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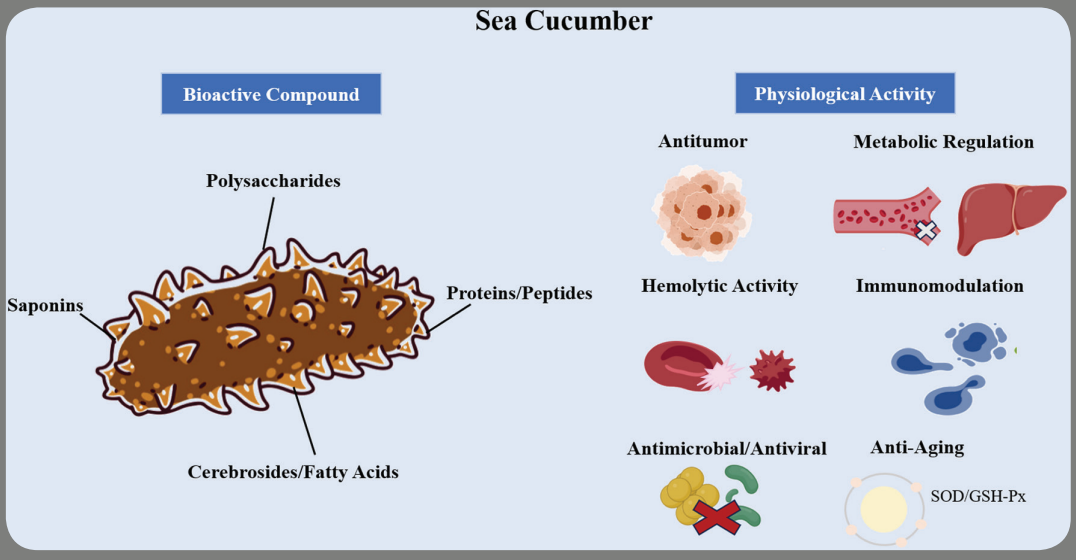

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Advancing food processing in Africa: Challenges, innovations and opportunities report on the IUFoST scientific roundtable

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Abstract

This contribution summarizes the outcome of a Scientific Roundtable Discussion webinar, jointly organized by the International Union of Food Science and Technology (IUFoST) and the Association of African Universities (AAU) on the topic of *Advancing Food Processing in Africa*. The event focused on addressing challenges such as post-harvest losses, especially of traditional and indigenous crops, limited infrastructure to scale up traditional processing technologies, difficulties in securing a consistent supply of quality ingredients for the food sector, and the confusion created by some food classification systems. Discussions emphasized the importance of multi-stakeholder collaborations to explore opportunities for value addition to agro-produce in Africa through sustainable and nutrition-focused food processing with retention of bioactive compounds, thereby enhancing food security and economic growth on the continent.

Keywords: Africa; (Traditional) food processing; Smallholder farmers; First mile approach; IUFoST Formulation & Processing Classification Scheme; Valorization; Bioactives.

1. Introduction

The Scientific Roundtable on Advancing Food Processing in Africa: Challenges, Innovations, and Opportunities, jointly organized by the International Union of Food Science and Technology (IUFoST) and the Association of African Universities (AAU), convened a diverse group of distinguished experts, researchers, policymakers, and participants from around the world. Held on January 21, 2025, this webinar explored strategies for scaling up food processing technologies in Africa to enhance food security, economic growth, and sustainability.

It was emphasized that food processing is not merely about transforming farm produce to palatable and shelf-stable products, but also has implications for food safety and public health. For example, it was discussed that African traditional food fermentations improve nutritional quality by increasing the digestibility of proteins and release of bioactive compounds, while improving the bioavailability of minerals and *de novo* synthesis of vitamins (Hotz and Gibson, 2007). Also, some of the microorganisms used in the fermentation process could health-promoting probiotics (Setta et al., 2020). Moreover, lactic acid bacteria of traditional fermentations produce antimicrobials such as lactic acid, propionic acid, diacetyl and bacteriocins, thus improving the safety and extending the shelf life of the product (Mokoena, et al., 2016). A key focus

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of these remarks that were given was the importance of traditional fermentation techniques in assuring food security and health. It was explained how these methods help eliminate toxic substances, such as cyanogenic glycosides in cassava, which can otherwise lead to fatal hydrogen cyanide poisoning. Dietary exposure to hydrogen cyanide from cassava products in Africa is well reported in the scientific literature (Padmaja and Steinkraus, 1995; Kobawila, et al., 2005; Nebiyu and Getachew, 2011; Apeh, et al., 2021). To underscore this point, references were made to food safety incidents including cases in Nigeria in 2024 (Azubuike, 2024), and Uganda in 2017 (Alitubeera, et al., 2019), where improper cassava processing resulted in deaths. These incidents, highlight the urgent need for scientific advancements and the scaling up of food processing technologies across the continent.

2. Scaling up traditional food processing technologies in Africa

It was emphasized that the deep cultural and economic significance of traditional food processing, noting that it is an integral part of African heritage and plays a vital role in sustaining local economies (Aworh, 2023). Addressing the challenges in traditional food processing, several key barriers to efficiency were highlighted. For example, many traditional methods rely on manual labour and localized equipment, resulting in inconsistencies in product quality. The lack of mechanization makes large-scale production difficult, limiting the potential for expansion. Additionally, many traditional foods and processing techniques struggle to meet regulatory and health standards, which hinders their acceptance in formal markets (Oguntoyinbo, 2014).

It was further stressed the need to scale up traditional food processing technologies, outlining the numerous benefits of doing so. Enhancing these technologies would significantly improve food security by increasing food availability, creating employment opportunities for young people in food processing industries, and enabling African food products to compete on the continent and in global markets. Strategic approaches to achieving this goal were then outlined. Investment in research and development was highlighted as being crucial for the advancement of food processing technologies, while capacity building through training programmes could empower small-scale processors. Public-private partnerships could attract the necessary investment and ensure market access, while governmental support through policies, tax incentives, and grants would provide the necessary financial and regulatory framework. Collaboration between research institutions and processors was also highlighted as essential for implementing new technologies effectively.

Successful examples of scaling traditional food processing were mentioned, including mechanized cassava processing in Nigeria, which has led to the production of high-quality cassava flour. The expansion of fermented dairy products from Ethiopia into markets beyond Africa, as well as advancements in drying and dehydration methods through the use of solar and hybrid dryers, were also mentioned. Additionally, standardisation of fermentation technologies has been introduced to ensure product consistency and safety.

In conclusion, it was emphasized that researchers, policymakers, industry leaders, and traditional food producers work together to scale up food processing technologies while preserving their authenticity and nutritional value. It was emphasized that a collaborative approach is essential for achieving sustainable food systems that benefit both local communities and the broader African economy.

3. Positioning processed and ultra-processed foods in food classification systems-policy and regulatory implications

The significance of food classification systems and their impact on food policy and regulation that support information to the public, whether through food labelling measures or overall consumer awareness initiatives was discussed. It was mentioned that food classification systems include the reliance on the level of processing as one of the parameters to categorize foods accordingly. The intent is to have a classification system addresses nutritional and societal impacts of dietary choices, but also touches on policies and decisions related to the need to address the possible inherent risks of chronic diseases which are associated with food.

The growing influence of food classification systems in shaping food regulatory policies, consumer awareness, and public health initiatives was highlighted, as reported (Moubarac, et al., 2014; Monteiro et al., 2018; Cuj et al., 2021). It was pointed out that while these systems aim to categorize foods based on processing levels (unprocessed or minimally processed foods, processed culinary ingredients, processed foods, and ultra-processed foods), they often oversimplify the complex relationship between food processing and health outcomes, given that the foods are subjected to more than processing but also formulation change, which includes added sugars, fats or salt. A word of caution against the tendency to associate all processed foods with negative health effects was noted, stressing that food processing encompasses a broad range of techniques that improve food safety, shelf life, and nutritional value. Processing includes a wide range of physical, chemical, and biological operations that enhance flavour, texture and even the accessibility to foods, which is crucial in Africa, where food processing is an important contributor to food safety and food security.

The misconceptions about processed foods, such as the belief that all processed foods are unhealthy, was stressed, as well as how such views can hinder the adoption of essential food preservation techniques like freezing and pasteurization. These methods play a crucial role in maintaining food safety and reducing food waste, particularly in regions like Africa where food security remains a pressing issue. Furthermore, the work of the IUFoST task force dedicated to developing a scientifically sound and methodologically robust food classification system was noted along with the importance of collaboration between food science and nutrition experts, citing ongoing partnerships with the International Union of Nutritional Sciences as a key step in ensuring that classification frameworks are well-informed and evidence-based.

The need for continued research and dialogue to refine food classification methodologies, support consumer education, and inform policy decisions such as front-of-pack labelling, marketing restrictions, and food taxation policies was emphasized. IUFoST's commitment to advancing food science for the benefit of global food security and public health was reiterated.

4. 'First mile approach to building a resilient and competitive food processing sector in Africa'

The role of food science and technology in advancing Africa's food processing sector was comprehensively analysed. The importance of integrating traditional knowledge with modern scientific approaches to enhance food security, nutritional quality, and economic opportunities across the continent was also emphasised. The key challenges facing the industry, including inadequate infrastructure, limited access to advanced processing technologies, and the need for regulatory harmonization across African nations

was highlighted. It was stressed that one of the challenges faced by food manufacturers in Africa is the challenge of securing ingredients/raw materials of consistent quality and quantity for their operations. Several examples of case studies across Africa on how the 'first mile approach' could improve the competitiveness and quality of food processing in Africa caried out by UNIDO was presented.

The first-mile approach – a reference to the setting up of produce collection or consolidation points within one mile of farms is an effective strategy used in the agricultural sector to control post-harvest changes to food products and limit product losses was discussed. The first mile approach, originally developed for the transportation/service delivery sector (Strover, 2000), has been applied in other sectors, including agriculture. It is particularly important for small-holder farmers, and works on the principle that a service (e.g., cold storage or drying facilities) will be located in a central location that is within one mile of selected farm locations. The relatively quick access to such facilities, coupled with training to farmers, goes a long way in assuring product quality and preventing or reducing post-harvest losses. The central location of these facilities also means that food manufacturers only need to visit these locations and have access to various products that are grouped, sorted, and processed or packaged based on certain desired criteria.

Several case studies and success stories of the use of the first mile approach in the food processing sector were provided. For example, the installation of a drying facility in an East African country, through the first mile approach, significantly reduced inconsistencies in quality of products supplied to the World Food Program. Other examples mentioned included the training of local farmers and establishment of a first mile locations for vanilla, moringa, and sesame production in Madagascar, Ethiopia, and Sudan respectively. The successes achieved in the aforementioned cases have led to private sector involvement and export of products from those countries. Importantly, when local farmers are given a demonstration of how first mile approaches work and the value it creates for the food supply chain, farmers are often self-motivated to adopt post-harvest practices that assure product quality and maintains the premium status of their produce.

Thus, to improve food processing in Africa, small holder farmers are important players that should not be ignored. There is also a need to look at the source of food ingredients and work with local farmers to establish first mile locations to act as collection points for their products, thereby increase the quality and quantities of raw materials, and competitiveness of farm produce.

5. Food processing for nutrition, diet and health, on refining the role of processing in food classification systems

The science-driven approach to food structuring and processing as a crucial enabler of sustainable food production and nutrition was explained. The necessity of understanding food structure at multiple levels, from molecular composition to macroscopic properties, to develop healthier, more efficient, and environmentally friendly food products, as mentioned in Joardder et al. (2017), was also emphasised. In this, three fundamental principles that guide innovations in food processing were outlined. The first was material and process engineering, which focuses on optimizing the conversion of raw materials through physical, chemical, and biological transformations. The second was sustainable food structuring, which leverages advanced technologies to design foods with enhanced nutritional profiles and improved functional properties. While, the third was digitalization and modelling, where computational tools are utilized to enhance process efficiency and predict food behaviour with greater accuracy. There was also a discussion on the transformative potential of alternative proteins, emulsions, and bio-based materials in developing novel food structures. The importance of food classification systems being scientifically grounded, cautioning against oversimplified categorizations that fail to capture the complexities of food processing was emphasized.

Some of the limitations of the NOVA classification system (proposed in Monteiro et al., 2018) were explained. Namely, 1) this system conflates 'formulation' with 'processing', 2) does not quantify the extent to which one might describe a product as being 'formulated' or 'processed', and 3) is based on a weak correlation between consumption of foods in certain NOVA food classes and health risks. The work of the IUFOST Task Force on Food Processing for Nutrition, Diet and Health in developing the IUFoST Formulation & Processing Classification (IF&PC) scheme was discussed. The IUFOST IF&PC scheme has recently been published (Ahrné, et al., 2025).

The key difference between formulation, i.e., systematic selection of relative quantities of ingredients for a food product, and processing, i.e., treatment of a food materials to achieve a desired effect was noted. The IF&PC scheme integrates the impact of formulation on nutrition value, and how this scheme does it was discussed. Case in point, to tease out the impact of formulation, the nutrient-rich food (NRF) index is used and computes the difference between quantities of 'qualifying nutrients' (i.e., those nutrients whose intake are encouraged, e.g., protein, fibre, vitamins, bioactive compounds, etc.), and 'disqualifying nutrients' (i.e., those nutrients whereby excessive intake are discouraged, e.g., sodium, added sugar, saturated fats) expressed as portion base (e.g., per 100 g of food or per kCal or per serve).

The impact of processing uses process analysis methodology, which relies on mass and energy balance entering and exiting a processing system. The changes in the NRF index of a food before and after processing therefore is the impact of processing on that food. The coupling of both parameters yields the food processing and formulation index (FPFI), which is a mathematical equation for the quantitative estimation of how various formulation and processing affects the nutrient value of food.

A classification matrix diagram was used to explain and give scenarios on how FPFI can be used in everyday settings. For example, the fortification of a food product with essential minerals would increase the qualifying nutrient factor in the equation, leading to a positive outcome on the NRF, while added sugars would do the reverse. When this product is then subjected to processing, e.g. thermal processing, any changes in the nutrient content can be reliable quantified to access how the nutrition of the product changed with processing.

There was also elaboration on how the IF&PC system could be extended and applied to consider other areas of interest to consumers such as anti-nutrient contents. With the aid of a classification matrix diagram, the speaker explained how the value of processing in reducing anti-nutrients in food could be quantified using the IF&PC. Other areas of application of this system include sustainability, palatability, safety, convenience, affordability, and digestibility.

The need for interdisciplinary collaboration between food scientists, engineers, industry partners, and policymakers was again noted and stressed. It was also emphasized that innovation in food processing must be driven by scientific research and technological advancements to ensure sustainable and nutrition-focused solutions that address global food challenges. Captured in Figure 1

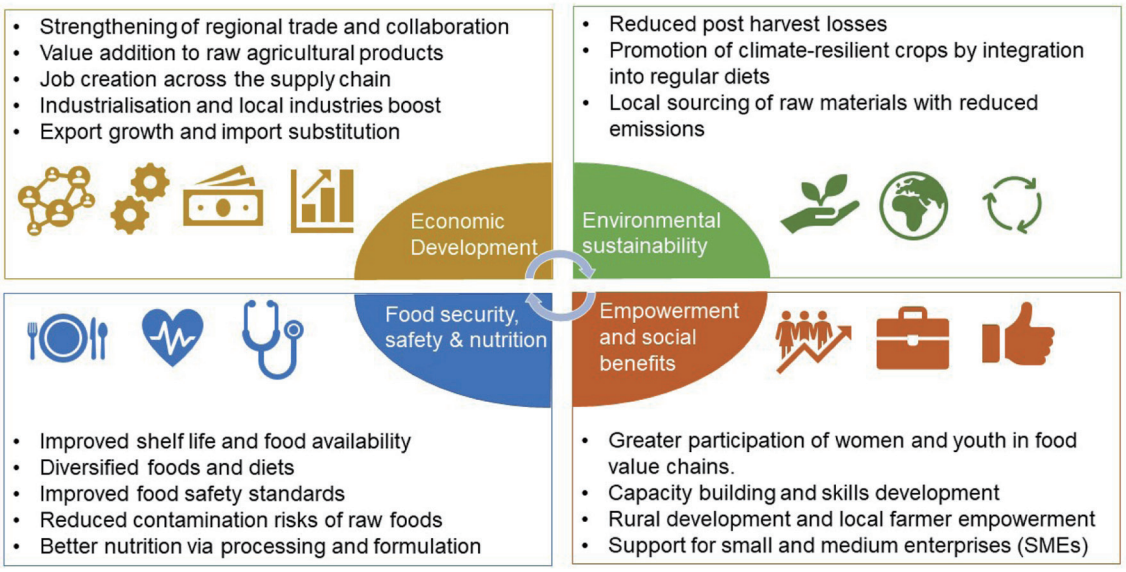

Figure 1. Some benefits of advancing food processing in Africa.

is a schematic showing some benefits of food processing and the interdependence among them.

6. Questions and answers

6.1. Technology communication and intellectual property in scaling up traditional food processing

Questions were asked about the technological advancements that have been made in the food processing area and what effective mechanisms there are to inform smallholder farmers of these. Additionally, a question was posed on how Intellectual Property (IP) rights are handled when scaling up traditional technologies. In response, it was proposed to establish technological incubation centres in rural areas to enhance knowledge transfer. The role of educational institutions including universities, was also noted, particularly in modifying curricula to incorporate entrepreneurship training, which would empower young graduates, even those outside food science disciplines, to engage in food processing. Short-term micro-credential courses were suggested as a means of equipping interested graduates with relevant skills.

Addressing the financial constraints of scaling up, the need for government and financial institutions to provide credit facilities to local food producers was mentioned. Encouraging investment in food processing by young entrepreneurs was also identified as a critical step toward sustainable growth. Regarding Intellectual Property, it was clarified that IP tools allow businesses to safeguard their innovations in the food industry. It was advised that innovators to file patents for their unique creations to establish ownership and secure their rights over the resulting benefits.

6.2. Sustainability of communal dryers and the role of hydroponics in Africa

The communal use of dryers in the first mile location was questioned as to how shared facilities were maintained and managed, to ensure sustainability. It was explained that communal dryers were not provided as donations but were granted to producer associations that co-invested in the purchase. Governance structures were established before the assets were acquired, and business plans were developed to ensure the facilities' long-term sustainability. The operational model included a pay-per-use system, ensuring that maintenance costs were covered. Typically, these facilities were handed over to cooperatives that were already engaged in commercial activities, such as aggregation and market supply.

Drawing from experience in Ethiopia, it was elaborated that capacity-building efforts before asset acquisition was necessary. When working with women's cooperatives, for example, knowledge-sharing programs were implemented by taking these women to learn from successful cooperatives, particularly in the coffee sector. This hands-on exposure ensured that beneficiaries understood management and operational aspects before receiving new infrastructure. It was further emphasized that capacity-building need to precede infrastructure provision, for sustainability.

The role of hydroponics in Africa's food industry was another topic of interest. On that topic, its potential was noted but it was stressed the need for gradual introduction through demonstration projects. An example was cited where solar pumping technology was introduced to Sudanese farmers in the sesame production trade. Initially hesitant, the farmers only adopted the technology after witnessing it in operation. It was noted that universities play a critical role in demonstrating new technologies, bridging the gap between research and practical applications. The need for financial support to help researchers take their innovations beyond academic institutions and into commercial markets was emphasized. It was proposed that governments and private sector partnerships should provide funding and policy support to facilitate technology transfer. Finally, the importance of linking smallholder farmers and SMEs with private sector players was highlighted, to ensure successful commercialization of research-based innovations.

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Review
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Pharmacokinetic profiles and improvement of resveratrol and derived stilbenes

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Abstract

Numerous studies have demonstrated the health-promoting benefits of resveratrol and its close derivatives in various aspects of disease prevention and management, yet due to their highly conjugated 1,2-diphenylethylene structural skeleton, the *in vivo* application of stilbenoids could be limited. Therefore, the metabolic profiles of these stilbene compounds warrant further attention and investigation. The bioavailability of a nutrient or a drug is significantly influenced by ADME (absorption, distribution, metabolism and excretion). In this review, we summarize the study results of drug metabolism and pharmacokinetics (DMPK) profiles of resveratrol and its close oligomeric derivatives, including oxyresveratrol, piceatannol, pterostilbene, rhaponticin, rhapontigenin and 2,3,5,4′-tetrahydroxystilbene-2-*O*-*β*-glucopyranoside (THSG). This review also addressees explored delivery strategies, such as stilbenoids-loaded nanoparticles or Pickering emulsions, to enhance their aqueous solubility, stability, and thus bioavailability.

Keywords: Resveratrol; Resveratrol related stilbenes; Bioavailability; DMPK profile; Pterostilbene.

1. Introduction

Resveratrol represents a family of monomeric stilbene phytochemicals heterogeneously distributed in plants, many of which are edible plants, usually fruits and vegetables such as grapes and berries. This family of 1,2-diphenylethylene derivatives is mainly found in dietary plants. In chemistry, resveratrol and its structural analogs are a group of stilbene compounds with benzene rings whose hydrogen atoms are replaced by varying numbers of hydroxyl and methoxy groups, among others (Peng et al., 2024; Wang, Zhao et al., 2020). It has been revealed that these stilbene compounds possess antioxidant and inhibitory effects on chronic inflammation (Soufi et al., 2015; Truong, Jun, and Jeong, 2018), which are closely associated with their potential protective effects in metabolic syndrome (Hou et al., 2019), particularly in diabetes and its complications (Huang et al., 2020; Peng et al., 2024). These stilbenoid compounds also exhibited beneficial effects in liver injury (Jia et al., 2019; Wu et al., 2019), brain diseases such as brain injury, dementia and Alzheimer's disease (Hornedo-Ortega et al., 2018; Pasinetti et al., 2015), cardiovascular disease (Fan et al., 2022; Breuss and Atanasov, 2019), anti-aging effect (Li, Li and Lin, 2018) and the modulation of gut microbiota is involved in the mitigation of some diseases (Koh et al., 2020).

However, despite potential health effects exhibited by phytochemicals from the stilbene family, their utilization as therapeutic candidates either as supplements or potential medications is hindered by a few factors. In the last few decades, researchers have studied the pharmacokinetic profile of resveratrol and its structural analogs, although much less investigation has been conducted for the latter. These works provide us with important insight into how they are absorbed and metabolized and identify potential factors contributing to their low bioavailability, such as poor solubility

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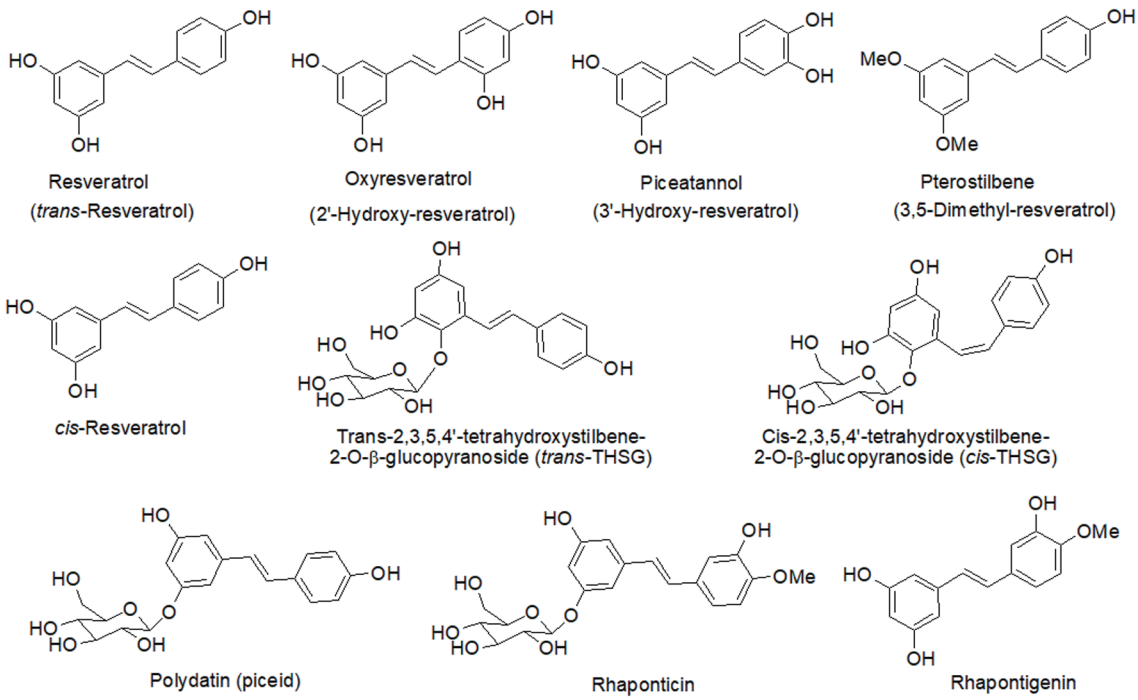

Figure 1. Chemical structures of resveratrol and its close derivatives.

and absorption and fast blood clearance. To solve these limitations associated with not only stilbenes but also many other natural phytochemicals, various formulation systems were created to enhance their stability against environmental stress and bioavailability.

To date, several delivery systems have been investigated for stilbenes compounds. A key strategy employed in these delivery methods is to protect these bioactive substances by encapsulating them within shell-like structures. This could be achieved by structuring lipid- or polymer-based nanoparticles, nanostructured lipid carriers (NLSc), or liposomes, among others approaches. Alternatively, strategies often focus on reducing the droplet size or improving the product's permeability through cell membrane to boost bioaccessibility. Nanoemulsions and Pickering emulsions are two representative choices.

In this review, focusing on several key stilbenes, namely resveratrol, pterostilbene, piceatannol, oxyresveratrol, polydatin, rhaponticin, rhapontigenin and THSG, we primarily summarize their pharmacokinetic profiles and current formulation strategies employed to enhance their bioavailability. Formulation such as nanoemulsions, nanoparticles and liposomes are highlighted. Additionally, their structural characteristics, dietary sources, bioactivities and associated mechanisms are briefly discussed.

2. Structure characteristics, dietary sources and brief bioactivities of resveratrol and its close derivatives

The study of structure-activity relationship starts with structural analysis. Stilbenes possess a basic C6-C2-C6 skeleton, comprising two phenyl rings linked by an ethylene group. The diversity among stilbenes arises from distinct substituents on these phenyl rings. As one or multiple hydrogen atoms on the basic skeleton are substituted by hydroxylation, glycosylation, and polymerization among others, more than 400 stilbene phytochemicals have been identified ((Pecyna et al., 2020; Shen, Wang, and Lou, 2009). The chemical structures of resveratrol and its close analogs are shown in Figure 1.

Resveratrol (*trans*-3,5,4′-trihydroxystilbene), present in plants like berries (e.g. grapes) and is rich in *Polygonum cuspidatum* (Tian and Liu, 2020). According to current reports, resveratrol possesses a few biological functions, ranging from antioxidant, cardioprotective and neuroprotective effects to anti-aging effect (Bastianetto, Ménard, and Quirion, 2015; Bonnefont-Rousselot, 2016; Li, Li, and Lin, 2018; Rauf et al., 2018).

Polydatin, a 3-glycoside derivative of resveratrol (Figure 1), is predominately found in the Vitaceae, Liliaceae, and Leguminosae families and its main plant source is *Polygonum cuspidatum*, which has a long history of use in traditional Asian herbal medicine (Imtiyaz et al., 2024). Similar to resveratrol, polydatin possesses multiple biological activities, such as cardio-protection (Huang et al., 2015), amelioration of diabetes nephropathy (Xie et al., 2012), neuroprotective effects (Chen et al., 2020; Li et al., 2012), and anti-cancer effects (Chen et al., 2020; Imtiyaz et al., 2024). Notably, comparing to resveratrol, polydatin has shown superior effects against oxidative stress *in vivo,* potentially by promoting the activity of serum superoxide dismutase, catalase and glutathione peroxidase (Wang et al., 2015).

Oxyresveratrol (*trans*-2,4,3′,5′-tetrahydroxystilbene, Figure 1) is a natural stilbenoid primarily found in the Moraceae family (mulberry family), particularly in *Artocarpus* (jackfruit etc.) and *Moru* genera (white mulberry etc.) (Likhitwitayawuid, 2021). Structurally, it differs from oxyresveratrol by having an extra hydroxyl group at the 2-position of resveratrol's mono-hydroxyl phenyl ring. While sharing similar biological activities with resveratrol (Likhitwitayawuid, 2021), this extra hydroxyl group brings oxyresvertrol stronger antioxidant and anticancer activities(Yang et al., 2019). Its diverse biological and pharmacological activities include anti-melanogenesis effects by inhibiting tyrosinase, anti-inflammatory

activity, gut microbiota modulation, and neuroprotective effects, among others (Likhitwitayawuid, 2021).

Pterostilbene (*trans*-3,5-dimethoxy-4′-hydroxystilbene, Figure 1), a natural 3,5-dimethylated analog of resveratrol with similar biological functions, is notably abundant in blueberries (Lin et al., 2020; Liu et al., 2020). The substitution of the two hydroxyl groups on the A-benzene ring of resveratrol with two methoxyl groups improves its lipophilicity and reduces its overall reaction with phase II metabolic enzymes like glucuronidase in the human body (Dellinger, Garcia, and Meyskens, 2014; Wang and Sang, 2018). Therefore, in comparison with resveratrol, pterostilbene may exhibit higher membrane permeability and metabolic stability, potentially resulting in better bioavailability (Dellinger, Garcia, and Meyskens, 2014; Kapetanovic et al., 2011; Liu et al., 2020).

Rhaponticin (*trans*-3,5,3′-trihydroxy-4′-methoxystilbene 3-*O*-*β*-D-glucoside), and its aglycone, rhapontigenin, are primarily found in various rhubarb species from the Rheum family and could accumulate up to 40.8 mg/g in the dry root of *Rheum rhaponticum* (Kolodziejczyk-Czepas and Czepas, 2019; Yang, Dai et al., 2024). Like other stilbenoids, these compounds occur naturally in both *cis* and *trans* form in plants, though chiefly in *trans* isoform. Rhapoticin shares a similar molecular structure with resveratrol, distinguished by a substituted glucose group at 3-position and a methyl group at 4′-position. Notably, rhaponticin exhibits strong anti-inflammatory effects. Both rhaponticin and rhapontigenin have been shown to effectively inhibit pro-inflammatory enzymes and transcription factors (Kageura et al., 2001; Kutil et al., 2015). Moreover, rhaponigenin demonstrates stronger inhibitory activity on NO production than resveratrol in macrophages (Yamamoto et al., 2017).

Structural analysis of resveratrol and its analogs enables us to recognize that structural variations, particularly the numbers and position of hydroxyl or methoxyl groups on the rings, are closely related to anti-inflammation, anti-oxidative and free-radical scavenging capacity, and other redox-related properties, which are believed to be pivotal in their overall bioactivities (Murias et al., 2005).

3. Major mechanisms involved in stilbenoids bioactivities

The antioxidant effects of resveratrol and its stilbene analogs have protected ROS-induced tissue damage in organs like the retina, kidney, heart, and nerves in diabetic complications in *in vivo* studies. Such protection is mediated by various mechanisms, including reduced production of reactive oxygen species (ROS) and nitric oxide (NO) level (Ates et al., 2007; Kutil et al., 2015), attenuated lipid peroxidation (Torres-Cuevas et al., 2021), downregulation of NADPH oxidase (Wu et al., 2016), elevated levels of glutathione (GSH), and detoxifying enzymes such as superoxide dismutase (SOD) (Fang et al., 2018; Sadi and Konat, 2016; Shen and Rong, 2015), glutamine synthetase (GS) (Zeng et al., 2016), catalase (CAT) (Kumar et al., 2007) and glutathione peroxidase (GPx) (Zhang et al., 2019). Key signaling pathways involved in these effects include Nrf2/HO-1 up-regulation (Kosuru et al., 2018; Li et al., 2019; Lv, Du, Zhang and Zhang, 2019; Zhang et al., 2021), PKC inhibition (Giordo et al., 2021), PI3K/Akt/GSK-3β/Nrf2 activation (Malik et al., 2019), Sirt1/FOXO3 modulation (Wang et al., 2017) and AMPK pathway (Wang, Li et al., 2020). Furthermore, rhapontigenin and polydatin have also shown anti-oxidative effect by reducing lipoxygenase (LOX) level (Ngoc et al., 2008) and activating SIRT1 (Liu, Liu, Xu and Ding, 2024; Kawakami et al., 2014).

Resveratrol and its related derivatives also exhibit effective inhibition on inflammation. Studies reveal that these oligomeric stilbenoids can suppress inflammation under *in vitro* and *in vivo* conditions and decrease the levels of inflammatory cytokines such as interleukin (I-1β), tumor necrosis factor (TNF)-α and IL-6 (Soufi et al., 2015; Cai et al., 2020). The inhibition of NF-κB with broad upstream and downstream targets is one of the primary anti-inflammation mechanisms (Huang, Xu et al., 2017; Soufi et al., 2015; Xie et al., 2012). Moreover, polydatin demonstrates anti-inflammation effects by regulating ICAM, ICAM-1, NLRP3 and the MAPK pathway (Lv, Du, Liu et al., 2019; Peritore et al., 2021). These stilbenoids also contribute to the inhibition of advanced glycation end products (AGEs) and their receptors (RAGE) (Xian et al., 2020). Furthermore, resveratrol stilbenoids have shown regulatory effects on inflammation-induced stress signals in microglia (Carey et al., 2013) and have attenuated cognitive behavioral dysfunction (Joseph et al., 2008). It was reported that compared to resveratrol, pterostilbene owns a better neuromodulatory activity in aging and Alzheimer's disease (Chang et al., 2011).

The anti-apoptotic effect represents another important bioactivity of these stilbenoids. Resveratrol and its closely derivatives significantly downregulate apoptotic markers sucha s caspase-3 and caspase-9 while upregulate Bcl-2, hence reducing cell apoptosis and associated tissue damages (Shah et al., 2019; Soufi et al., 2015; Li et al., 2019; Wang, Li et al., 2020). These actions involves the modulation on several signaling pathways, namely the PI3K/Akt/FOXO3a, (Wu et al., 2017), NLRP3 (Li et al., 2018), microRNA-29b/specificity protein 1 (Zeng et al., 2017) and AMPK/Sirt1/PGC-1α (Li et al., 2017) pathways. In addition, the stimulation of autophagy (Huang, Ding et al., 2017), attenuation of related endoplasmic reticulum stress (Guo et al., 2015), and activation of inositol requiring 1alpha (IRE-1α) /JNK pathways (Wang et al., 2011; Malaguarnera, 2019) could also contribute to the actions of these resveratrol-associated stilbenoids.

Beyond the bioactivities described above, resveratrol and associated stilbenes have also demonstrated effects in alleviating pathological changes, such as the inhibition of angiogenesis and fibrosis. For example, in diabetic retinopathy, stilbenoids compound can suppress the proliferation and migration of endothelial cells (Rokicki et al., 2014; Shen and Rong, 2015). The AMPK and PI3K pathways are suggested to contribute to the inhibition of retinal epithelial cell migration (Chan et al., 2013). Extensive studies on resveratrol have been conducted, revealing its effective inhibition of growth factors including vascular endothelial growth factor (VEGF) and transforming growth factor (TGF)-β1 in retinal and renal cells as well as in diabetic retinopathy rats , thereby mitigating pathological angiogenesis (Chen et al., 2019; Kim et al., 2012; Wen et al., 2013). Studies have also revealed that resveratrol can attenuate renal fibrosis by regulating AMPK/NADPH oxidase 4/ROS pathway (He et al., 2016), decreasing fibronectin production (Gong et al., 2020), inhibiting p38 MAPK and TGF-β1 (Qiao et al., 2017), suppressing endothelial to mesenchymal-transition (EndTM) by activating SIRT1 (Du et al., 2021), and inhibiting PKC/NADPH oxidase/ROS pathway (Giordo et al., 2021). Similarly, pterostilbene and *trans*-2,3,5,4′-tetrahydroxystilbene (*trans*-THSG) effectively reduced fibronectin secretion by down-regulating TGF-β/drosophila mothers against decapentaplegic protein 1 (Smad1) pathway (Zhang, Ren et al., 2019) and inhibiting renin-angiotensin system (Chen, Yang et al., 2016). Moreover, resveratrol inhibited myocardial fibrosis through its antioxidant function (Wang et al., 2018). It exhibited strong anti-proliferative effects on mouse cardiac fibroblast cells through ROS/ERK signaling pathway and ameliorated myofibroblast cell differentiation through the ROS/ERK/TGF-β/periostin pathway (Wu, Li et al., 2016). Similar

effects were observed in oxyresveratrol, pterostilbene and *trans*-2,3,5,4′-tetrahydroxystilbene, as they exhibited hepatic protective activity via inhibiting liver and renal fibrosis (Long et al., 2019; Yang et al., 2021) by elevating antioxidant activity, inhibiting expression of TGF-β and its downstream activation of ERK1/2 and Smad1/2 (Yang et al., 2021; Zhan et al., 2021).

4. Bioavailability and pharmacokinetics

4.1. Resveratrol and polydatin

Following oral administration, resveratrol undergoes rapid absorption in the gastrointestinal (GI) tract, with peak plasma concentrations (C_{max}) occurring within the first 30 min and 1.5–2 h after low and higher doses in fasting status, respectively (Table 1). Notably, fed states can greatly affect influences its pharmacokinetics. For instance, C_{max} can be delayed from 3 h to 5 h post-administration in mice or rats when switching from a standard to a high-fat diet (Huang et al., 2019). Urine and feces analyses estimate the absorption of resveratrol in both human and rats at around 75%, mainly attributed to transepithelial diffusion (Li et al., 2003; Soleas et al., 2001; Walle et al., 2004). However, its oral bioavailability is limited due to extensive first-pass metabolism in the intestine and liver, and neither dose escalation nor repeated dosages significantly improved it (Soleas et al., 2001; Walle, 2011). Depending on the solubilizing formulation systems, the absolute oral bioavailability of resveratrol exhibits considerable variability ranging from 2.6% to 46.4% (Ha et al., 2021). While it is challenging to detect the free form of resveratrol in circulating plasma due to extremely low concentration, tissue accumulation has been observed in the kidney, liver, brain, and intestine (Huang et al., 2019; Walle, 2011). This finding is consistent with its relatively high volume of distribution (V_d), indicating substantial extravascular distribution of resveratrol (Huang et al., 2019; Walle et al., 2004). Metabolic studies reveal that resveratrol's major metabolites post-absorption are glucuronides and sulfates, suggesting the involvement of enterohepatic circulation (Wenzel and Somoza, 2005). Unlike resveratrol which is absorbed in the intestine primarily through transepithelial diffusion, polydatin has an additional glucose group at the 3-position. While this enhance its solubility, it may concurrently impede its absorption into the bloodstream as active glucose transporters are required (He et al., 2007). Nevertheless, polydatin concentration in rat serum was observed to be 3–4 times higher than that of resveratrol following a 200 mg/kg oral does (Wang et al., 2015). Upon oral co-administration of polydatin and resveratrol in the form of *Ramulus Cinnamomi* extract, both compounds were quickly absorbed, reaching T_{max} within 0.5 h. However, polydatin showed a 4.7-fold greater AUC0-t and 2.5-fold higher Cmax with a shorten t1/2, suggesting an enhanced absorption but more rapid elimination in the blood (Yang, Wang et al., 2024). Notably, it is crucial to recognize that other co-existing components within this herbal extract may have profound impacts on the pharmacokinetic profiles of these two components (Yang, Wang et al., 2024).

4.2. Pterostilbene

The pharmacokinetics of resveratrol and pterostilbene has been comprehensively compared by Wang and Sang (2018). As illustrated in Table 1, pterostilbene exhibits a superior pharmacokinetic profile over resveratrol due to dimethyl ether structure, manifesting as higher metabolic stability and bioavailability (Azzolini et al., 2014; Liu et al., 2020; Wang and Sang, 2018). Moreover, dose-escalation lead to an increase in T_{max}, suggesting that the pterostilbene absorption is a capacity-limited process (Yeo, Ho, and Lin, 2013). The distribution of pterostilbene in various tissues has been reported, with Deng et al. (2015) specifically highlighting its ability to pass through the blood-brain barrier. In parallel, another research reveals that pterostilbene concentrations in certain tissues can be much higher than that in blood, potentially explaining its bioactivity despite low circulating plasma level (Azzolini et al., 2014). In mice, rats, and human, pterostilbene undergoes phase II metabolism, with sulfation and glucuronidation being the dominant metabolic pathways. In particular, a comparative study in human liver microsomes demonstrated pterostilbene's better metabolic stability over resveratrol, as more than 75% of pterostilbene remained unchanged while 68% of resveratrol went through glucuronidation (Dellinger, Garcia, and Meyskens, 2014). Elimination of pterostilbene occurs mainly through renal and hepatic excretion. Notably, increasing the dosage resulted in a reduced plasma elimination rate, leading to non-linear pharmacokinetics, which indicates the saturation of related enzymes (Yeo, Ho, and Lin, 2013).

4.3. THSG

The pharmacokinetic profile of THSG remains comparatively underexplored. Limited studies in rats show that THSG can be rapidly absorbed after oral administration, with a T_{max} of 10–30 min and a half-elimination time ($T_{1/2e}$) of 50–120 min (Table 1) depending on the dose (Dong et al., 2014; Sun et al., 2018; Zhao, Cheng, et al., 2013). THSG is widely distributed following absorption, mainly to the heart, kidney, liver, and lung (Zhao, Zhang et al., 2013). This widespread tissue distribution is also supported by a V_d of approximately 3.94 L/kg, thereby suggesting its potential biological function at extrahepatic sites (Zhao, Cheng, et al., 2013). While one study reported that around 81% THSG was present as phase II metabolites (Dong et al., 2014), other two studies revealed a low recovery of unchanged THSG, implying that it was primarily excreted as metabolites through feces, most likely as monoglucuronides (Zhao, Cheng, et al., 2013; Zhao, Zhang, et al., 2013).

4.4. Piceatannol

In rat, the total area under the curve (AUC) of piceatannol (include both intact and conjugate form) is lower than that of resveratrol. However, the ratio of intact piceatannol AUC to total AUC was 3.7- to 4.3-fold higher than that of resveratrol (Table 1), suggesting greater metabolic stability of piceatannol (Setoguchi et al., 2014). While phase I metabolism appears to be insignificant in the metabolism of piceatannol, it can be metabolized to a few compounds following intravenous administration, through glucuronidation, sulfation, and methylation, with piceatannol-monoglucuronide being the most abundant (Dai et al., 2020; Setoguchi et al., 2014). Also, piceatannol can be effectively converted to active monomethylated derivatives, rhapontigenin and isorhapontigenin, after oral administration, compensating for oxyresveratrol's lower plasma exposure as parent compound (Dai et al., 2020). Although piceatannol undergoes extensive hepatic glucuronidation and exhibits a relatively low absolute bioavailability of 6.99 ± 2.97% (Dai et al., 2020), it has a relatively high V_d of 10.76 L, indicating its wide distribution in tissues (Roupe et al., 2006). It also possesses a long half-life of 2–3 h and is predominantly eliminated via the hepatic pathway with a limited clearance rate (Dai et al., 2020; Roupe et al., 2006). Moreover, the pharmacokinetic pro-

Table 1. Pharmacokinetic values of resveratrol and its derived stilbenes

Stilbenoids	Solubility	Bioavailability	C_{max} (μg/mL)	Time to C_{max}	V_d (L/kg)	$T_{1/2}$ (h)	AUC (μg h/ mL)	Major metabolites	References
Resveratrol	<0.05 mg/mL	2.6–46% (human); 20–29% (rat, dose 50 mg/kg)	0.073–0.539	30 min low dosage; 1.5–2 h high dose	2.24–2.49	1 to 3	5.05 (mice); 0.179 ± 0.079 (human)	Glucuronides Sulfates	Das et al., 2008; Ha et al., 2021; Kapetanovic et al., 2011; Sergides et al., 2015; Johnson et al., 2011; Wang and Sang, 2018; Wenzel and Somoza, 2005; Yang, Wang et al., 2024
Polydatin	0.16 mg/mL	2.9%	12.63	0.33 h	63.83	2.48 ± 0.99	14.31 ± 0.35	Glucuronides	Yang, Wang et al., 2024
Pterostilbene	0.018 mg/mL	80% (rat, dose 20 mg/kg)	0.415–1.038	90–300 min	2.5–3.0	1.57 ± 0.37	4.43 – 11.9 (mice)	Sulfates; Glucuronides	Sun et al., 2018; Dellinger, Garcia, and Meyskens, 2014; Kapetanovic et al., 2011; Remsberg et al., 2008
Trans-THSG	10 mg/mL	na	31.91	10–30 40min	3.94	0.83 - 2	639.9 ± 347.2 (rat)	Monoglucuronides	Zhao, Cheng et al., 2013; Zhao, Zhang et al., 2013
Piceatannol	<0.5 mg/mL	6.99 ± 2.97% (human); 50.7% (rat, dose 10 mg/kg)	2.0	15 min	10.76	4.23	508.8	Monoglucuronide; Sulfates; methylation; rhapontigenin	Roupe et al., 2006; Setoguchi et al., 2014
Oxyresveratrol	0.6 mg/mL	10–15%	1.5	1–2 h	105	1.70 ± 0.77	5.43 ± 1.02 (rat)	monoglucuronide and monosulfate	Huang et al., 2010; Hu et al., 2014; Junsaeng et al., 2019
Rhaponticin	0.109 mg/mL	0.03%	1.71	10.32 min	62.02	3.0 ± 1.35	52.8	Rhapontigenin monoglucuronide and monosulfate	Roupe et al. (2006); Zhao et al., 2012
Rhapontigenin	0.11 mg/mL	na	0.18	52.5 min	0.011	3	42	monoglucuronide and monosulfate	Roupe et al., (2006); Navarro-Orcajada et al., 2023

file of piceatannol can be improved when co-administrated with α-cyclodextrin (Inagaki et al., 2016).

4.5. Oxyresveratrol

Based on limited data, oxyresveratrol was rapidly absorbed from the GI tract after oral administration, with a short T_{max} of 15 min. It is further excreted in both bile and urine, predominantly as monoglucuronide and monosulfate (Huang et al., 2010; Huang et al., 2008). Specifically, oxyresveratrol-2-*O*-*β*-*D*-glucuronide has been identified as the primary glucuronide metabolite in human liver and intestine (Hu et al., 2014). Despite a relatively low absolute bioavailability of around 10% after a single oral dose, oxyresveratrol demonstrates a long residence time in circulation. While dose escalation was correlated with a significantly improved absolute bioavailability, consecutive doses for 1 week did not yield further enhancement (Chen, Yeo et al., 2016). The pharmacokinetic profile of oxyresveratrol was improved when used in combination with piperine (Junsaeng et al., 2019).

4.6. Rhaponticin and rhapontigenin

The glucose group on the rhaponticin enhance its solubility compared to oxyresveratrol. However, rhaponticin's oral bioavailability was remarkably low, at only 0.03%, primarily due to its rapid metabolism (Zhao et al., 2012). Roupe et al. (2006) reported that rhapontigenin, administrated at a dosage of 10 mg/kg, has a half-life around 3 h in rat plasma, implying a fast elimination from blood, although it is longer than that of resveratrol. The C_{max} of rhapontigenin in murine plasma was only 8.91 μg/mL 5 min after a 100 mg/kg oral dosage (Zhao et al., 2012) while a lower plasma concentration of rhaponticin at 1.71 μg/mL was observed. Overall, both rhaponticin and rhapontigenin exhibit characteristics of poor solubility and rapid metabolism (Zhao et al., 2012).

In brief, the therapeutic promise of resveratrol and its close derivatives is often hindered by their pharmacokinetic profiles, characterized by poor oral bioavailability and rapid metabolism and clearance. As the dominance of Phase II metabolism appears as a recurring theme for stilbene metabolism, rapid first-pass metabolism is identified as a major contributor to their low bioavailability. However, studies reported above also demonstrate that structural variation often result in different metabolic patterns and that in some cases, stilbene absorption is a capacity-limited process by which higher dose can mitigate the first-pass barrier. Neverthelss, rhaponticin, despite possessing a higher solubility, still suffers from exceptionally low bioavailability, hence suggesting that improved solubility alone is insufficient to guarantee a favorable pharmacokinetic profile if rapid metabolism and clearance predominates. Therefore, close examination and in-depth understanding of stilbene's metabolic fates are crucial for effective formulation strategies.

5. Improvement of metabolic profiles

In addition to the pharmacokinetic limitations, the poor aqueous solubility and instability against environmental conditions such as moisture, light, heat or high temperature, oxygen and the combination of two or more above factors are also involved (He et al., 2018; Zhang et al., 2014; Zupančič et al., 2015). Therefore, improvement of their water solubility and bioavailability using different strategies is vital for unlocking their preventive or therapeutic potentials. Since structural modification aimed at enhancing bioavailability may interfere with essential physiological functions and introduce uncertain toxicity, research have focused on developing diverse oral delivery systems, such as the recent trends on nano-based systems (Peng et al., 2018).

5.1. Summary of phytochemical delivery systems

Among nano-based systems, fabrication of stilbenoids-loading nanoparticles is a theme of current relevance as it allows the use of broad biocompatible materials, particularly food-grade materials which are considered safe to use. This nanotechnology enables researchers to design a smart stilbene delivery system in which the timing and spot for the deconstruction of the coating layer can be controlled by choosing materials with different chemical properties. In general, owing to the protection from the coating layer, fabricated nanoparticles of stilbenoids have a higher resistance to the change in pH and metabolic enzymes during digestion and metabolism, thus contributing to better stability, prolonged residence time, and achieving an improved controlled-release profile. Meanwhile, reduced particles size and modified surface facilitate an enhanced cellular uptake of encapsulated compounds. Coupled with increased solubility, these attributes collectively improve their overall bioavailability. Some original research and reviews that reported the improvement in the stability, solubility and/or bioavailability of stilbenoids' nanoemulsions or nanoparticles are summarized in Table 2 and also in the following list: resveratrol (Mohseni et al., 2019; Santos et al., 2019; Yang, Wang, et al., 2019), pterostilbene (Liu et al. 2019; Peng et al., 2018; Tzeng et al., 2021; Zou et al., 2021), THSG (Liu et al., 2022), piceatannol (Aljabali et al., 2020), and oxyresveratrol (Sangsen et al., 2016). Moreover, in the last five years, research focusing on the comparison of bioactivities between free form stilbenoids and their nanoparticles in various disease models (Chung et al., 2020; Yang, Wang, et al., 2019) have gradually emerged, among which a more potent effect of nanoparticles in diabetes and diabetic complications was also indicated (Dong et al., 2019; Mohseni et al., 2019).

5.2. Examples of stilbene delivery systems

5.2.1. Resveratrol

A near-spherical shaped nanosuspension of resveratrol was prepared containing 0.38% of polyvinylpyrrolidone K17 and surfactant F188 (3.63%) and study of in vivo pharmacokinetics revealed that the bioavailability of resveratrol suspension was improved with the increase of Cmax and AUC values by 3.35- and 1.27-fold comparing to that of resveratrol alone, respectively (Hao et al., 2015). In a solid lipid nanoparticle system with stearic acid as the solid-lipid core, the encapsulation efficiency of resveratrol reached 79.9%. In the oral feeding to Wistar rats, the resulted nanoparticles of SLN-resveratrol exhibited an initial burst release followed by a sustained resveratrol release in natural conditions. Both AUC and C_{max} of SNL-resveratrol were increased compared to those of resveratrol suspension, and $t_{1/2}$ was 2.37 and 11.51 h for suspension and SNL of resveratrol respectively. In terms of efficacy, oral administration of SLN- resveratrol exhibited stronger effect in preventing weight loss and reducing blood glucose levels compared to RES alone (Pandita et al., 2014).

The application of emulsion formulation of resveratrol has been widely explored. Employing liquid and semi-solid self-emulsifying drug delivery systems, micro-emulsions of resveratrol was cre-

Table 2. Delivery examples of resveratrol and close stilbenoid compounds

Stilbene compounds	Delivery methods	Core components	Evaluation model	Results	References
Resveratrol	Nanosuspension	Polyvinylpyrrolidone, mannitol	Wistar rats	Bioavailibilty (AUC & C_{max})↑ ,Vd↓	Hao et al., 2015
	Nanoemulsion	long-chain triglycerides	Wistar rats	Bioavailibilty (AUC & C_{max})↑ , absorption↑, permeability↑	Singh and Pai, 2015; 2016
	Polymer nanoparticles	carboxymethyl chitosan	Wistar rats	Bioavailibilty (AUC & C_{max})↑, t_{max}↑	Zu et al., 2014
	Protein nanoparticles	Zein	Wistar rats	Bioavailibilty (AUC & C_{max})↑, t_{max}↑	Penalva et al., 2015
	Solid lipid nanoparticles	Stearic acid	Wistar rats	Bioavailibilty (AUC & C_{max})↑, t_{max}↑, Cl↓	Pandita et al., 2014
	Cyclodextrin	β –cyclodextrin	SD rats	Cmax↑	Das et al., 2008
Polydatin	Liposome	Lethicin, cholesterol	SD rats	AUC↑, T_{max}↑, $T_{1/2↑}$, MRT↑	Zhang et al., 2024
Oxyresveratrol	Microemulsion	Tween80®, Labrasol®	Wistar rats	Bioavailibilty (AUC & C_{max})↑	Sangsen et al., 2016
	Bioenhancement	Piperine	Wistar rats	Bioavailibilty↑(2–6 fold), C_{max}↑	Junsaeng et al., 2019
Piceatannol	Emulsion	α-Cyclodextrin	SD rats	Bioavailibilty (AUC_{0-3h} & C_{max})↑	Inagaki et al., 2016
Pterostilbene	Nanoemulsion	Lipids: MCT, sunflower oil or olive oil	Caco-2 cell monolayer	Bioaccesibility↑; Apparent permeability coefficient↑	Liu et al., 2019
	Nanoparticle	Soybean lecithin, D-alpha-tocopheryl polyethylene glycol succinate	N/A	Solubility↑ anti-tumor acitivity↑	Zou et al., 2021
Trans-THSG	Nanoparticle	Carboxymethyl chitosan and chitosan hydrochloride	Simulated environmental stress	Heat and solar radiation stability↑; Improved pH release profile	Liu et al., 2022
	Vesicles-containing gel system	Oleic acid, calcium chloride, sodium alginate	Franz diffusion cell	Trasdermal flux↑	Lai et al., 2020
Rhaponticin	Vesicles-containing gel system	Polyethylene glycol; Cholesterol, egg phosphatidylcholine, hydrogenated soybean phosphatidylcholine	Female nude mice	C_{max} ↓, T_{max} ↑, $T_{1/2}$ ↑ AUC_{0-tn}↑	Sun and Zhao, 2012
	Emulsion	Folic acid	Balb/c mice	Water solubility↑ receptor affinity↑; Therapeutic effect↑, toxicity↓	Liang et al., 2013

Cl: clearance; MCT: medium chain triglyceride; MRT: mean residence time; SD: Spraw-Darley.

ated with a droplet size of approximately 100 nm. An *ex vivo* study with the jejunum of rats demonstrated that the permeability of the micro-emulsion resveratrol was significantly enhanced, showing an 8.5-fold increase for the resveratrol-nanoemulsion compared to a resveratrol dispersion in an ethanolic medium with the preservation of the intestinal functional viability (Mamadou et al., 2017).

The encapsulation of resveratrol in liposomes has also demonstrated effectiveness. A study by Soo et al. (2016) developed a dual carrier system to co-encapsulate pure resveratrol with cyclodextrin-resveratrol inclusion complexes in both the lipophilic and hydrophilic compartments of liposomes. The final formulation exhibited a particle size of 131 ± 1.30 nm, a polydispersity index of 0.089 ± 0.005, and a zeta potential of −2.64 ± 0.51 mV. In contrast to free resveratrol and conventional liposomal formulations that showed a drug release profile of 40–60%, this nanoformulations exhibited complete (100%) drug release within 24 h (Soo et al., 2016). Recently, different particle sizes of resveratrol nanoliposomes were created using a thin-film hydration technique and results showed that small size liposomes (<100 nm) more effectively enhanced aqueous solubility, cellular permeability and cellular antioxidant activity (Baek, Jeong and Lee, 2023).

5.2.2. Polydatin

A liposome formulation of polydatin has been developed using a membrane dispersion method (Zhang et al., 2024). The liposome was prepared from lethicin, DSPE-PEG-2000 and cholesterol. The resulted polydatin liposome had a sustained release of polydatin, prolonged *in vivo* circulation time and dramatically improved bioavailability. Comparing to free polydatin, the yielded polydatin liposome had an increased T_{max} (60 min *vs* 30 min), $T_{1/2}$ (5.15 ± 0.47 *vs* 1.02 ± 0.07 h), AUC (13.03 ± 0.30 *vs* 3.58 ± 0.14 mg h/mL), and mean residence time (MRT, 12.02 ± 2.28 *vs* 3.32 ± 1.20 h) in SD rats bioavalability evaluation (Zhang et al., 2024). Moreover, the liposome of polydatin demonstrated more effective hypoglycemic effects than that of free form.

5.2.3. Oxyresveratrol

Formulation of oxyresveratrol has been challenging for its low aqueous solubility, poor bioavailability and instability. Cyclodextrins were often used for oxyresveratrol encapsulation to improve bioavailability and stability. Solid lipid nanoparticles were also used to entrap oxyresveratrol to improve stability and bioavailability (Likhitwitayawuid, 2021). Sangsen et al. (2016) examined the influence of different ratios of surfactants in oxyresveratrol microemulsifyings on the bioavailability enhancement and they found that C_{max}, AUC and relative bioavailability were increased dramatically compared to free form of oxyresveratrol. For example, for oxyresveratrol delivered in the microemulsion using high percentage of Tween80®, C_{max} increased to 2.36 µg/mL from 0.66 µg/mL of unformulated oxyresveratrol (Sangsen et al., 2016). Another bioavailability-enhancing method was the combination of oxyresveratrol with a bioenhancer piperine (Junsaeng et al., 2019). The combination increased oxyresveratrol bioavailability by 2–6 folds, and the C_{max} reached 1.5 mg/mL within 1–2 h after oral dosing to rats.

5.2.4. Piceatannol

α-Cyclodextrin has been used in the emulsion formulation of piceatannol (Inagaki et al., 2016). When mixed with artificial gastric juice, the solubility of piceatannol was higher as a result of co-formulation with α-cyclodextrin and reached 35.0 ± 0.8 mM immediately, while that of piceatannol in its free form is about 10 folds lower (3.2 ± 0.1 mM). The bioavailability of piceatannol was significantly enhanced, indicated by the increase in $AUC_{0\text{-}3h}$ from 4.8 to 7.0 µmol h/L or more, depending on the amount of α-cyclodextrin used among different formulations. The C_{max} value of piceatannol increased from 2.5 µmol/L in its free form to 5.3, 4.8 and 5.8 µmol/L when formulated with low, medium and high concentration of α-cyclodextrin in emulsions, respectively (Inagaki et al., 2016).

5.2.5. Pterostilbene

By encapsulating pterostilbene in oil-in-water nanoemulsion through high pressure homogenization, Liu et al. (2019) reported delivery systems that enhanced its solubility and bioavailability and demonstrate the potential impact of lipid compositions on the stability and performance of nanoemulsion. To mimic the *in vivo* digestion process, pterostilbene loaded-nanoemulsions was exposed to simulated stomach and intestinal conditions and after digestion, the bioaccessibility of pterostilbene in MCT oil-nanoemulsion(98.7 ± 3.9%) was significantly higher than control (< 20%) and other two nanoemulsions that utilized oils with a higher content of long-chain fatty acids such as sunflower oil (41.3 ± 0.4%) and olive oil (32.9 ± 1.9%) . In addition, Caco-2 cells were used to simulate intestinal epithelial cell barrier in gut and further assess the effect of nanoemulsion formulation on the permeability and bioavailability of pterostilbene. The apparent permeability coefficients of pterostilbene in micelles obtained from digesta of MCT based-nanoemulsion was the higher than that of unencapsulated pterostilbene by 5.7-fold, reaching 8.21 ± 2.09 × 10^{-6} cm S^{-1}, which was attributed to higher solubility of pterostilbene in MCT oil.

5.2.6. THSG

To address THSG's sensitivity to environmental stress, a nanoparticle composed of carboxymethyl chitosan and chitosan hydrochloride was developed. This formulation not only improved its stability against heat and solar radiation but also exhibited a great controlled release profile under gastrointestinal pH condition post-administration, thus offering a potential solution for overcoming the current limitations of THSG (Liu et al., 2022). Regarding the enhancemnet of THSG's bioavailability especially intestinal absorption, one investigated strategy is its co-administration with polysaccharides from *Ophiopogon japonicus*. This approach resulted in a improved solubility and stability of THSG in aqueous solution and further improvement in metabolic profile, increasing the T_{max}, C_{max} and $AUC_{0\text{-}tn}$ to 3.5-, 1.45-, and 2.32-fold, respectively, relative to pure THSG. However, these were accompanied with a slight decrease in permeability (Sun et al., 2018). Another explored strategy is to apply THSG in transdermal drug delivery system through skin. In a recent study, a gel system was developed wherein THSG was loaded in oleic acid-containing vesicles, which were further incorporaed into a complex gel matrix. This novel gel system successfully increased the transdermal flux of THSG by about 4-fold, potentially due to the disruption of stratum corneum integrity (Lai et al., 2020).

5.2.7. Rhaponticin and rhapontigenin

Offering multiple health benefits, rhaponticin and rhapontigenin are considered as great candidates for dietary supplement. However,

rhaponticin's poor aqueous solubility and fast metabolism remain significant challenges that must be solved for further development. Therefore, several formulation methods were tested to improve their characteristics. PEGylated liposome, commonly employed in pharmaceutical research and industry, has been utilized to construct carrier for rhaphoticin and improve its solubility. Compared to oral administration of free rhaponticin, rhaponticin encapsulation by PEGylated liposome resulted in a smaller C_{max} (0.7-fold), longer T_{max} (4.5-fold) and $T_{1/2}$ (1.9-fold), and higher $AUC_{0\text{-}tn}$ (5.45-fold), indicating a slower release, yet higher concentration of rhaponticin, and better capacity to withstand plasma clearance. Further *in vivo* study also revealed a stronger anti-tumor effect compared to free rhaponticin, potentially attributed to the delivery of a larger amount of rhaponticin and higher efficiency in cellular uptake (Sun and Zhao, 2012). Another formulation strategy to enhance rhaponticin bioavailability invovles the synthesis of folate-targeted rhaphonticin conjugate. Due to its linkage with folic acid, this conjugate exhibited improved water solubility and a high affinity for folate receptor-positive cell. Spontaneous release of rhaponiticin inside endosomes upon disulfide bond reduction was confirmed, and results from an *in vivo* tumor model showed an enhanced therapeutic effect, along with reduced toxicity (Liang et al., 2013).

6. Conclusion

Resveratrol and related stilbenoids, characterized by a highly conjugated 1,2-diphenylethylene structural skeleton, are widely distributed in food sources. These phytochemicals have been reported to provide health benefits across various conditions, including but not limited to diabetes and its complications, brain and cardiovascular diseases. However, their utilization as therapeutic candidates, either as dietary supplements or as potential pharmaceutical interventions, needs further investigation. Despite extensive research on the pharmacokinetic behaviors of resveratrol over recent decades, comparatively limited works were conducted for other stilbenoid compounds. Collective studies reveal that resveratrol and related compounds share common characteristics such as poor absorption, extensive metabolism and rapid elimination in blood. These characteristics remain primary challenges for the in-depth discovery of stilbenes' true biological effects, as therapeutically relevant concentrations often cannot be reached when delivered in their free form. Therefore, rising trends of investigation on formulation strategies and delivery systems have been observed. Currently, common approaches including nanoparticles, liposome and nanoemulsions are applied, and they are proved effective in improving stability against environmental stress and enhancing bioavailability, either through increased solubility or enhanced pharmacokinetic profiles such as permeability and absorption. However, further validation using *in vivo* study is needed given the prevalence of *in vitro* study in some case studies. In addition, beyond the aforementioned formulation strategies, alternative formulation methods, or the combination of multiple forms of delivery systems are also promising approaches for future research. For instance, incorporating nanoparticles into hydrogel may further enhance their release profiles and allow desired characteristics such as targeted release in the intestinal tract, topical application, or in-situ administration, thereby providing sustained and localized drug delivery with greater efficacy.

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Research progress on bioactive components and their activities in Sea buckthorn

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Abstract

Sea buckthorn (*Hippophae rhamnoides L.*) is a naturally occurring dual-use plant for both medicine and food. It contains numerous bioactive compounds demonstrating significant health-promoting effects, including anti-inflammatory, antioxidant, and blood lipid-regulating activities. China currently possesses the world's largest sea buckthorn resources. The full and rational utilization of these resources holds substantial importance for enhancing public health and stimulating local economic development. While research on the bioactive compounds and efficacy of sea buckthorn has garnered increasing attention in recent years, a systematic review of this knowledge remains lacking. This article comprehensively summarizes the rich profile of bioactive compounds in sea buckthorn and their documented health benefits. It aims to provide a foundation for the further application and development of sea buckthorn within the pharmaceutical, functional food, and related industries.

Keywords: Sea buckthorn; Bioactive components; Vitamin; Antioxidant; Anti-inflammatory.

1. Introduction

As an ecologically and economically valuable plant, sea buckthorn has garnered significant attention due to its rich natural bioactive constituents (Guo et al., 2017; Criste et al., 2020). Its potential in nutritional supplementation and disease management positions it as a multidisciplinary research hotspot. Berries and leaves are rich in bioactive compounds, constituting a valuable medicinal and edible plant resource (Wang et al., 2022; Fu et al., 2016). Sea buckthorn is gaining popularity as a functional food owing to its rich nutritional profile. Furthermore, sea buckthorn exhibits several advantageous biological traits, including tolerance to cold, drought, and poor soils (Liu et al., 2014; Luo et al., 2023). Consequently, it possesses significant ecological value in windbreak and sand fixation, soil and water conservation, and soil improvement. Specifically, its cultivation can mitigate sandstorms, enhance the ecological environment, and facilitate subsequent vegetation restoration.

Based on its significant nutritional and ecological value, China has emerged as a major global grower of sea buckthorn, with extensive cultivation concentrated in its central and western regions. The abundant sea buckthorn resources have spurred considerable research interest into its functional properties. Consequently, the bioactive properties of sea buckthorn have been extensively investigated (Wang et al., 2022; Luo et al., 2023). This article provides a comprehensive synthesis of current research advances concerning the bioactive components of sea buckthorn and their associated health benefits. It aims to facilitate and support the further valorization of this valuable plant resource.

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Table 1. Main bioactive components in sea buckthorn

Type	Composition	Plant parts	Bioactivities	References
Flavonoids	Kaempferol	Fruit	Antioxidant, Lipid-lowering	Shi, 2025
	Quercetin	Fruit	Antioxidant, Anti-cancer	Liu et al., 2012
	Isorhamnetin	Fruit	Antioxidant, Anti-inflammatory	Ren et al., 2023; Wu et al., 2024
	Rutin	Fruit	Antioxidant Anti-cancer	Chen et al., 2014
	Myricetin	Fruit	Antioxidant	Li et al., 2024
	Apigenin	Fruit	Antioxidant, Lipid-lowering	Zhou et al., 2020
	Hippophaeoside	Fruit	Antioxidant, Anti-inflammatory	Wu et al., 2024; Ji et al., 2023
Vitamins	A	Fruit	Anti-inflammatory	Yan et al., 2021
	B2	Leaf	Anti-inflammatory	Liu et al., 2025
	C	Fruit	Antioxidant	Yan et al., 2021
	E	Fruit	Antioxidant, Lipid-lowering	Olas, 2016; Hu et al., 2021
	Carotenoids	Fruit	Antioxidant	Hu et al., 2021
Fatty Acids	linoleic acid	Seed	Antioxidant, Lipid-lowering	Saeidi et al., 2016; Maria et al., 2021
	linolenic acid	Seed	Antioxidant, Lipid-lowering	Bouras et al., 2017; Cui et al., 2022
	oleic acid	Seed	Antioxidant	Ren et al., 2020; Maria et al., 2021
Polysaccharides	Glucose	Fruit	Antioxidant, Anti-cancer	Zhao et al., 2024
	fructose	Fruit	Antioxidant, Anti-inflammatory	Zhao et al., 2024; Shi et al., 2024
	sucrose	Fruit	Antioxidant, Anti-inflammatory	Lin et al., 2024; Zhao et al., 2023
Amino acids	Aspartic acid	Leaf	Anti-cancer	Shang et al., 2023
	Tryptophan	Leaf	Anti-cancer	Wang et al., 2022
	Glutamic acid	Leaf	Anti-cancer, Antioxidant, Lipid-lowering	Liu et al., 2022; Sharma et al., 2018
	Arginine	Leaf	Anti-cancer, Lipid-lowering	Deng et al., 2024
	Tyrosine	Leaf	Anti-cancer, Gut microbiota modulation	Li et al., 2024; Kong et al., 2024
Others	Phenolic acids	Leaf	Anti-cancer, Gut microbiota modulation	Ge et al., 2023; Tian, 2023
	Sterols	Fruit	Anti-inflammatory, Antioxidant	Asofiei et al., 2019
	Alkaloids	Fruit	Anti-cancer, Lipid-lowering	Tang, 2022

2. Major chemical components

China accounts for over 90% of global sea buckthorn cultivation, reflecting its abundant resources (Fu et al., 2022). Sea buckthorn berries are rich in diverse bioactive compounds, primarily including flavonoids, vitamins, polysaccharides, fatty acids, phenolic acids, and minerals, show as in Table 1 and Figure 1. Among these, certain compounds have received considerable research attention due to their specific physiological functions (Chandra et al., 2018). These compounds confer antioxidant, anti-inflammatory, and glucose-regulating properties, enabling broad applications across industries (Zhang et al., 2017; Niyazi et al., 2020).

3. Research and applications of bioactive components in sea buckthorn

Based on its excellent biological activity, sea buckthorn has been widely used in the fields of food and medicine. And its biological activity relies on its rich variety of active ingredients (Xiong et al., 2022). Sea buckthorn constitutes a high-value multipurpose tree species integrating phytopharmaceutical potential, nutraceutical applications and ecological engineering significance.

3.1. Flavonoids

Flavonoids, as primary bioactive constituents in sea buckthorn, predominantly accumulate in its leaves at concentrations ranging from 310 to 1,238 mg/100g – significantly exceeding levels in fruits. Currently, 49 flavonoids have been isolated from sea buckthorn, mainly including quercetin, isorhamnetin, kaempferol, and their stems (Liu et al., 2012; Shi, 2025). As the main component of sea buckthorn, flavonoids demonstrates remarkable antioxidant capacity, effectively scavenging free radicals such as DPPH, superoxide anions (O_2^-), and hydroxyl radicals (·OH) in *vitro* (Ren et al., 2023; Zhou et al., 2020; Chen et al., 2014). Furthermore, studies have shown that flavonoids extracted from sea buckthorn leaves can enhance the vitality of PC-12 cells by preserving membrane integrity, attenuating intracellular oxidative stress, mitigating mitochondrial dysfunction, and reducing apoptotic rates (Wang

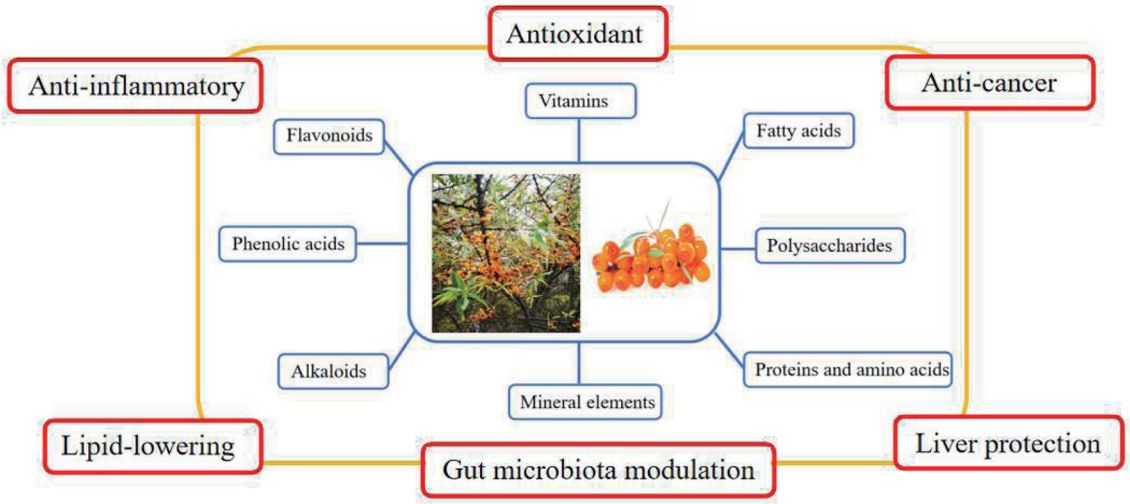

Figure 1. The main biocative components and activities in sea buckthorn.

et al., 2007; Li et al., 2024). Anti-inflammatory is one of the biological activities of sea buckthorn flavonoids. Sea buckthorn flavonoids can significantly enhance the phagocytic ability of LPS induced Raw 264.7 cells by inhibiting the release of cytokines NO and inflammatory factors IL-6, TNF - α, and COX-2 to suppress inflammation (Wu et al., 2024; Liu et al., 2025). Furthermore, due to its anti-proliferative properties, sea buckthorn flavonoids have shown great potential in anti-cancer applications. Notably, sea buckthorn flavonoids display anti-cancer potential through anti-proliferative effects via p53-mediated apoptosis (Guo et al., 2017; Rana and Gulliya, 2019). And it can also significantly inhibit the proliferation of human prostate cancer PC-3 cells and human liver cancer HepG2 cells in *vitro* by regulating the expression of Bax and Bcl-2 proteins, blocking the cell cycle (Zhao et al., 2018; Bai and He, 2021; Hao et al., 2022). Many researchers have proved that sea buckthorn flavonoids also can reduce the risk of diabetes by avoiding glucose absorption or improving glucose tolerance (Wang et al., 2022; Alqudah et al., 2023). Quercetin can reduce the ability of diabetes rats to absorb glucose, making it comparable to the absorption level of normal rats (Gao et al., 2017). Sea buckthorn flavone can significantly increase the levels of insulin and liver glycogen in ALX induced diabetes mice, and alleviate abnormal lipid metabolism, which may be closely related to cholesterol conversion and outflow (Zhu et al., 2021; Ji et al., 2023).

3.2. Vitamins

Sea buckthorn is a natural source of vitamins, rich in various vitamins, mainly including vitamin A, vitamin C, vitamin E, and vitamin F. Crucially, its vitamin C content surpasses most horticultural crops by 3- to 10-fold, attributable to the ascorbate oxidase-mediated stabilization system that minimizes oxidative degradation (Yan et al., 2021; Zhu et al., 2024; Liu et al., 2025). Vitamin A in sea buckthorn exists as provitamin A carotenoids, primarily β-carotene, which undergoes enzymatic conversion to retinol in humans. Sea buckthorn oil contains exceptionally high carotenoid concentrations, ranging from 54 to 9,265 mg per 100 g. Vitamin A sufficiency confers multisystem protection through enhanced tumor immunosurveillance, atherosclerosis prevention, improved thyroxine conversion efficiency, heavy metal chelation capacity and telomere maintenance (Yan et al., 2021; Liu et al., 2025). The high content of vitamin C endows sea buckthorn with antioxidant, lipid metabolism regulating, and immune enhancing functions. Vitamin E shows the effects of regulating cholesterol metabolism, promoting capillary proliferation, and improving cardiovascular and cerebrovascular diseases (Olas, 2016; Hu et al., 2021).

3.3. Fatty acids

All parts of sea buckthorn contain certain fatty acids, including linoleic acid, lauric acid, palmitic acid, oleic acid (Saeidi et al., 2016; Bouras et al., 2017; Ren et al., 2020). Most of them are unsaturated fatty acids that are easily absorbed by the human body, accounting for more than 70%. Sea buckthorn oil has various physiological activities and is a natural pain relieving medicine (Zielinsk and Nowak, 2017; Balkrishna et al., 2019; Maria et al., 2021). It can improve the body's immune function, promomte tissue regeneration and healing, and has good applications in the treatment of skin injuries (Cocetta et al., 2021; Cui et al., 2022). Furthermore, sea buckthorn oil has antidepressant effects and can significantly improve the mental state of the human body (Chen et al., 2023; Cui et al., 2022). The latest researches show that sea buckthorn oil has good antioxidant activity, regulating substance metabolism in the liver, alleviating liver damage caused by CCl_4, and demonstrate a good therapeutic effect on viral hepatitis (Hao et al., 2023). In addition, sea buckthorn oil promote the excretion of mercury from the kidneys and alleviate oxidative damage to the liver, and relieve liver damage in acute and subacute cadmium contaminated rats (Sheng et al., 2021; Xu et al., 2023).

3.4. Polysaccharides

One of the main components of sea buckthorn fruit is sugar and glycosides, mainly composed of glucose, fructose, and sucrose (Zhao et al., 2024; Lin et al., 2024). Researchers have found that sea buckthorn polysaccharides exhibit good antioxidant capacity in *vitro* and can effectively eliminate free radicals such as ABTS and DPPD (Liu et al., 2021; Zhao et al., 2023). Sea buckthorn polysaccharides can alleviate effect on acute liver injury induced by various drugs such as LPS in mice (Zhang et al., 2017; Marciniak et al., 2021; Zargar et al., 2022). It exerts polypharmaco-

logical effects through multiple pathways and targets (Lin et al., 2024). Therefore, sea buckthorn is expected to be developed as a new type of functional food or drug to improve liver injury. Recent investigations have established sea buckthorn polysaccharides as potent immunomodulatory agents with significant anti-inflammatory properties, garnering substantial research attention (Shi et al., 2024). Sea buckthorn fructose can activate the phagocytic function of macrophages, release pro-inflammatory factors, regulate the TLR4/MyD88/NF-κB signaling pathway, and participate in various physiological processes such as inflammatory response, oxidative stress, and immune regulation in the body (Mahahan et al., 2018; Li et al., 2019). Sea buckthorn polysaccharides can significantly enhance the immune function of cyclophosphamide induced immunocompromised mice, and increase the levels of TNF - α, IL-6, interferon - γ, and NO in spleen tissue (Ning et al., 2021). In addition, the expression of TLR4, MyD88, and p-MAPK7 can be expressed to reduce the levels of inflammatory and apoptotic factors in cells, and up regulate the expression of immunoglobulin to alleviate inflammatory reactions and exert anti-inflammatory and immune regulatory effects. Biological activities such as reducing anti-tumor, blood sugar, improving lipid metabolism disorders, and resisting obesity were observed with sea buckthorn polysaccharides (Attri and Goel, 2018; Tascioglu et al., 2021).

3.5. Proteins and amino acids

Compared with sea buckthorn fruit, sea buckthorn seeds are a high-quality plant protein resource, and have a higher protein content (Tan et al., 2018; Shang et al., 2023). Globulin, albumin, and alcohol soluble protein are the three types of sea buckthorn protein with relatively high content, which are mainly influenced by the planting area and extraction method (Liu et al., 2022; Wang et al., 2022). Sea buckthorn protein contains a rich variety of amino acids, including 8 essential amino acids for the human body, which endows it with many excellent biological activities. Studies have shown that sea buckthorn protein or its enzymatically hydrolyzed peptides have the effects of regulating blood glucose, antioxidation, and improving gut microbiota (Yuan et al., 2018; Sharma et al., 2018). Feeding sea buckthorn seed protein could reduce blood sugar in db/db diabetes mice, and this result was also verified in diabetes mice (Liu et al., 2022). Sea buckthorn protein can enhance the expression of AMP activated protein kinase (AMPK) and silent information regulator 1 in the liver by regulating the level of inflammatory factor C-reactive protein (CRP), interleukin-6 (IL-6), nuclear factor - (NF-κβ), tumor necrosis factor-α (TNF-α), thereby reducing the expression of low glucose 6-phosphatase (G-6-P), glycogen synthesis. Down regulation of hepatic glycogen synthase kinase-3β (GSK-3β) and carnitine palmitoyltransferase 1α (CPT1α) expression ameliorated insulin resistance, consequently improving hyperglycemia and polyuria in diabetic murine models (Shu et al., 2020; Deng et al., 2024; Kong et al., 2024). In addition, seabuckthorn protein can also regulate the composition and structure of intestinal flora, increase the abundance of *bifidobacteria*, *lactobacilli*, and *bacteroides*, reduce the abundance of *Clostridium globosum*, and improve diabetes symptoms (Chang et al., 2019; Kou et al., 2023; Li et al., 2024).

3.6. Other components

Sea buckthorn also contains phenolic acids, alkaloids, and mineral elements, which play important roles in promoting and maintaining overall health (Guo et al., 2017; Asofiei et al., 2019). Sea buckthorn polyphenols have been proven to have good effects in anti-cancer, improving gut microbiota, lowering blood lipids, and protecting the liver (Ge et al., 2023; Tian, 2023). And sea buckthorn alkaloids demonstrated good ability and nutritional health benefits (Ding et al., 2023). The mineral profile in sea buckthorn includes nutritionally significant levels of Ca, K, P, Mg, Na and 11 essential trace elements (Fe, Mn, I, Cu, Zn, Se, Cr, Mo, Co, Ni). These elements serve as enzymatic cofactors and metabolic modulators, particularly in energy transduction pathways (Tian et al., 2018; Tang, 2022).

4. Conclusion

As a typical medicinal and edible substance, sea buckthorn is rich in various chemical components and has multiple medicinal effects, which is in line with people's pursuit of the concept of natural and healthy food. At present, seabuckthorn has been widely used in various fields such as food, medicine, and health products. Comprehensive characterization of its phytoconstituents, particularly fruit pulpolisaccharides and leaf flavonol glycosides provides the molecular foundation for developing targeted functional products with validated health claims.

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Conflict of interest

All authors declare that there is no conflict of interest.

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Components and bioactivities of sea cucumber: an update

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Abstract

Sea cucumbers (holothurians), a classic marine invertebrate echinoderms, were found worldwide mainly as benthic organisms attached to sediments on the ocean floor. In particular, Sea cucumbers have long been esteemed as a valuable food due to their unique high nutritional value. Bioactives from Sea cucumbers play a crucial role in favor of human wellness. In this review, we collected recent research advances regarding this topic. First, we summarized the bioactive components from sea cucumbers including saponins, polysaccharides, polypeptides, proteins, fatty acids, cerebrosides, and gangliosides. Furthermore, we also outlined the role of sea cucumbers in anti-tumor, metabolic disease prevention , immune regulation and anti-aging. In a word, this review intends to cause further attention on the researches and development of sea cucumbers on the basis of the health protective mechanisms associated with the bioactives.

Keywords: Sea cucumbers; Bioactive compounds; Health benefits; Diseases.

1. Introduction

Sea cucumbers have long been esteemed as a valuable food due to their unique high nutritional value. Recent studies indicate that they play an important role in maintaining health and preventing diseases, making them a quintessential functional food. Sea cucumbers primarily inhabit tropical or subtropical marine rocky reef areas from 0 to 28°C. As benthic organisms, they clung to sediments, rocks, or algae, and feed on tiny organisms or other organic matter in coral sand(He and Li, 2015; Aminin et al., 2015). Belonging to the *phylum Echinodermata* and the class *Holothurioidea*, sea cucumbers are invertebrates further classified into three subclasses, six orders, and 24 families (He and Li, 2015). Currently, more than 900 species of sea cucumbers have been identified worldwide, yet only around 40 species are considered edible. To date, over 140 species have been discovered in China, of which 21 are edible. These 21 edible species include: from the order *Dendrochirotida*, the family *Cucumariidae* such as *Pentacta quadrangularis*, *P. inornata,* and *P. anceps*; from the order *Aspidochirotida*, the family *Holothuriidae* including *Bohadschia marmorata*, *B. argus*, *B. graeffei*, *Actinopyga lecanora*, *A. mauritiana*, *A. miliaris*, *Holothuria nobilis*, *H. leucospilota*, and *H. scabra*; from the order *Aspidochirotida*, the family *Stichopodidae* comprising *Apostichopus japonicus*, *Thelenota ananas, T. anax*, *Stichopus chloronotus*, *S. variegatus*, *S. horrens*, and *S. flaccus*; and from the order *Apodida*, the family *Synaptidae* including *Acaudina molpadioides* and *Paracaudina chinensis var. ransonnetii*. The geographical distribution of sea cucumbers is characterized by a gradual decrease in both diversity and abundance from the equator toward the poles.

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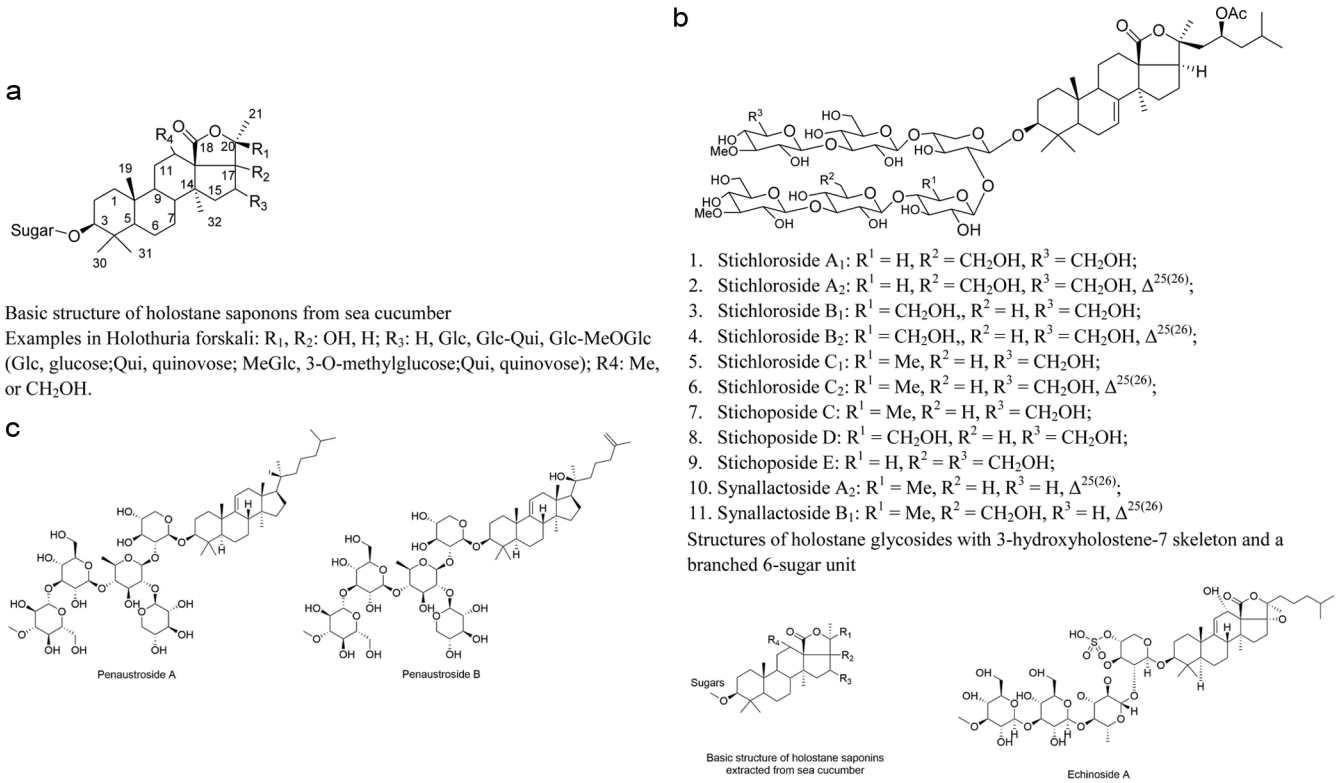

Figure 1,. Structures of saponins extracted from sea cucumber.

The Indian Ocean and the western Pacific host the greatest variety and number of sea cucumbers. Notably, *Apostichopus japonicus* is found in northern China, while the remaining edible species predominantly inhabit the southern marine areas near Hainan Island and the Xisha Islands (He and Li, 2015; Zhang et al, 2012).

The edible portion of sea cucumbers consists primarily of their body wall, which is nutritionally rich in proteins while being low levels of carbohydrates and fats. Moreover, this marine delicacy provides an excellent source of vitamins, including vitamin A, B1, B2, and B3—as well as minerals such as calcium (Ca), magnesium (Mg), iron (Fe), copper (Cu), and zinc (Zn). Additionally, it contains beneficial trace elements like manganese (Mn), selenium (Se), molybdenum (Mo), cadmium (Cd), and strontium (Sr) (Shi et al., 2016). These nutrients play a vital role in human growth and overall health. The biological functions of sea cucumbers are largely attributed to their unique bioactive compounds. For example, Hu et al. analyzed the protein content and amino acid composition across 8 sea cucumber species, revealing that a distinct amino acid profile characterized by a low lysine/arginine ratio, a feature associated with cholesterol-lowering effects (Wen et al., 2010). Sea cucumbers are rich in bioactive substances that exhibit diverse physiological activities, including anti-angiogenic, antitumor, anticoagulant, antihypertensive, anti-inflammatory, antimicrobial, antioxidative, and antithromboticeffects (He and Li, 2015; Shi et al., 2016; Bordbar et al., 2011)[3,4,6]. This review focus on summarizing the structural characteristics and physiological activities of these bioactive compounds, thereby providing a theoretical foundation and practical insights to guide future research on sea cucumber derived bioactive substances and their health physiological activities. Additionally, this work supports the development of functional foods or dietary supplements leveraging these marine resources.

2. Bioactive components

Sea cucumbers contain various bioactive components, including saponins, polysaccharides, polypeptides, proteins, fatty acids, cerebrosides, and gangliosides. Many of their physiological activities (e.g., immune regulation, antitumor effects, antidiabetic effects, and lipid-lowering properties, etc.) are closely linked to these active ingredients.

2.1. Saponins

Sea cucumber saponins (Figure 1) are a unique triterpenoid compounds characterized by their structural complexity. This complexity arises from variations in the substituent types and positions on the aglycone ring, as well as differences in the types, numbers, and linkage order of the attached sugars moieties (Zhao et al., 2018). Typically, these saponins have a relative molecular weight of approximately 1,000 Daltons, classifying them among the larger bioactive substances (Han et al., 2008; Kalinin, 2000). Structurally, each sea cucumber saponin features i an oligosaccharide chain attached to the C-3 position of the aglycone. In terms of solubility, these componds are readily soluble in polar solvents such as water, methanol, and aqueous ethanol, but exhibit low solubility in nonpolar organic solvents such as benzene and diethyl ether.

The aglycone moiety of sea cucumber saponins contains of 30 carbon atoms and is predominantly of the holostane type, characterizing by a pentacyclic nucleus with adjacent rings connected in a *trans* configuration. Based on the position of the lactone ring, sea cucumber saponins are classified into two types:Saponins with an aglycone comprise of lanostane-3β-ol with a γ(18,20)-lactone

in the E-ring of the pentacyclic triterpene are referred to as holostane saponins (Figure 1b). For instance, Stichlorosides (Figure 1b), isolated and identified from *Eupentacta fraudatrix, Holothuria lessoni, Bohadschia marmorata, Stichopus chloronotus* and *Staurocucumis liouvillei* belong to this category. Penaustroside A and Penaustroside B from *Pentacta australis* (Figure 1c) belong to non-holostane-type triterpenoid oligoglycosides. Notably, holostane saponins represent the majority of identified sea cucumber saponins accounting for over 70 out of more than 100 known structures. (Miyamoto et al., 1992). Conversely, if the lactone ring is located at the C-18(16) position or if the aglycone lacks a lactone ring entirely, the saponin is termed a non-holostane saponin. Cumarioside G2, isolated and identified from Eupentacta fraudatrix, is an example of a non-holostane saponin, a group that is less commonly found (Avilov et al., 1994).

At the C-3 carbon atom, an oligosaccharide side chain composed of 2–6 monosaccharide units is generally attached. This side chain exhibits structural diversity, existing either linear or branched configurations, and is composed of various monosaccharides such as glucose, 3-O-methylglucose, xylose, 3-O-methylxylose, and quinovose. There are significant differences among various sea cucumber saponins regarding both the number and the types of monosaccharides constituting the oligosaccharide chain. The hydroxyl groups on certain monosaccharides are often undergo sulfation, with one or several hydroxyl groups being esterified by sulfate moieties. Additionally, a hydroxyl group at the C-3 position of the aglycone forms a glycoside bond with the sugar via a β-O-glycosidic linkage. Hydroxyl or acetyl groups are usually attached to the C-12, C-16, and C-17 positions of the aglycone. Furthermore, one or more double bonds are typically present at positions such as Δ7(8), Δ8(9), Δ9(11), Δ24(25), and Δ25(26) of the aglycone, and conjugated double bonds occasionally appear on the side chain (Miyamoto et al., 1992).

2.2. Polysaccharides

Sea cucumber polysaccharides consitute another nutritionally and physiologically significant component of the body wall. Their content varies differently among species, generally accounting for about 6% of the total organic matter in dried sea cucumbers, while some species may contain as much as 31% polysaccharides. The composition andabundance of polysaccharides are key indicators for assessing the nutritional quality of sea cucumbers. Based on the structural characteristics of the sugar chains, sea cucumber polysaccharides can be classified into two categories. One category comprises glycosaminoglycans (GAGs), also termed acidic mucopolysaccharides, are heteropolysaccharides with branched structures. Their sugar residues primarily consist of D-N-acetylgalactosamine, D-glucuronic acid, and L-fucose, with a relative molecular weight ranging between 40,000 and 50,000. The other category consists of holothurian fucans (HF), which are linear homopolysaccharides predominantly of L-fucose residues, with a higher molecular weight of 80,000 and 100,000. Although these two types differ in monosaccharide composition and chain structure, both types undergo partial sulfation of hydroxyl groups on their sugar chains. Variations in sulfation sites and degrees contribute to structural diversity and variations in molecular weight, complicating the purification and structural characterization (Shi et al., 2016; Yin et al, 2009).

2.3. Proteins and peptides

Proteins are the most abundant organic component in the sea cucumber body wall, accounting for approximately 90% of its weight. These proteins are composed of 18 amino acids, linked by peptide bonds, with glycine, glutamic acid, and arginine being relatively abundance. Among these, seven essential amino acids required for human nutrition are present. Sea cucumber peptides refer to low-molecular-weight polypeptides, typically composed of 3-10 amino acids, obtained through protease hydrolysis of fresh sea cucumbers (He and Li, 2015). These peptides are predominantly collagen-derived peptides, along with glycopeptides, neuropeptides, antimicrobial peptides, and so on (Liu et al., 2010). Due to their lower molecular weight, these peptides exhibit a broader range of physiological activities compared to intact sea cucumber proteins, including lowering blood pressure and lipid levels, preventing atherosclerosis, enhancing fatigue resistance, exhibiting antimicrobial and antiviral properties, boosting immune function, as well as delaying aging and providing antioxidant activity. Moreover, in terms of solubility, stability, and bioavailability through digestion and absorption, these peptides outperform sea cucumber proteins, leading to significantly higher bioavailability compared to conventional sea cucumber products (Zhong et al., 2007).

2.4. Other components

Sea cucumbers contain 0.24–0.83% lipid substances, including fatty acids, gangliosides, cerebrosides, and others. The predominant fatty acids are of the structural types 16:0, 18:0, 20:1, 20:4 (n-6), and 20:5 (n-3). Variations in fatty acid composition exist among sea cucumbers from different marine habitats. For instance, those inhabiting temperate waters have approximately 15.5% branched-chain fatty acids, a higher content of 20:5 (n-3), and a lower content of 20:4 (n-6), whereas sea cucumbers from tropical waters contain only about 1% branched-chain fatty acids. Approximately 12.5–29.0% of the fatty acids are phospholipids, such as gangliosides and cerebrosides (Yuan et al., 2008). Cerebrosides have been shown to possess various biological activities, including inhibition of tumor cell growth, anti-HIV effects, and hepatoprotective properties. To date, 29 distinct cerebrosides have been isolated and identified from species such as Stichopus, Apostichopus, and Holothuria.

Research by Gowda et al. (Gowda et al., 2008) has demonstrated that lectins isolated from different sea cucumber species exhibit remarkable diversity in their biological activities. For example, lectins isolated from Stichopus display potent hemolytic activity against both human and murine erythricytes, while those obtained from Holothuria scabra show additional antibacterial properties. Liu et al.(Liu, 2008) purified a Stichopus lectin with a molecular weight of 31,000 that exhibits unique biochemical properity. This lectin's agglutination of rabbit erythrocytes is specifically inhibited by bovine thyroglobulin, but remains unaffected by D-fructose, D-mannose, or D-glucose. Moreover, its activity demonstrates exceptional stability, being independent of metal cations such as Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, or by EDTA, mataining functionality across a broad pH values ranging from 4.0 to 10.14, and retaining hemagglutinating capacity even after exposure to 90°C for 30 minutes.

Additionally, sea cucumbers contain pigments—primarily naphthoquinones, carotenoids, melanins, porphyrins, echinochrome, and astaxanthin—as well as organic compounds such as methionine, taurine, and niacin, and trace elements including Fe, Cu, Zn, Mn, Se, Mo, Ge, and Sr. These substances are essential for human growth and development, fulfilling irreplaceable physiological functions (Zhang et al., 2012).

Table 1. The antitumor activities of various sea cucumber saponins summarized on the basis of a previous report

Glycoside	Species	Physiological Activities
Philinopside A	*P. quadrangularis*	Reduces cell viability, induces apoptosis, and inhibits angiogenesis as well as tumor growth both in vitro and in vivo.
Philinopside E		
Patagonicoside A	*P. graeffei*	Reduces cell viability; inhibits cell adhesion, migration, metastasis, and invasion; induces apoptosis; causes cell cycle arrest; and inhibits tumor cell growth in vivo.
Echinoside A		
24-dehydro echinoside A		
Colochiroside A	*Colochirus anceps*	Reduces cell viability and inhibits tumor cell growth in vivo.
Intercedenside A	*Mensamaria intercedens*	Reduces cell viability and inhibits tumor cell growth in vivo.
Okhotoside B1	*Cucumaria okhotensis*	Reduces cell viability.
Okhotoside B2		
Okhotoside B3		
Frondoside A	*C. frondosa; C. okhotensis*	Reduces cell viability, inhibits cell proliferation, induces cell cycle arrest and apoptosis; inhibits cell migration and invasion; suppresses metastasis and angiogenesis; reduces tumor growth in vivo; and enhances the inhibitory effects of other antitumor agents.
Stichoposide C	*T. anax*	Induces apoptosis and inhibits tumor growth in vivo.
Cucumariosides A2	*C. japonica*	Inhibits cell proliferation, causes cell cycle arrest, induces apoptosis; inhibits cell metastasis and invasion; reduces tumor growth in vivo; and enhances the tumor growth-inhibitory effects of other agents.

3. Physiological activities

3.1. Antitumor activity

Numerous bioactive components in sea cucumbers exhibit antitumor properties, Among which saponins have been the most extensively studied due to their potent antitumor activity (Aminin et al., 2015; Kalinin et al., 2008; Zou et al., 2005) (Table 1). As early as 1976, Pettit *et al*. (Pettit et al., 1976) isolated and purified three active compounds, namely Stichostatin 1, Thelenostatin 1, and Actinostatin 1, from the Cuvierian tubes of sea cucumbers. These pioneering findings revealed the compounds' remarkable ability to inhibit the proliferation of both mouse leukemia cells (P-388, L-1210) and human oral carcinoma cells (KB). Zhang et al. (S.-Y. Zhang et al., 2006) isolated and identified three novel triterpene glycosides—Fuscocinerosides A, B, and C—from the metabolites of H. fuscocinerea. These compounds not only exhibited cytotoxicity against tumor cells but also inhibited angiogenesis. Recent studies have further confirmed the broad-spectrum antitumor potential of sea cucumber saponins,with compelling evidence showing their significant growth-inhibitory effects on multiple cancer cell lines, including human gastric cancer cells, mouse leukemia cells, prostate cancer cells, ovarian cancer cells, liver cancer cells, Hela cells, and colorectal cancer cells (Wang et al., 2006; Wu et al., 2007). Dong et al (2012) isolated and identified eight sea cucumber saponins from Pearsonothuria graeffei, with structure-activity relationship studies revealing a critical finding:the length of the glycoside chain attached to the aglycone correlate significantly influences antitumor activity. lAdditionally, structural modifications on the side chain—such as hydroxylation, the presence of double bonds or epoxy structures—can reduce the antitumor efficacy of sea cucumber saponins. Avilov et al. (Avilov et al., 2000) isolated and identified four monosulfated triterpene saponins from Pentamera calcigera: Cucumarioside G2, Calcigerosides B, Calcigerosides C1, and Calcigerosides C2. Among these, Calcigerosides B, C1, and C2 are novel triterpene saponins. The desulfated derivatives of Calcigerosides B, C1, and C2 exhibited strong inhibitory activities with IC50 values under 50 μg/mL againstmultiple cancer cells: P-388 (mouse leukemia cells), A-549 (human lung cancer cells), HT-29 (human colon cancer cells), and Mel-28 (human malignant melanoma cells).

Philinopside E (Tian et al., 2007) inhibits angiogenesis by interacting with the extracellular domain of the kinase insert domain-containing receptor (KDR) for vascular endothelial growth factor (VEGF). This interaction blocks the binding of KDR to VEGFand downstream signaling, thereby suppressing neovascularzation. Tong *et al*. (Tong et al., 2005) isolated a sea cucumber saponin, Philinopside A, from P. quadrangulari, which exhibits multiple receptor tyrosine kinases, including the VEGF receptor, fibroblast growth factor receptor-1 (FGFR-1), platelet-derived growth factor receptor-β (PDGFR-β), and epidermal growth factor receptor (EGFR). This multi-receptor inhibition induces apoptosis in both tumor cells and tumor-associated endothelial cells, leading to reduced tumor volume in mouse sarcoma (S-180) models. Intercedensides A, B, and C, isolated by Zou et al. (Z.-R. Zou et al., 2003) from Mensamaria intercedens, exhibit potent antitumor activity against 10tumor cell lines, including Lewis lung carcinoma and S-180, with ED50 values ranging from 0.6–4.0 μg/mL.

Another key bioactive component in the sea cucumber body wall is polysaccharides. Janakiram *et al*. (Janakiram et al., 2010) demonstrated that Frondanol A5, a glycolipid extract from Cucumaria frondosa, inhibits colon cancer with an IC50 value of 0.13 mg/mL, while showing no toxicity normal human cells. Gao *et al*.

(Gao et al., 2008) investigated the effects of acidic polysaccharides from *Stichopus japonicus* on the HepG2 human liver cancer cell line. They found that these polysaccharides suppress HepG2 proliferation in a time- and dose-dependent manner by downregulating the expression of the Bcl-2 gene and upregulating nm23-H1 gene expression (Lu et al., 2010). Furthermore, Song et al. (Song et al., 2013) employed a diethylnitrosamine (DEN)-induced rat model of hepatocellular carcinoma to evaluate the therapeutic effects of Stichopus japonicus acidic polysaccharides administered via intraperitoneal injuction and oral gavage. Their findings revealed that these polysaccharides significantly suppressed serum alpha-fetoprotein (AFP) and proliferating cell nuclear antigen (PCNA), expression, promoted p21 expression, enhanced cellular immunity, and inhibited DEN-induced hepatocarcinoma growth.

Sugawara *et al*. (Sugawara et al., 2006) treated human colorectal adenocarcinoma cells (DLD-1), human colon cancer cells (WiDr), and human colon adenocarcinoma cells (Caco-2) with sea cucumber cerebrosides, observing chromatin condensation, increased Caspase-3 activity and subsequent apoptosis. Wang (Wang, 2007)[36] isolated collagen-derived peptides from Stichopus japonicus (Japanese sea cucumber) and administered the homogenized peptide solution via oral gavage to mice with transplanted tumors models. The treatment significantly suppressed the growth of S-180 sarcoma, increased spleen and thymus indices, and elevated serum hemolysin levels in tumor-bearing mice, suggesting that these peptides enhanc immune function to control and eliminate tumor cells. Zhou *et al*. treated sea cucumber (S. japonicus) proteins with trypsin followed by ultrafiltration membrane filtration, to obtain the peptide LSCP-2, which significantly inhibited the growth of gastric cancer cells (SGC-7901) and human breast cancer cells (MCF-7) (Zhou et al., 2012). Du *et al*. (Du et al., 2012) investigated the antitumor activity of sea cucumber cerebrosides in S-180 sarcoma-bearing mice and found that a dose of 50 mg/kg reduced tumor weight by 45.24%, prolonged survival time by 55.28%, downregulated the expression of Bcl-2 and Bcl-xL, and upregulated Bax, Cytochrome C, Caspase-9, and Caspase-3 in tumor cells. These results indicate that sea cucumber cerebrosides induce apoptosis via the mitochondrial apoptosis pathway.

3.2. Improvement of metabolic diseases

Rodriguez *et al*. (Rodríguez et al., 2000) demonstrated that dietary supplementation with sea cucumber polypeptides in rats resulted in increased serum high-density lipoprotein (HDL) levels and decreased serum triglyceride concentrations compared to the control group, indicating the lipid-modulating bioactivity of these peptides. Taboada *et al*. (Taboada et al., 2003) observed that rats fed with sea cucumber- supplemented diets for 16 consecutive days exhibited significantly lower triglyceride levels than those receiving casein. In addition, the sea cucumber diet was associated with enhanced activities of intestinal maltase, lactase, alkaline phosphatase, and leucine aminopeptidase, while decreasing hepatic leucine aminopeptidase and glutamyl transpeptidase activities compared to the casein group. These results suggest that sea cucumber contains active ingredients capable of modulating lipid metabolism and influencing enzymatic activies in both intestinal and hepatic tissues.

Zhao *et al*. (Zhao et al., 2009) utilized enzymatic hydrolysis to break down sea cucumber body wall proteins and subsequently applied membrane separation techniques to obtain an angiotensin I-converting enzyme (ACE) inhibitory peptide (MEGAQEAQGD). At a dose of 3 μmoL/kg, this peptide significantly reduced blood pressure in spontaneously hypertensive rats. Wang *et al*. (Wang et al., 2009) administrated sea cucumber to rats via oral gavage, observing a marked reduction in lipid content in both the blood and liver, along with an increase in fecal fat content. These findings indicate that sea cucumber can inhibit lipid absorption and promote lipid degradation, thereby improving overall lipid metabolism.

Furthermore, Hu *et al*. (Hu et al., 2009) found that supplementation with sea cucumber saponins effectively attenuated obesity-related metabolic disturbances in rats. The treatment not only suppressed adipose tissue accumulation, particularly in perirenal fat mass,but also significantly lowered the concentrations of total cholesterol (TC) and triglycerides (TG) in both serum and liver. The study also demonstrated a direct inhibitory effect on fatty acid synthase (FAS). In addition, Xu *et al*. (Xu et al., 2011) investigated the impact of cerebrosides isolated from Acaudina molpadioides (AMC-2) on fatty liver in rats. Adding 0.03% and 0.006% AMC-2 to drinking water significantly decreased hepatic triglyceride (TG) and total cholesterol (TC) levels, seppressed stearoyl-coenzyme A desaturase (SCD) mRNA expression and activity, and reduced de novo lipogenesis, thereby ameliorating non-alcoholic fatty liver disease in rats.

3.3. Hemolytic activity

Sea cucumber saponins also exhibit significant hemolytic activity, a property intrinsically related to their unique molecular structure. These saponins bind to unsaturated sterols at the 5(6) position of biological membranes, forming saponin–steroid complexes that disrupt plasma membrane integrity by inducing large pore formation. At low concentrations (e.g., in the blood), smaller pores formed on erythrocyte membranesthat selectively permit K^+ efflux, ultimately leading to cellular dysfunction and membrane rupture (Hu et al., 2005; Kalinin et al., 1996). Kalinin (Kalinin, 2000) observed that higher saponin concentrations induce larger pores form capable of facilitating the leakage of macromolecules like amino acids, resulting in complete cellular disruption and hemolysis. In his study of the relationship between saponin structure and hemolytic activity, Kalinin et al. (Kalinin et al., 1996) investigated the structural basis of saponin-induced hemolysis via K^+ efflux kinetics, demonstrating a positive correlation between K^+ leakage rate and pore density. For instance, sulfation at C-4 of xylose (first sugar in the glycan chain) or C-6 of glucose (third sugar) enhances K^+ efflux. Methylation at C-3 of the terminal sugar in the glycan chain also exacerbates K^+ loss. A 7(8)-double bond in the aglycone combined with C-16 ketone substitution reduces the rate of K^+ efflux. Substitutions at different positions may alter the structure of sea cucumber saponins, ultimately determining their membrane-disruptive capacity and hemolytic potential.

Xiong et al. (Xiong et al., 2008) extracted Nobiliside A from Holothuria nobilis and investigated its hemolytic properties. At a low concentration (0.41 μg/mL), Nobiliside A caused hemolysis in 5% of red blood cells, while complete hemolysis was observed at a higher concentration (16.0 μg/mL). Notably, pre-incubation with cholesterol abolished hemolysis, confirming sterol-dependent pore formation (Anisimov, 1987). Due to their strong hemolytic effects, sea cucumber saponins are unsuitable for intravenous administration use. Structural engineering to minimize sterol binding may reduce toxicity and enable therapeutic applications.

Fonseca et al. (Fonseca et al., 2009) isolated a sulfated fucan and a fucosylated chondroitin sulfate from Ludwigothurea grisea and studied their effects on coagulation, thrombosis, and hemorrhage. They found that both compounds possess anticoagulant activity, albeit via entirely different mechanisms. Fucosylated chondroitin sulfate exerts its anticoagulant effect by inhibiting heparin cofactor II (HC-II), whereas sulfated fucan targets both antithrombin and HC-II to suppress thrombin. The sulfated fucan

effectively inhibited venous thrombosis at lower doses, while FCS was more potent against arterial thrombosis. Li Zhiguang et al. (Li et al., 2000) examined the effects of glycosaminoglycans (GAGs) on human venous endothelial cells and found that GAGs reduced the procoagulant activity and tissue factor expression in these cells while increasing the expression of thrombomodulin. Chen et al. (Chen et al., 2012) isolated a sulfated deoxy-galactan fucan (Fucan-Ib) and a fucosylated chondroitin sulfate (fCS-Ib) from the Mexican sea cucumber Isostichopus badionotus. Fucan-Ib demonstrated excellent anticoagulant and antithrombotic activities by acting on thrombin, while fCS-Ib exerted its anticoagulant effects via heparin cofactor II.

3.4. Immune regulatory activity

Aminin et al. (Aminin et al., 2001) reported that injection of saponins extracted from C. japonica into mice stimulated innate immune responses by enhanceing the phagocytic activity and increasing the release of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), while promoting antibody formation. Cumaside, a monosulfated triterpene glycoside isolated from C. japonica by Aminin et al. (Aminin et al., 2011), has been shown to possess radioprotective properties. Echinoside A can activate metallothionein activity and protect against CCl_4-induced liver damage in mice (Itoh et al., 1997). Moreover, sea cucumber polysaccharides have been found to enhance cellular immunity and can improve immune deficiency conditions. Huang et al. (Huang et al., 2001) isolated a polysaccharide (designated Dichotomous Sea Cucumber Polysaccharide-1) from dried sea cucumbers and demonstrated that it accelerates the secretion of interleukin-2 (IL-2), thereby promoting the proliferation of mouse splenic lymphocytes in vitro. The polysaccharide also increases spleen and thymus indices and enhances the delayed-type hypersensitivity response, indicating a marked immunostimulatory effect.

3.5. Antifungal and antiviral activity

Kumar et al. (Kumar et al., 2007) systematically evaluated 4 antifungal activities-Marmoratoside A, 17-α-hydroxy impatienside A, Marmoratoside B, and 25-acetoxy bivittoside D against 20 fungal strains, revealing particularly potent antifungal activity for Marmoratoside A and 17-α-hydroxy impatienside A with IC50 values ranging form 0.7 and 2.81 μM. Sedov et al. (Sedov et al., 1990) examined the effects of saponins derived from Cucumaria on various Gram-negative bacteria. In vivo, mice infected with different Gram-negative pathogens were treated via intraperitoneal injection of the saponins, achieving a cure rate of up to 90% when administered via intraperitoneal injection. In 1976, Kitagawa et al. (Kitagawa et al., 1976) isolated the saponin Holotoxin A and B from Stichopus japonicus,which were subsequently developed into effective treatments for tinea peids. Contemporary research has expanded our understanding of these antimicrobial effects, with multiple studies confirming the inhibitory activity of sea cucumber saponins against diverse pathogens including yeast, Salmonella, and Candida albicans (Pacheco et al., 2000; Mourão et al., 1998; Cong et al., 2006; Xiao et al., 2005; Wang et al., 2004). With ongoing research into the antifungal properties of sea cucumber saponins, they are poised to become a new focus in the development of antifungal drugs.

Researches demonstrated the significant antiviral and antimicrobial potential of bioactive compounds derived from sea cucumbers. Minamiguchi K et al. (Minamiguchi et al., 2003) conducted a comparative study showing that sea cucumber glycosaminoglycan (sc-GAG) exhibited potent anti-herpetic activity against herpes simplex virus (HSV) with an half maximal effective concentration (EC_{50}) of 10 μg/mL , representing a 2.5-fold greater potency than the conventional antiviral drug vidarabine (EC_{50} = 25 μg/mL).. Additionally, Schillaci et al. (Schillaci et al., 2013) isolated a peptide fragment with a molecular weight of 5 kDa from the coelomic fluid of H. tubulosa. At a concentration of 3.1 mg/mL, this peptide inhibited biofilmformation *Staphylococcus aureus* and *Pseudomonas aeruginosa*. These findings collectively underscored the diverse therapeutic applications of sea cucumber-derived compounds in combating viral infections and microbial biofilms.

3.6. Anti-aging effects

The accumulation of reactive oxygen species (ROS) in the body, promptly cleared, can cause oxidative tissues damage and accelerate aging if not efficiently eliminated. Fang Kun (Fang, 2013) demonstrated that papain-hydrolyzed fresh sea cucumbers peptides exhibited significant antioxidant potential, scavenging 25.10% of superoxide anions. When neutral protease A.S.1398 was employed for hydrolysis, the resulting peptides achieved a 77.00% hydroxyl radical scavenging activity. Wang et al. (Wang et al., 2010) isolated various peptide fractions of different molecular weights from autolyzed sea cucumbers via membrane separation and compared their antioxidant activities with that of vitamin C and all four peptide fractions demonstrated to display stronger antioxidant activity in a dose-dependent manner. Additionally, Wang et al (Wang, 2007) reported that Japanese Stichopus collagen peptides (AJCP) could enhance the activities of superoxide dismutase, glutathione peroxidase, and catalase in mice, while reducing malondialdehyde levels. Moreover, AJCP significantly increased the total hydroxyproline content in the skin, repaired damaged collagen fibers, and provided a protective effect against collagen degradation, suggesting its potential as a therapeutic agent for oxidative stress-related tissue damage and aging..

3.7. Other activities

During the peri-ovulatory period, the saponin content in sea cucumbers rises, which has been shown to inhibit the maturation of sea cucumber oocytes and prolongs their reproductive period. Mats et al. (Mats et al., 1990) isolated Holotoxins A1 and B1 from Stichopus japonicus, revealing that this mixture not only suppresses ovulation but also stimulates uterine contractions. In another study, sulfated polysaccharides extracted from Stichopus were combined with fibroblast growth factor (FGF) and applied to rat neural stem cells. This results demonstrated a notable enhancement in both proliferation and differentiation efficiency compared to FGF treatment alone. This effect possibly attributed to the ability of Stichopus sulfated polysaccharides to reduce apoptosis, prolong cell survival, and promote neurogenesis. Furthermore, Zhang et al. (Y. Zhang et al., 2010) demonstrated that sea cucumber sulfated polysaccharides at a concentration of 500 ng/mLstimulated the proliferation of neural stem/progenitor cells (NSPCs). Likewise, they also enhanced the proliferative effect of fibroblast growth factor-2 (FGF-2) on NSPCs, likely via reducing apoptosis in NSPCs.

4. Summary

Sea cucumbers contain a variety of bioactive substances that are beneficial to human health. To the date, various bioactives have

been isolated and identified. The separation and purification of sea cucumber compounds are particularly challenging due to the abundance of structural isomers. Current methods rely on multi-step chromatography, gel filtration, and prerarative HPLC. These methods are complex, time-consuming, and solvent-intensive, often resulting in low amounts of the target compounds. Therefore, establishing simplified, high-efficiency methods for isolation of sea cucumbers will greatly promote the identification of new bioactive substances and facilitate the development of functional foods.

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Elicitor-induced enhancement of bioactive content and α-amylase inhibition in cluster bean sprouts

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Abstract

This study investigates the effects of elicitor treatments on bioactive compounds and α-amylase inhibitory activity in *Cyamopsis tetragonoloba* sprouts. Elicitors like glutamic acid, ascorbic acid, chitosan, and their combination were used on seeds to check their effects on germination, radicle length, phytochemical content, and antioxidant activity. Glutamic acid and a combination of 50 mg/kg low-molecular-weight chitosan with 5 mM glutamic acid significantly increased protein, phenol, and ascorbic acid contents. The highest DPPH radical scavenging activity (15.57%) was observed in sprouts treated with 50 mg/kg chitosan and 5 mM glutamic acid, while no notable ferric reducing antioxidant power (FRAP) activity was recorded. Elicited sprouts showed significantly higher α-amylase inhibitory activity than non-germinated seeds, with the chitosan-glutamic acid combination achieving the highest inhibition (53.92%). These findings underscore the potential of elicitors to enhance the nutritional and functional properties of cluster bean sprouts, warranting further research into specific phenolic compounds and other bioactive.

Keywords: α-Amylase inhibitory activity; Bioactive compounds; Cluster bean; Elicitors; Germination; Glutamic acid.

1. Introduction

Legumes such as beans, lentils, and peas are prized worldwide for their nutrient-dense composition, offering starch, dietary fiber, protein, healthy fats, and essential minerals (Lin and Lai 2006). However, antinutritional factors like phytates, tannins, and lectins can hinder nutrient absorption. Germination, a straightforward bioprocessing method, reduces these compounds by enzymatically breaking them down, enhancing nutrient bioavailability, digestibility, and taste (Urbano et al., 2005). It also decreases antinutritional factors while boosting bioactive compounds, which contribute to antioxidants, anti-inflammatory, and other health-promoting properties in legume sprouts (Gan et al., 2017).

To further increase bioactive compound levels, elicitors—compounds that activate plant defense mechanisms—are applied during germination. Elicitors are either biotic (e.g., chitosan, glutamic acid, salicylic acid) or abiotic (e.g., NaCl, light, temperature stress) and stimulate phytochemical production by mimicking stress responses (Baenas et al., 2014). According to Tang et al. (2021), chitosan elicitor was found to have a large accumulation of vitamin C, total phenolic, and total flavonoid content in the soybean sprouts (Tang et al., 2021). Treatment with glutamic acid has been shown to speed up sprouting because it offers an extra source of nitrogen for effective embryonic growth (Ampofo and Ngadi 2021). Using ascorbic acid as an elicitor, sprouted kidney beans, fava beans, and peas suggested de NOVO synthesis of phenolic chemicals. It is generally known that ascorbic acid promotes the phenyl propanol and pentose phosphate pathways, which in turn causes the production of phenolic compounds in legumes (Uchegbu and Amulu 2015). Biotic elicitors include phytohormones, amino acids, and polysaccharides, while abiotic ones involve salts, ultrasound, or high pressure (Liu et al., 2019). These enhance seedling growth and the synthesis of compounds like flavonoids, phenolic acids, and amino acids, which show α-amylase inhibitory activity

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(Limón et al., 2014; Teoh and Das 2018). Therefore, considering the response exerted by the elicitors on the seeds during germination, ascorbic acid, glutamic acid and combination of chitosan with glutamic and lactic acid were selected for the present study.

With the rising global prevalence of diabetes, there is an urgent need for effective therapeutic strategies. Guidelines from the American Diabetes Association and the European Association for the Study of Diabetes recommend α-amylase and α-glucosidase inhibitors to manage blood glucose levels by reducing carbohydrate absorption (Yan et al., 2019). This study focuses on *Cyamopsis tetragonoloba* (cluster bean), a nutrient-rich legume from the Fabaceae family, containing vitamins, amino acids, flavonoids, and phenolic compounds (Khare 2004). While cluster bean extracts have shown various health benefits in prior research, the effects of germination and elicitation on its sprouts are underexplored. This study examines the phytochemical composition, antioxidant activity, and in vitro α-amylase inhibitory activity of germinated cluster bean seeds treated with various elicitors, aiming to highlight their potential as functional foods with enhanced nutritional and bioactive properties.

2. Materials and methods

2.1. Seed procurement and cleaning

Seeds of *Cyamopsis tetragonoloba* (L.) Taub (Variety - Gujarat Vegetable Guar 11-GVG 11_Anand Bahar) were obtained from the Main Vegetable Research Station, Anand Agricultural University, Anand, Gujarat, India (Latitude 22.5523° N, Longitude 72.9240° E) in September 2023. Upon collection, seeds were meticulously cleaned to remove debris and stored in an airtight container.

2.2. Chemicals

Chemicals and reagents used in this study were sourced as follows: α-amylase porcine pancreas (5 U/mg), ascorbic acid, chitosan (Low MW), 3,5-dinitrosalicylic acid (DNSA), gallic acid, glutamic acid, and 2,4,6-tripyridyl-S-triazine (TPTZ)were purchased from SRL Pvt Ltd India. α, α-diphenyl-β-picrylhydrazyl (DPPH) and α-amylase from malt (RM 638) were procured from Himedia Pvt Ltd India. All other chemicals were of analytical grade.

2.3. Elicitor treatments

Elicitor concentrations were selected based on the study by Burguieres et al., (2007). The elicitors employed included 500 µmol/L ascorbic acid, 5 mM glutamic acid, a combination of 50 mg/kg LMW chitosan with 5 mM glutamic acid, and a combination of 50 mg/kg LMW chitosan with 5 mM lactic acid. Distilled water (DW) was used as the control. Elicitor solutions were freshly prepared in DW and applied daily by spraying onto seeds during germination.

2.4. Germination of cluster bean seeds

The germination with elicitors was carried out as described by Limón et al., (2014). The cluster bean seeds were initially washed with DW to remove surface impurities and subsequently immersed in a 0.05% sodium hypochlorite solution for 30 minutes for disinfection. After thoroughly rinsing with tap water to neutralize pH and eliminate residual sodium hypochlorite, the seeds were soaked in DW for 6 hours with periodic stirring. Hydrated seeds were then distributed into five trays lined with filter paper for elicitor treatment. Elicitor solutions were applied daily, and germination was conducted at 37 °C in a hot air oven for 72 hours. Radicle length and germination rates were recorded, followed by drying of sprouts at 40 ± 2 °C for 48 hours. Dried sprouts were ground into fine powder and stored at −20 °C until further use.

2.4.1. Germination percentage

The germination percentage (%) was determined by dividing the number of germinated seeds by the total number of seeds started, multiplied by 100, using the formula:

$$\text{Germination percentage}(\%) = \frac{\text{Number of Germinated seed}}{\text{Total number of seeds}} \times 100$$

This calculation was performed for each respective tray containing the seeds treated with different elicitors and the control group.

2.4.2. Aqueous sample preparation

One gram of powdered sprouts from each group (elicited and control) was suspended in 10 mL of DW, vortexed for 10 minutes, and centrifuged at 1,391 g for 10 minutes. The resulting supernatant was stored at −20 °C for subsequent analyses.

2.5. Phytochemical analysis

The protein content was determined using BSA as a standard and expressed as mg/g of BSA equivalents following Lowry et al., (1951). A known amount of sample was added to the alkaline copper solution, incubated, and then folin reagent was added to the test tube in the dark and incubated for half an hour. The blue color absorbance was measured at 660 nm. Phenol content was measured with gallic acid as a standard and reported as mg/g of gallic acid equivalents as described by Thimmaiah (1999). A known amount of sample was combined with FCR reagent and incubated for three minutes at room temperature. Twenty percent sodium carbonate was added in tubes and kept in a boiling water bath for one minute. The absorbance of the reaction mixture was measured at 650 nm after the tubes had cooled. Total ascorbic acid content was quantified following the method of Schaffert and Kingsley (1955). The seed samples were treated with thiourea and DNPH reagent, followed by incubation in a boiling water bath at 100 °C for 15 minutes and cooled under tap water. 85% chilled sulphuric acid was added in the treated sample and further incubated for 15 minutes in an ice bath. The absorbance of the reaction mixture was measured at 515 nm.

2.6. Antioxidant activity

Antioxidant activity was assessed using DPPH radical scavenging and FRAP assays. The DPPH test was carried out in accordance with Brand-Williams et al. (1995). The seeds aqueous extracts were incubated with DPPH solution (0.1 mM in methanol) in dark for 30 minutes at 37 °C and absorbance was measured at 517 nm against blank. Using the following formula, the scavenging activity was determined:

$$\text{DPPH Scavenging activity}\ (\%) = \frac{\text{A of control-A of sample}}{\text{A of control}} \times 100$$

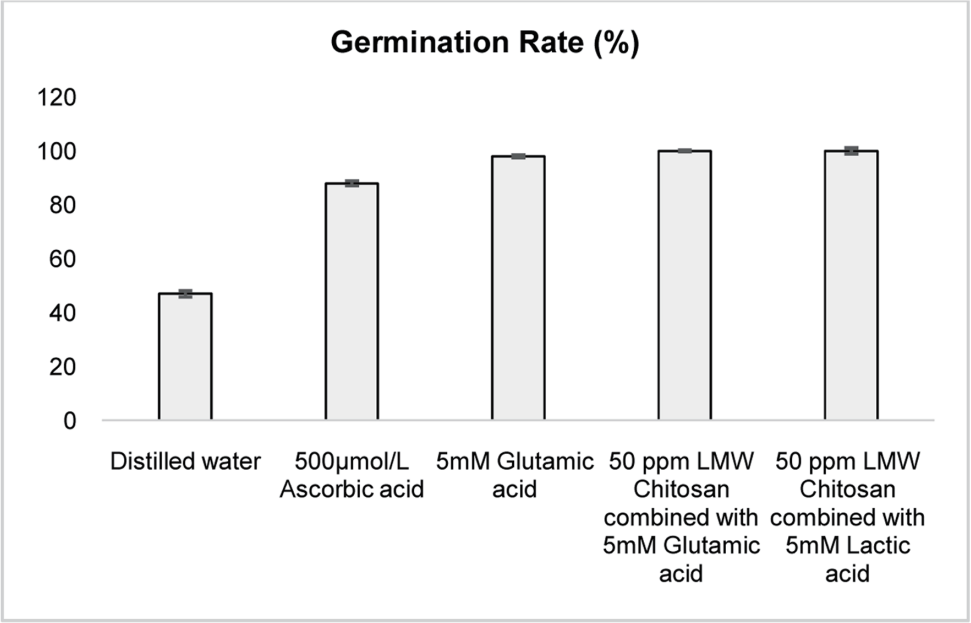

Figure 1. Germination percentage of cluster bean seeds following various chemical elicitor treatments. All the values are expressed as Mean ± SEM (n = 3).

The FRAP assay used ascorbic acid as the reference standard and was conducted according to Benzie and Strain (1996) procedure. Aqueous samples were mixed with FRAP solution (containing TPTZ, FeCl3 and acetate buffer) incubated for 30 min at 37 °C in the dark. The absorbance readings were taken at 593 nm wavelength.

2.7. α-Amylase inhibitory activity

The α-amylase inhibition assay was conducted following a modified method based on Nair et al., (2013). Triplicate samples were incubated with 100 µl of α-amylase from porcine pancreas (5 U/mg) or α-amylase from malt source and incubated at 37 °C for 10 minutes. Subsequently, 50 µl of 1% soluble starch solution was added to each sample and further incubated for 10 minutes at 37 °C. The reaction was terminated by adding 100 µl of 96 mM DNSA reagent and boiling the mixture at 100 °C for 5 minutes, followed by cooling to room temperature. Diluted samples were then analyzed for absorbance at 540 nm using a spectrophotometer.

The α-amylase inhibitory activity (%) of each sample was calculated using the formula:

$$\text{áamylase inhibitory activity \%} = \frac{\text{A of control} - (\text{A of Sample} - \text{A of Sample blank})}{\text{A of control}} \times 100$$

where: A of control is the absorbance of the control containing substrate and enzyme, A of sample is the absorbance of the sample containing sample, enzyme, and substrate. A of sample blank is the absorbance of the sample and substrate.

2.8. Statistical analysis

Statistical evaluations were conducted using one-way ANOVA followed by Tukey's test using SPSS (version 16.0). Results are presented as Mean ± SEM (n = 3), and statistical significance was set at $p < 0.05$.

3. Results and discussion

3.1. Germination percentage and radicle length

All elicited groups of *Cyamopsis tetragonoloba* seeds exhibited germination percentages ranging from 47% to 100%, with the highest rate (100%) observed in seeds treated with a combination of chitosan and lactic acid or glutamic acid combined with chitosan (Figure 1). Radicle lengths in elicited samples ranged from 3.9 cm to 5.8 cm, with the longest (5.8 cm) recorded in seeds treated with 50 ppm chitosan and 5 mM lactic acid, and the shortest (3.2 cm) in distilled water (DW)-treated seeds (Figure 2). Germination, a critical developmental stage, involves biochemical changes driven by endogenous enzyme activation, enhancing nutritional quality compared to non-germinated seeds (Nkhata et al., 2018). The 100% germination rate in chitosan-lactic acid and chitosan-glutamic acid treatments suggest these elicitors significantly boost germination efficiency, likely by stimulating metabolic activity. The increased radicle length, particularly with chitosan and lactic acid, indicates improved early seedling growth, consistent with Tang et al., (2021), who reported enhanced nutritional and physiological functions in soybean sprouts treated with 0.01% chitooligosaccharide, and Zayed et al., (2017), who demonstrated nano-chitosan's role in promoting germination under salt stress in bean plants.

3.2. Phytochemical analysis

Protein content significantly increased in all elicited groups compared to non-germinated seeds and the DW control (Table 1). Glutamic acid-treated seeds exhibited the highest protein content

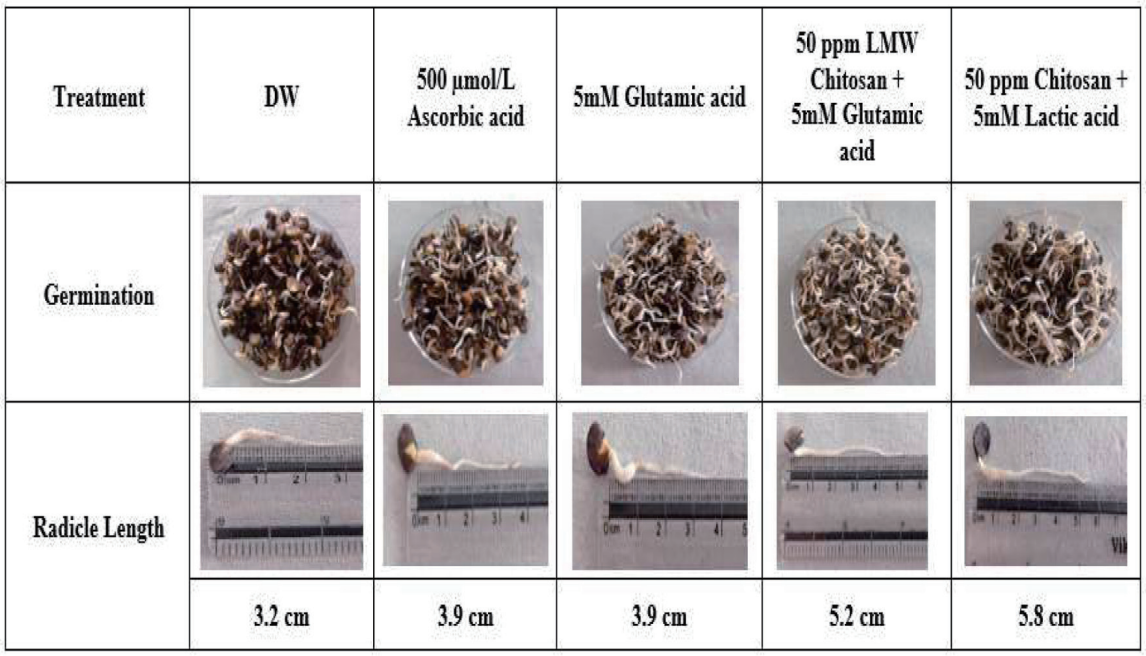

Figure 2. Germination and radicle length of cluster bean sprouts after treatment with various elicitors.

(285.94 ± 3.763 mg/g), followed by glutamic acid combined with chitosan (266.487 ± 1.675 mg/g), with statistically similar values ($p < 0.05$). DW and ascorbic acid-treated seeds showed comparable protein contents, while non-germinated seeds had the lowest (115.173 ± 0.7185 mg/g). Phenol content ranged from 12.12 to 15.95 mg/g gallic acid equivalent, with the highest in glutamic acid-treated seeds (15.95 ± 0.15 mg/g) and glutamic acid combined with chitosan (14.7 ± 0.34 mg/g) (Table 2). Statistically similar phenol contents were observed in non-germinated, DW, ascorbic acid, and lactic acid with chitosan-treated seeds. Ascorbic acid content was statistically similar across non-germinated seeds (10.58 ± 0.13 mg/g), glutamic acid with chitosan (11.16 ± 0.60 mg/g), and lactic acid with chitosan (10.63 ± 0.28 mg/g), with the lowest in control and glutamic acid-treated seeds (Table 1).

Germination enhances legume nutritional quality by altering bioactive compound profiles (López-Martínez et al., 2017). The significant protein increase, particularly with glutamic acid, likely results from endogenous protease activation, hydrolyzing storage proteins into soluble forms, and new protein synthesis during germination (Chen et al., 2017). This aligns with Świeca et al., (2014), who reported enhanced protein in chitosan-treated lentil sprouts, and Oh (2003), who observed similar effects in brown rice. The elevated phenol content in glutamic acid-treated seeds correlates with findings by Dueñas et al., (2015) and Ampofo and Ngadi (2021), who noted increased phenolic composition in elicited kidney and common bean sprouts, respectively. The rise in total phenol content can be linked to the activation of endogenous hydrolases, including phenylalanine ammonia-lyase in phenylpropanoid pathway, which is a crucial enzyme in phenol synthesis (Mao et al., 2024). While a detailed profile of specific phenolic compounds was not analyzed in this study, the focus on total phenol content is justified, as it is a widely accepted indicator of phenolic accumulation during germination and elicitation (Świeca et al., 2014; Pérez-Ramírez et al., 2018). Future studies could employ HPLC to characterize specific phenolic classes, such as flavonoids or isoflavonoids, to further elucidate elicitation effects. The limited varia-

Table 1. Phytochemical Analysis of Non-Germinated Cluster Bean Seeds and Germinated Seeds Treated with DW and Various Elicitors for 72 Hours

Treatment	Protein content (mg BSAE/g)	Total Phenol Content (mg GAE/g)	Total Ascorbic acid (mg AA/g)
Non-Germinated	115.173 ± 0.718^{a}	12.851 ± 0.118^{a}	10.586 ± 0.138^{cb}
DW	198.767 ± 2.726^{b}	12.783 ± 0.307^{a}	8.066 ± 0.201^{a}
500 µmol/L Ascorbic acid	195.257 ± 0.303^{b}	12.126 ± 0.240^{a}	9.715 ± 0.341^{b}
5 mM Glutamic acid	285.946 ± 3.763^{e}	15.957 ± 0.152^{c}	8.180 ± 0.170^{a}
50 ppm LMW chitosan with 5 mM Glutamic acid	266.487 ± 1.675^{d}	14.713 ± 0.349^{b}	11.163 ± 0.606^{c}
50 ppm LMW chitosan with 5 mM Lactic acid	207.780 ± 2.205^{c}	12.443 ± 0.349^{a}	10.632 ± 0.280^{bc}

Phytochemical analysis of non-germinated and germinated cluster bean seeds (*Cyamopsis tetragonoloba*) treated with distilled water and various elicitor solutions for 72 hours. The table presents the total protein content, total phenolic content, and total ascorbic acid content in the different treatment groups. Data are expressed as Mean ± SEM (n = 3). Significant differences ($p < 0.05$) between treatment groups are indicated by different superscript letters. Protein Content is expressed as mg/g of BSA equivalents, Phenolic Content is expressed as mg/g of gallic acid equivalents, Ascorbic Acid Content is expressed as mg/g of ascorbic acid equivalents.

Table 2. Pearson's Correlation Coefficients of Antioxidant and Phytochemical Content in Cluster Bean Sprouts

Parameter	DPPH	FRAP
Protein	0.848^{**}	-0.739^{**}
Phenol	0.544^{*}	-0.138^{ns}
Total ascorbic acid	0.158^{ns}	0.273^{ns}

ns: – nonsignificant and *, ** - correlation is significant at $p < 0.05$, $p < 0.01$ levels respectively.

tion in ascorbic acid content suggests minimal elicitor impact, possibly due to insufficient germination duration, as longer sprouting periods elevate ascorbic acid in legumes (Uppal and Bains, 2012).

3.3. Antioxidant activity

DPPH inhibitory activity of sprouted samples (20 mg/ml) ranged from 4.83% to 15.57%, with the highest in glutamic acid (15.17 ± 0.78%) and glutamic acid combined with chitosan (15.57 ± 1.56%) treatments, showing statistically similar values ($p < 0.05$) (Figure 3). No significant improvement in ferric reducing antioxidant power (FRAP) was observed across treatments, with the highest activity in non-germinated seeds (68.08 ± 0.1 mg/g ascorbic acid equivalent) and the lowest in chitosan and lactic acid-treated seeds (32.49 ± 0.86 mg/g) (Figure 3). Elicitor application enhances antioxidant activity and secondary metabolite production, improving sprout health benefits (Szulc et al., 2024). The high DPPH activity in glutamic acid and chitosan-treated seeds, correlating with elevated protein and phenol contents ($p < 0.01$; $p < 0.05$), suggests these compounds drive antioxidant properties, as supported by Solanki et al., (2024) in Lab Lab sprouts. The lack of FRAP improvement, with higher activity in non-germinated seeds, may reflect metabolism of specific phenolic compounds during sprouting (Dueñas et al., 2015). The absence of correlation between phenol or ascorbic acid content and FRAP activity likely stems from differences in phenolic composition and structural complexity, as antioxidant activity depends on hydroxyl group configuration (Jing et al., 2012).

3.4. α-Amylase inhibitory activity

All elicited sprout extracts demonstrated superior α-amylase inhibitory activity compared to controls (Table 3). Aqueous extracts (10 mg/ml) exhibited inhibition ranging from 10.45 ± 0.87% to 49.55 ± 1.39% with malt as the enzyme source and 1.75 ± 0.55% to 53.92 ± 1.51% with porcine pancreas. Treatments with glutamic acid, glutamic acid combined with chitosan, and lactic acid with chitosan achieved up to 54% inhibition, outperforming the ~31.76% reported for non-germinated cluster bean seeds (Riaz et al., 2022) and ~20% for elicited Indian beans (Solanki et al., 2024). These differences may stem from cultivar variations or improved extraction techniques. The strong performance of glutamic acid and lactic acid with chitosan treatments suggests enhanced phenolic content, which correlates with elevated α-amylase and DPPH inhibitory activities. Similar findings were noted in chitosan-treated barley sprouts, where prolonged germination reduced activity, highlighting the need to optimize elicitor dosage and timing (Ramakrishna et al., 2017). Elicitation with glutamic acid and chitosan markedly improves the bioactive profile and α-amylase inhibitory activity of *Cyamopsis tetragonoloba* sprouts, subtly supporting their potential as functional foods for applications like diabetes management, aligning with previous legume sprout studies.

4. Conclusion

The application of elicitors such as glutamic acid, chitosan, and

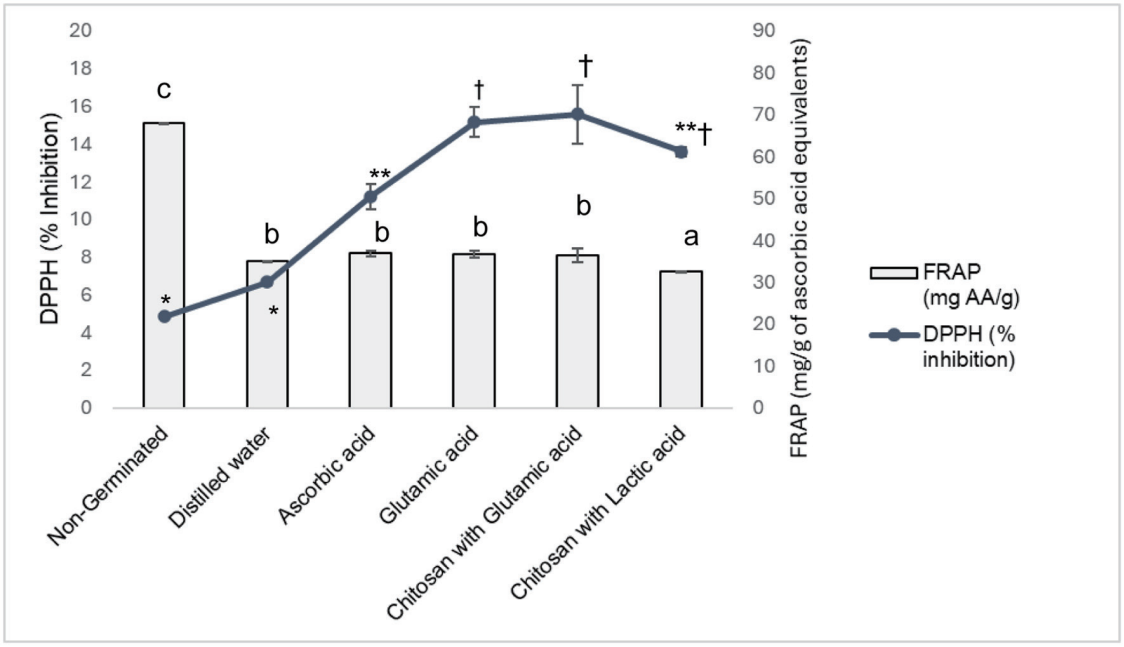

Figure 3. Antioxidant potential of cluster beans on treatment with varied elicitors. All values represent the Mean ± SEM (n = 3). Different lower-case letters within the FRAP clustered column on the graph indicate significant differences among elicitor treatments. For DPPH *, **, and † markers on the stacked line, significant differences among elicitor treatments are indicated.

Table 3. α-Amylase Inhibition Potential of Non-germinated and Elicited Cluster Bean Sprouts

Sample 10mg/ml	α-amylase inhibitory activity using malt source (% inhibition)	α-amylase inhibitory activity using porcine pancreas source (% inhibition)
Non-Germinated	10.453 ± 0.876[a]	1.750 ± 0.556[a]
Distilled water	16.300 ± 1.599[b]	13.280 ± 2.426[b]
500 µmol/L Ascorbic acid	40.350 ± 1.656[c]	42.810 ± 1.031[c]
5 mM Glutamic acid	49.39 ± 1.233[d]	49.053 ± 0.640[de]
50 ppm LMW chitosan with 5 mM Glutamic acid	49.553 ± 1.394[d]	53.920 ± 1.518[e]
50 ppm LMW chitosan with 5 mM Lactic acid	49.510 ± 1.424[d]	45.980 ± 0.381[cd]

All the values are the Mean ± SEM (n = 3). Different lower-case letters in column indicate the significant difference for the different elicitor treatment.

their combinations significantly enhances the α-amylase inhibitory activity and bioactive compound content in *Cyamopsis tetragonoloba* sprouts, surpassing the performance of non-germinated seeds. These treatments elevate phenolic and protein levels, contributing to improved antioxidant and enzyme-inhibitory properties, which highlight the potential of elicited sprouts as nutrient-rich functional foods. The superior inhibitory activity observed, particularly with glutamic acid and chitosan combinations, underscores the efficacy of targeted elicitation in optimizing sprout functionality. Future research should identify specific phenolic compounds driving α-amylase inhibition and optimize elicitor dosages and germination times to maximize bioactivity. In vivo studies and assessments of processing stability and sensory properties are also needed to support the use of elicited cluster bean sprouts in functional foods.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

All authors contributed to the study conception and design. Ms. Komal Solanki and Ms. Rushna Mansuri were involved in carrying out experiments, data collection, data analysis and manuscript writing. Dr. Krutika contributed to conceptualization, data analysis, and project administration. All authors read and approved of the final manuscript.

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Quantification of 3,3-dimethyl-1-butanol (DMB) in olive oil: a rapid and novel method

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Abstract

A novel headspace gas chromatography-mass spectrometry (HS-GC/MS) method for identifying and quantifying 3,3-dimethyl-1-butanol (DMB), a bioactive compound, in extra virgin olive oil was developed. In this study, solvents with similar properties to DMB but potentially different molecular masses, namely isopropanol, n-butanol, n-pentanol, and 2-pentanol, were tested and 2-pentanol was selected as the internal standard due to both chromatographic separation and distinctive mass spectrum pattern. The method for DMB determination and quantification was validated according to Eurochem CITAC Guide. Sensitivity, specificity, linearity, accuracy, and precision parameters were evaluated. The validation process included an assessment of the method's robustness and repeatability, ensuring that it produces reliable results for future analyses of olive oil. A linear regression equation was developed for DMB concentrations ranging from 100 to 5,000 µg/L, expressed as $y = x.11.462.10^{-6}$, where y represents the peak area ratio and x is the DMB concentration in µg/L. This equation has a high correlation coefficient of 0.9989 (R^2), and the uncertainty budget was estimated to be 8.22% at a confidence level of k = 2. In addition, the lower concentration range of 0 to 100 µg/L was characterized by the equation $y = x.17.860.10^{-6}$ which has a correlation coefficient of 0.9983 (R^2). The uncertainty of DMB measurements in extra virgin olive oil was estimated to be 7.0% at a confidence level of k = 2. This new method was applied to determine the presence of DMB in various olive oil samples from Greece and Türkiye. The DMB values for olive oil samples were: N1(Plus health blue multi varietal) 9.7 µg/L, N2 (Plus health DMB multi varietal) 11.4 µg/L, N3 (Fyllikon first harvest organic) 8.3 µg/L, N4 (Plus health green multi varietal) 7.8 µg/L, N5 (Agourelaio early harvest organic) 6.8 µg/L, N6 (unknown) 4.8 µg/L, N7 (Armonia monovarietal organic) 4.8 µg/L and N8 (Edremit type olive oil from Kırkağaç, Manisa) 8.8 µg/L and N9 (Market product in Türkiye) 1.5 µg/L.

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Keywords: Virgin olive oil; 3,3-dimethyl-1-butanol (DMB); Novel method for DMB determination; DMB values in olive oil; Oleocanthal; Trimethylamine N-oxide (TMAO).

1. Introduction

Olive oil, a natural juice obtained by mechanical means from the fruit of the tree *Olea europaea L*., plays a crucial dietary role in the Mediterranean culture for thousands of years (Kiritsakis and Karamesinis-Kiritsakis, 2017). However, in recent years this popularity has started to spread worldwide (Kiritsakis et al., 2020a). Numerous studies have highlighted olive oil as a significant source of antioxidants based on its phenolic profile, which includes phenolic acids and alcohols, polyphenols, lignans, secoiridoids, oleacein, and oleocanthal (Kiritsakis et al., 2020b). The biological effects of olive oil components include protection against cardiovascular diseases, anti-inflammatory action, and neuro- and endothelial protection. Both *in vitro* and *in vivo* studies in humans and animals have been conducted to better understand the metabolism and bioactivity of olive oil phenolics (Kiritsakis et al., 2024; Micheli et al.,2023; Manach et al., 2004). Antioxidants from olive oil are not only absorbed by the body but can also bind to the lining of the digestive tract, contributing to its health benefits (Manach et al., 2004).

Consumption of extra virgin olive oil (EVOO) is associated with the prevention of cardiovascular diseases (CVDs) and certain types of cancer. This is linked to its well-balanced fatty acid composition, with oleic acid being the main component, as well as the presence of minor compounds such as polar phenols, tocopherols, and sterols. These characteristics make olive oil a premium functional food (Keceli Mutlu et al., 2017; Kiritsakis and Keçeli Mutlu, 2023).

Recently, a new compound, namely 3,3-dimethyl-1-butanol (DMB) in extra virgin olive oil, was found to play a key role in inhibiting the enzyme TMA-lyase, involved in the formation of trimethylamine N-oxide (TMAO). TMA is produced by the microbiome from foods such as red meat and then is absorbed into the bloodstream and oxidized to TMAO in the liver. TMAO seems to facilitate the development of atherosclerosis in animal models fed on a diet rich in animal protein (Wang et al., 2015). TMAO, an intestinal microbiota-dependent metabolite, is associated with inflammatory diseases such as atherosclerosis, whose immunologic processes mirror those of rheumatoid arthritis (RA). Gut microbiota-dependent metabolite TMAO is associated with poor prognosis in pulmonary arterial hypertension (Yang et al., 2022).

Researchers in the Cleveland Clinic, Ohio, USA and the University of California, Los Angeles, USA, who conducted experiments on mice, reported that dietary supplementation with DMB safely inhibited atherosclerosis (plaque buildup in arteries) and significantly lowered the production of TMAO (Wang et al., 2015). A pre-clinical study by Wang (2015) showed that DMB reduced TMAO levels, inhibiting atherosclerosis in animal models. Thus, DMB is an orally active inhibitor of trimethylamine (TMA) and TMAO (Wang et al., 2015).

Gut microbiota-derived metabolites, including TMA, TMAO, and short-chain fatty acids (SCFAs), have been linked to hypertension (Hsu et al., 2021). Treatment with DMB during pregnancy and lactation has been shown to rescue hypertension, providing insights into the therapeutic potential of DMB as a microbiome-based metabolite treatment for the prevention of hypertension of developmental origins (Hsu et al., 2021). Researchers have suggested that if future studies in humans show similar effects, DMB could lead to new strategies for preventing or even treating heart disease and stroke, two of the leading causes of death globally.

TMAO has also been linked to aging, cognitive impairment, and other brain disorders. A study by Mao (2021) for the first time provided direct evidence that repeated DMB exposure produces significant effects on the social dominance of adult mice without any effects on anxiety, depression-like behavior phenotype, or memory formation. This highlights the regulatory effects of gut-brain interaction on social behavior (Mao et al., 2021).

Trimethylamine N-oxide has been demonstrated to promote vascular inflammation across various cardiovascular diseases. Notably, microbial populations involved in TMAO metabolism are elevated in patients with pulmonary hypertension (PH) (Huang et al., 2022). In a murine model of heart failure (HF), Wang et al. (2020) reported that treatment with DMB significantly reduced circulating TMAO levels and mitigated both structural and electrical cardiac remodeling. As a gut microbiota-derived metabolite, TMAO is linked to increased cardiovascular (CV) risk due to its role in vascular dysfunction. Yet, this pathological effect can be attenuated through DMB supplementation (Casso et al., 2022).

Since no data on DMB analysis in olive oil has been published, this work was undertaken to establish a method for determining DMB and its presence in olive oil. The method developed here was applied internationally for DMB analysis for the first time to detect its presence in olive oil.

2. Materials and methods

2.1. Chemicals

The chemicals used included 2-pentanol (99.7%, Sigma-Aldrich, 8.07501.0500, Dusseldorf, Germany), 3,3-dimethyl-1-butanol (DMB) (≥99.3%, TCI, D1333, Tokyo Japan), 1-butanol (≥99.4%, Sigma-Aldrich, 360465-2.5L, Dusseldorf, Germany) and ethanol (≥99.9%, ISOLAB chemicals, LR0090606ANQ, Eschau, Germany). Stock solutions of 2-pentanol (internal standard) and DMB 4000 mg/L and 100 mg/L were prepared in ethanol.

2.2. Preparation of stock and calibration solutions

The stock solution of DMB at 100 mg/L was added to a 20 mL headspace vial to obtain final concentrations of 10, 15, 20, 50, 100, 250, 500, 750, 1,000, 2,500, and 5,000 µg/L in 500 µL of oil. Then, 6.25 µL of 4,000 mg/L 2-pentanol in ethanol stock solution was quickly added to the vial to obtain an internal standard concentration of 50 mg/L. The required amount of Riviera-type olive oil devoid of DMB was added to the vials to make a final solution volume of 500 µL. The cap of the headspace vial was quickly placed, tightly closed with a clamp, and vortexed in a Heidolph device for 1 min at room temperature. Prepared samples were stored at ambient temperature in the absence of sunlight until the time of analysis.

2.3. Olive oil sample preparation

Nine (9) olive oil samples numbered N1-N9 were used to apply the

method. Samples N1-N7 were donated by Sakellaropoulos Organic Farms (Sparta, Greece), and sample N8 was provided by Ahmet C Goren's farm from Kırkağaç-Manisa, Türkiye, whereas N9 was the commercial olive oil sample obtained from the Turkish Market. A volume of 493.75 µL of olive oil sample was spiked by using 6.25 µL of 4,000 mg/L 2-pentanol solution (internal standard), so a final concentration of 50 mg/L was obtained. The heads space (HS) vial was sealed up and vortexed on a Heidolph device for 1 min at room temperature. Prepared samples were stored at ambient temperature in the dark until analysis.

2.4. Analysis of headspace gas chromatography mass spectrometry (HS-GC/MS)

The HS-GC/MS analyses were carried out with a Thermo Scientific TriPlus 500 Head Space, connected to Thermo TSQ 9610 MS-MS system on a VF-WAXms (V, Varian) capillary column (60 m × 0.25 mm, 0.25 µm film thickness) where helium is used as carrier gas (1.0 mL/min). The headspace equipment was programmed for a 20 mL vial; the samples were incubated for 10 min at 120 °C with a medium shaking rate. The vial was pressurized to 90 kPa for 1.0 min. The loop was enabled, set to 120 °C with a pressure of 70 kPa, and allowed to equilibrate for 1 min. Injection mode was configured as MHE with an injection time of 1.0 min. The GC oven temperature was initially maintained at 40 °C for 2 min, then increased to 120 °C at a rate of 15 °C/min, and held at 120 °C for 12.67 min. The total analysis duration was 20 min. A retention time window of 2 min was selected. The split ratio was adjusted to 10:1, and the injector temperature was set at 120 °C. The MS transfer line temperature was maintained at 280 °C, while the ion source temperature was set to 250 °C. The mass spectra were acquired in SIM mode. SIM parameters were set at m/z 45 and 55 for 2-pentanol and m/z 57 and 69 for DMB, corresponding to retention times of 9.94 and 12.07 min, respectively.

2.5. Method validation

The developed method for DMB quantification in olive oil was validated according to the Eurachem Citac Guide (Eurachem, 2012; Magnusson and Ornemark, 2014). Experiments for the validation of the method were carried out by spiking DMB into the olive oil samples as described in the preparation of stock and calibration solutions section. Matrix-matching calibration samples were prepared using DMB-free olive oil samples with added DMB. Two calibration curves were obtained covering low and high concentration levels. The five-point calibration curve was obtained in the lower concentrations of 10, 15, 25, 50, and 100 µg/L. The seven-point calibration curve was obtained in 100, 250, 500, 750, 1,000, 2,500, 5,000 µg/L levels. Each sample of both calibration series included 2-pentanol as an internal standard solution (IS), which was obtained by plotting the peak area ratio (analyte/IS) versus the expected analyte concentration for both. The method validation parameters of the current method were selected as specificity, linearity, accuracy, precision, repeatability, recovery, robustness, the limit of detection (LOD), the limit of quantitation (LOQ), and stability (Ozer et al., 2024; Shah et al., 2024; Magnusson and Ornemark, 2014).

2.5.1. Specificity

The ICH (International Council for Harmonization) (ICH, 2025) guideline defines specificity (widely known also as Selectivity) as "the ability to assess the analysis in the presence of components accurately." Therefore, the specificity of the method was evaluated by performing six replicates of HS-GC/MS measurements of blank samples (water), non-spiked DMB-free blank olive oil, and standard mixture solution separately (ICH, 2025). No peaks were observed in the blank matrixes for IS (2-pentanol) at RT 9.94 min and target compound (DMB) at 12.07 in the GC/MS chromatogram of olive samples (Figures S1–S3).

Table 1. Method accuracy parameters of determination of DMB in Olive oil by HS-GC/MS

	Concentration level		
Concentration (µg/L)	100	1,000	5,000
Recovery (%)	100.9	101.6	99.6
RSD (%)	6.4	4.1	1.8

2.5.2. Linearity

Two calibration curves were prepared using the matrix-matched methodology. Meanwhile preparing this calibration curve for the higher concentrations, solutions of DMB at concentrations of 100, 250, 500, 750, 1,000, 2,500, and 5,000 µg/L were used on the DMB free matrix with 50 mg/L as the internal standard at each concentration level and, linear least-squares regression analysis was performed, correlation coefficients (R2) was determined as 0.9989 and, linear regression equation was obtained as $y = x.11.462.10^{-6}$ where y is peak area ratio, X is the DMB concentration in µg/L. The calibration curve for the lower levels was obtained by preparing DMB solutions at 10, 15, 20, 50, 100 µg/L concentrations in the DMB free matrix with 50 mg/L of 2-pentanol as internal standard at each level, correlation coefficient (R2) was determined as 0.9983 and, linear regression equation was obtained as $y = x.17.860.10^{-6}$. The linear range of the method was worked as 0–100 and 100 - 5,000 µg/L (Figures S4 and S5, respectively, Table S1). Herein, Statistical analysis was performed in Microsoft Excel spreadsheets (See Figures S4 and S5 for supporting information).

2.5.3. Repeatability, accuracy, precision and recovery

Samples (n-6) with three different DMB concentration levels (100, 1,000, and 5,000 µg/L) were prepared to assess the method's performance. These points cover the matrix's low, medium, and high analyte concentrations. RSD of the recovery values and recovery values themselves obtained from the six replicates of the olive oil matrix provided information on the method accuracy (Figures S6–S8, Table 1, Table S2). The data's recovery and relative standard deviation (RSD) were calculated as shown below.

$$\%\ \text{Recovery} = \frac{\text{Recovered concentration}}{\text{Injected concentration}} \times 100$$

$$\%\ \text{RSD} = \frac{\text{Standard deviation}}{\text{Mean}} \times 100$$

2.5.4. Limit of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ values were estimated using spiked standard solutions (obtained with dilutions) to the matrix at significantly lower mass fractions such as 1, 2, and 10 µg/L. The developed HS-GC/

MS method was applied to the six spiked samples from each level, and the LOD and LOQ values were calculated by multiplying the standard deviation (SD) by 3 and 5, respectively. As a result, the LOD value of the method reported here for DMB was determined as 1.91 µg/L, while it was deemed appropriate to determine 5 times this value for the LOQ value (Figures S4, S5, and S9).

2.5.5. Robustness

The method's robustness patterns were assessed for three concentration levels (100, 1,000, and 5,000 µg/L) by changing the mobile phase flow rate in the range 0.90–1.00–1.10 (ml/min) and the HS oven thermostat temperature in the range of 115–120–125 °C. The high selectivity properties of HS-GC/MS measurements showed no significant change in the analytes' peak areas and resolution. RT changed with flow rate according to our expectations. The HS-GC/MS measurements are presented in Figures S10–S12.

2.6. Uncertainty assessment

The primary sources of uncertainty for the developed method were estimated according to the Eurochem/Citac Guide and literature data (Magnusson and Ornemark, 2014; Ozbay et al., 2023; García-Alegría et al., 2023). The bottom-up approach methodology for uncertainty budget estimation was considered initially. The potential uncertainty sources were sample weighting, glassware volume and pipetting, standard purity, calibration curve, experiment recovery, and method repeatability. Some sources were neglected due to their minor effect on the total uncertainty budget; the identified primary sources of uncertainty are repeatability, calibration curve, and recovery. Uncertainty was determined as 8.2% for 100–5,000 µg/L and 7.0% for 10–100 µg/L range. The combined uncertainty (UCombined) was estimated as follows: The primary source of uncertainty budget comes from the experiment's recovery, calibration curve, and repeatability.

$$u_{Combined} = \sqrt{\left(u_{standard}\right)^2 + \left(u_{weighing}\right)^2 + \left(u_{recovery}\right)^2 + \left(u_{curve}\right)^2 + u_{repeatability}}$$

The expanded uncertainty ($U_{Expanded}$), giving a 95% confidence level was evaluated using a coverage factor k of 2 as follows:

$$U_{Expanded} = u_{Combineds} \times k$$

3. Results and discussion

3.1. Optimization of headspace gas chromatography mass spectrometry (HS-GC/MS) measurements

The HS-GC/MS analyses were carried out by using a Thermo Scientific TriPlus 500 HeadSpace, connected to Thermo TSQ 9610 MS-MS system on a VF-WAXms (V, Varian) capillary column (60 m × 0.25 mm, 0.25 µm film thickness). Different solvents were used to choose the best for the evaluation of the proposed method and the identification and determination of the 3,3-dimethyl-1-butanol (DMB). This target compound has a molecular mass (MW) of 102.18 g/mol, and its boiling temperature (Tb) is 143.0 °C. Compounds with similar chemical structures, close molecular weight, and boiling temperature were tested as candidates for internal standard use. The compounds were 1-butanol (MW = 74.12 g/mol, Tb = 117.7 °C, SIM parameters were determined as m/z 45 and 55) and 2-pentanol (MW = 88.15 g/mol, Tb = 119.3 °C). The study was continued with 2-Pentanol because it has a retention time (RT = 9.94 min) closer to DMB's (RT = 12.07 min), and it has a more intense peak than 1-butanol (RT = 10.22 min). The Head Space oven temperature was adjusted to 120 °C to match the boiling temperature of 2-pentanol, the component with the lowest boiling temperature, and avoid evaporation and interference with other non-target compounds. We also tested the nonpolar type column, DB-5 (Restek Corp., Phase: RTX-5 SUMS) capillary column 30 m × 0.25 mm (0.25 mm film thickness), but the chromatograms observed, showed peaks with a tailing effect. Thus, we decided to use a polar column instead of a nonpolar one for this measurement.

In order to select the calibration range for the developed method, firstly, 1 and 10 mg/L DMB solutions were prepared in water, and single-point calibration was performed by performing HS-GC/MS analysis. Then, olive oil samples were determined, and the approximate DMB concentration range of olive oil samples was estimated. In light of the obtained data from this experiment, it was that the DMB concentration in olive oil samples was found to be at µg/L level. To ensure accuracy, the calibration range was selected as 10 - 5,000 µg/L (Figures S4 and S5, Table S1). Additionally, experiments for estimating the µg/L level, limit of detection (LOD), and limit of quantification (LOQ) values were included in the validation parameters. Then, validation parameters were selected as specificity, linearity, accuracy, precision, repeatability, recovery, robustness, the limit of detection (LOD), the limit of quantitation (LOQ), and stability.

Specificity experiments of the method were performed with six replicate measurements of blank samples (water), non-spiked DMB-free blank olive oil, and standard mixture solution individually. No peak appears at 9.94- and 12.7-min retention time in the absence of internal standard and DMB, respectively. (a). Peak d is higher than curve b because in d, the olive oil contains 20 µg/L of DMB, while in b, it contains 10 µg/L of DMB (Figure S9 in supporting this information). Thus, we can clearly say that our developed method has strong specificity for DMB measurements in olive oil.

In this study, in order to determine the linear working range of the method, and to minimize the matrix effect - although not necessarily considering the technique applied and the scarcity of interferences - the matrix-matched calibration methodology was used, and six different samples from each level were prepared by measuring them six times. As a result of the experiments, the calibration curve's correlation coefficient (R2) was found to be 0.9983, while the linear regression equation was $y = x.17.860.10^{-6}$ (See Table 2).

In order to determine the robustness of the developed method, measurements were repeated with different flow rates at different DMB contents. When the flow rate increased from 0.9 ml/min to 1.1 mL/min in three different samples with different DMB concentrations (100, 1,000, and 5,000 µg/L), the internal standard and DMB peaks appeared earlier, as expected. However, no significant change was detected in the measured values due to the high selectivity of the mass spectrometer. For this reason, no trial was conducted regarding the change in the column oven temperature by 3–5 °C, which is the other factor affecting the RT change. As a result, it was shown that this new measurement method reported here is robust and repeatable even if the flow rates and retention time data of the analytes change at 0.9, 1.0, and 1.1 mL/min.

By using the developed HS-GC/MS method herein, DMB amounts in extra virgin olive oil samples obtained from the Sparta region of Greece and Kırkağaç from Türkiye were determined, and the experimental results are given in Tables S2 and S3 (S3

Table 2. Method validation parameters of determination of DMB in Olive oil by HS-GC/MS

Paramater	Data	Data
Linear regression equation	$y = x.11.462.10^{-6}$	$y = x.17.860.10^{-6}$
Linearity range (µg/L)	100–5,000	10–100
R^2	0.9992	0.9983
Recovery AVG (%)	101.63 (at 1,000 µg/L)	100.86 (at 100 µg/L)
Recovery SD (%)	0.63 (at 1,000 µg/L)	3.82 (at 100 µg/L)
LOD (µg/L)		1.91
LOQ (µg/L)		9.55
$U_{expanded}$ %	±8.22	±7.0

The method's precision was determined by repeating the measurement six times at three concentration levels (100, 1,000, and 5,000 µg/L) prepared to assess the method's performances for determining DMB. The average RSD values were 6.4, 4.0, and 1.8%, respectively. On the other hand, the recoveries of each level were determined as 100.9, 101.6, and 99.6 % for the same concentration levels. As expected, the lower concentration levels' RSD and recovery levels were higher than the high-concentrated ones.

in Supplementary material). Considering these results, DMB was detected in nine extra virgin olive oil samples. Among these, the highest DMB amount was detected in the N2 coded Plus health DMB Multivarietal olive oil sample. Meanwhile, the lowest level was observed in the samples named Armonia Monovarietal Organic with N6 and N7 codes (see Table 3). Similarly, the amount of DMB in olive oil sample N8 directly obtained from the producer after olive oil pressing was found to be 8.8 µg/L. However, the olive oil obtained from the market was found to contain 1.5 µg/L. The GC-MS chromatograms of the HS-GC/MS measurements of samples are given in the supplementary material between Figures S13–S21, the chromatograms of the blank sample, 100 µg/L DMB spiked olive oil sample and Plus health DMB Multivarietal olive oil sample are given in Figure 1.

4. Application of the method

The method developed in this study was successfully applied to routine measurements of DMB in extra virgin olive oil (for the measurement chromatogram, see Figure 1). The method developed here is robust and specific that can be applied for DMB measurement in olive oil samples. Though the boiling temperature of the olive oil is around 300 °C, and the headspace oven temperature was set to 120 °C, the oil vapors produced are carried across the different parts of the HS-GC/MS system, affecting peak intensities, so the periodic maintenance of the ion source and injector sections cleaning after every 300 injections is recommended.

In the sample of extra virgin olive oil purchased from the Turkish market, approximately six times less DMB was detected compared to the fresh olive oil obtained from Türkiye. This study did not include a market survey of DMB content across various olive oils; therefore, we analyzed only a single product. However, we plan to conduct further research to evaluate the DMB levels in a broader range of market samples in future.

Table 3. Amount of DMB (3,3-dimethyl-1-Buthanol) in the tested olive oil samples*

Sample code	Amount of DMB (µg/L)	U Expanded Uncertainty k = 2 (µg/L)
N1	9.7	0.68
N2	11.4	0.80
N3	8.3	0.58
N4	7.8	0.55
N5	6.8	0.55
N6	4.8	0.33
N7	4.8	0.34
N8	8.8	0.50
N9	1.5	0.11

*N1 (Plus health blue multivarietal), N2 (Plus health DMB multivarietal), N3 (Fyllikon first harvest organic), N4 (Plus health green multivarietal), N5 (Agourelaio early harvest organic), N6 (unknown), N7 (Armonia monovarietal organic), N8 (Edremit type olive oil from Kırkağaç, Manisa), N9 (Market product in Türkiye).

5. Conclusion

Olive oil, a key component of the Mediterranean diet, has played a significant role in nutrition and health for thousands of years. Numerous scientific studies indicate the importance of specific constituents within olive oil for human well-being. Most of these constituents have been identified and characterized through various analytical methods. The development of this new headspace gas chromatography/mass spectrometry (HS-GC/MS) method encourages further studies on the presence of this compound in olive oil and the factors that affect its concentration. Our new method will likely find wide applications in olive oil research and quality as well as in health assessment. Subsequent studies using this method are likely to influence the marketing of olive oil and its production, storage, and packaging technology.

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Supplementary material

Figure S1. Specificity measurements for 2-pentanol and DMB in water: (a) absence of 2-pentanol in water, (b) 50 mg/L 2-pen-

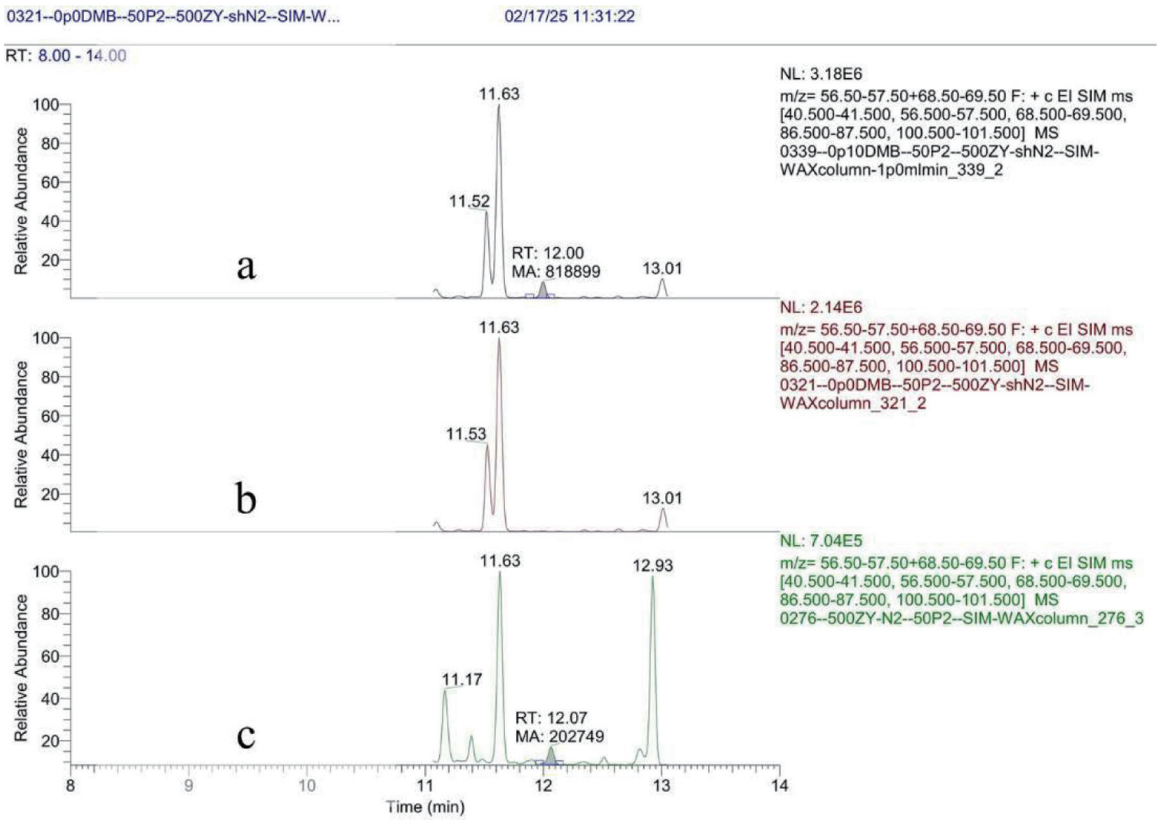

Figure 1. Chromatograms for 100 µg/L DMB in blank olive oil (a), blank olive oil (b), Olive oil sample N2 (c).

tanol in water, (c) absence of DMB in water, (d) 50 mg/L DMB in water.

Figure S2. Specificity measurements for 2-pentanol and DMB in blank olive oil: (a) absence of 2-pentanol in blank olive oil, (b) 50 mg/L 2-pentanol in blank olive oil, (c) absence of DMB in blank olive oil, (d) 50 mg/L DMB in blank olive oil.

Figure S3. Specificity measurements for 2-pentanol and DMB in standard mixture solution: (a) pure water, (b) 50 mg/L 2-pentanol, (c) pure water, (d) 50 mg/L DMB.

Figure S4. Calibration points for a range of 10 – 5,000 µg/L of DMB (passes through the origin).

Figure S5. Calibration points for a range of 0 – 100 µg/L of DMB.

Figure S6. Accuracy control for 100 µg/L DMB samples (RT = 11.71 min) in blank olive oil

Figure S7. Accuracy control for 1,000 µg/L DMB (RT = 12.01 min) samples in blank olive oil

Figure S8. Accuracy control for 5,000 µg/L DMB (RT = 12.06 min) samples in blank olive oil

Figure S9. Determination of LOD using chromatography data. Blank Olive oil sample (a), with 10 µg/L (b), 15 µg/L (c) and 20 µg/L (d) of DMB sample chromatograms.

Figure S10. Robustness control for 100 µg/L DMB samples at different mobile phase flow rate: 0.9 ml/min (a), 1.0 ml/min (b), 1.1 ml/min (c).

Figure S11. Robustness control for 1 000 µg/L DMB samples at different mobile phase flow rate: 0.9 ml/min (a), 1.0 ml/min (b), 1.1 ml/min (c).

Figure S12. Robustness control for 5,000 µg/L DMB samples at different mobile phase flow rate: 0.9 ml/min (a), 1.0 ml/min (b), 1.1 ml/min (c).

Figure S13. Olive oil sample N1 with 50 mg/L of P2 (top) and DMB (bottom) chromatogram, 1st injection.

Figure S14. Olive oil sample N2 with 50 mg/L of P2 (top) and DMB (bottom) chromatogram, 1st injection.

Figure S15. Olive oil sample N3 with 50 mg/L of P2 (top) and DMB (bottom) chromatogram, 1st injection.

Figure S16. Olive oil sample N4 with 50 mg/L of P2 (top) and DMB (bottom) chromatogram, 1st injection.

Figure S17. Olive oil sample N5 with 50 mg/L of P2 (top) and DMB (bottom) chromatogram, 1st injection.

Figure S18. Olive oil sample N6 with 50 mg/L of P2 (top) and DMB (bottom) chromatogram, 1st injection.

Figure S19. Olive oil sample N7 with 50 mg/L of P2 (top) and DMB (bottom) chromatogram, 1st injection.

Figure S20. Olive oil sample N8 with 50 mg/L of P2 (top) and DMB (bottom) chromatogram, 1st injection.

Figure S21. Olive oil sample N9 with 50 mg/L of P2 (top) and DMB (bottom) chromatogram, 1st injection.

Table S1. Calibration points for a range 0 µg/L – 5,000 µg/L, observed peak area ratio and calculated DMB amount in the calibration sample.

Table S2. HS-GC/MS measurement results for 100*, 1,000 and 5,000 µg/L in blank olive oil with the Recovery and RSD values were estimated.

Table S3. HS-GC/MS measurement results for Olive oil samples N1-N9.

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The effect of L-carnitine and catechin together with calories restriction on the reduction of body fat and blood lipids

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Abbreviations: AMPK, AMP-activated protein kinase; BMI, Body mass index; BCS, Bovine calf serum; C/EBPα, CCAAT/enhancer-binding protein alpha; DMSO, Dimethyl sulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMEM, Dulbecco's Modified Eagle's Medium; FFA, Free fatty acid; GPDH, Glycerol-3-phosphate dehydrogenase; HDL-C, High-density lipoprotein cholesterol; LDL-C, Low-density lipoprotein cholesterol; PPARγ, Peroxisome proliferator-activated receptor gamma; PBS, Phosphate-buffered saline; TC, Total cholesterol; TG, Triglyceride

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Abstract

Obesity has become a critical health problem worldwide, and it's associated with other health issues. Weight loss supplement and caloriese restriction from the daily diet can be implemented to help reduce body weight. L-carnitine and catechin can help reduce body weight by upregulating fatty acid metabolism and altering lipid formation. This study was to assess the effect of L-carnitine and catechin together with calories restriction on body weight and lipid profile. 3T3-L1 cell line was used to assess the effect of L-carnitine and catechin on lipid accumulation. For the human study, a total 30 subjects with obesity were recruited and divided into 2 intervention groups, one is calories restriction group and the other one is L-carnitine and catechin together with calories restriction. Result shows that L-carnitine and catechin significantly reduced fat accumulation in the 3T3-L1 cell model. In the human study, L-carnitine and catechin together with calories restriction significantly reduced body weight, and body fat and improved the lipid profile of the subject, and showed better effect than calories restriction only.

Keywords: L-Carnitine; Catechin; Body fat; Lipid profiles; 3T3-L1 adipocytes.

1. Introduction

Nowadays obesity is a global health concern due to lifestyle changes (Engin, 2017). Imbalanced diet and less physical activity increase the risk of obesity. Obesity is defined as over accumulation of body fat throughout the body which is associated with increased risk of several health problems such as heart disease, diabetes, hypertension, liver disease, etc (Mayoral et al., 2020; Rush and Yan, 2017). Dietary control, increasing physical exercise, surgery, and drug therapy could be used as a common countermeasure for obesity (Talenezhad et al., 2020). Dietary supplements especially from natural sources can help to combat obesity.

L-carnitine is an amino acid in several animal products such as meat, fish, and milk (Pekala et al., 2011). L-carnitine can promote the β-oxidation of fatty acid by promoting the transportation of

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long-chain fatty acid into mitochondria (Choi et al., 2020; Longo et al., 2016). Catechin in green tea is known to affect lipid metabolism by suppressing peroxisome proliferator-activated receptor gamma (PPARγ), glycerol-3-phosphate dehydrogenase (GPDH), CCAAT/enhancer-binding protein alpha (C/EBPα) and inhibit adipogenesis in 3T3-L1 cell lines (Furuyashiki et al., 2004; Mochizuki and Hasegawa, 2004; Ogawa et al., 2010; Shin, Ghimeray, and Park, 2014). Animal study gets positive results regarding the effect of catechin on fat accumulation and lipid metabolism by activating AMP-activated protein kinase (AMPK) and suppressing PPARγ (Yamasaki et al., 2013; Choi et al., 2020; Thomaz et al., 2022).

L-carnitine and catechin were found to have anti-obesity effect respectively, but no study about the combined use of two both on obesity improvement. This study will focus on L-carnitine and catechin treatment on adipogenesis in 3T3-L1 cell lines and evaluate the use of L-carnitine and catechin together with calories restriction on the reduction of body fat and lipid profile of the obese subjects and also compared with the claori restriction group.

2. Materials and methods

2.1. Sample

The sample used in this study was provided by Venken Biotech Co., Ltd., Taipei, Taiwan, China. Testing sample contained 0.3 g of carnitine and 80–120 mg of catechins for each tablet. The weiht of one tablet was 0.7 g.

2.2. 3T3-L1 cell culture

Mouse fibroblast (3T3-L1) cell lines were purchased from ATCC (ATCC CL-173) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% bovine calf serum (BCS) in a humidified incubator (37 °C) containing 5% CO_2. Differentiation of 3T3-L1 cells into mature adipocytes carried out after cell reach confluency using DMEM containing 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1 mM DEXA and 10 mg/mL insulin at 37 °C humidified incubator with 5% CO_2.

2.3. Cell viability measurement

3T3-L1 cell viability was measured by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. 3T3-L1 pre-adipocytes and mature adipocytes were cultured in a 96-well plate and treated with various concentrations of tablet contents (0; 0.1; 0.2; 0.25; 0.5; 1 ng/mL) for 24 hours. After 24 hours, samples were removed and MTT solution (0.5 mg/mL in phosphate-buffered saline (PBS)) was added for 4 hours. At the end of incubation, the MTT solution was removed and 100 μL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystal. Absorbance was read at 570 nm and relative cell viability (%) was compared with untreated cells.

2.4. Lipid accumulation measurement using oil-red staining

Mature adipocytes in 24-well plate and treated with different concentrations of sample for 24 hours. After treatment, cells were washed with PBS twice and fixed using 10% formaldehyde solution for 1 hour. After fixation, cells were washed with PBS and incubated with 0.5% oil red solution for 15 minutes. Cells were washed with dd H2O to remove unbound oil red solution and isopropanol was added to dissolve the oil red. 100 μL solution from each treatment was transferred to 96-well and the absorbance was obtained at 490 nm. Lipid accumulation was calculated by comparing treated cells with untreated cells.

2.5. Clinical trial study

This study was carried out from May to September 2022 at Chung Shan Medical University, Taichung, Taiwan, China. This study was conducted for 14 weeks, 12 weeks for intervention and the other 2 weeks for follow-up.

2.6. Ethics approval

This study received approval from the Institutional Ethics Committee of Chung Shan Medical University Hospital. Every subject enrolled in this study received written informed consent before the start of the study. The study was registered with the number of CS1-21170.

2.7. Inclusion and exclusion criteria

Subjects enrolled for this study were greater than 20 years old and obese (body mass index (BMI) ≥27). Subjects with severe chronic disease, secondary obesity, endocrinopathy, pregnancy, or in the lactation period were excluded from this study. Subjects who took another supplements for weight loss or medication affecting lipid metabolism were also excluded.

2.8. Subjects characteristics measurement and blood sampling

Subjects met the inclusion criteria were randomized into 2 groups (control for calories restriction, supplement for the intervention of L-carnitine and catechin together with calories restriction). Each subject in the supplement group took 2 tablets after lunch and 2 tablets after dinner. Anthropometric measurements, blood sample, and a 3-day dietary record were obtained at week 0, 4, 8, 12, and follow-up period. Blood samples were taken using a tube with a clot activator to separate the serum and a tube with K_3EDTA to separate the plasma for various blood markers and antioxidant index.

2.9. Outcomes

The primary outcomes of this study were a reduction of body weight and body fat compared to baseline. Secondary outcomes were the changes in the lipid profile of the subject (triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), free fatty acid (FFA)) and related hormone regulation of lipid metabolism (e.g. leptin and adiponectin). All the measurements of adiponectin, leptin, TEAC, TBARS, GSH and the above biochemical analyses were obtained by using the method of this laboratory[28].

2.10. Statistical analysis

All results from this study were analyzed using the Statistical Pack-

age for the Social Sciences (SPSS) version 25 for Windows: SPSS Inc, Chicago). The data were expressed as mean ± standard deviation. The results of the cell study were analyzed using the one-way ANOVA statistical method followed by the Duncan multiple comparison test. For the clinical trial, a paired t-test statistical method was applied, and the one-way ANOVA was used followed by the Duncan post hoc test to examine possible differences between groups within the same period. Non-quantitative indicators were analyzed using descriptive statistics. All statistical results were considered statistically significant with $p < 0.05$

3. Results

3.1. Effect on cell viability and lipid accumulation

Sample supplement didn't show toxicity effect on both 3T3-L1 pre- and mature adipocytes up to concentration 0.5 ng/mL (Figure 1a and b). Treatment with supplement significantly reduced the amount and size of oil droplets inside the cell ($p < 0.05$) (Figures 2 and 3).

3.2. Subject recruitment and distribution

A total of 30 subjects were included in this study. Each group consisted of 15 persons. The subjects baseline characteristics was shown in Table 1. There was no significant difference in baseline between the groups.

3.3. Change in energy and anthropometric characteristics over time

Subjects in two groups of this study were asked to reduce 250 kcal from their total energy intake every day (Table 2). Energy and carbohydrates were clearly reduced in both groups. As for body weight, BMI and hip circumference for two groups showed a significant reduction (Table 3). These changes were also significantly

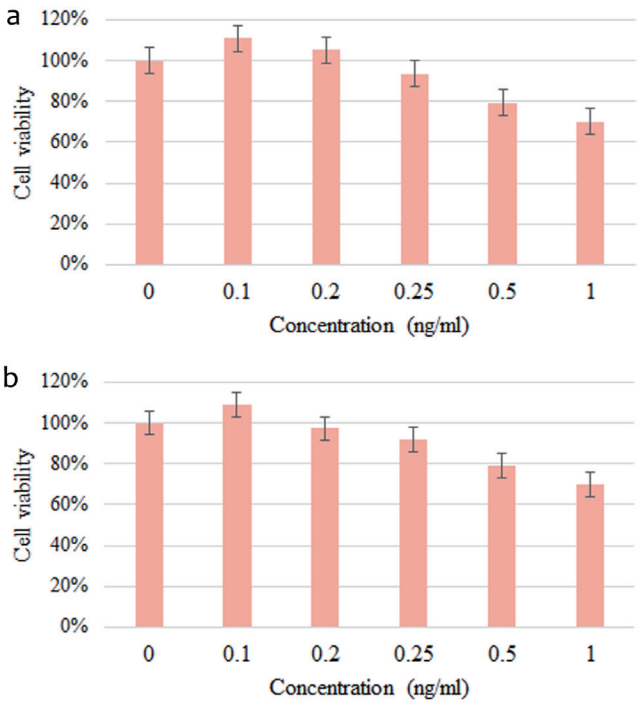

Figure 1. The effect of supplement on the survival rate of a) 3T3-L1 preadipocytes; b) 3T3-L1 mature adipocytes. Values are expressed as mean ± SD

reflected in reducing total body fat and waist circumference, but only found in supplement group.

3.4. Change in blood lipid profile and glucose

As shown in Table 4, two groups didn't show any significant change in total cholesterol (TC) and triglyceride (TG) levels over time. Free fatty acid (FAA) levels were found very greatly in-

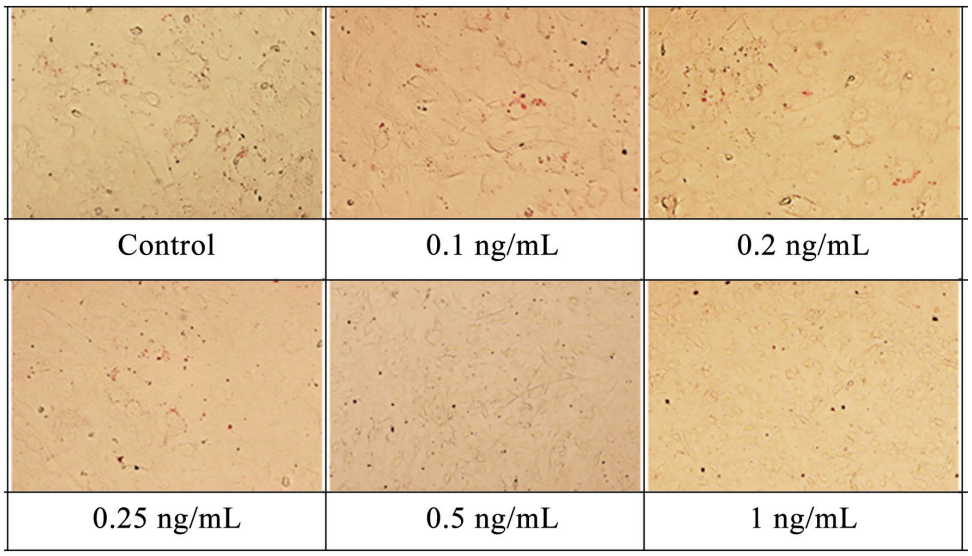

Figure 2. The effect of supplement on 3T3-L1 mature adipocyte cell stained by the oil-red staining microscopic view at 400X magnification.

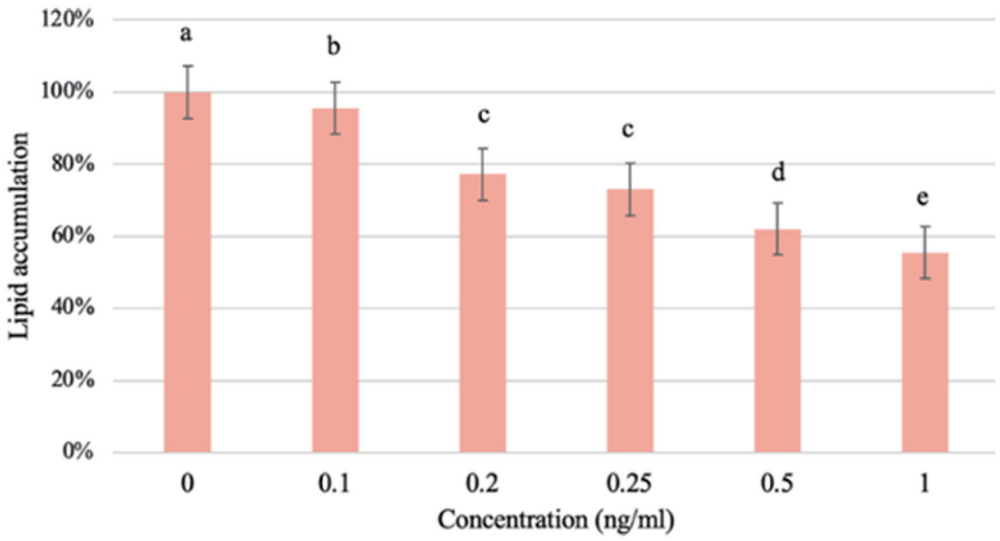

Figure 3. The effect of supplement on the lipid accumulation of 3T3-L1 mature adipocyte. Cell value is expressed as mean ± SD, Different superscript letters show significant difference ($p < 0.05$)

creased after intervention of supplement. Blood glucose showed no difference in two groups.

3.5. Changes in blood pressure

Supplement significantly reduced SBP after 8 weeks of intervention, but no change in the control group (Table 5).

3.6. Effect on cardiac, liver and kidney function

There was no change for all parameters in both groups (Table 6).

3.7. Change in adiponectin and leptin

The change in adiponectin and leptin levels of all groups were shown in Table 7. Both groups showed a significant increase in adiponectin levels after intervention ($p < 0.05$), but no difference on leptin in two groups.

3.8. Change in the antioxidant index over time

Supplement group showed a significant increase in plasma antioxidant capacity and a significant reduction in thiobarbituric acid reactive substances ($p < 0.05$), and a great increase in glutathione levels after intervention (Table 8), but no difference in control group.

4. Discussion

This study was focused on the reduction of lipid accumulation by

Table 1. Baseline subject's characteristic

	Control group (n = 15)	Supplement group (n = 15)
Gender		
Male	6	6
Female	9	9
Age (years)		
20–29	3	4
30–39	3	3
40–49	4	3
50–59	4	3
60 above	1	2
Average age	43.60 ± 15.02	41.13 ± 15.65
Height (cm)	164.53 ± 9.23	164.20 ± 8.87

Average age and height data represent as Mean ± SD for each group.

Table 2. Change of calories and macronutrients intake of subjects over time

	Week 0	Week 4	Week 8	Week 12	Follow-up
Total energy (Kcal)					
Control group	1,883.03 ± 429.18	1,617.23 ± 551.72*	1,656.73 ± 652.35*	1,650.52 ± 723.26*	1,629.54 ± 555.82*
Supplement group	1,922.27 ± 608.06	1,684.00 ± 454.24*	1,657.87 ± 348.50*	1,630.34 ± 329.81*	1,622.76 ± 419.98*
Protein (%)					
Control group	16.10 ± 1.92	17.55 ± 4.20	17.65 ± 3.96	17.58 ± 3.10	17.59 ± 2.63
Supplement group	16.20 ± 2.15	17.48 ± 3.08	17.40 ± 3.21	17.09 ± 2.78	16.92 ± 3.29
Lipid (%)					
Control group	39.07 ± 9.22	41.30 ± 10.96	40.67 ± 10.34	39.50 ± 7.77	38.08 ± 7.59
Supplement group	38.88 ± 8.25	39.23 ± 8.24	40.62 ± 7.18	41.83 ± 6.88	39.88 ± 8.04
Carbohydrate(%)					
Control group	42.57 ± 10.55	38.30 ± 12.68*	39.30 ± 10.56*	40.88 ± 9.01*	42.29 ± 9.21*
Supplement group	42.91 ± 8.42	41.23 ± 8.54*	40.01 ± 9.01*	39.11 ± 8.11*	41.20 ± 9.44*

Values are expressed as Mean ± SD. *Significant difference ($p < 0.05$) compared with week 0 within the same group.

using L-carnitine and catechin together with calories reduction. To assess whether supplement can reduce lipid accumulation, the 3T3-L1 cell line was used. According to the results, supplement can be categorized as a non-toxic supplement on pre- and mature adipocytes up to 1 ng/mL (>70%). Oil red staining showed that the treatment of supplement on 3T3-L1 mature adipocytes could significantly reduce fat accumulation inside the cell. The accumulation of red color was significantly reduced when the sample concentration was increased, this indicated less and smaller oil droplets inside the cell. This result was aligned with several previous studies that investigate L-carnitine and catechin as individual compounds (Furuyashiki et al., 2004; Mochizuki and Hasegawa, 2004; Lee et al., 2006). L-carnitine promotes the transportation of long-chain fatty acid into mitochondria thus pushing β-oxidation of fatty acid in mitochondria (Longo et al., 2016; Theodoridis et al., 2022).

On the other hand, catechin treatment on mature adipocytes shown to upregulate lipolysis by the cAMP/PKA pathway (Lee et al., 2006; Jiang et al., 2019). cAMP activation could further promote intracellular protein kinase A and activate hormone-sensitive lipase and adipose triglyceride lipase which increase intracellular lipid breakdown (Cho et al., 2014; Jiang et al., 2019). Catechin is also known to downregulate the activity of PPARγ thus reducing adipocyte differentiation and adipogenesis (Wang et al., 2014; Lefterova et al., 2014; Ma et al., 2018).

This clinical trial was conducted from May to September 2022 and divided into 12 weeks of intervention and a 2 weeks of follow-up period. During the intervention period, all subjects were asked to take the supplement and also reduce 250 kcal from their daily energy intake. Reduction of the energy intake was based on their daily energy intake from previous weeks. Most subjects reduced carbohydrate intake but not lipid intake. According to dietary guidelines from the Ministry of Health and Welfare, daily lipid intake is around 20–30%, but lipid was around 39% which was some higher than the recommendation in this study. This could be due to most of the subjects purchase their meals outside and it's difficult to control their lipid intake. The compliance rate for supplement group was around 87% throughout the study.

The data showed that calories reduction can significantly reduce body weight and additional supplement could help to reduce more body weight over time. Excess energy from diet was the main factor to contribute the body weight gain (Romieru et al., 2017). Even our result showed a reduction of body weight but not body fat in control group. Supplement might play a role in reducing body fat due to its active components. As mentioned earlier, both cathecin and L-carnitine can affect lipid metabolism and help to reduce body weight and fat.

Barzegar et al. (2013) showed that daily L-carnitine supplementation (2 g/day) together with low energy diet (−500 daily energy intake) for 8 weeks significantly reduce body weight, body fat percentage, leptin, and inflammatory markers (IL-6 and hs-CRP) compared to placebo with low energy diet. Different meta-analysis studies conclude that 2 grams of daily L-carnitine is sufficient to help reduce body weight and BMI (Askarpour et al., 2020; Talenezhad et al., 2020). L-carnitine supplementation is also known to affect lipid profile (TG, TC, LDL-C, HDL-C). A meta-analysis from Asbaghi et al. (2020) showed that a dose less than 3,000 mg/day, significantly reduces TC and LDL-C levels. Fathizadeh et al. (2019) also showed similar result on reducing TG, TC, and LDL-C and increase HDL-C levels.

Catechin has been proposed as a hypolipidemic and anti-obesity agent with several ways of action such as inhibiting adipogenesis, inducing apoptosis of adipocytes, and interfering with lipid metabolism (Huang et al., 2014). Brown et al. (2011) demonstrated the effect of decaffeinated green tea extract (containing 400 mg catechin, twice a day) significantly affect body weight and LDL-C levels after 6 weeks of intervention. Another study also shows that dried herbal capsules with a total dose of catechin (840 mg/day) reduced the body weight and BMI of obese subjects (Lenon et al., 2012). A combination of catechin and inulin is shown to reduce body weight, BMI, total fat mass, and waist and hip circumference after treatment for 8 weeks (Yang et al., 2012). Catechin belongs to the flavonoids mostly is found in tea and shows strong antioxidant properties. Our result showed a clear increase in plasma antioxidant capacity, which aligned with the result obtained from Venkatakrishnan et al. (2018) by using enriched catechin green tea and oolong tea (780 and 640 mg catechin respectively) for 12 weeks to increase plasma antioxidant activity and enzyme levels, and also reduce body weight, body fat and improve blood lipid profile.

Table 3. Change of anthopometric measurements in subjects

	Week 0	Week 4	Week 8	Week 12	Follow-up
Body weight (kg)					
Control group	80.99 ± 13.54	80.15 ± 13.24*	79.71 ± 12.85*	79.69 ± 13.78**	79.89 ± 13.66*
Supplement group	79.35 ± 14.84	78.75 ± 14.78**	78.49 ± 14.57**	78.46 ± 14.62**	77.98 ± 14.36**
ΔBody weight (kg)					
Control group		−0.85 ± 1.27^{A}	−1.29 ± 1.93^{A}	−1.31 ± 1.47^{AB}	−1.10 ± 1.49^{A}
Supplement group		−0.60 ± 0.73^{A}	−0.87 ± 0.97^{A}	−0.89 ± 0.84^{B}	−1.37 ± 2.66^{A}
BMI (kg/m^2)					
Control group	29.76 ± 2.57	29.42 ± 2.50*	29.29 ± 2.45*	29.27 ± 2.64**	29.34 ± 2.62*
Supplement group	29.19 ± 3.15	28.98 ± 3.23**	28.87 ± 3.16**	28.84 ± 3.11**	28.93 ± 3.22**
ΔBMI (kg/m^2)					
Control group		−0.34 ± 0.50^{A}	−0.47 ± 0.67^{A}	−0.49 ± 0.57^{AB}	−0.42 ± 0.55^{B}
Supplement group		−0.21 ± 0.24^{A}	−0.32 ± 0.35^{A}	−0.35 ± 0.31^{B}	−0.26 ± 0.49^{B}
Body fat (%)					
Control group	35.49 ± 5.56	35.01 ± 5.98	34.45 ± 5.62	34.58 ± 5.33	34.70 ± 5.39
Supplement group	36.20 ± 4.79	35.05 ± 4.76**	34.81 ± 4.78**	35.27 ± 4.90**	35.22 ± 4.73*
ΔBody fat (%)					
Control group		−0.48 ± 2.48^{A}	−1.04 ± 2.46^{A}	−0.91 ± 2.02^{B}	−0.78 ± 1.93^{B}
Supplement group		−1.15 ± 1.16^{A}	−1.39 ± 1.42^{A}	−0.93 ± 1.68^{B}	−0.98 ± 1.52^{B}
Fat-Free Mass (%)					
Control group	64.51 ± 5.56	64.97 ± 5.37	64.98 ± 5.99	64.44 ± 5.67*	65.72 ± 5.71
Supplement group	63.79 ± 4.78	64.54 ± 5.01**	64.95 ± 4.76**	64.82 ± 5.06**	65.20 ± 4.78*
ΔFat-Free Mass (%)					
Control group		0.47 ± 2.46^{A}	1.21 ± 2.45^{A}	1.19 ± 2.00^{B}	0.96 ± 1.82^{A}
Supplement group		1.16 ± 1.16^{A}	1.41 ± 1.44^{A}	0.95 ± 1.67^{B}	1.46 ± 2.29^{A}
WC (cm)					
Control group	94.61 ± 8.16	94.43 ± 8.26	94.39 ± 7.99	93.50 ± 9.36	94.15 ± 9.30
Supplement group	93.85 ± 8.86	93.00 ± 9.34*	93.45 ± 9.38*	92.70 ± 8.89*	92.67 ± 8.99*
HC (cm)					
Control group	106.73 ± 4.78	105.23 ± 4.70*	105.10 ± 4.58*	103.97 ± 4.89**	104.53 ± 4.79**
Supplement group	106.37 ± 7.86	105.55 ± 8.01*	104.95 ± 8.09**	104.67 ± 8.20**	104.77 ± 8.28**
MAMC (cm)					
Control group	31.82 ± 3.85	31.67 ± 3.49	31.52 ± 3.49	31.00 ± 3.57	30.98 ± 3.40
Supplement group	32.91 ± 2.88	32.77 ± 2.79	32.65 ± 2.80	32.40 ± 2.74	32.47 ± 2.72
TSF (mm)					
Control group	17.07 ± 6.83	16.80 ± 7.06	16.97 ± 7.05	16.50 ± 6.59	16.72 ± 6.52
Supplement group	11.97 ± 5.50	11.03 ± 3.15	11.03 ± 3.76	11.27 ± 3.48	11.23 ± 3.47

Values are expressed as Mean ± SD. *Significant difference when compared with week 0 within the same group, $p < 0.05$ **Significant difference when compared with week 0 within the same group, $p < 0.01$. Different superscript letters are significantly other among the groups in the Δ value with Week 0 ($p < 0.05$). WC: Waist circumference; HC: Hip circumference; MAMC: Mid-arm muscle circumference; TSF: Triceps skin fold.

This was the first clinical trial by using the mixture of L-carnitine and catechin, results clearly showed the combined effective compounds really worked well on obesity improvement and blood lipid regulation. When comparing with the past studies about L-carnitine or catechin, this mixture could show additive even synergistic effect.

Table 4. Change of blood lipids and glucose in subjects

	Week 0	Week 4	Week 8	Week 12	Follow-up
TG (mg/dL)					
Control group	136.47 ± 58.05	136.47 ± 78.17	164.40 ± 134.21	138.73 ± 43.66	139.57 ± 70.76
Supplement group	124.20 ± 58.62	119.20 ± 71.51	126.20 ± 62.56	121.67 ± 64.17	140.80 ± 94.84
TC (mg/dL)					
Control group	189.93 ± 43.51	183.73 ± 38.26	187.20 ± 32.83	186.53 ± 39.81	187.67 ± 37.95
Supplement group	229.27 ± 92.24	225.40 ± 84.64	230.40 ± 90.36	226.40 ± 102.81	224.27 ± 91.99
LDL-C (mg/dL)					
Control group	117.89 ± 36.66	114.50 ± 36.11	106.37 ± 30.91	110.37 ± 34.62	109.80 ± 34.97
Supplement group	145.33 ± 76.96	148.33 ± 72.07	145.80 ± 66.59	143.25 ± 87.12	140.66 ± 73.57
HDL-C(mg/dL)					
Control group	45.03 ± 9.12	44.08 ± 9.16	42.31 ± 8.43	44.54 ± 7.31	44.33 ± 8.28
Supplement group	52.12 ± 8.54	49.66 ± 8.79	48.95 ± 7.23	48.68 ± 9.11	49.63 ± 10.92
FFA (mmol/L)					
Control group	0.49 ± 0.19	0.64 ± 0.15	0.58 ± 0.17	0.49 ± 0.29	0.51 ± 0.19
Supplement group	0.52 ± 0.16	0.68 ± 0.14**	0.63 ± 0.13**	0.62 ± 0.19**	0.61 ± 0.19*
FBG (mg/dL)					
Control group	94.00 ± 9.25	93.47 ± 10.62	91.60 ± 9.08	91.07 ± 9.72	91.97 ± 9.74
Supplement group	97.33 ± 14.85	95.07 ± 14.60	95.67 ± 16.67	98.60 ± 17.93	96.40 ± 19.78
HbA1C (%)					
Control group	5.50 ± 0.47	5.55 ± 0.43	5.47 ± 0.38	5.49 ± 0.41	5.46 ± 0.42
Supplement group	5.59 ± 0.47	5.59 ± 0.48	5.58 ± 0.52	5.53 ± 0.51	5.56 ± 0.57
Insulin (μIU/mL)					
Control group	20.19 ± 8.17	19.67 ± 7.94	20.44 ± 15.75	21.10 ± 11.54	18.84 ± 8.52
Supplement group	19.76 ± 11.62	17.97 ± 9.60	17.76 ± 8.53	20.15 ± 11.32	16.91 ± 12.34
HOMA-IR					
Control group	4.70 ± 2.07	4.65 ± 2.29	4.64 ± 3.35	4.87 ± 2.99	4.39 ± 2.38
Supplement group	5.01 ± 3.85	4.33 ± 2.62	4.39 ± 3.03	5.26 ± 4.18	4.14 ± 3.22

Values are expressed as mean ± SD. *Significant difference when compared with week 0 within the same group, $p < 0.05$ **Significant difference when compared with week 0 within the same group, $p < 0.01$ Different superscript letters are significantly different among the groups in the Δvalue with week 0 ($p < 0.05$).

Table 5. Blood pressure of subjects

	Week 0	Week 4	Week 8	Week 12	Follow-up
SBP (mmHg)					
Control group	128.00 ± 14.30	127.07 ± 13.60	126.33 ± 13.25	124.87 ± 18.60	126.20 ± 17.03
Supplement group	129.53 ± 17.09	124.13 ± 18.26	120.53 ± 16.46**	121.60 ± 16.89**	120.13 ± 15.84**
DBP (mmHg)					
Control group	83.53 ± 8.94	84.93 ± 10.59	81.67 ± 11.60	81.20 ± 10.19	81.27 ± 10.73
Supplement group	79.20 ± 15.61	78.47 ± 14.39	79.00 ± 13.60	77.47 ± 12.78	78.13 ± 13.93
Pulse (bpm)					
Control group	75.33 ± 7.58	75.13 ± 8.43	75.40 ± 7.37	77.00 ± 6.60	75.33 ± 7.19
Supplement group	73.07 ± 10.00	73.33 ± 9.09	71.13 ± 6.79	72.93 ± 9.09	76.00 ± 10.49

Values are expressed as Mean ± SD. *Significant difference when compared with week 0 within the same group, $p < 0.05$ **Significant difference when compared with week 0 within the same group, $p < 0.01$ SBP: Systolic blood pressure DBP: Diastolic blood pressure.

Table 6. Cardiac, liver and kidney function in subjects

	Week 0	Week 4	Week 8	Week 12	Follow-up
CPK (IU/L)					
Control group	100.07 ± 38.18	121..40 ± 61.79	104.40 ± 59.45	92.60 ± 46.47	103.10 ± 60.03
Supplement group	134.40 ± 71.23	131.00 ± 67.41	137.07 ± 102.39	133.13 ± 104.52	121.47 ± 77.97
LDH (IU/L)					
Control group	136.80 ± 17.03	143.80 ± 24.59	144.47 ± 21.33	145.53 ± 21.40	139.90 ± 21.08
Supplement group	141.80 ± 24.10	148.67 ± 25.12*	146.80 ± 27.21*	149.47 ± 27.50*	142.00 ± 24.72
GOT (IU/L)					
Control group	24.87 ± 7.35	26.20 ± 7.09	28.73 ± 13.8	25.33 ± 10.44	26.10 ± 8.81
Supplement group	20.40 ± 4.29	22.20 ± 7.55	21.80 ± 7.12	22.33 ± 7.90	21.33 ± 7.52
GPT (IU/L)					
Control group	30.33 ± 20.40	31.27 ± 15.40	34.73 ± 24.75	34.40 ± 32.22	33.90 ± 25.68
Supplement group	23.80 ± 8.39	25.60 ± 12.74	23.53 ± 11.22	25.73 ± 14.02	25.27 ± 12.27
BUN (mg/dL)					
Control group	11.60 ± 2.59	12.33 ± 2.23	12.20 ± 3.12	13.93 ± 3.63	12.37 ± 2.56
Supplement group	12.60 ± 4.12	12.53 ± 3.80	12.67 ± 2.72	12.07 ± 3.65	11.27 ± 2.37
Creatinine (mg/dL)					
Control group	0.85 ± 0.22	0.84 ± 0.20	0.84 ± 0.21	0.84 ± 0.20	0.83 ± 0.21
Supplement group	0.81 ± 0.13	0.83 ± 0.12	0.81 ± 0.12	0.80 ± 0.11	0.81 ± 0.11
UA (mg/dL)					
Control group	6.39 ± 1.76	6.80 ± 1.65	6.44 ± 1.42	6.37 ± 1.62	6.45 ± 1.61
Supplement group	5.95 ± 1.37	5.95 ± 1.15	5.93 ± 1.01	5.99 ± 1.01	6.47 ± 1.35

Values are expressed as Mean ± SD. *Significant difference when compared with week 0 within the same group, $p < 0.05$ **Significant difference when compared with week 0 within the same group, $p < 0.01$. CPK: Creatine Phosphokinase, LDH: Lactic dehydrogenase.

5. Conclusions

According to the 3T3-L1 model study, L-carnitine and catechin treatment significantly reduced lipid accumulation. Clinical trial strongly suggested that calories restriction together with the supplement (L-carnitine and catechin) significantly reduced body weight and body fat and improved blood lipid profile in obese subjects.

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Conflict of interest

The authors declare that they have no conflicts of interest

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Table 7. Change of adiponectin and leptin in subjects

	Week 0	Week 4	Week 8	Week 12	Follow-up
Adiponectin (μg/mL)					
Control group	2.77 ± 1.44	3.12 ± 1.56*	3.08 ± 1.88*	3.31 ± 1.87*	3.42 ± 1.91*
Supplement group	4.37 ± 3.15	4.59 ± 3.09*	4.68 ± 3.41*	4.71 ± 3.45*	4.47 ± 3.07*
Leptin (ng/mL)					
Control group	17.70 ± 12.42	17.17 ± 11.29	19.51 ± 14.85	19.84 ± 9.81	17.52 ± 9.76
Supplement group	21.21 ± 17.83	16.81 ± 9.44	16.26 ± 8.36	15.38 ± 7.96	16.60 ± 12.08

. Values are expressed as Mean ± SD. *Significant difference when compared with week 0 within the same group, $p < 0.05$ **Significant difference when compared with week 0 within the same group, $p < 0.01$.

Table 8. Antioxidant status of subjects

	Week 0	Week 4	Week 8	Week 12	Follow-up
TEAC (μM)					
Control group	4.01 ± 0.17	4.09 ± 0.19	4.02 ± 0.20	4.16 ± 0.36	4.15 ± 0.33
Supplement group	4.03 ± 0.12	4.08 ± 0.19*	4.53 ± 0.17*	4.94 ± 0.46*	5.32 ± 0.44*
TBARS (μM)					
Control group	1.00 ± 0.35	0.96 ± 0.32	0.87 ± 0.25	0.81 ± 0.19	1.02 ± 0.38
Supplement group	1.17 ± 0.35	0.88 ± 0.31*	0.73 ± 0.22*	0.63 ± 0.25*	0.87 ± 0.19*
GSH (ng)					
Control group	5.97 ± 0.07	5.99 ± 0.05	6.03 ± 0.04	6.05 ± 0.07	6.03 ± 0.05
Supplement group	5.88 ± 0.07	5.97 ± 0.06*	6.02 ± 0.04*	6.05 ± 0.05*	6.06 ± 0.05*

Values are expressed as Mean ± SD. *Significant difference when compared with week 0 within the same group, $p < 0.05$ **Significant difference when compared with week 0 within the same group, $p < 0.01$. TEAC: Trolox equivalent antioxidant capacity, TBARS: Thiobarbituric acid reactive substances, GSH: Glutathione.

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Botanicals impact the bifidogenic effect and metabolic outputs of *in vitro* fiber fermentation by gut-derived microbiota in individual-specific ways

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Abstract

Fortification of products frequently consumed by a large proportion of society provides an attractive strategy to close the "fiber gap" and may have the potential to concomitantly reverse the detrimental health effects exacerbated by our modern diets. Besides prebiotic fibers, products can contain other functional components, e.g. botanicals. However, most studies have investigated functional components in isolation. The impact of other components present in functional product blends on the bifidogenic effect typically exerted by prebiotic fibers are largely unexplored. Here, we investigated the fiber and botanical blends included in OLIPOP, a functional soda, in an *in vitro* gut fermentation model. Our data revealed that the blend of inulins and resistant dextrins promoted growth of bifidobacteria across gut microbiota from four donors, even those with small initial populations. In addition, botanicals interacted with fiber fermentation in donor-specific ways, in some cases strongly enhancing fermentation rate and production of short-chain fatty acids.

Keywords: Fiber augmentation; Beverages; Botanicals; Prebiotic; Microbiome; Butyrate.

1. Introduction

Carbohydrate intake in modern Westernized diets has significantly shifted in fiber content and composition compared with ancestral human diets, both with hunter-gather and agricultural traditions (De Filippo et al., 2010; Schnorr et al., 2014). Current hunter-gatherer populations, such as the Hadza of Tanzania, typically consume upwards of 100 g of fiber per day and approximately 70% of their caloric intake is plant-derived (Smits et al., 2017). By contrast, the modern-day population overwhelmingly consumes easily-digested carbohydrate types, such as simple starches (54%) and sugars (36%). Currently, Westernized diets are associated with high intakes of saturated fat and sucrose and are low in total fiber intake. Collectively, the consumption of a Western type diet represents a growing health risk for metabolic diseases such as diabetes and diseases of the gut, such as inflammatory bowel disease (Statovci et al., 2017). Other literature describes that Western diets and lifestyles are associated with cancer and less diverse gut microbiomes (O'Keefe et al., 2015; Vangay et al., 2018). Conversely, mounting scientific evidence suggests that increasing total fiber intake is beneficial both for supporting digestive, gut microbiome and overall health (Makki et al., 2018; Reynolds et al., 2019). Specifically, in the U.S., the Academy of Nutrition and Dietetics recommends that higher daily dietary fiber intakes may reduce risk of multiple chronic diseases, including type 2 diabetes, cardiovascular disease, and some cancers, and lower body weight (Quagliani and Felt-Gunderson, 2017). Consequently, the Academy suggests a minimum daily fiber intake of 14 g per 1,000 kilocalories for adult

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women and men, equaling 25 and 38 g/day, respectively (Dahl and Stewart, 2015).

Despite changes to nutrition policy, governmental reimbursement of healthier foods, and insurance programs incentivizing healthy food purchasing, modern societies fail to consume sufficient total fiber and diversity of fiber types on a daily basis. Furthermore, modern consumers are choosing increasingly restrictive dietary patterns (gluten-free, grain-free, wheat-free, etc) which may be leading to reduced daily fiber intake (Quagliani and Felt-Gunderson, 2017). Unfortunately, daily nutrition intake surveys analyzed from National Health and Nutrition Examination Survey (NHANES) cycles 2013–2018 indicate Americans on average only consume between 9–10 g/1,000 kcal of total fiber per day, which translates to less than 1 in 10 of the population meeting minimum suggested fiber intake levels. This shortfall between recommended and actual fiber intake is commonly referred to as the "fiber gap." Fiber fortification of foods and beverages can potentially bridge this gap and may, in this way, contribute to reduced incidence of chronic disease in industrialized urban societies. Even the CODEX Alimentarius Commission has aligned its definition to support bridging the fiber gap (Jones, 2014). To this end, one rapidly growing category in the beverage sector is functional soda, which aims to provide consumers with healthier alternatives to traditional soda, often by reducing sugar and adding various prebiotic fibers. Such approaches hold promise for modulating population-scale fiber intake because the selection of a high-fiber alternative with a similar sensory profile within the same product category often requires relatively minimal behavioral adaptation. This ease of adoption by consumers may explain the rapid growth of this product category. Moreover, success in this product category likely serves as a model for fiber augmentation/sugar replacement strategies in other categories.

The mechanisms by which fibers exert beneficial health-promoting properties are multi-factorial, but include increased production of short-chain fatty acids (SCFAs), stimulation of beneficial microbial taxa (e.g. bifidobacteria), reduction in the production of nitrogenous and otherwise pro-inflammatory metabolites, ammonia, amines, and some phenolic compounds (Makki et al., 2018). SCFAs produced by gut bacteria through fiber fermentation support healthy gut epithelial barrier and immune function. Beyond the role of SCFAs in gut barrier integrity, dietary fibers interacting in the digestive tract mechanically stimulates the gut epithelium to secrete mucus. Depletion of dietary fiber consumption compromises gut barrier integrity, which increases the risk for acute infection and chronic disease (Desai et al., 2016). Moreover, high fermentable fiber consumption provides substrates for the growth and maintenance of beneficial microbial populations in the colon. However, leveraging fiber supplementation to bridge the fiber gap requires a more thorough mechanistic understanding of the relationship between microbiome compositional changes, metabolite production, and human physiology.

Dietary fiber is defined by the FDA in 21 C.F.R.§101.9(c)(6)(i) as "non-digestible soluble and insoluble carbohydrates (with 3 or more monomeric units), and lignin that are intrinsic and intact in plants; or isolated or synthetic non-digestible carbohydrates (with 3 or more monomeric units) determined by FDA to have physiological effects that are beneficial to human health." Some dietary fibers are termed "prebiotic" according to the International Scientific Association for Probiotics and Prebiotics ("ISAPP") definition (Gibson et al., 2017), which means that they are selectively utilized by gut microbes; the concept of a prebiotic has recently been expanded to include molecules outside the carbohydrate class (e.g. polyphenols). Fibers that are also designated prebiotics are often termed "prebiotic fibers" on consumer products. Thus, "dietary fiber" is a heterogeneous category of plant carbohydrates, having wildly diverse physical and chemical structures and associated physiochemical properties (solubility, viscosity). It is very likely that specific fiber structures exert divergent impacts directly on the host and indirectly via its gut microbiota. For example, even though they are all glucans, a set of commercial mixed-linkage α-glucans, resistant dextrins, and polydextroses produced as supplemental fibers for food products fermented to distinct metabolic and microbial outcomes; however, some taxa exhibited specificity for some glucans across donors (Romero Marcia et al., 2021). Successful utilization of fibers and prebiotics in designing fiber-supplemented food and beverage products to improve health via the microbiome at population scales requires much greater insight into such mechanistic linkages among fine fiber structure and microbiota.

Importantly, fiber fermentation *in vivo* does not occur in a vacuum; fibers are fermented in the context of many other compounds that might impact their utilization by microbes and metabolic fate in the colon. Some functional foods and beverages deliberately employ plant extracts (hereafter, "botanicals") and probiotics, prebiotics or postbiotics, with the goal of enhancing their health benefits. Increasing our understanding of a botanical extract's influence on the microbiome and human health is important given their inclusion in numerous dietary supplements or functional food and beverage products in recent years (https://tinyurl.com/krmet4vs). Furthermore, botanical extracts being included in dietary supplements or functional food and beverage products is not unique; frequently, companies are creating multi-ingredient products including botanicals and prebiotic fibers. Although mechanistic and human health outcomes are widely studied for prebiotic fibers, there is a paucity of scientific literature assessing the effects of botanical extracts on the gut microbiome or broader human health. Moreover, to the best of our knowledge, no microbiome studies have been performed elucidating the combined interaction effects of botanical extracts and prebiotic fiber within a final product formulation. Developing mechanistic and human clinical trial datasets on a product's functional blend to complement research on isolated ingredients is necessary to understand microbiome and beneficial human health effects.

In vitro batch culturing systems mimicking fermentation by colonic microbiota are a valuable tool to screen the impact of product formulations on the microbiome prior to the execution of larger clinical studies (Yao et al., 2020). Here, we assessed the impact of the prebiotic fibers and botanicals in OLIPOP, a functional soda, on gut microbiome composition and functionality, by employing an anaerobic batch culturing system inoculated with fecal slurry from 4 donors. Through the inclusion of study arms containing OLIPOP formulations with and without added botanical extracts, the interaction impacts of these important dietary components could also be investigated.

2. Materials and methods

2.1. Donor selection

Fecal donors were recruited for this study under oversight from the Purdue University Institutional Review Board under protocol IRB-2020-1650. Four donors were selected that satisfied the following criteria: 1. between 18–39 years old, 2. a normal or overweight BMI ($18.5 < BMI < 30$), 3. consumed their normal diet for the last two weeks, 4. did not take any antibiotics within the last 12 months, 5. did not consume any fiber, prebiotic, or probiotic prod-

ucts within the last 3 months, and 6. are not heavy alcohol drinkers (consumption limited to 5 or less alcoholic drinks per day). Donors selected had no history of gastrointestinal or chronic metabolic diseases and had not had a major gastrointestinal surgery in the past 5 years or a major bowel resection ever. Fecal samples were collected from two male and two female donors aged 20–39. Both males (Donors 1, 38 years old and 2, 27 years old) and one female (Donor 4, 28 years old) reported diets consistent with omnivory, whereas the other female (Donor 3, 32 years old) reported a vegetarian diet.

2.2. Fermentation substrates

Carbon sources were provided by OLIPOP, Inc. shipped on ice, and immediately placed at 4°C. Two different carbon sources were used to supplement the medium used during the fecal fermentations: a blend of fibers (cassava root fiber, chicory root inulin, and Jerusalem artichoke inulin; "the OLISMART fibers") reconstituted in sterile water, and the same reconstituted OLISMART fibers combined with a mixture of botanicals (extracts from nopal cactus, marshmallow root, calendula flower, and kudzu root). We maintained the concentrations of fibers and botanicals equivalent to those found in any complete OLIPOP soda product found at retail - 9 g of dietary fiber per one 355 ml can. This equated to a total substrate loading of 0.025 g/ml, or 2.5% carbohydrate (w/v).

2.3. In vitro fermentation

One liter of the basal fermentation medium contained the following substances: 0.001 g resazurin, 0.10 g Na_2SO_4, 0.40 g urea, 0.45 g KCL, 0.468 g NaH_2PO_4, 0.47 g NaCl, and 0.865 g Na_2HPO_4 and autoclaved (121°C for 30 minutes). Heat sensitive compounds CaCl2, $MgCl_2$, 1 mL cysteine hydrochloride (0.25 g/L) and 1 mL of 1000X P1 metal solution were added via 0.22 µM filter sterilization. Per liter, the P1 metal solution was composed of 34.26 g H_3BO_3, 4.32 g $MnCl_2 \cdot 4H_2O$, 0.315 g ZnCl2, 44 mg $Na_2MoO_4 \cdot 2H_2O$, 3 mg $CuSO_4 \cdot 5H_2O$, 12.15 mg $CoCl_2 \cdot 6H_2O$, 259 mg $NiCl_2$, 0.28 ml EDTA (10 mM), 1 ml $FeCl_3 \cdot 6H_2O$ (3.89 mg/ml in 0.1MHCl). The media was fortified with 200 µM sterilized amino acids (10 µM each) and 1% (v/v) ATCC vitamin supplement (ATCC MD-VS; Hampton, NH) and finally pH controlled at 7.0. The amino acid mixtures (200 µM final concentration in media) contained 20 proteinogenic amino acids, including: alanine, glycine, isoleucine, leucine, proline, valine, phenylalanine, tryptophan, tyrosine, aspartic acid, glutamic acid, arginine, histidine, lysine, serine, threonine, cysteine, methionine, asparagine and glutamine at final concentrations of 10 µM each.

The base media was used as a blank, with either OLISMART (prebiotic fibers and botanicals mentioned above) or OLISMART excluding botanicals solutions added for experimental conditions. All fermentations were performed in an anoxic chamber (Coy Laboratory Products Inc., Great Lake, MI, USA) supplied with nitrogen, carbon dioxide, and hydrogen (90%, 5%, and 5% respectively). The media were placed in the anoxic chamber 24 h before the fermentation in order to reduce oxygen levels.

Fecal samples were obtained from donors in the early morning using a custom fecal collection kit. Fecal samples were collected in sterile 50 mL Falcon tubes and stored immediately on ice. Fecal inocula were anoxically prepared as previously described (Yao et al., 2020) with the following modifications. Briefly, fecal aliquots were diluted 1:5 in medium and homogenizing via rapid pipetting and vortexing. The fecal slurry was further diluted in a 1:20 ratio with the corresponding medium type (no additions, fibers, fibers and botanicals) and poured over 4 layers of cheesecloth to remove large particles, yielding a final fecal slurry consisting of a 1:100 dilution of fecal material. 5.0 mL of the prepared fecal slurry was placed in 15 mL Balch tubes and sealed with a butyl rubber stopper plus an aluminum seal. The Balch tubes were placed in an incubator at 37°C, shaking at 10,000 × *g*. Fermentation cultures were harvested in triplicate for each of the 3 media at timepoints 2, 4, 8, 12, 18, and 24 h. Two aliquots of 1.5 mL were used from each tube for downstream SCFA analysis and DNA extraction, and pH was measured on the remaining spent media.

2.4. DNA extraction and sequencing

Upon donation of fecal samples on ice, three tubes were filled with raw fecal material (~500 mg), and three more tubes were filled with 1.5 mL of 1:100 feces to medium mixture and immediately placed at −80°C. During the fermentation, three fermentation tubes were sacrificed for each condition at each timepoint, and the product was immediately placed at −80°C in 1.5 mL aliquots. Raw feces, baseline blanks (medium with fecal slurry at the initiation of the experiment), and fermentation products (fiber, fiber and botanicals) at each timepoint were then sent to Diversigen™ for DNA extraction via PowerSoil Pro (Qiagen), automated for high throughput processing on the QiaCube HT (Qiagen), using Powerbead Pro Plates (Qiagen) with 0.5 mm and 0.1 mm ceramic beads. Samples were quantified with Quant-iT PicoGreen dsDNA Assay (Invitrogen). Libraries were prepared with a procedure adapted from the Illumina DNA Prep kit. For shallow sequencing, libraries were sequenced on an Illumina NovaSeq using single-end 1×100 reads. DNA sequences were filtered for low quality (Q-Score < 30) and length (<50), and adapter sequences were trimmed using cutadapt.

2.5. Metagenomic analysis

A conda virtual environment was created on Purdue University's Bell cluster (CentOS 7) with Python version 3.7.10. All jobs were run using Dell compute nodes with two 64-core AMD Epyc 7662 "Rome" processors and 256 GB of available memory. Sequences were downloaded as raw FASTQ files from Diversigen and FastQC (v0.11.9) was used to analyze initial metagenomic reads and determine if trimming and filtering were required. Reads were taxonomically assigned using MetaPhlAn (v3.0.7) and PhyloPhlAn (v3.0.2), which use reference-based algorithms to taxonomically identify reads up to the species level. To generate raw abundance calculations of taxonomic representation per sample, reads were summed at the highest specificity of classification. Relative abundances were then computed using total library size as a normalization factor for each sample. Reads without a taxonomic classification were removed from further analysis.

Alpha and Beta diversities were calculated by phyloseq (v1.30.0) using relative abundances of taxonomically classified reads as input. Beta diversity was calculated using the Bray-Curtis, Jaccard, and UniFrac methods. HUManN (v3.0) was used to profile the abundance of microbial pathways in the sequenced communities to determine metabolic potential.

2.6. Short-chain fatty acid (SCFA) analysis

SCFAs were measured at each of the timepoints in triplicate us-

ing a gas chromatograph (Nexis GC-2030, Shimadzu) with a flame-ionization detector and Stabilwax-DA column (Crossbond Carbowax polyethylene glycol; Restek, Inc.). The parameters of the analysis were as follows: 30 m long by 0.25 mm diameter column with 0.25μM film thickness, 230°C injector temperature, and 100°C initial oven temperature with a max column temperature of 260°C. 0.5 μL of sample were injected and measured for 23.43 mins in triplicate for each sample. External standards were used to back-calculate sample concentrations of acetate (Thermo Fisher catalogue number A38S), propionate (A258), butyrate (AC108111000), isobutyrate (AC122520250), and isovalerate (AA A18642AU).

Frozen aliquots consisting of 1.5 mL of fermentation product were thawed at room temperature and spun at 13,000 ×*g* for 10 minutes. Supernatant was mixed in a 4:1 ratio with 4-methylvaleric acid (Thermo Fisher catalogue number AAA1540506) in 6% *v/v* phosphoric acid and copper sulfate pentahydrate; these additional products added to the sample made up the internal standard.

2.7. Statistical analyses

An analysis of variance (ANOVA) was independently run on acetate, butyrate, and propionate concentrations with factors including donor, timepoint, and media source to test main effects. The base R (version 2021.09.1) function *aov()* was used to determine the main effects each independent variable had on acid production. 2-group t-tests through base R (function *t.test()*) were used to test for significant acid production differences at specific timepoints for each donor individually. Pairwise multiple testing and p-value adjustments were performed using Tukey's honest significant difference (R stats library; function *TukeyHSD()*) to determine statistically significant acid production for each media type across each donor. Significant alpha diversity changes were also investigated with factors including Donor, Time, and Sample using *aov()* and *TukeyHSD()* R functions.

Metagenomic reads were quantified at the taxonomic level of family and subjected to ANOVA with read count as the dependent variable and media condition and donor as the interacting independent variables. Each donor's abundance data was independently analyzed for condition effects on family-level read-count information. The base R function *aov()* was used to model the effects and *TukeyHSD()* (stats library) was used with a confidence level of 0.95 to determine significant differences across each group.

The R session, code, and input files required to replicate each of our plots, as well as raw input are available as supplementary information.

3. Results

3.1. OLISMART fibers are strongly fermented by gut microbiota but fermentation rate responses to botanicals are donor-specific

All *in vitro* fermentations containing OLISMART fibers proceeded rapidly, as measured by medium acidification, and reached terminal acidities (pH 3.5–4.0) within 12 h of inoculation for donors 1 and 2, whereas pH continued to modestly decline in cultures from donors 3 and 4 for the duration of the experiment (Figure 1). Interestingly, the effect of botanicals on fermentation rate was donor-specific; botanicals substantially increased the fermentation rate in donor 1 and donor 3 cultures, had little or no impact on donor 2 culture rates, and significantly slowed acid generation in donor 4 cultures. In contrast, control fermentations to which no carbon source was added did not display a significant acidification, with pH values remaining between 6–7 for the entire duration of the experiment (data not shown). Thus, although fermentative responses to OLISMART fibers by gut microbiota were very similar across donor microbiota, the interactions of fibers with botanicals were determined by donor context.

3.2. Short-chain fatty acid production levels are donor and botanical specific

Fermentation of OLISMART fibers across all donors was strongly acetogenic, approaching or exceeding 60 mM by the end of the fermentation (Figure 2a). The effect of botanicals on fiber fermentation was donor specific, generally in ways that reflected overall acid production trends; acetogenesis of donor 1 and donor 3 microbiota were significantly stimulated by botanicals at 12 h ($p < 0.05$) while donor 4's microbiota was not significantly affected. In contrast, acetogenesis by donor 4's microbiota was strongly delayed by botanicals for the first 18 h, thereafter showing significant botanical stimulation of acetate at 24 h ($p < 0.05$); these communities produced no meaningful amounts of acetate until 12 h post-inoculation. Interestingly, acids were still being produced in these communities (see below); the pH of these cultures dropped below 5 by the 8-h time point. Further, the acetogenesis rate in botanical-amended cultures increased with time, until the end of the experiment. These data suggest substantial rewiring of the metabolism of OLISMART fibers by the donor 4 microbiota, and more minor influences on the donor 1 and 3 communities.

Unlike acetate production, production of propionate and butyrate from fermentation of OLISMART fibers was strongly donor- and botanical-dependent. Donor 1's microbiota produced far more butyrate than propionate, and both levels appeared enhanced by the presence of botanicals (Figure 2; butyrate: ~14 mM with and ~7 mM without, $p < 0.05$; propionate: ~7 mM with and ~4.5 mM without, $p < 0.05$). Isobutyrate was also detectable only in donor 1 cultures with botanicals, which was also true for isovalerate (with the exception of t = 2 h, where this latter metabolite was also found in fermentations lacking botanicals). By contrast, fermentations containing donor 2 microbiota produced modestly more butyrate than propionate, and the production rates and amounts of these metabolites were similar with and without botanicals ($p > 0.05$ for all timepoints except propionate at 12 h [likely due to limited number of replicates]); isovalerate and isobutyrate were undetectable in these cultures (data not shown). Donor 3's microbiota were substantially more propiogenic than butyrogenic when OLIPOP fibers were fermented alone, and produced both significantly more butyrate (~6 mM vs. ~4 mM) and propionate (upwards in some cases of 15 mM vs. a maximum of ~8 mM) when botanicals were present (Figure 2). ANOVA using acid concentration as the dependent variable revealed significant main effects of media, timepoint, and acid when comparing donor 3 butyrate and propionate SCFA data ($p < 0.01$ for all independent variables). Again, donor 4's microbiota showed strong metabolic rewiring with botanicals; though slightly more propiogenic than butyrogenic without botanicals, when botanicals were added the production of butyrate dropped significantly ($p < 0.05$ for all timepoints except t = 2 h) and propionate production was delayed, though it reached higher substantially concentrations at the end of the fermentation compared with fibers alone. We hypothesize that much of the SCFA production difference in donor 4 cultures was due to the accumulation of lactate, a common metabolic product of bifidobacteria, which was not measured in

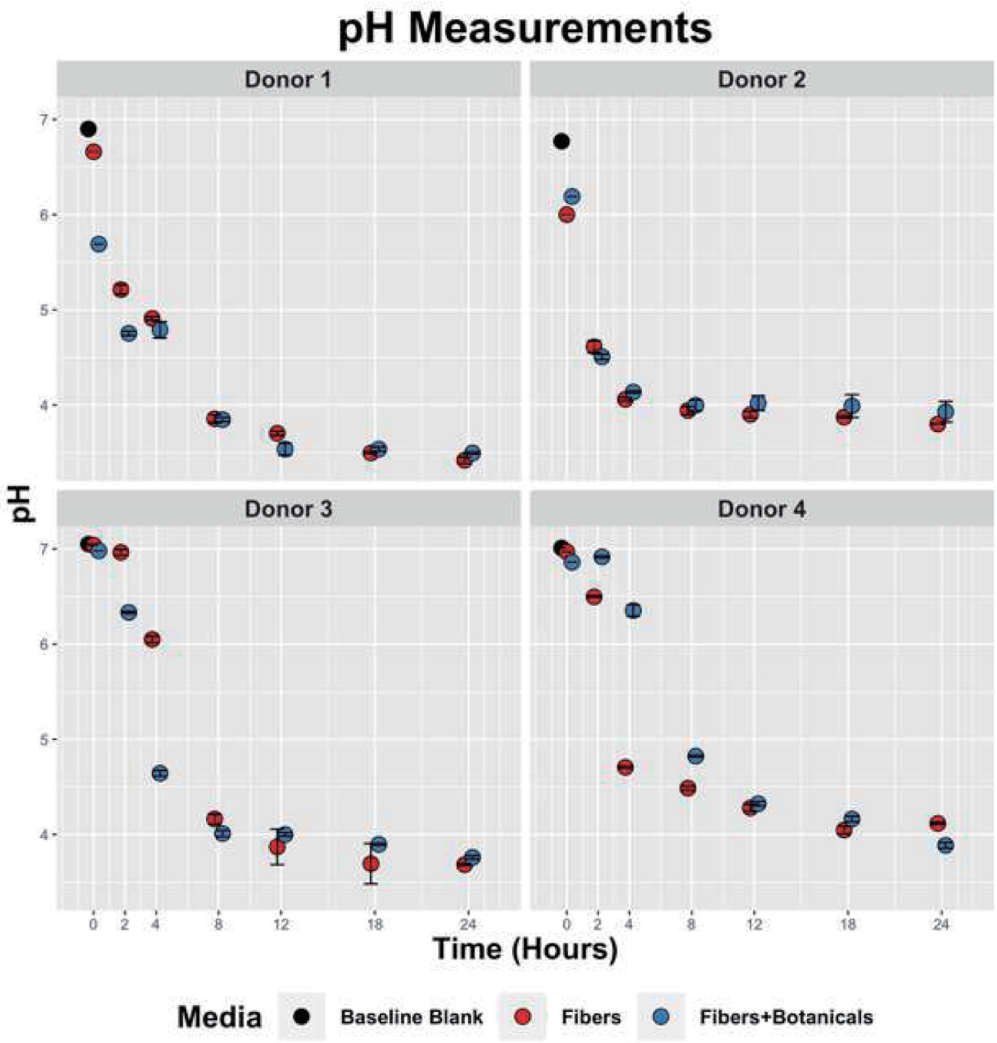

Figure 1. Temporal pH reduction by fecal slurries containing OLISMART fibers or OLISMART fibers supplemented with botanicals.

the study but is consistent with the magnitude of observed bifidobacterial blooms (similarly with acetate). Interestingly, donors that were strongly responsive to botanicals were the only ones in which BCFAs (isobutyrate, isovalerate) could be detected (data not shown), with maximum isobutyrate and isovalerate levels of 0.10 µM and 0.30 µM for Donor 1 and values of 0.11 µM and 0.18 µM for Donor 2, respectively.

3.3. Fermentation of OLISMART fibers is bifidogenic across donors but the extent is individually modulated by botanicals

Although the microbial community composition responses to fermentation were individual and depended somewhat upon initial community composition (Figure 3), bifidobacteria expanded dramatically in relative abundance across all four donor populations during fermentation of OLISMART fibers either with or without added botanicals ($p < 0.001$, ANOVA using family read count as dependent variable and media type as independent variable). Metagenomic read counts generated for the *in vitro* fermentations revealed that across donors, members of phylum Actinobacteria, or more specifically of the family Bifidobacteriaceae significantly ($p < 0.001$, ANOVA) bloomed when grown on OLISMART fibers, with and without botanicals (Figure 4a). This rapid relative growth of bifidobacteria came at the expense of diverse members of Bacteroideaceae, Lachnospiraceae, and Ruminococcaceae in all donors, as well as Akkermansiaceae specifically for donor 4 (Figure 4b) ($p < 0.001$, ANOVA). It should be noted that reduction in the relative abundances of microorganisms does not imply that their absolute numbers are reduced, but rather that they are not growing sufficiently rapidly to keep up with the average growth rate of the community. Thus, these data should be interpreted that the growth rate of the bifidobacteria was substantially greater than other taxa in the community, regardless of the starting bifidobacterial population size. Not surprisingly, such strong expansion in one taxonomic group inherently led to significant ($p < 0.001$, ANOVA) time-dependent reduction in alpha diversity (a combination of species richness and evenness) over the 24-h fermentation, as small populations of organisms fall below the limit of detection (reducing observed richness of the community given identical sampling effort) and the evenness of the community is reduced by the bloom in a single taxon (Figure 5). Notably,

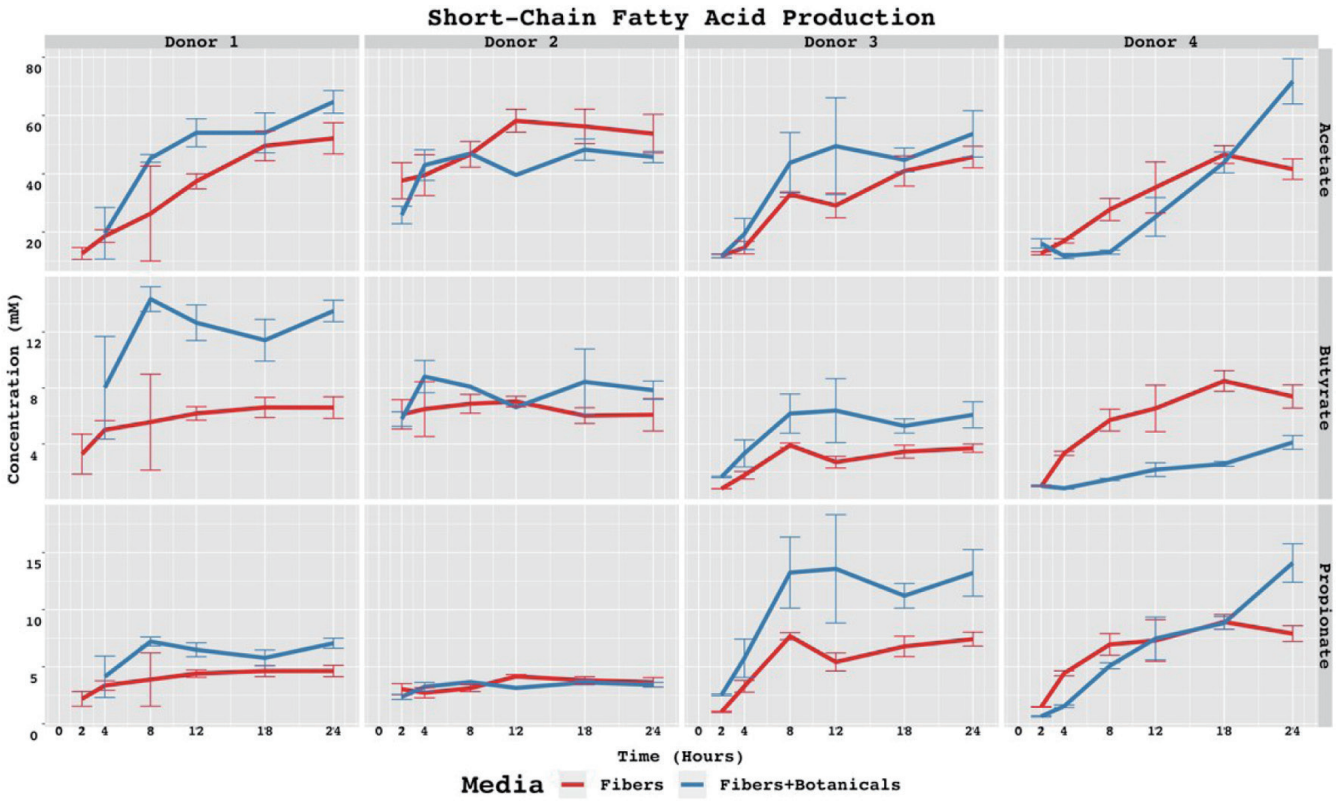

Figure 2. SCFAs concentrations in baseline controls and fiber fermentations with or without added botanicals. Statistically significant differences are calculated by pairwise t-tests with $p < 0.05$. Symbol style: p-value < 0.05 (*). Asterisk (*) above specific timepoints (x-axis) indicates statistical significance for timepoint; asterisk (*) in top right corner of panel indicates statistical significance at every timepoint for the donor-acid combination.

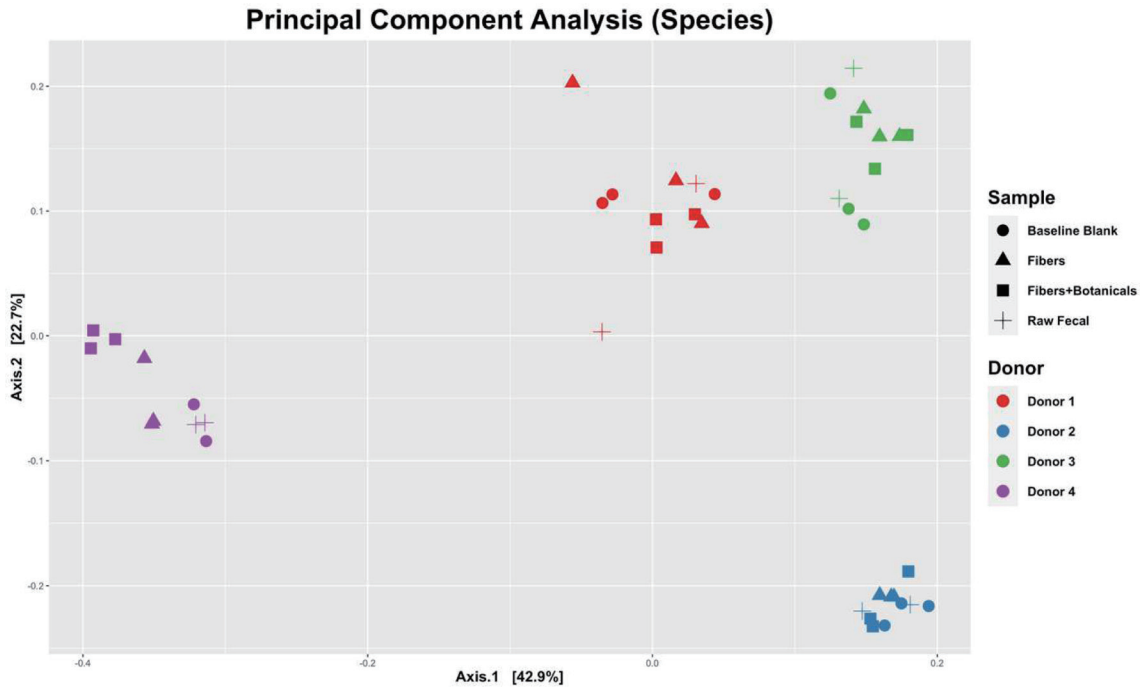

Figure 3. Principal components of analysis (PCoA) plot displaying UniFrac beta-diversity relationships among fecal samples, controls, and fermentations by donors.

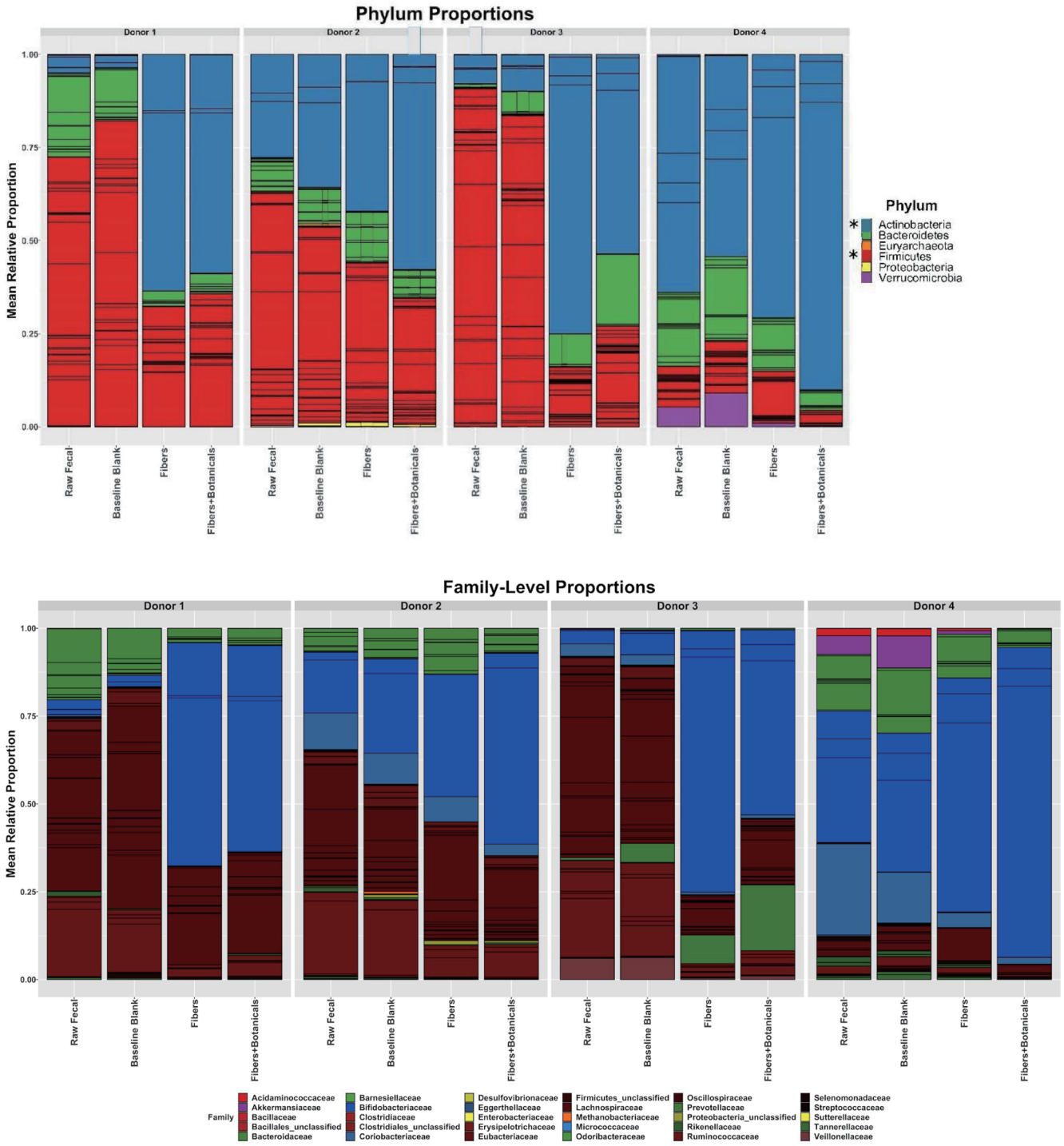

Figure 4. Relative abundances of metagenomic reads from fecal samples, baseline blanks, and fermentations, assigned by bacterial families. Individual OTUs are represented by the internal bars within a family's color. An asterisk (*) beside legend symbols in panel A denotes significant donor condition effects ($p < 0.001$, ANOVA) in the same time-dependent direction for all 4 donors.

this reduction in alpha diversity was seen across all donors and no significant donor effects were observed ($p > 0.1$, ANOVA + Tukey HSD test). These data suggest that OLISMART fibers are strongly selective for bifidobacteria *in vitro* and this bifidogenic effect is observed even when initial endogenous populations of bifidobacteria are small.

Mapping of bifidobacterial reads to the species level revealed strong, significant expansion of *B. adolescentis* populations, and this species was the most abundant bifidobacterial species after fermentation of OLISMART fibers, both with and without bo-

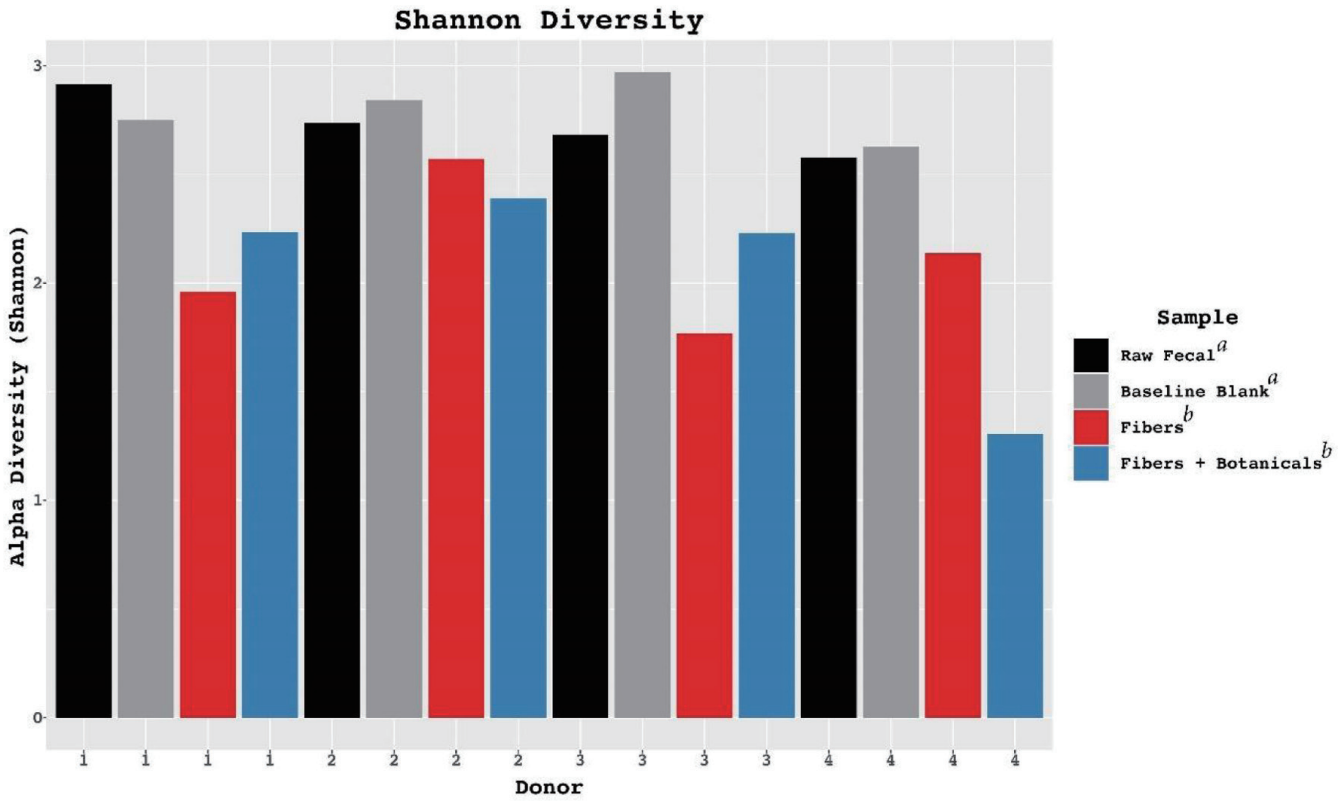

Figure 5. Alterations in Shannon diversity across donors in controls and fermentations compared with fecal samples. Letters *a* and *b* in the figure legend represent significantly different groups based on ANOVA results.

tanicals, in fermentations using all donors' microbiota (Figure 6). Interestingly, the magnitude of the bifidogenic effect and the species selected were individual and dependent in some cases upon botanicals. With donor 1 and 3 microbiota, the initially small *B. longum* population was substantially increased after fermentation of OLISMART fibers, with and without botanicals. In contrast, this species was already more abundant in donor 2 and 4 microbiota pre-fermentation but was not increased (in relative abundance) by fermentation. Further, donor 2 and 3's microbiota showed time-dependent increases in *B. pseudocatenalatum*, with donor 2's *B. pseudocatenalatum* showing significant ($p < 0.05$, ANOVA) increases from timepoint 0 in only the botanical supplemented media while donor 3 saw significant increase in both conditions post-fermentation. *B. longum* showed a significant time-dependent increase ($p < 0.001$, ANOVA) in both donor 1 and 3, with donor 3 also exhibiting a significant increase in abundance in the presence of botanicals versus fiber-supplemented media alone ($p < 0.001$, pairwise t-test). Interestingly, in donor 2 fermentations the *B. longum* population was outcompeted by *B. adolescentis* when fermenting OLISMART fibers alone but was retained (and the entire bifidobacterial population increased) when botanicals were present. Donor 4's initially large population of bifidobacteria contained sizable amounts of *B. adolescentis, B. longum,* and *B. bifidum*; in this case, only *B. adolescentis* markedly increased in relative abundance post-fermentation ($p < 0.01$, ANOVA) and the magnitude of this improvement was further increased in the presence of botanicals ($p < 0.01$, pairwise t-test). Less abundant bifidobacterial populations were influenced by botanicals in donor-specific ways. For example, in fermentations with donor 1's microbiota, only fermentations containing the OLISMART fibers and botanicals resulted in the detection of *B. pseudocatenulatum.* Together, these data suggest that relative growth rates of bifidobacterial species are governed by donor context and the presence of botanicals in the fermentation.

4. Discussion

Fermentation of dietary components and their gut microbiome and metabolome effects are often investigated in isolation, whereas product formulations often contain blends of several functional ingredients. For example, research has been separately conducted on the beneficial effects of prebiotic fibers (Makki et al., 2018; Qin et al., 2023) and to a lesser extent for botanicals (Cefalu et al., 2008; Choi et al., 2020; Xu et al., 2022) but, to our knowledge, no research is published on the inter-related effects of both functional ingredient categories. To this end, we investigated this concept by employing OLIPOP, a widely consumed functional soda containing a mixture of 3 fibers and 4 botanicals, in *in vitro* fermentations of the prebiotic fibers in OLIPOP with and without the paired botanicals. As established previously (Yao et al., 2020) inter-replicate measurement differences in our proof of concept study were very small, suggesting the *in vitro* system employed here was technically robust for reproducible fermentation by gut microbiota.

Across all 4 donors employed, independent of initial community size and composition, a strong bifidogenic response and concomitant elevated SCFA production levels were established in fermentations containing the fiber mixture, either in the presence or

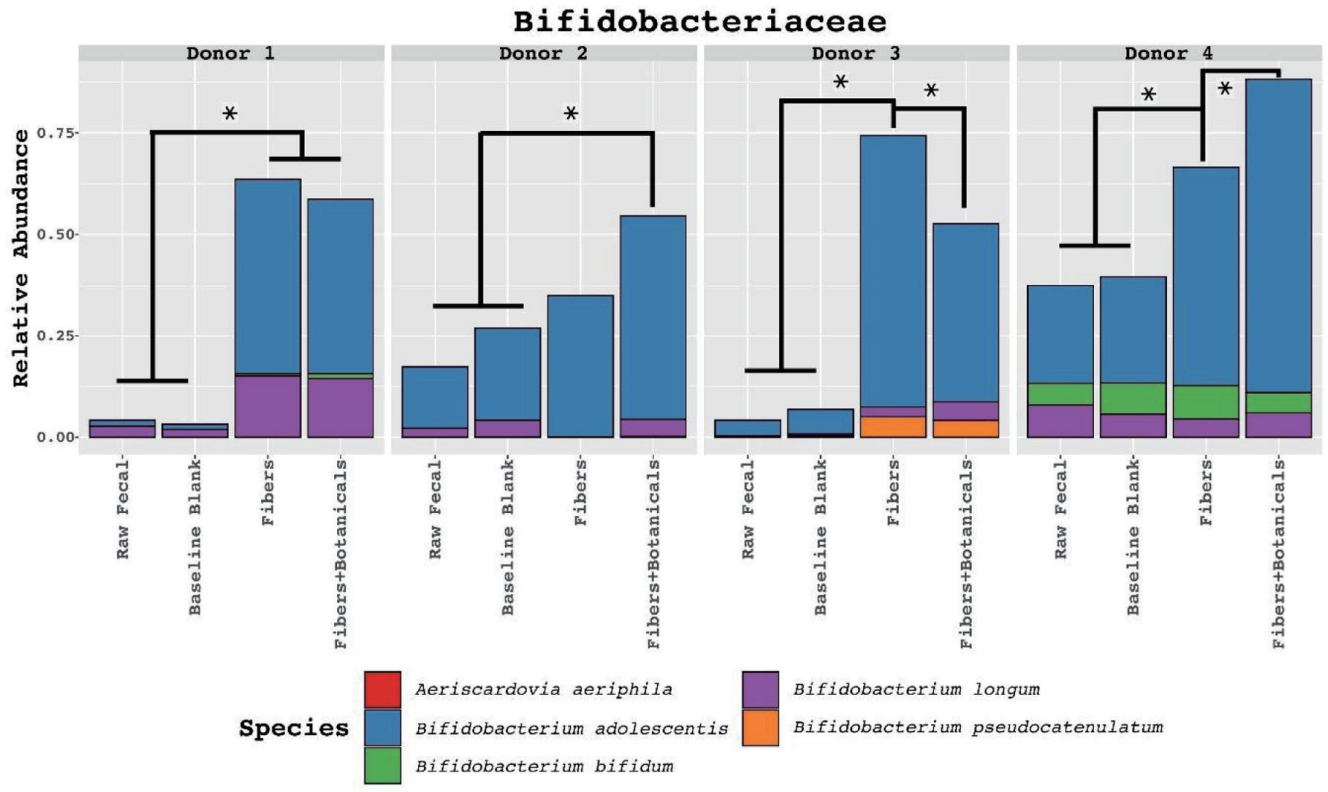

Figure 6. Relative abundances of metagenomic reads derived from fecal samples, controls, and fermentations assigned to family *Bifidobacteriaceae* and classified to the species level. Colored boxes in top-left of each facet represents significant fiber-dependent abundance increase on the species level. Statistically significant differences determined by ANOVA pairwise comparisons test (read-count as dependent and media type as the independent variable, split by donor) with $p < 0.001$. Symbol style: p-value < 0.001 (*).

absence of the botanical blend. Importantly, at the species level, the bifidobacterial community composition displayed subtle interpersonal differences in fermentation responses. This is a common observation and not surprising considering the fact that individuals harbor distinct endogenous bifidobacterial populations (Voreades et al., 2014; Arboleya et al., 2016; Tierney et al., 2023). The consistency of the bifidogenic effect might be aided by the presence of multiple fibers in the blend, which may differentially stimulate the growth of distinct species. Hence, in addition to the general benefit of elevated SCFA levels triggered by fiber fermentation by any bifidobacterial species, species-specific health effects of bifidobacteria may play a complementary, donor-dependent role. To this end, fermentation of the fiber mixture by fecal microbiota resulted in increased levels of *B. longum*, *B. adolescentis*, or *B. pseudocatenulatum*, but not to the same extent in all donors. Recent studies suggest that *B. adolescentis* might be beneficial against constipation (Wang et al., 2017) and support kidney stone management (Abratt and Reid, 2010). Furthermore, it has been hypothesized that *Bifidobacterium* species have anti-obesogenic properties. While our study did not seek to measure the impact of adding live bacteria to assess obesity protective benefits, earlier work suggested potentially supportive effects when ingesting *Bifidobacterium pseudocatenulatum* strain CECT 7765 in a high-fat diet-fed obese mice model (Moya-Perez et al., 2015). They report addition of *B. pseudocatenulatum* resulted in lower serum cholesterol, triglyceride, and glucose levels and also reduced insulin resistance and positively impacted glucose tolerance. Given the significant global rise in obesity, prediabetes and type 2 diabetes rates, the observed increase of *B. pseudocatenulatum* in our study warrants further investigation to assess translational effects in human clinical studies using the botanical and prebiotic fiber blend from this study. Similarly, *B. longum* has been reported to protect against inflammatory bowel disease (Yao et al., 2021). Taken together, this suggests that while prebiotic fibers, such as those employed in this study, are likely to broadly elicit health benefits across individuals, these benefits may be personalized by individuals' unique microbiome communities.

Recently, consumer food and beverage or supplements have evolved from containing single to multiple prebiotic and/or fibers components. However, research efforts to assess the interactive microbiome or health effect of multiple microbiome-active fibers is still in its infancy. Inulin is one of the most widely utilized prebiotic fiber substrates in functional foods and beverages, as well as supplements, principally due to favorable cost structure, substantial scientific research and compliant global regulatory position. Few *in vitro* or *in vivo* studies have examined the interactive beneficial microbiome or human health effects of combining inulin with other prebiotic or fiber substates, though these interactions are likely to be very influential on their behavior in the gut. Koecher et al., examined fermentation profiles both *in vitro* and in humans for fructo-oligosaccharides (FOS), inulin, gum acacia, and pea fiber alone or blended (Koecher et al., 2014). Additionally, Lecerf et al. sought to examine the differential effects of either xylo-oligosaccharide (XOS) alone or inulin-and-XOS mixture in a small human cohort (Lecerf et al., 2012). Interestingly, in this study XOS or XOS plus inulin did exhibit microbiome differences, yet crucial physiological effects were also observed when combinations were

employed. Both of these studies indicate potentially beneficial effects of combining inulin and other prebiotic fibers ranging from increased SCFAs (Koecher et al., 2014) to attenuation of pro-inflammatory responses (Lecerf et al., 2012). Combinations of inulin and resistant dextrin, which was used in the present study, have not been previously investigated for microbiome or digestive health benefits. Cai et al. examined a milk powder with or without inulin and resistant dextrin blend on markers for type 2 diabetes (Cai et al., 2018). Studies combining inulin, resistant dextrin, and botanicals or, more broadly, any botanical and prebiotic fiber blends do not exist to our knowledge in the peer-reviewed scientific research. These prior studies and our findings indicate that products including prebiotic fiber blends need to be investigated to complement beneficial health impacts reported for isolated prebiotic fibers. Blending of fibers with other functional components such as botanicals could also lead to interaction effects, which need to be considered when investigating potentially beneficial microbiome and metabolomic impacts. Our proof-of-concept study suggests a potential need for the inclusion of study arms for each of the possible combinations of functional ingredients.

5. Conclusion

We sought to determine whether interaction effects of a functional blend would arise by conducting an *in vitro* batch culture fermentation experiment utilizing multiple donor's fecal microbiota. Despite the fact our study encompassed gut microbial communities from a modest set of donors, it argues for the design of future clinical studies investigating the effects of blends of microbiome-active components to contain study arms investigating individual components and the full functional blends to measure potential interaction effects. Food-as-medicine interventions and dietary recommendations policy indicate that providing patients with medically tailored meals enabling achievement of daily recommended fiber intake led to beneficial health outcomes across multiple chronic conditions (Graff et al., 2023; Vedantam et al., 2023). As such, our study provides a roadmap for the functional beverage space and beyond, to rationally design studies and formulate products that result in beneficial health or microbiome impacts, with the ultimate aim to generate functional foods that bridge the fiber gap.

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Data Availability Statement

The metagenomic datasets for this study can be found in the NCBI BioSamples Repository: using accession number PRJNA950354.

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Conflict of interest

PAB and NV consult for OLIPOP and receive compensation in turn. They did not accumulate the data nor perform the data analyses. SRL serves on the health advisory board of OLIPOP.

Author contributions

NV and SRL conceived the study and directed the overall project. DGD carried out the experiments and, together with SRL, performed the data analyses, visualization and interpretation. DGD, PAB, NV, and SRL wrote the manuscript.

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Allyl isothiocyanate confers resistance against low-pH stress conditions to RGM1 gastric normal epithelial cells

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Abstract

In this study, we attempted to elucidate the effects of ally isothiocyanates (AITC) on stress resistance. RGM1 cells, derived from the normal gastric mucosa of rats, were pretreated with AITC (0-30 μM) 24 hr or 48 hr prior to post-treatment with AITC (0 or 20 μM), or both. While approximately 90% of the vehicle-pretreated cells died by a posttreatment with AITC, pretreatments with AITC (10-30 μM), especially 24 hr prior and double pretreatments, exhibited striking cytoprotective effects. AITC, as a xenobiotic, increased the amounts of reactive oxygen species and insoluble proteins. On the other hand, double pretreatments with AITC markedly upregulated the mRNA expression levels of anti-oxidative, detoxification, and molecular chaperone genes for homeostasis. Interestingly, pretreatments with AITC (10 and 15 μM) significantly mitigated low-pH, but not high-pH, stress conditions, which may involve the activation of phosphoinositide 3-kinase and Na^+/H^+ exchanger. Taken together, we show here that multiple exposures to AITC can confer a stress resistance phenotype, including adaptation to acidic pH, by upregulating the expressions of self-defensive enzymes. Therefore, this study implies the importance of continuous ingestion of phytochemicals for efficiently increasing the stress resistance capacity against harmful chemicals.

Keywords: Isothiocyanate; pH stress; Hormesis; Oxidative stress; Detoxification.

1. Introduction

Isothiocyanates (ITCs) are produced from glucosinolates by the function of myrosinase in many cruciferous vegetables. They have an electron-deficient carbon that is susceptible to the electrophilic addition by anti-oxidative glutathione (GSH), leading to its consumption and thus increased oxidative stress conditions. Based on this biochemical property, ITCs at high concentrations or excess doses have been shown to have harmful effects, as demonstrated by cellular and experimental rodent models. For instance, benzyl ITC (BITC) induces both chromosome aberrations and sister chromatid exchanges in Chinese hamster ovary (CHO) cells (Musk et al., 1995). Both 0.1% phenethyl ITC (PEITC) and BITC in the diet of F344 rats lowered the urinary pH and aggravated the pro-inflammatory scores in the urinary bladder (Akagi et al., 2003). In addition, the fetal and placental weights of rats treated with 25 and 50 mg/kg BITC were significantly lower than those of the control (Adebiyi et al., 2004).

On the other hand, ITCs are known to have diverse biofunctions, including anti-cancer (Gupta et al., 2014), anti-oxidation (Li et al., 2015), and detoxification (Abdull Razis et al., 2018) activities. ITCs at moderate concentrations oxidize and/or react with the thiol moiety of Kelch-like ECH-associated protein 1 (Keap1) to liberate its partner transcription factor, nuclear factor-erythroid 2-related factor 2 (Nrf2), for up-regulating many adaptive enzymes (Baird and Yamamoto, 2020). This biochemical property of ITCs accounts for the mechanisms underlying their cancer preventive activities, in which activated procarcinogens are detoxified by the functions of the Nrf2-dependent enzymes (Kwak and Kensler, 2010). It is worth noting that sulforaphane, an ITC found in broccoli, at low concentrations increased the cell proliferation of

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astrocytes *via* the p38 mitogen-activated protein kinase pathway, whereas it showed inhibition at higher concentrations (Yang et al., 2022). In addition, Okulicz et al. treated diabetic rats with allyl ITC (AITC, 2.5, 5 and 25 mg/kg) for 2 weeks and found that AITC at the highest dose caused pancreatic amylase and lipase drops and thyroid gland hypertrophy, whereas blood glucose levels were significantly reduced at 2.5 and 5 mg/kg (Okulicz et al., 2021). These phenomena are consistent with the concept of hormesis, in which exogeneous and endogenous stressors at moderate doses show beneficial effects while high doses are harmful, along with a biphasic response (Calabrese et al, 2024).

Phytochemicals, including ITCs, are the secondary metabolites of plants that are substantially xenobiotics to animals. Therefore, ingestion of phytochemicals increases the expression of detoxification enzymes (Reuland et al., 2013). Additionally, some anti-oxidation enzymes are also up-regulated as many phytochemicals have been uncovered to be pro-oxidants (Fernando et al., 2019; Canedo-Santos et al., 2022; de Roos and et al., 2015). Therefore, treatments with phytochemicals at moderate concentrations may increase the stress resistance capacity by up-regulating self-defensive enzymes. To ascertain this hypothesis, in the present study, we examined whether repetitive treatments with AITC, a pungent in Japanese horseradish or *Wasabi*, can cause phenotypic changes in RGM1 rat gastric normal epithelial cells.

2. Materials and methods

2.1. Cells and reagents

RGM1 rat gastric normal epithelial cells were kindly provided by Prof. Mitsugu Akagawa (University of Tokushima), who purchased them from RIKEN BioResource Research Center (Tsukuba, Japan). Dulbecco's Modified Eagle's Medium (DMEM)/Nutrient Mixture F-12 Ham was purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific (Walthman, MA). All other reagents were purchased from FUJIFILM Wako Pure Chemical (Osaka, Japan) and were of special grade unless specified otherwise.

2.2. Cytotoxicity test

RGM1 cells were maintained at 37°C in 50% DMEM/Ham's F12 media with 10% FBS, penicillin (100 units/ml), and streptomycin (100 µg/ml), and dimethyl sulfoxide (DMSO) was used as a vehicle. For the cytotoxicity assay, RGM1 cells (1 x 10^6/mL) were preincubated in 200 µL of 50% DMEM/Ham's F12 media on a 96-well plate overnight. After washing the cells twice with phosphate-buffered saline (PBS), they were exposed to the first AITC pretreatment (0–30 µM) for 24 hr and to the second one for another 24 hr, which was followed by posttreatment with AITC (0 or 20 µM). After 24 hr, cell viability was measured by using Cell Counting Kit-8 (DOJINDO Laboratories, Kumamoto, Japan).

2.3. Reactive oxygen species (ROS) detection

RGM1 cells (5 x 10^5/mL) were seeded on eight consecutive chamber slides (Thermo Fisher Scientific) and precultured in 200 mL of DMEM/Ham's F12 medium containing 10% FBS overnight. After washing the cells twice with PBS, the well was replaced with 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA, DOJINDO Laboratories, 10 µM) dissolved in serum-free DMEM and incubated at 37°C for 30 min. Then, the cells were washed twice with PBS again, and each sample (0.5% DMSO, 10 or 30 µM AITC, or 200 µM H_2O_2) was added and incubated at 37°C for 30 min. After washing twice with PBS, the chamber was removed, and a coverslip was mounted with 50% glycerol/PBS and observed using a confocal laser microscope (FV3000, Olympus, Tokyo, Japan). For semi-quantification, RGM1 cells (5 x 10^5/mL) were preincubated in 200 µL of 50% DMEM/Ham's F12 medium containing 10% FBS on a 96-well black plate (clear bottom, Thermo Fisher Scientific) overnight. After washing the cells with PBS, FBS-free DMEM containing DCFH-DA (final concentration: 10 µM) was added and incubated at 37°C for 30 min. Then, the cells were washed twice with PBS again, and each sample was added and incubated at 37°C for 30 min. After washing with PBS, the fluorescence of media was measured at Ex505 nm and Em525 nm by a microplate reader (Synergy H1, BioTek, Winooski, VT).

2.4. Protein solubility assay

Proteostress was evaluated by the method as previously reported (Suihara et al., 2021). Briefly, RGM1 cells (1 x 10^6/mL) were precultured in 50% DMEM/Ham's F12 media containing 10% FBS on a 24-well plate overnight. Each well was washed twice with PBS, replaced with FBS-free DMEM, and each sample (0.5% DMSO or 30 µM AITC) was added. After incubation at 37°C for 0, 2 or 4 hr, the cells were washed twice with PBS and extracted with regular lysis buffer (BD Pharm Lyse™, BD Biosciences, Franklin Lakes, NJ) or high detergency lysis buffer (regular lysis buffer containing 2% sodium dodecyl sulfate, SDS) for determination of the amounts of the soluble and the whole protein, respectively, which was quantified by the BCA method. The amounts of insoluble proteins were estimated by subtracting those of soluble proteins from whole ones.

2.5. Real-time reverse transcription polymerase reaction (RT^2-PCR)

RGM1 cells (1 x 10^6/mL) were preincubated in 1 mL of 50% DMEM/Ham's F12 medium containing 10% FBS on a 12-well plate overnight. After washing the cells twice with PBS, the media were replaced by FBS-free DMEM containing AITC (0 or 15 µM) and incubated for 24 hr. After another washing, the cells were exposed to posttreatment with AITC (0 or 20 µM), and the total RNA was extracted using the RNeasy Mini Kit™ (Qiagen, Venlo, The Netherlands). Total RNA concentrations were calculated by measuring the absorbance at 260 nm. cDNA was synthesized using total RNA (1 µg) with 25 mM $MgCl_2$ (4 µL), PCR buffer (Takara Bio, Kusatsu, Japan) (2 µL), 2 mM dNTP (Takara Bio) (2 µL), 40 U/µL RNase inhibitor (Takara Bio) (0.5 µL), 5 U/µL AMV RTase (Takara Bio) (1 µL) and 1 g/L oligo-dT adaptor primer (Sigma-Aldrich, 1 µL) by RT reaction, which was performed by using a Programable Thermal Controller PTC-100 (MJ Research, St. Bruno, Canada). The conditions of cDNA synthesis were as follows: 30°C for 10 min, 55°C for 30 min, 95°C for 5 min, and 4°C for 15 min. For PCR, 5 µL cDNA was mixed with a sense- and antisense-primer (10 pmol, 4.5 µL), synthesized by Thermo Fisher Scientific, Power SYBR Green PCR Master Mix (25 µL) and ultrapure water (11 µL). PCR was performed using a 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA) and the thermal conditions were as follows: 50°C 2 min and 95°C 2 min for 1 cycle, and 95°C for 15 sec, 60°C for 30 sec, 72°C for 30 sec for 99 cycles. The

Table 1. Primers used for RT^2-PCR

Gene	Primer	Sequence (5′-3′)
Catalase	Sense	CCCAGAAGCCTAAGAATGCAA
	Antisense	TCCCTTGGCAGCTATGTGAGA
HO-1	Sense	CGTGCAGAGAATTCTGAGTTC
	Antisense	AGACGCTTTACGTAGTGCTG
HSP27	Sense	GGCAAGCACGAAGAAAGG
	Antisense	GATGGGTAGCAAGCTGAAGG
GST-α	Sense	CTGCAGCAGGGGTGGA
	Antisense	CTCTCTCCTTCATGTCCTTCC
β-actin	Sense	CGTCATACTCCTGCTTGCTG
	Antisense	GTACGCCAACACAGTGCTG

relative mRNA levels were estimated by the ΔΔCt method. The sequences of the primers are shown in Table 1.

2.6. pH stress assay

RGM1 cells (1 x 10^6/mL) were preincubated in 200 µL of 50% DMEM/Ham's F12 medium containing 10% FBS on a 96-well plate overnight. After washing the cells twice with PBS, they were pretreated with AITC (0, 2.5, 5, 10 and 15 µM) for 1 hr. After washing, the media were replaced by FBS-free DMEM, the pH of which was adjusted to 4.6, 7.4, or 9.8, and the cells were incubated for 1 hr. After washing the cells, the media were replaced to FBS-free DMEM (pH 7.4) followed by a 24 hr-incubation. Cell viability was determined as described above.

2.7. pH determination

For extracellular pH measurement, RGM1 cells (1 x 10^6/mL) were preincubated in 200 µL of 50% DMEM/Ham's F12 medium containing 10% FBS on a 24-well plate overnight. After washing the cells twice with PBS, they were pretreated with AITC (0 or 15 µM) for 1 hr. After washing, the media were replaced by FBS-free DMEM, in which the pH was adjusted to 4.6. After incubation for 1 hr, the media pH was returned to 7.4, followed by a 24 hr-incubation. The pH of medium was measured using a handy pH meter (LAQUAtwin pH-11B, HORIBA, Kyoto, Japan). For intracellular pH determination, RGM1 cells (1 x 10^6/mL) were preincubated in 200 µL of 50% DMEM/Ham's F12 media containing 10% FBS on a 96-well black plate (Thermo Fisher Scientific) overnight. After washing the cells with HEPES, FBS-free DMEM containing BCECF-AM (final concentration: 5 µM) was added and incubated for 30 min. After washing with HEPES, the fluorescence of medium was measured at Ex490 nm and Em535 nm by a microplate reader (Synergy H1). To make a calibration curve, RGM1 cells (1 x 10^6/mL) on a 96-well black plate were incubated in FBS-free DMEM, and then nigericin (DOJINDO Laboratories, final concentration: 10 µg/mL) Nigericin, an electroneutral K^+/H^+ ionophore derived from *Streptomyces hygroscopicus*, was added to equilibrate intra- and extracellular pH (Kaneko et al., 1991) to make a calibration curve. After 10 min, BCECF-AM (5 µM) was added, and the fluorescence of medium was measured using the same method as the above. A linear calibration curve regarding the intracellular pH was obtained in a range of pH 5.5 and 7.9 in the media (data not shown).

2.8. Statistical analysis

Each experiment was performed at least 3 times and the values are shown as the mean ± standard deviations (SD). Statistically significant differences between the groups for each assay were determined using Student's *t*-test. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Cytoprotective effects of AITC

ITCs are xenobiotics to animals and thus increase the expression of xenobiotic-metabolizing enzymes (Keum et al., 2005). Therefore, pretreatments with ITCs may potentiate cytoprotective activity toward xenobiotics, including ITCs themselves at cytotoxic concentrations. In addition, we were interested in the issue of how long the cytoprotective effects of ITCs can be prolonged. To test these questions, we pretreated RGM1 gastric normal mucosa cells from rats with AITC (0–30 µM) 24 hr or 48 hr prior to posttreatment with AITC (20 µM), or both. We selected pH 4.6 as an acidic stress condition because AITC could not protect RGM1 cells cultured at the pH of gastric juice (pH 1–2, data not shown). As shown in Figure 1, posttreatment with AITC (20 µM) with double vehicle pretreatments resulted in drastic cell death (viability <10%). On the other hand, pretreatments with AITC under several conditions dramatically protected cells from cytotoxicity. For instance, viability of the cells pretreated with AITC 24 hr and 48 hr (both 20 µM) before posttreatment were 85% and 72%, respectively. Double pretreatments at 20 and 30 µM were found to be most protective as compared with each single pretreatment, whereas the concentrations of AITC pretreatments 24 hr prior to posttreatment and those of double pretreatments to exhibit significant cytoprotection were notably lower than those of 48 hr. Meanwhile, it is notable that the cytoprotective effects of AITC pretreatments 24 hr before posttreatment abolished at the highest concentration of 30 µM.

3.2. AITC induces oxidative stress and proteostress

ITCs, including AITC, have been reported to induce oxidative stress that is involved in its neuroprotective effects *via* Nrf2-de-

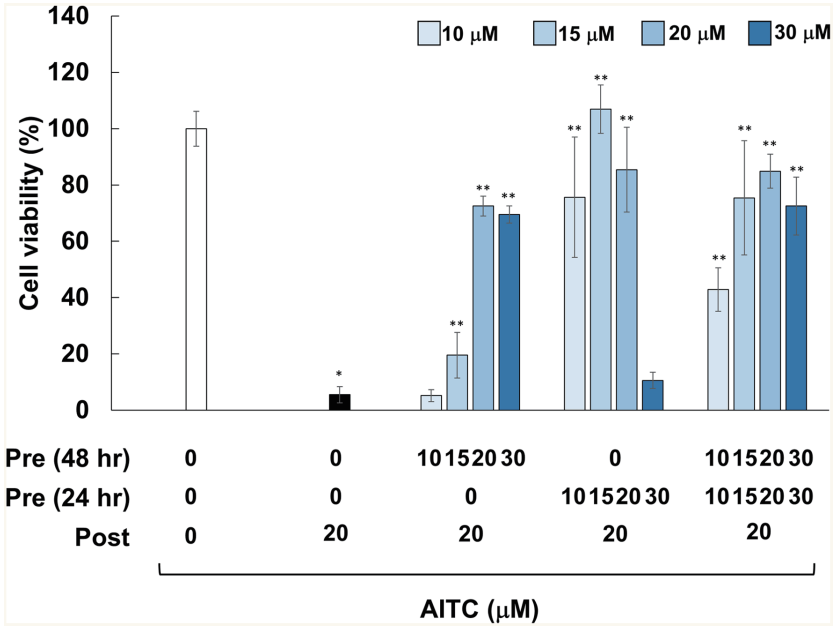

Figure 1. Effects of pretreatments with AITC on cytotoxicity induced by AITC posttreatment. RGM1 cells (2 x 10^5/200 μL) were exposed to the vehicle (DMSO) or AITC (10–30 μM) for 24 hr. After washing, the cells were treated with the vehicle or AITC (10–30 μM) for another 24 hr, and then incubated with the vehicle or AITC (20 μM) for 24 hr, followed by measurement of cell viability. The data are shown by means ± SD (n = 4), *p < 0.05 versus Control (vehicle treatments at 3 times), **p < 0.05 vs. AITC (20 μM) after vehicle treatment twice (black bar) in Student's t-test.

pendent mechanisms (Calabrese and Kozumbo, 2021). As shown in Figure 2a, treatment with AITC (10 and 30 μM) for 30 min resulted in significant generation of DCFH-DA-positive cells, a hallmark of ROS generation, as compared with the vehicle control. We also explored their properties to induce proteostress (Suihara et al., 2021; Valentine et al., 2019) because ITCs are known to covalently bind both the cysteine and lysine residues of biological proteins to form protein adducts (Kumar and Sabbioni, 2010). Cellular proteins were extracted before and after AITC treatments using conventional lysis buffer or that containing 2% SDS, and resultant cell lysates were designated as the soluble and the whole proteins, respectively. The amounts of insoluble proteins were estimated by subtracting those of soluble proteins from whole ones. Treatment with AITC (30 μM) for 2 and 4 hr significantly decreased the amounts of soluble proteins and accordingly increased the insoluble proportions (Figure 2b).

3.3. AITC increased expression of self-defensive genes

We then examined whether AITC increases the mRNA expression levels of the anti-oxidation enzymes (catalase and HO-1), the molecular chaperone (heat shock protein 27, HSP27), and the phase II detoxification enzyme (GSH-*S*-transferase-α, GST-α). In addition, we compared the effects of single and double treatments on these expressions since double treatments were distinctly cytoprotective (Figure 1). RGM1 cells were pretreated with AITC (0 or 15 μM) for 24 hr, and then exposed to another AITC treatment (0 or 20 μM) for 6 hr, which was followed by RT^2-PCR. Interestingly, the combination of pre- and posttreatment with AITC significantly upregulated the expressions of catalase and GST-α, whereas each treatment was ineffective (Figure 3a and d). In addition, while posttreatment alone significantly increased both HO-1 and HSP27 expressions, double treatments were significantly more inducible (Figure 3b and c).

3.4. AITC confers resistance to low-pH stress

Subsequently, we investigated whether AITC provides resistance capacity against low-pH stress conditions because RGM1 cells are derived from the normal gastric mucosa, where they are exposed to acidic pH in pathological conditions, such as gastritis. After being pretreated with AITC (0–15 μM) for 1 hr, RGM1 cells were incubated in the media of pH 4.6, pH 7.4 or pH 9.8 for 1 hr. Then, the cells were recovered in the media of pH 7.4 for 24 hr, followed by viability determination. As shown in Figure 4a, AITC (10 and 15 μM) significantly suppressed the decrease of cell viability in acidic media. Intriguingly, however, AITC did not protect cells from alkaline pH stress conditions, and rather, it concentration-dependently decreased cell viability (Figure 4b).

3.5. Effects of AITC on the extracellular and intracellular pH

Given the aforementioned results, we measured both the extra- and intracellular pH before and after AITC exposure. After being treated with AITC (0 or 15 μM) in media of pH 7.4 for 1 hr, RGM1 cells were incubated in pH 4.6 for 1 hr. Then, the cells were recovered in pH 7.4 for 24 hr. Both extra- and intracellular pH were measured 1 hr after AITC exposure, 1 hr after replacing with pH 4.6 media, and 24 hr after recovery. As a result, AITC did not affect the extracellular pH in both acidic and neutral conditions (Figure 5a). For intracellular pH measurement, BCECF-AM, which is hydrolyzed to the membrane impermeable BCECF (Garrido et al., 1996), was used as a pH indicator. Interestingly, while not having affected intracellular pH in neutral media, pretreatment with AITC significantly suppressed pH reduction, and this tendency was maintained 24 hr after recovery. Meanwhile, Na^+/H^+ exchanger (NHE) has been reported to play an essential role in the adaptation against acidic stress conditions in RGM1

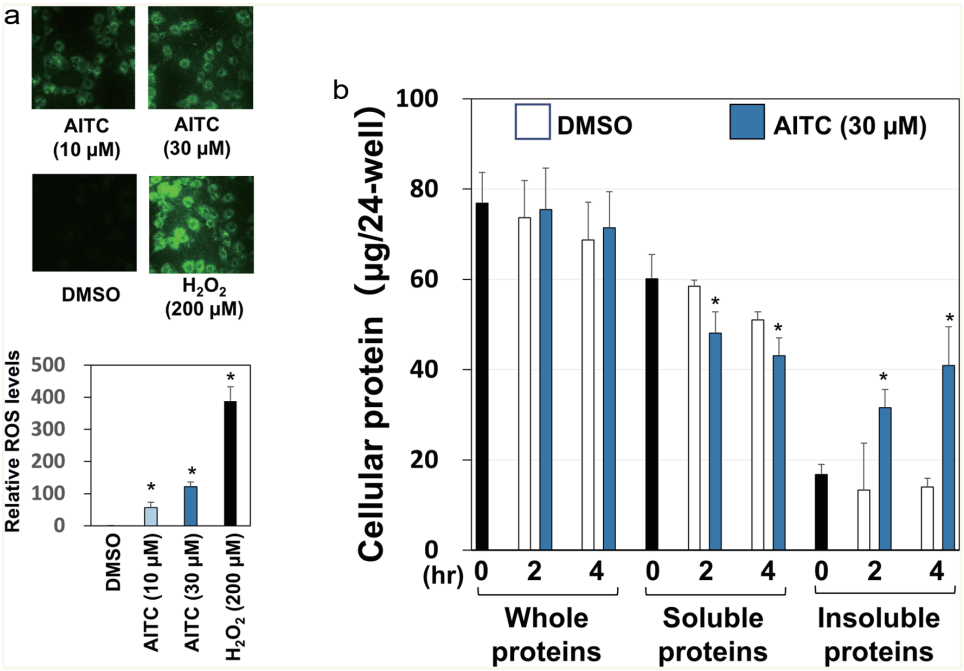

Figure 2. Oxidative stress and proteostress induced by AITC. Panel A, RGM1 cells (5 x 10^5/mL), seeded on eight consecutive chamber slides, were exposed to DCFH-DA (10 μM) for 30 min. Then, each sample (10 or 30 μM AITC, 200 μM H_2O_2, 0.5% DMSO) was added and incubated for 30 min. After the chamber was removed, a coverslip was mounted with 50% glycerol/PBS and observed using a confocal laser microscope for detecting intracellular ROS generation. For semi-quantification, RGM1 cells (2 x 10^5/200 μL) were preincubated on a 96-well black plate (clear bottom) overnight. After washing, DCFH-DA was added and incubated for 30 min. Then, the cells were washed, and each sample was added and incubated at 37°C for 30 min. After washing with PBS, the fluorescence of the media was measured at Ex505 nm and Em525 nm by a microplate reader. Panel B, after RGM1 cells (5 x 10^5/mL) were precultured on a 24-well plate overnight, each sample (30 μM AITC or 0.5% DMSO) was added and incubated for 0, 2 or 4 hr. Then, the cells were washed and extracted with regular lysis buffer or high detergency lysis buffer (regular lysis buffer with 2% SDS) for soluble and whole protein determination, respectively, which was quantified by the BCA method. The amounts of insoluble proteins were estimated by subtracting those of the soluble proteins from the whole ones. The data are shown by means ± SD (n = 3), *$p < 0.05$ versus DMSO by Student's *t*-test.

cells (Furukawa and Okabe, 1997). In addition, phosphatidylinositol-3 kinase (PI3K) may be responsible for the activation of NHE (Furukawa et al., 1999). Therefore, we attempted to examine their roles in the mechanisms underlying the protective activity of AITC against acidic stress conditions using pharmacological inhibitors. As shown in Figure 5b, both LY294002 (10 μM, PI3K inhibitor) and amiloride (100 μM, NHE inhibitor) significantly disrupted the protective effects of AITC.

4. Discussion

AITC has long been anticipated to mitigate chemical stress conditions by upregulating phase II detoxification enzymes (Munday et al., 2002). For instance, this phytochemical may contribute to the reduction of toxicity derived from mycotoxins in feed and food (Adegbeye et al., 2020). However, to the best of our knowledge, there has been no study that examined whether pretreatment with AITC can protect against toxicity induced by AITC itself. As shown in Figure 1, pretreatments with AITC in most of the experimental conditions dramatically protected RGM1 gastric normal epithelial cells from cytotoxicity induced by posttreatment with AITC. Pretreatment with AITC 24 hr before posttreatment was more protective than that with 48 hr, suggesting that cytoprotective effects may have decayed within 24 hr after pretreatment. In addition, biphasic responses, a hallmark of hormesis, are noticeable for each pretreatment because their cytoprotective effects reached peaks at a concentration of 15 or 20 μM, and then declined or abolished at 30 μM (Figure 1). Meanwhile, it may be confusing that, while posttreatment with AITC alone at a concentration of 20 μM was highly cytotoxic, pretreatments at the same concentration was not cytotoxic, and rather apparently protective. These data were well reproduced, and the reason for these puzzling results could be because serum starvation and the lack of chemical stimuli during 48 hr before AITC posttreatment reduced cellular defensive ability.

ITCs evoked pro-oxidative responses in biological systems (Valgimigli et al., 2009; Sestili et al., 2015), which corresponds with our present results (Figure 2a). In addition, electrophilic ITCs covalently bind cysteine and lysine residues to form cellular protein adducts (29,30), which is described as proteostress. In accordance with these findings, several electrophilic phytochemicals and their metabolites, including curcumin (Valentine et al., 2019), zerumbone (Ohnishi et al., 2013), and the *o*-quinone metabolite of (-)-epigallocatechin-3-*O*-gallate (Suihara et al., 2021), have been demonstrated to induce proteostress. Taken together, our results showing the increase of insoluble cellular proteins by AITC (Figure 2b) may be associated with the formation of protein adducts.

In response to the oxidative stress and proteostress induced by AITC, RGM1 cells are forced to activate self-defensive machinery for homeostasis and survival. In fact, treatments with AITC upregulated mRNA expression levels of anti-oxidative enzymes (catalase and HO-1), detoxification enzyme (GST-α), and molecular chaperone (HSP27) (Figure 3). It is important to indicate that double treatments are more effective than each single one.

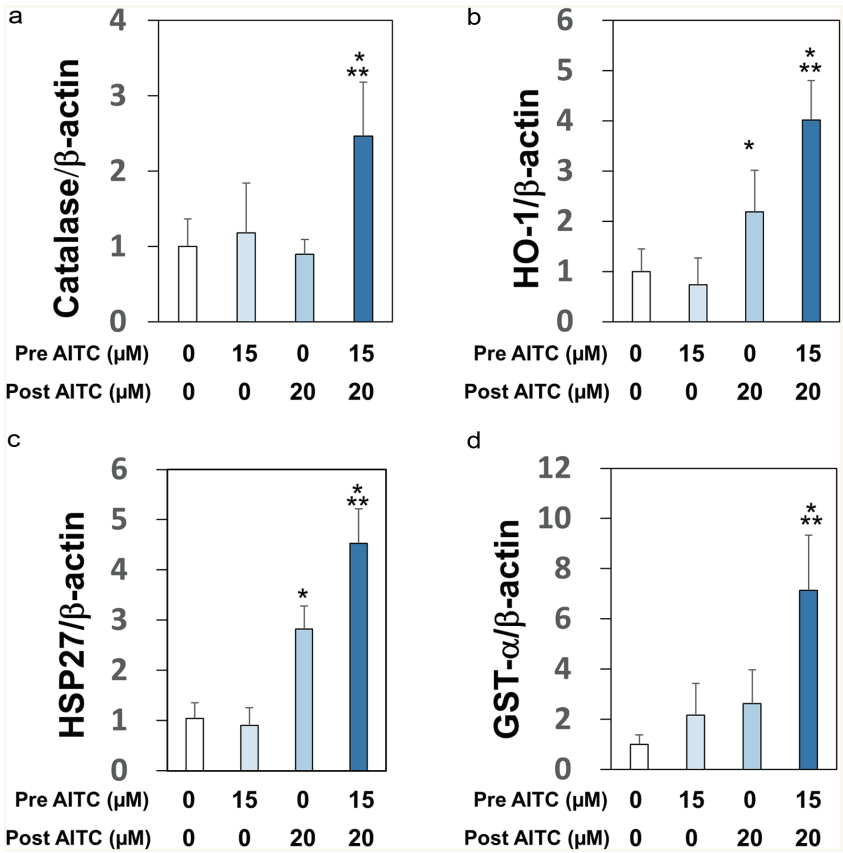

Figure 3. Upregulations of mRNA expression levels of self-defensive enzymes. RGM1 cells (1 x 10^6/mL) preincubated on a 12-well plate overnight were exposed to AITC (0 or 15 μM), and incubated for 24 hr. After another washing, the cells were exposed to AITC (0 or 20 μM). cDNA was synthesized using total RNA (1 μg). PCR was performed with a sense- and antisense-primer for catalase (Panel A), HO-1 (Panel B), HSP27 (Panel C), GST-α (Panel D), and β-actin (internal standard). The data are shown by means ± SD (n = 4), *p < 0.05 versus vehicle, **p < 0.05 versus post AITC (20 μM) by Student's t-test.

Although the mechanistic reason(s) for these additive effects remains to be elucidated, it is worth noting that increased histone acetylation promotes the formation of euchromatin (uncompacted form of chromatin) and thereby rapidly facilitates protective gene expression under stressful conditions (Gu et al., 2013). Interestingly, pretreatment with trichostatin A, a euchromatin formation inducer (Williams et al., 2011), significantly protected RGM1 cells from AITC-induced cytotoxicity (data not shown). In addition,

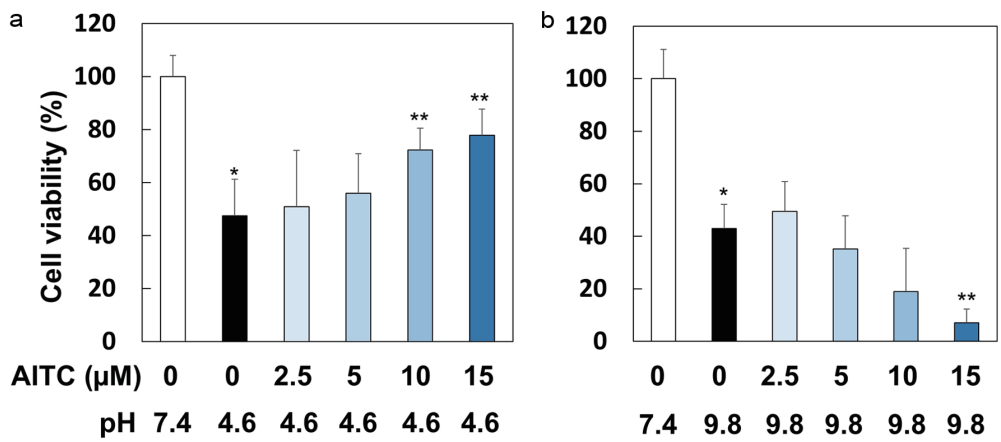

Figure 4. AITC protected RGM1 cells from acidic but not alkaline stress conditions. RGM1 cells (2 x 10^5/200 μL) were preincubated on 96-well plate overnight. After washing, they were pretreated with AITC (0, 2.5, 5, 10 and 15 μM) for 1 hr, and then the media were replaced by FBS-free DMEM, in which pH was adjusted to 7.4, 4.6, or 9.8, followed by incubation for 1 hr. Then, the media were replaced to FBS-free DMEM (pH 7.4) followed by a 24 hr-incubation. The data are shown by means ± SD (n = 6–8), *p < 0.01 versus pH 7.4, **p < 0.05 versus AITC (0 μM), pH 4.6 or pH 9.8 by Student's t-test.

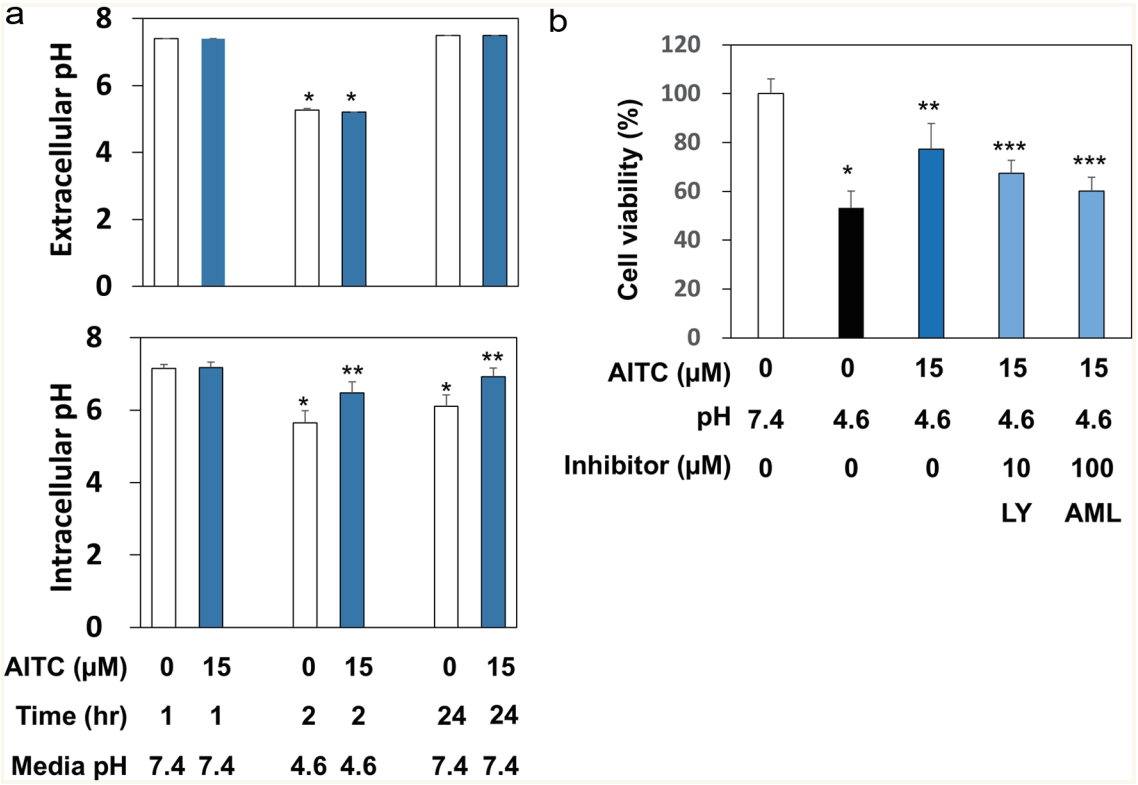

Figure 5. AITC regulated the intracellular pH possibly by targeting PI3K and NHE. Panel A, for extracellular pH measurement, RGM1 cells (1 x 10^6/mL) were preincubated on a 24-well plate overnight. After washing, the cells were pretreated with AITC (0 or 15 μM) for 1 hr. Then, the media were replaced by FBS-free DMEM, in which the pH was adjusted to 7.4, 4.6, or 9.8. After incubation for 1 hr, the pH of medium was measured. For intracellular pH determination, RGM1 cells (2 x 10^5/200 μL) were preincubated on a 96-well black plate (clear bottom) overnight. After washing, FBS-free DMEM containing BCECF-AM (final concentration: 5 μM) was added and the cell were incubated for 30 min. After washing, the fluorescence of the media was measured at Ex490 and Em535 nm. The data are shown by means ± SD (n = 4), *p < 0.05 versus pH 7.4, 1 hr, **p < 0.05 versus AITC (0 μM), 2 hr or 24 hr by Student's t-test. Panel B, RGM1 cells (2 x 10^5/200 μL) were preincubated on a 96-well plate overnight. After washing, the cells were pretreated with the vehicle, LY294002 (10 μM, PI3K inhibitor), or amiloride (100 μM, NHE inhibitor) for 30 min and then exposed to AITC (15 μM) for 1 hr. After washing, the media were replaced by FBS-free DMEM, in which the pH was adjusted to 4.6, and the cells were incubated for 1 hr. After washing the cells, the media were replaced with FBS-free DMEM (pH 7.4) followed by a 24 hr-incubation. Cell viability was determined as described above. The data are shown by means ± SD (n = 4), *p < 0.05 versus pH 7.4, **p < 0.05 versus AITC (0 μM), pH 4.6, ***p < 0.05 versus AITC (15 μM), pH 4.6 by Student's t-test. LY, LY294002 (PI3K inhibitor); AML, amiloride (NHE inhibitor).

AITC has been reported to increase histone acetylation to promote euchromatin formation in several types of cultured cells (Lea et al., 2001; Mitsiogianni et al., 2020). Collectively, we hypothesized that AITC may have promoted euchromatin formation *via* epigenetic mechanisms and thereby increased the capacity for stress adaptation, which is currently under investigation.

We attempted to examine whether AITC exhibits cross-resistance, which has been reported to be associated with epigenetic mechanisms (Horowitz et al., 2017). Notably, AITC protected RGM1 cells from low-pH, but not high-pH, stress conditions by regulating the intracellular pH (Figures 4 and 5). Previous reports have shown that the epidermal growth factor protected cells from acidic stress conditions *via* PI3K for activation of NHE (Furukawa et al., 1997, 1999), a membrane-bound Na^+/H^+ exchanger. Our present results with pharmacological inhibitors (Figure 5) suggest the involvement of both PI3K and NHE in the regulation of acidic pH. Importantly, several ITCs, including AITC, have been shown to activate PI3K in many types of cells (Yang et al., 2020; Du et al., 2024; Jakubíková, et al., 2005; Liu et al., 2017). Meanwhile, our present results may contribute to the understanding of the mechanisms underlying the preventive effects of an ITC on gastritis (Yanaka, 2006) where oxidative and acidic stresses become dominant.

Upregulation of anti-oxidation and detoxification enzymes, together with molecular chaperones, by AITC (Figure 3) is quite reasonable because it is a xenobiotic and has pro-oxidative and proteostress properties (Figure 2). On the other hand, the acquisition mechanisms of low-pH stress resistance may not be directly associated with the stress-inducing properties of AITC. However, it can be described as cross-resistance since AITC did not affect the intracellular pH in a normal culture condition (Figure 5a). Delgado-Jarana et al. have previously reported cross-resistance, in which hyperosmotic stress conditions conferred tolerance against oxidative stress in yeasts (Delgado-Jarana et al., 2006). Also, Spitz and Li have shown that heat shock increased the adaptive capacity against oxidative stress in CHO cells (Spitz et al., 1987). However, as far as we know, this study is the first to demonstrate cross-resistance by a phytochemical. Further studies that aim to uncover other types of cross-resistance conferred by phytochemicals are warranted.

Adaptation is the fundamental mechanism provided with every organism or homeostasis and survival. In principle, continuous exposures to xenobiotics at a tolerable level frequently result in decay of responsiveness. For example, repetitive exposure to anti-

cancer drugs often lead to reduced therapeutic effects, and similar phenomena are known for antibiotics, steroids, and insulin, *etc*. Likewise, unfavored taste and smell of vegetables, most of which are derived from phytochemicals, gradually become bearable with increasing age. For instance, tolerance to capsaicin, a pungent in chill pepper that infants and children tend to dislike, is associated with the release of β-endorphin to induce reward effects (Bach et al., 1995). Such adaptation generally occurs after repetitive and long-time ingestion of them. ITCs are the major sharp and pungent flavors of the cruciferous family of plants, although their effects on β-endorphin release remain to be clarified. In any case, the tolerance to AITC in cruciferous vegetables, including *Wasabi*, might also be related to the increased detoxification ability, which is presumably acquired through its continuous ingestion. Our present data (Figure 3d) showing marked expression levels of GST-α by double AITC treatments may support this hypothesis.

5. Conclusions

We have demonstrated that pretreatments with AITC notably protected RGM1 cells from cytotoxicity induced by its posttreatment. The underlying protective mechanisms may involve the increased expression of self-defensive enzymes, which were derived from the oxidative stress and proteostress induced by AITC. Increased activities of defensive enzymes derived from continuous ingestion of phytochemicals may strengthen our ability to detoxify environmental toxins, and such an adaptive situation has been coined as 'Chemical training' (Murakami, 2024).

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Data availability

The data that support the findings of this study are available from the corresponding author (A.M.) upon reasonable request.

Conflict of interest

There are no conflicts to declare.

Author contributions

A.M. designed and directed this study. S.K. and A.M. performed the experiments and analyzed the data. S.K., A.I. and A.M. discussed the experimental results and contributed to the preparation of the final version of this manuscript.

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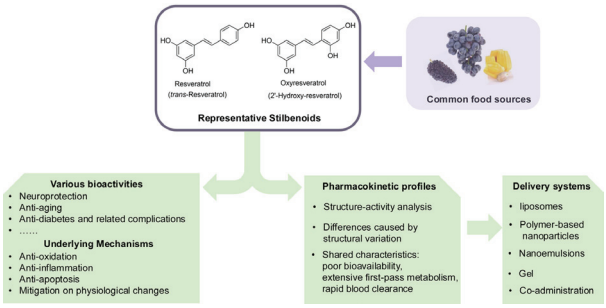
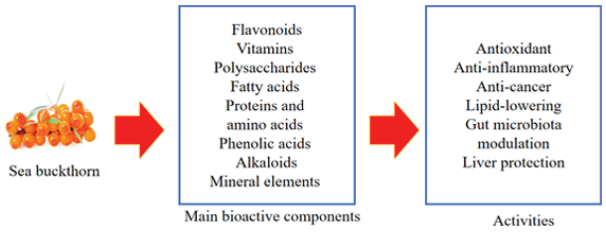
Journal of Food Bioactives

The Journal of Food Bioactives (JFB), a publication of the International Society for Nutraceuticals and Functional Foods (ISNFF), aims to bring together the results of fundamental and applied research on food bioactives, functional food ingredients, nutraceuticals and natural health products that are known to possess or perceived to have health-promoting properties.

Meeting Report

Reviews





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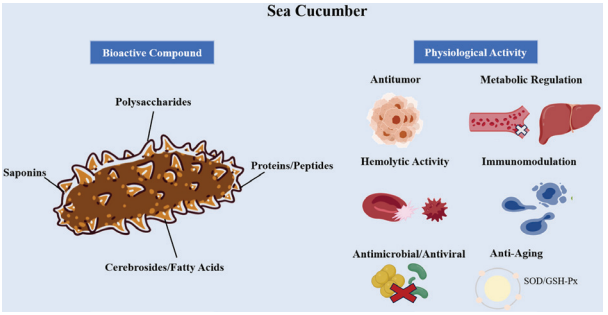
Components and bioactivities of sea cucumber: an update

Xinru Yang, Hui Zhao, Yanfei Liu, Jie Pan, Guliang Yang, Qi Tang

Sea cucumbers (holothurians), a classic marine invertebrate echinoderms, were found worldwide mainly as benthic organisms attached to sediments on the ocean floor.



Original Research

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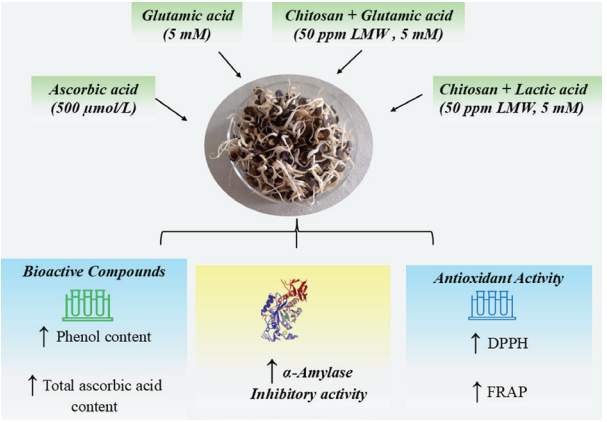
Elicitor-induced enhancement of bioactive content and α-amylase inhibition in cluster bean sprouts

Komal Solanki, Rushna Mansuri,
Krutika Saurabh Abhyankar

This study investigates the effects of elicitor treatments on bioactive compounds and α-amylase inhibitory activity in *Cyamopsis tetragonoloba* sprouts.



40

Quantification of 3,3-dimethyl-1-butanol (DMB) in olive oil: a rapid and novel method

Apostolos Kiritsakis, Rifat Gimatdin, Hasan Yavuz Gören, Nikos Sakellaropoulos, Konstantinos Kiritsakis, Charalampos Anousakis, Fereidoon Shahidi, Ahmet Ceyhan Gören

A novel headspace gas chromatography-mass spectrometry (HS-GC/MS) method for identifying and quantifying 3,3-dimethyl-1-butanol (DMB), a bioactive compound, in extra virgin olive oil was developed.

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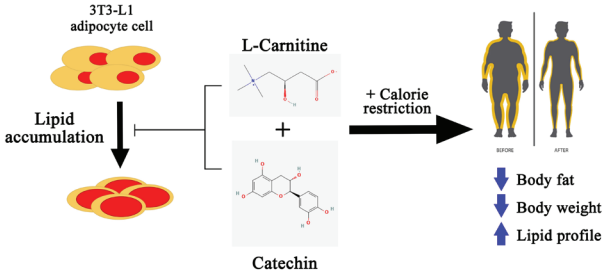
The effect of L-carnitine and catechin together with calories restriction on the reduction of body fat and blood lipids

Chi-Hua Yen, Yohanes Tandoro, Yu-Hsuan Liu, Bo-Kai Chen, Asif Ali, You-Cheng Shen, Chin-Kun Wang

Obesity has become a critical health problem worldwide, and it's associated with other health issues.

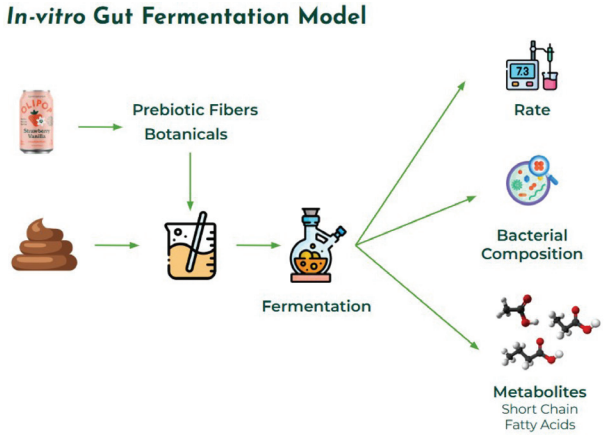


57

Botanicals impact the bifidogenic effect and metabolic outputs of *in vitro* fiber fermentation by gut-derived microbiota in individual-specific ways

Dane G Deemer, Noah Voreades, Peter A Bron, Stephen R Lindeman

Fortification of products frequently consumed by a large proportion of society provides an attractive strategy to close the "fiber gap" and may have the potential to concomitantly reverse the detrimental health effects exacerbated by our modern diets.



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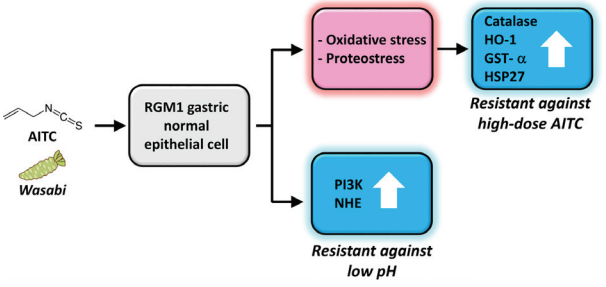
Allyl isothiocyanate confers resistance against low-pH stress conditions to RGM1 gastric normal epithelial cells

Shiho Kawaguchi, Akari Ishisaka, Akira Murakami

In this study, we attempted to elucidate the effects of ally isothiocyanates (AITC) on stress resistance.



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