses achados subsidiaram o desenvolvimento de uma patente inédita: um suplemento alimentar contendo miRNAs artificiais (miR-122, miR-30b, miR-29b, entre outros) produzidos em *E. coli* e formulados para aumentar a tolerância de peixes a baixas temperaturas. O método patenteado combina:

- Design computacional de miRNAs (AmiRNA Designer),
- Expressão heteróloga em biorreatores (com vetor LITMUS 38i),
- Inativação térmica da biomassa bacteriana, e
- Encapsulação em ração com adjuvantes lipídicos.

A formulação age modulando vias metabólicas (ex.: glicólise) e antioxidantes, oferecendo uma solução não transgênica, oral e sazonal – diferencial frente a abordagens como injeção ou modificação genética direta. A escolha de *E. coli* para a patente justifica-se pela escalabilidade industrial, embora futuros estudos possam adaptar o processo para *R. sulfidophilum*, aproveitando seu perfil de segurança e compatibilidade com ambientes aquáticos.

Impacto e Perspectivas

Esta pesquisa avança em duas frentes:

- Científica: Consolida *R. sulfidophilum* como plataforma emergente para produção de RNAs não codificantes, com insights sobre design de vetores e processamento de miRNAs.
- Tecnológica: Oferece um produto comercializável para mitigar perdas na aquicultura, com potencial adaptação a outros estresses (ex.: doenças, hipóxia).

Como próximos passos, sugere-se:

- Desenvolver *R. sulfidophilum* geneticamente modificada para expressar miRNAs;
- Validar a produção dessas moléculas;
- Produzir o suplemento contendo a cepa produtora do miRNA, e administrar em espécies padrão de experimentação (*D. rerio*) e comerciais (ex.: *Salmo salar*, *Oreochromis niloticus*);
- Explorar a produção em *R. sulfidophilum* para reduzir custos;
- Investigar sinergias com outras plataformas (ex.: probióticos).

Conclui-se que a integração entre engenharia genética microbiana e nutrição animal – como demonstrado aqui – abre novas rotas para a biotecnologia aplicada à aquicultura, alinhando inovação e sustentabilidade.

6 REFERÊNCIAS

ABDELKHALEK, N. K. M. et al. Protective role of dietary Spirulina platensis against diazinon-induced Oxidative damage in Nile tilapia; Oreochromis niloticus. Environmental Toxicology and Pharmacology, v. 54, p. 99-104, 2017. ABO-AL-ELA, H. G. RNA interference in aquaculture: A small tool for big potential. Journal of Agricultural and Food Chemistry, v. 69, n. 15, p. 4343-4355, 2021.

ALAGAWANY, M. et al. Nutritional applications of spirulina and chlorella in farmed fish: A review. Aquaculture, p. 736841, 2021.

BARRAZA-GUARDADO, R. H. et al. The use of microalgae as partial constituents of growout feeds for tilapia (Oreochromis niloticus). Biotecnia, v. 22, n. 1, p. 135-141, 2020.

BARROSO, R. M., Muñoz, A. E. P., & Cai, J. Social and economic performance of tilapia farming in Brazil. Embrapa Pesca e Aquicultura-Fôlder/Folheto/Cartilha(INFOTECA-E), 2019. BARTEL, D. P. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell, v. 116, n. 2, p. 281-297, 2004.

BHUJEL, R. C. A review of strategies for the management of Nile tilapia (Oreochromis niloticus) broodfish in seed production systems, especially hapa-based systems. Aquaculture, v. 181, n. 1-2, p. 37-59, 2000.

BIZUAYEHU, T. T.; BABIAK, I. MicroRNA in teleost fish. Genome biology and evolution, v. 6, n. 8, p. 1911-1937, 2014.

BLÖDORN, E. B. et al. MicroRNA roles and their potential use as selection tool to cold tolerance of domesticated teleostean species: A systematic review. Aquaculture, p. 736747, 2021.

BOSCOLO, W. R. et al. Composição química e digestibilidade aparente da energia e nutrientes da farinha de resíduos da indústria de filetagem de tilápias, para a tilápia do Nilo (Oreochromis niloticus). Ciência Rural, v. 38, p. 2579-2586, 2008. BROUGHTON, J. P. et al. Pairing beyond the seed supports microRNA targeting specificity. Molecular cell, v. 64, n. 2, p. 320-333, 2016.

CAMPOS, V. F. et al. MicroRNAs e seu papel no desenvolvimento embrionário. Ciência Rural, v. 41, p. 85-93, 2011.

CASTILHO-BARROS, L. et al. Economic feasibility of tilapia culture in southern Brazil: A small-scale farm model. Aquaculture, v. 515, p. 734551, 2020.

CHOU, B. S.; SHIAU, S. Y. Optimal dietary lipid level for growth of juvenile hybrid tilapia, Oreochromis niloticus x Oreochromis aureus. Aquaculture, v. 143, n. 2, p. 185-195, 1996. CHEN, Q. et al. SIDT1-dependent absorption in the stomach mediates host uptake of dietary and orally administered microRNAs. Cell research, v. 31, n. 3, p. 247-258, 2021.

CUI, Y.; WAN, H.; ZHANG, X. miRNA in food simultaneously controls animal viral disease and human tumorigenesis. Molecular Therapy-Nucleic Acids, 23, 995-1006. 2021.

CYRINO, J. E. P. et al. Aquicultura, segurança alimentar e qualidade do pescado.Aquaculture Brazil. V.21. p.11-16. 2019.

DA SILVA, L. S. et al. Capillary electroporation affects the expression of miRNA-122-5p from bull sperm cells. Gene, v. 768, p. 145286, 2021.

DE MORAES, C. R. F.; DE ATTAYDE, J. L.; HENRY-SILVA, G. G. Stable isotopes of C and N as dietary indicators of Nile tilapia (Oreochromis niloticus) cultivated in net cages in a tropical reservoir. Aquaculture Reports, v. 18, p. 100458, 2020.

DE OLIVEIRA, E. G. et al. Produção de tilápia: mercado, espécie, biologia e recria. Embrapa Meio-Norte-Circular Técnica (INFOTECA-E), 2007.

DEL POZO-ACEBO, L. et al. Bovine milk-derived exosomes as a drug delivery vehicle for miRNA-based therapy. International journal of molecular sciences, v. 22, n. 3, p. 1105, 2021. FERNANDES-SILVA, M. M. et al. Exercício físico e microRNAs: novas fronteiras na insuficiência cardíaca. Arquivos Brasileiros de Cardiologia, v. 98, p. 459-466, 2012.

FAO - Food and Agriculture Organization of the United States. The State of World Fisheries and Aquaculture 2024 – Blue Transformation in action. Rome. 2024. https://doi.org/10.4060/cd0683en

FURLANETO, F. P. B.; AYROZA, D. M. M. R.; AYROZA, L. M. S. Análise econômica da produção de tilápia em tanques-rede, ciclo de verão, região do médio Paranapanema, Estado de São Paulo, 2009. Informações Econômicas, v. 40, n. 4, p. 5-11, 2010. GANDIKOTA, M. et al. The miRNA156/157 recognition element in the 3′ UTR of the Arabidopsis SBP box gene SPL3 prevents early flowering by translational inhibition in seedlings. The Plant Journal, v. 49, n. 4, p. 683-693, 2007.

GANDHI, G. et al. The potential role of miRNA therapies in spinal muscle atrophy. Journal of the Neurological Sciences, p. 117485, 2021.

GISMONDI, A. et al. Plant miR171 modulates mTOR pathway in HEK293 cells by targeting GNA12. Molecular Biology Reports, v. 48, n. 1, p. 435-449, 2021.

HOARE, R. et al. Oral vaccination of Nile tilapia (Oreochromis niloticus) against francisellosis elevates specific antibody titres in serum and mucus. Fish & Shellfish Immunology, v. 113, p. 86-88, 2021.

ISMAIL, M. S. et al. Feed-based vaccination regime against streptococcosis in red tilapia, Oreochromis niloticus x Oreochromis mossambicus. BMC veterinary research, v. 12, n. 1, p. 1-6, 2016.

KHURANA, P.; GUPTA, A.; VARSHNEY, R. Diet-Derived Exogenous miRNAs As Functional Food Components: Facts and New Perspectives. Preprints. 2021. LEE, R. C.; FEINBAUM, R. L.; AMBROS, V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell, v. 75, n. 5, p. 843-854, 1993.

LI, D. et al. A Timely Review of Cross-Kingdom Regulation of Plant-Derived MicroRNAs. Frontiers in Genetics, v. 12, p. 702, 2021. LI, Z.; XU, R.; LI, N. MicroRNAs from plants to animals, do they define a new messenger for communication?. Nutrition & metabolism, v. 15, n. 1, p. 1-21, 2018.

LISBOA, V. et al. Piscicultura marinha brasileira: desafios e perspectivas do seu desenvolvimento no estado do Ceará. Sistemas & Gestão, v. 15, n. 2, p. 113-122, 2020. MORI, M. A. et al. Extracellular miRNAs: from biomarkers to mediators of physiology and disease. Cell metabolism, v. 30, n. 4, p. 656-673, 2019.

NICO, L.G.; SCHOFIELD, P.J.; NEILSON, M.E. "Oreochromis niloticus (Linnaeus, 1758)" . Food and Agriculture Organization, United Nations . 2019. Acessado em 20/08/2021 as 23:40 h. <http://www.fao.org/fishery/culturedspecies/Oreochromis_niloticus/en#tcNA014C>.
O'BRIEN, J. et al. Overview of microRNA biogenesis, mechanisms of actions, and circulation. Frontiers in endocrinology, v. 9, p. 402, 2018.

PAULINO, R. R. et al. Optimal dietary linoleic acid to linolenic acid ratio improved fatty acid profile of the juvenile tambaqui (Colossoma macropomum). Aquaculture, v. 488, p. 9-16, 2018. PENG, C.; WANG, Y. MicroRNAs as new players in endocrinology. Frontiers in endocrinology, v. 9, p. 459, 2018.

PLANT, K. P.; LAPATRA, S. E. Advances in fish vaccine delivery. Developmental & Comparative Immunology, v. 35, n. 12, p. 1256-1262, 2011.

POOLSAWAT, L. et al. O xilo-oligossacarídeo dietético melhorou o crescimento, a utilização de nutrientes, a microbiota intestinal e a resistência da tilápia a doenças (Oreochromis niloticus x O. aureus). Animal Feed Science and Technology , v. 275, p. 114872, 2021.

RAGAP, H. M.; KHALIL, R. H.; MUTAWIE, H. H. Immunostimulant effects of dietary Spirulina platensis on tilapia Oreochromis niloticus. Journal of Applied Pharmaceutical Science, v. 2, n. 2, p. 26, 2012.

RAZA, S. H. A. et al. MicroRNAs mediated environmental stress responses and toxicity signs in teleost fish species. Aquaculture, p. 737310, 2022.

REBOUÇAS, P. M. et al. Influence of thermal oscillation on pisciculture water. Journal of Animal Behaviour and Biometeorology, v. 2, n. 2, p. 35-42, 2020.

RUPAIMOOLE, R.; SLACK, F. J. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. Nature reviews Drug discovery, v. 16, n. 3, p. 203-222, 2017.

SAINT-PAUL, U. Native fish species boosting Brazilian’s aquaculture development. Acta of Fisheries and Aquatic Resources, v. 5, n. 1, p. 1-9, 2017.

SAQUIB, M. et al. Exogenous miRNA: A Perspective Role as Therapeutic in Rheumatoid Arthritis. Current Rheumatology Reports, v. 23, n. 6, p. 1-7, 2021.

SARKER, P. K. et al. Towards sustainable aquafeeds: Evaluating substitution of fishmeal with lipid-extracted microalgal co-product (Nannochloropsis oculata) in diets of juvenile Nile tilapia (Oreochromis niloticus). PLoS One, v. 13, n. 7, p. e0201315, 2018.

SCORVO FILHO, J. D. et al. A tilapicultura e seus insumos, relações econômicas. Revista Brasileira de Zootecnia, v. 39, p. 112-118, 2010. SILVA, D. C. P. et al. Papel dos miRNAs na Fisiopatologia das Doenças Cardiovasculares. Arquivos Brasileiros de Cardiologia, v. 111, p. 738-746, 2018.

SIRINGI, J. O.; TUROOP, L.; NJONGE, F. Growth and biochemical response of Nile tilapia (Oreochromis niloticus) to spirulina (Arthrospira platensis) enhanced aquaponic system. Aquaculture, v. 544, p. 737134, 2021.

TAKEUCHI, T. et al. Effect on the growth and body composition of juvenile tilapia Oreochromis niloticus. Fisheries Science, v. 68, n. 1, p. 34-40, 2002.

VALENTI, W. C. et al. Aquicultura no Brasil: passado, presente e futuro. Aquaculture Reports , v. 19, p. 100611, 2021.

WOLDEMARIAM, N. T. et al. Expanding the miRNA repertoire in Atlantic salmon; discovery of IsomiRs and miRNAs highly expressed in different tissues and developmental stages. Cells, v. 8, n. 1, p. 42, 2019.

XIA, Y. et al. Efeitos da suplementação de probióticos na dieta sobre o crescimento, saúde intestinal e resistência a doenças de juvenis de tilápia do Nilo (Oreochromis niloticus). Nutrição Animal , v. 6, n. 1, pág. 69-79, 2020.

XIE, D. et al. Effects of dietary carbohydrate and lipid levels on growth and hepatic lipid deposition of juvenile tilapia, Oreochromis niloticus. Aquaculture, v. 479, p. 696-703, 2017.

XIONG, Y. et al. Deep sequencing of the tilapia (Oreochromis niloticus) liver transcriptome response to dietary protein to starch ratio. Aquaculture, v. 433, p. 299-306, 2014.

YU, Y. et al. MicroRNAs: The novel mediators for nutrient-modulating biological functions. Trends in Food Science & Technology, 2021. ZHOU, W. et al. Research Progress on the Regulation of Nutrition and Immunity by microRNAs in Fish. Fish & Shellfish Immunology, v.113, p. 1-8, 2021.

ZHOU, X. et al. Effect of treatment with probiotics as water additives on tilapia (Oreochromis niloticus) growth performance and immune response. Fish physiology and biochemistry, v. 36, n. 3, p. 501-509, 2010.