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Bioactivity of Secreted Compounds Made by Fermented Vegetable-Associated Lactobacilli

By

LEI WEI

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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Dissertation Abstract

Lacto-fermented fruits and vegetables (FFVs) have gained popularity for their positive impact on health, however, insights of the mechanistic details underpinning the benefits of FFVs are limited. This dissertation aimed to first examine the current evidence on the effects of whole FFVs consumption and the health-promoting potentials of the constituents in FFVs (Chapter 1). Next, the capacity of fermented cabbage to ameliorate inflammation-induced intestinal barrier disruption was investigated using an *in vitro* Caco-2 monolayer model (Chapter 2). Furthermore, this dissertation focused on Plantaricin EF (PlnEF), a bacteriocin peptide produced by *Lactiplantibacillus plantarum*, a member of FFV microbiomes, as a case study for assessing the feasibility of encapsulation for intestinal delivery of bioactive peptides (Chapter 3) and elucidating the dual functionality of PlnEF as an effector molecule with intestinal barrier protective property (Chapter 4) and antimicrobial activity (Chapter 5). Consumption of whole FFVs was associated with improvement of metabolic and immune functions and reduction of oxidative stress, which overlaps with the bioactivity of fermentation derived metabolites found in FFVs (Chapter 1). Despite the enticing support for the health benefits of whole FFVs and their bioactive constituents, more mechanistic studies focusing on the influences of individual bioactive components in the context of the whole FFV food are needed. To help bridge this gap, the capability of fermented cabbage and fermentation-derived metabolites to mitigate cytokine-induced intestinal barrier permeabilization in Caco-2 monolayers was examined (Chapter 2). My findings revealed that fermented cabbage, but not raw cabbage in brine, prevented cytokine-induced barrier disruption, and this activity that was attributed to the distinct metabolomes and specific metabolites after fermentation. To focus in on specific bioactive compounds expected to be present in FFV, this dissertation evaluated the effectiveness of encapsulation in protecting the

peptides, PlnEF, during simulated gastrointestinal (GI) transit (Chapter 3). The results revealed that although the cross-linked calcium alginate microcapsules (CLAMs) were not able to protect PlnEF from gastric pepsin degradation, mixing CLAMs-encapsulated PlnEF in food (peanut butter) was successful in retaining active PlnEF through simulated GI digestion. This dissertation also showed that PlnEF exhibited a putative prophylactic effect in preventing disruptions to intestinal barrier permeability in Caco-2 cells, likely attributed to the modulation of tight junction proteins and other PlnEF-receptor mediated pathways (Chapter 4). Lastly, the antibacterial activity of PlnEF was shown by the increased the lag phase times of a PlnEF sensitive *L. plantarum* strain in a dose-dependent manner (Chapter 5). A spontaneous, PlnEF-resistant mutant of *L. plantarum* with a mutation in the Mg^{2+} efflux protein, CorC, contained higher intracellular Mg^{2+} levels regardless of the presence of PlnEF, pointing to the roles of intracellular Mg^{2+} in the heterogenous cellular response against the bacteriocin. In summary, this dissertation markedly contributes to the understanding of the effects of bioactive compounds produced by FFVs-associated LAB on intestinal barrier integrity. The findings showed that FFVs can serve as a feasible dietary source of bioactive constituents with beneficial impact on intestinal barrier function. This work also underlines the complexities of these microbe-host and microbe-microbe interactions mediated through microbial metabolites.

Chapter 1. Lacto-fermented fruits and vegetables: bioactive components and effects on human health

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Abstract

Lacto-fermented fruits and vegetables (FVs), produced through the non-alcoholic, fermentative energy conservation metabolism of diverse lactic acid bacteria (LAB), have served as staples of human diet throughout history. Along with their desirable organoleptic properties and improved shelf-life and safety compared to the raw ingredients, lacto-fermented FVs may confer health benefits. Herein, we discuss the impacts of consuming whole lacto-fermented FVs, including kimchi, sauerkraut, fermented olives, fermented fruits, and fermented juices, through insights gained from randomized controlled trials (RCTs). Furthermore, we review evidence gained from human, animal, and *in vitro* studies on the potential health-promoting mechanisms of the bioactive constituents derived from lactic acid fermentation (e.g., organic acids, phenolic compounds, amino acid derivatives, bacteriocins) and beneficial live microbes in lacto-fermented FVs. These studies highlighted the positive influences of whole lacto-fermented FVs and their bioactive constituents on anti-obesogenic, anti-diabetic, anti-inflammatory, and anti-oxidative properties. The potential benefits of lacto-fermented FV foods are described alongside perceived risks, including exposure to foodborne pathogens. More RCTs are needed to assess the bioavailability of specific bioactive compounds from lacto-fermented FVs to determine whether sufficient quantities can remain stable during GI transit and exert the proposed physiological effects. Development of starter culture strains, with the capability to produce desired bioactive compounds during FV fermentation, along with experimentally tested recipes and resources, can help making lacto-fermented FVs a feasible and safe dietary means of delivering fermentation enriched bioactive compounds and beneficial live microbes.

Introduction

Fermented foods are defined as foods and beverages made through desired microbial growth and enzymatic conversions of food components (Marco et al., 2021). The importance of fermented foods in the human diet is evident in that it is estimated that approximately one-third of all foods consumed globally are fermented (Campbell-Platt, 1994). Archeological and other records suggest that these foods also have a long history of production, well before the development of agriculture. Besides their desirable organoleptic properties, fermented foods frequently have longer shelf-lives, provide improved food safety (Ross et al., 2002; Gänzle, 2020), and confer health benefits (Marco et al., 2017; Diez-Ozaeta & Astiazaran, 2022; Caffrey et al., 2024).

It is estimated that there are more than 5,000 varieties of fermented foods (Tamang et al., 2016; Gänzle, 2022). Because of the significant compositional differences between these foods, they are often classified based on the main ingredients (Tamang et al., 2020; Tamang et al., 2020, 2016). To this regard, fermented foods are made from nearly every kind of food substrate, including milk, meat and other animal tissues, fish, and plants. Fermented plant-based foods can be further divided based on whether they are made using fruits and vegetables, cereal grains, tubers or roots, legumes, nuts, or seeds (Aidoo, 1986; Wuyts et al., 2020). These plant-based fermented foods differ from each other by their preparation steps and the types of microbes needed. Whereas many of these foods require yeasts (for example, bread and wine) or molds (for example, tempeh and soy sauce), others rely more on bacteria (for example, kimchi and sauerkraut).

Lactic acid bacteria (LAB) are the microorganisms needed to produce non-alcoholic fermented fruits and vegetables, or here called “lacto-ferments”. Although LAB are found in

diverse nutrient-rich ecosystems (De Filippis et al., 2020; Duar et al., 2017; George et al., 2018; Martino et al., 2016), including the mammalian digestive tract, and some strains are applied as probiotics, LAB are even better understood for their roles in food fermentations (Gänzle, 2022; Valentino et al., 2024; Wang et al., 2021). Taxonomically, LAB members belong to the order *Lactobacillales* and encompass 78 genera including lactobacilli, *Leuconostoc*, *Lactococcus*, *Oenococcus*, *Streptococcus*, *Enterococcus*, and *Pediococcus* (Schoch et al., 2020). Many of these genera were recently renamed due to the reclassification of the *Lactobacillus* genus (Zheng et al., 2020). They are named LAB because of their formation of lactic acid as a sole or the main end-product of fermentation energy conservation metabolism (George et al., 2018; Wang et al., 2021; Zheng et al., 2020).

Lacto-fermented fruits and vegetables (FV) have been staples in human diets for thousands of years (Medina-Pradas et al., 2017). These foods have been essential for the preservation of readily perishable, but highly nutritive, FV foods (Ross et al., 2002). Recently, there has been a rise in home fermentation in the US (Ramos et al., 2022) and a surge in consumer interests in the health benefits of these foods (Future Market Insights, 2022; Nielson-Stowell, 2020; Vinderola et al., 2023). However, lacto-fermented FV were occasionally associated with foodborne outbreaks and there is the potential for the LAB to produce undesired compounds like biogenic amines (Barbieri et al., 2019). Therefore, this review examines the microbial diversity and ecology, health benefits and bioactive compounds, and safety of lacto-fermented FVs.

Microbial composition and variation in lacto-fermented FVs

One of the most common ways of making lacto-fermented FV is to mix the ingredients in salt or a brine solution to reach between 2% to 10% w/v NaCl and then allowing the mixture to ferment in a closed container, creating a low-oxygen environment. Although LAB are found on the surface of vegetable foods, ranging from < 10 cells/g to $10^{8.5}$ cells/g, they are typically minor constituents of fruit and vegetable microbiomes (Yu et al., 2020). Instead for FV foods, the microbial composition is dominated by bacteria in the Proteobacteria (Pseudomonadota) phylum, including *Sphingomonas, Pantoea and Pseudomonas* (Sohrabi et al., 2023; Wicaksono et al., 2023; **Figure 1**). These bacteria are the dominant members of the plant leaf surface (phyllosphere) core microbiota (Sohrabi et al., 2023) and are found in a range of 10^3 to 10^8 cells/g (Jackson et al., 2013; Rastogi et al., 2012; Remus-Emsermann et al., 2014). On fruits, yeasts are found including, *Saccharomyces, Pichia,* and *Candida* (Chanprasartsuk et al., 2010; Di Cagno et al., 2010; Di Cagno et al., 2013) in a range of 10^2 to 10^6 cells/g (Di Cagno et al., 2008). However, for some foods like olives with high phenolic contents, the microbial numbers can be quite low (Medina-Pradas & Arroyo-Lopez, 2015; Pereira et al., 2006).

Shortly after the preparing the food for fermentation, LAB begin to grow and within a couple days can reach high numbers, ranging from 10^3 to 10^9 cells/g as reviewed in (Rezac et al., 2018). The LAB that eventually dominate the ferment are typically a part of the plant substrate microbiota and/or from the environment (e.g., kitchen surfaces, producer facility, etc.) (Einson et al., 2018; Miller et al., 2019), although for some ferments, the inoculation of a prior ferment (backslopping) or industrial starter cultures are used. In all scenarios however, there is typically a succession of LAB growth. Heterofermentative LAB such as species of *Leuconostoc* and *Weissella* dominate in the early phase of most plant-based fermentations (Gänzle, 2015).

Heterofermentative LAB possess the phosphoketolase pathway for fermentation energy conservation metabolism and yield a mixture of lactate, acetate, ethanol, and CO_2 end-products (Gänzle, 2015). Subsequently, homofermentative LAB species abundance increases during the later stages of some lacto-fermented FVs, such as *Lactiplantibacillus plantarum* in sauerkraut *Lactiplantibacillus pentosus* in olives, *Latilactobacillus sakei* in kimchi and *Pediococcus* species in vegetable (such as cabbage and cucumber) fermentation (Gaudioso et al., 2022; Wuyts et al., 2018; Zabat et al., 2018). Homofermentative LAB oxidize glucose using the Embden-Meyerhof pathway to pyruvate with lactate as the main end-product (Gänzle, 2015).

Fungi are also members of the lacto-fermented FV microbiota. Molds are an indicator of a failed fermentation and occurrence of unwanted spoilage (Bavaro et al., 2017; Ezeonu, 2017). Yeast overgrowth can also cause spoilage (Ballester et al., 2021), for instance, resulting in excessive softening of olives (Golomb et al., 2013; Zaragoza et al., 2017), off-flavors (Lanza, 2013), and fruit damage due to excessive CO_2 production (Lanza, 2013). Yeasts, such as *Saccharomyces*, *Pichia* and *Candida*, can be beneficial members of lacto-FV fermentations, such as olives, and can play important roles in the degradation of polyphenols, flavor generation, production of esters and volatile compounds, and texture maintenance through pectinase activity (reviewed in Arroyo-Lopez et al., 2008; Arroyo-Lopez et al., 2012; Di Cagno et al., 2010).

Species of acetic acid bacteria (AAB) have been found in lacto-fermented fruit juices such as nata de coco (fermented coconut water) (Gomes et al., 2018). AAB, such as *Acetobacter* spp. and *Gluconobacter* spp., are strict aerobes within the *Acetobacteriaceae* family that are well-known for their important role in "aerobic fermentation" of vinegars (De Roos & De Vuyst, 2018). Lastly, bacteriophages have been found in lacto-fermented FVs, such as sauerkraut (Yoon et al., 2002) and fermented cucumbers (Lu et al., 2012, 2020), targeting

fermentation isolates such as *Leuconostoc mesenteroides*, *L. plantarum* (Lu et al., 2010; Park et al., 2011). However, their importance within fermented FV microbial communities remains to be characterized (Paillet & Dugat-Bony, 2021).

Although LAB dominate lacto-fermented FV foods, there is also considerable species variation between different ferment types (Hutkins, 2007; Tamang et al., 2020; Tamang & Lama, 2022; Tamang et al., 2016). The specific microorganisms found in lacto-fermented FVs can vary depending on factors, such as the type of vegetable or fruit being fermented, the specific fermentation conditions used like salinity (M.-A. Lee et al., 2021; Lin et al., 2021), pH (McMurtrie et al., 2019), temperature (Cabello-Olmo et al., 2020; He et al., 2020) and time, the use of starter cultures, the prevailing microbiota on the starting ingredients. Previous studies have shown that strain-level differences can occur in lacto-fermented FVs (A. O. Yu et al., 2021) and that there is likely both cross feeding (Stadie et al., 2013; Stevens et al., 2023) and antagonistic interactions (Choi et al., 2018) in these foods. These types of interactions have been shown for several fermented foods (Ivey et al., 2013; Sieuwerts et al., 2008) and are being investigated as models to understand community assembly and interactions (Louw et al., 2023; Wolfe & Dutton, 2015).

Effects of lacto-fermented FVs on health

Broadly, fermented foods have been associated with the alleviation of chronic metabolic and inflammatory disorders (Gille et al., 2018; Paul et al., 2023) and improving mental (Aslam et al., 2020) and digestive health (Bell et al., 2018; Dimidi et al., 2019; Garnas, 2023; Leeuwendaal et al., 2022; Mukherjee et al., 2024). With regards to fermented FVs, kimchi, sauerkraut, table olive, fruit, and juice have been investigated in at least one human study. The findings of those

studies along with those from complementary animal and *in vitro* models are described below (**Figure 2**).

Kimchi

Randomized controlled trials (RCTs) investigating the health benefits of kimchi consumption have been recently reviewed by Song et al., (2023). Among the 11 RCTs (total 608 participants included in the trials) selected from prospective clinical studies of kimchi consumption and systemically reviewed, there was a consensus that kimchi intake is associated with lower lipids, cholesterol levels, and body fat and improved colon health (Song et al., 2023). Additional cohort, cross-sectional, and retrospective clinical studies also reported anti-diabetic, anti-obesogenic, and cholesterol-lowering properties of kimchi (An et al., 2013; Kim et al., 2018; Kim et al., 2011; Park et al., 2014). To this regard, a cross-sectional analysis of the Korean Health Examinees (HEXA) Study data from 2004 to 2013 (n = 115,726) concluded that consumption of 1 to 3 servings of kimchi a day is associated with a lower risk of obesity (Jung et al., 2024). That study found that baechu kimchi (cabbage kimchi) intake is correlated with a lower obesity prevalence in men, whereas kkakdugi (radish kimchi) is linked with a lower prevalence of abdominal obesity in both men and women (Jung et al., 2024). These findings are also supported by studies performed in animal and cell culture models showing that kimchi may protect against oxidative stress (J. M. Park et al., 2021), inflammatory perturbations (Moon et al., 2023), and support lipid and immune homeostasis (Cui et al., 2015; Islam & Choi, 2009; Jo et al., 2015; Kim et al., 2018; S. E. Park et al., 2020).

Sauerkraut

The potential for sauerkraut to alleviate symptoms of Irritable Bowel Syndrome (IBS) was studied in a randomized, double-blinded, intervention pilot study (Nielsen et al., 2018). It was found that both pasteurized and unpasteurized sauerkraut significantly improved IBS symptoms ($p < 0.04$), as noted through the IBS-Symptom Severity Score assessing abdominal distension, abdominal pain, satisfaction with bowel habits, and the extent to which symptoms affect the quality of life (Nielsen et al., 2018). This suggested that live microbes in the sauerkraut were not needed for alleviating IBS symptoms (Nielsen et al., 2018). More recently, a six-week, parallel arm, pilot trial with 31 women was conducted to compare the effects of regular consumption of fermented cabbage or cucumber (100 g/day, 5 days per week), as compared to no vegetables or pickled (non-fermented) cabbage or cucumber, on serum markers of inflammation (C-reactive protein (CRP), TNF-α, lipopolysaccharide binding protein (LBP)) and effects on the gut microbiota (Galena et al., 2022). While there were no significant differences in the levels of CRP, TNF-α, and LBP, daily fermented cabbage consumption led to the enrichment of *Faecalibacterium prausnitzii* (nearly doubled compared to baseline) (associated with anti-inflammatory properties) and a reduction in the proportions of *Ruminococcus torques* (by at least 2-fold in 9 out of 10 participants compared to baseline) (associated with insulin resistance) (Galena et al., 2022). Lastly, the animal and *in vitro* studies found that sauerkraut has antioxidative (Krajka-Kuzniak et al., 2011; Xu et al., 2022), immunomodulatory (Zubaidah et al., 2020), anti-carcinogenic (Raak et al., 2014), and anti-inflammatory (Huang et al., 2020; Siddeeg et al., 2022) properties.

Table olives

One pilot study with healthy subjects (n = 25) reported that consuming 12 table green olives per day for thirty days resulted in significantly reduced serum IL-6 levels, plasma malondialdehyde (a biomarker of oxidative stress), and body fat mass percentage at the end of the intervention compared to baseline levels (Accardi et al., 2016). In hypertensive rats, systolic and diastolic blood pressure was transiently reduced compared to controls after a single dose of fermented table olives (3.85 g/kg body weight) during a short-term (1 day) study (Franco-Ávila et al., 2023). During a long term (7 week) study, daily intake of fermented table olives (3.85 g/kg body weight) reduced the systolic and diastolic blood pressure in hypertensive rats starting during week 2 compared to the controls (Franco-Ávila et al., 2023). In another table olive feeding study in rats, plasma levels of malondialdehyde and angiotensin II, two metabolites associated with oxidative stress and inflammation, were found to be significantly lower (Gómez-Contreras et al., 2023).

Fermented fruits

RCTs assessing the effects of lacto-fermented fruits on human health are scarce. One RCT (n = 90) found that consumption of lacto-fermented apple puree by individuals with cardiovascular disease risk factors increased HDL cholesterol and reduced trimethylamine-N-oxide (TMAO) levels compared to when apple puree was provided (Tenore et al., 2019). While the number of clinical studies investigating the effects of lacto-fermented fruits needs to be expanded, findings from animal and human cell culture studies suggest that lacto-fermented fruits, such as fermented blueberries (Sivapragasam et al., 2023), contain beneficial bioactive activity. In a diet-induced obese (DIO) rat model, a 7-week, concurrent administration of lacto-

fermented blueberries at three dosages (26, 52, and 78 ml/kg/day) with a high-fat diet (HFD) resulted in a dose dependent reduction in serum triglyceride and total cholesterol levels and significantly higher HDL-C levels compared to HFD controls (Jiang et al., 2024). Feeding healthy and hypertensive rats freeze-dried blueberries fermented by *L. plantarum* DSM 15313 (2 g/day) along with the tannase-producing *L. plantarum* strain DSM 15313 (10^9 CFU/day) for 4 weeks reduced the systolic and diastolic blood pressure compared to baseline (Ahrén et al., 2015). Examination of *L. casei* CICC 20280-fermented blueberry pomace during *in vitro* simulated digestion and colonic fermentation showed that the ferment resulted in significantly increased proportions of *Bifidobacterium* and *Lactobacillus* genera by nearly 2-fold and elevated lactic acid and butyric acid levels compared to blueberry pomace (Cheng et al., 2020).

Fermented fruit and vegetable juices

One RCT evaluated the effects of fermented FV juice consumption on human health. This single blind RCT of healthy adults (n = 89) after consuming lacto-fermented grape juice (250 or 500 ml per day) for 40 days found significantly lower levels of serum lipid peroxidation product (diene conjugate, malondialdehyde, and homocysteine) levels compared to baseline, while this change was not observed among individuals who did not drink fermented grape juice (Amoutzopoulos et al., 2013). Despite the paucity of human studies on fermented juices, animal and cellular studies examining biologically active compounds, sensory qualities, and functional properties of fermented FV juices have revealed their antioxidant activity as well as anti-diabetic, anti-obesogenic, and anti-inflammatory characteristics of these foods compared to the raw ingredients (Szutowska, 2020). In a DIO rat model, male rats administered cabbage-apple juice fermented with *L. plantarum* strain EM (10 ml//kg body weight, daily for 8 weeks) along with a

HFD had reduced body weight, liver and white fat pad weights, and levels of serum triglyceride, total cholesterol, LDL-cholesterol, insulin, glucose, and leptin compared to DIO control rats (S. Park et al., 2020). In a study with obese mice, administration of a fermented mixed vegetable juice containing either *Companilactobacillus allii* WiKim39 or *Lactococcus lactis* WiKim0124 exhibited reduced weight gain and liver fat accumulation and had lower expression of genes encoding sterol regulatory element binding protein-1c (SREBP-1c), CCAAT/enhancer binding protein-α (C/EBP-α), PPAR-γ, FABP4, and FAS and higher expression of genes encoding for lipolytic enzymes, ATGL and HSL (Lee et al., 2023). Lastly, in a rat model of type 2 diabetes (T2D), fermented carrot juice containing *Lacticaseibacillus rhamnosus* GG (LGG) had improved metrics of hyperglycemia, including lower fasting blood glucose and insulin and improved kidney morphology (Hu et al., 2019).

Bioactive compounds produced or modified during FV lacto-fermentations

To elucidate how fermented FVs can affect human health in ways distinct from the foods used to make them, it is necessary to understand the specific compounds enriched, depleted, or modified during fermentation and how those compounds affect intestinal and systemic responses. FVs contain mono- and disaccharides as well as dietary fiber, micronutrients, and phytochemicals such as vitamins, phenolic compounds, and carotenoids (de la Rosa et al., 2009; Septembre-Malaterre et al., 2018). The concentrations of the nutritive and non-nutritive bioactive compounds before and after fermentation of some raw FVs have been investigated, highlighting the variations in the levels of these compounds depending on the FV matrix, fermentation conditions, and bacterial strains present (Paramithiotis et al., 2022; Septembre-Malaterre et al., 2018). These compounds, encompassing organic acids, phenolic compounds, amino acid

derivatives, bacteriocins, as well as other minerals, vitamins, glucosinolates, and lipids are discussed below (**Figure 3**).

Lactate and acetate

LAB produce lactic acid and acetic acid using homofermentative and heterofermentative energy conservation metabolism (Gänzle, 2015). Lactic acid, in its two enantiomers (D- and L-lactic acid), and acetic acid accumulate up to 1% v/v in the final product of lacto-fermented FVs. In four types of commercial kimchi from five producers, D- and L-lactic acid were found in similar ranges from 17 to 87 mM (Yoon et al., 2005). In the same study, five brands of commercial sauerkraut were found to contain significantly higher concentrations of D-lactic acid (69 to 104 mM) than kimchi, while the concentrations of L-lactic acid were similar to kimchi (74 to 82 mM) (Yoon et al., 2005). Acetic acid was found in concentrations between 0.66 to 80 mM in different lacto-fermented FVs, including sauerkraut (Gaudioso et al., 2022; Gustaw et al., 2021), fermented cucumber (Kao & Lin, 2023), fermented broccoli, carrot, pepper, and beet (Kiczorowski et al., 2022). Accounting for the serving size, acetate concentrations in pickled onion, sauerkraut, kimchi, and gherkin range between 247 to 872 mg per serving (3.29 – 24.11 mg/g) (Gill et al., 2021).

The production of lactic and acetic acids during FV fermentations is important for the determining the overall microbial composition of the food, mainly due to the capacity of these organic acids to inhibit other bacteria. Lactic and acetic acid synthesis results in reducing the pH of the FV foods, ranging from pH 3.5 to 4, within a couple days after the start of the fermentation (Di Cagno et al., 2016; Gaudioso et al., 2022; Kao & Lin, 2023). The reductions in pH and antimicrobial properties of the organic acids inhibit the growth of the dominant plant-associated

microbiota, most notably in the Proteobacteria (Pseudomonadota) phylum. Weak organic acids like lactic acid are more likely to lead to cytoplasmic acidification and proton motive force disruption in sensitive bacteria (Reis et al., 2012). While the antimicrobial effects of lactic and acetic acids vary (Cheng et al., 2003; Ryu et al., 1999) depending on intrinsic and extrinsic factors such as temperature (Breidt et al., 2007), pH (Kreske et al., 2008), dissolved oxygen (Lu et al., 2011), acid tolerance mechanisms of the target bacterial strains (Jones et al., 2020), generally a combination of organic acids was found to be more effective at inhibiting food-borne pathogens and spoilage microbes (Adams & Hall, 1988; Ji et al., 2023; Sun et al., 2021).

Besides antimicrobial effects, lactate may also affect human health. A single oral dose of a lactate (25 g/L) racemic mixture was found to slow gastric emptying and increase the feeling of "fullness" one hour after consumption by healthy male volunteers (n = 10) compared to intravenous DL-lactate administration (Pedersen et al., 2022). Furthermore, the oral DL-lactate resulted in reduced plasma concentrations of acylated ghrelin and elevated GLP-1, insulin, glucagon starting 30 min after consumption (Pedersen et al., 2022). Lactate is an activating ligand for the hydroxycarboxylic acid receptor 1 (HCA1/GPR81) (Blad et al., 2011; Offermanns, 2014). DL-lactate was also shown to exert anti-inflammatory (Iraporda et al., 2015; H. C. Zhou et al., 2022), anti-oxidative (Kahlert et al., 2016; H. C. Zhou et al., 2022), intestinal barrier protective benefits *in vitro* (Luo et al., 2022). These effects are further supported by animal model studies, wherein DL-lactate administration dampened pro-inflammatory responses (Iraporda et al., 2016), promoted circulatory and phagocytotic function (McDonald et al., 2020; Yan et al., 2022), stimulated intestinal barrier repair (Y. Yu et al., 2021), and reduced the severity of gut epithelial damage induced by colitis (Li et al., 2024; H. C. Zhou et al., 2022) and other perturbations (Y. S. Lee et al., 2018).

Acetate is also bioavailable when ingested and has health modulatory effects. Firstly, the absorption of acetate was shown in a pharmacokinetic study, wherein the consumption of apple cider vinegar (~25.8 mmol of acetate) was found to resulted in two-fold higher levels of acetate in blood plasma when measured 60 min later compared to participants who consumed a control drink (Gill et al., 2021). In a rat model of T2D, higher concentrations of cecal acetate, as well as butyrate and propionate, were found after consumption of a carrot juice fermented using *L. rhamnosus* strain LGG (Hu et al., 2019). That juice also contained 7.11 mM acetate, 9.72 mM propionate, 10.78 mM butyrate (Hu et al., 2019), suggesting that other SCFAs may also be present in some lacto-fermented FVs.

Meta-analyses of human studies examining the effects of acetate ingestion (e.g., through vinegar intake) found that daily consumption of acetate (750 to 3600 mg for an average of 8 weeks) led to reductions in fasting blood glucose in volunteers who were healthy or with metabolic disorders (e.g., T2D, obesity) (Cherta-Murillo et al., 2022; Valdes et al., 2021). In animal studies, consumption of acetate is associated with reduced inflammation (Iraporda et al., 2015), weight loss (Hernandez et al., 2019; Launholt et al., 2020), improved insulin sensitivity and reduced blood glucose level (Santos et al., 2019), and increased satiety (Frost et al., 2014). Including acetate (200 mM) in the drinking water of mice with hypertension was associated with reductions in blood pressure, cardiac fibrosis, and renal fibrosis (Marques et al., 2017). Mice consuming acetate (846.8 mM) supplemented into a HFD gained less body weight and had lower liver weight and hepatic triglyceride accumulation (Shimizu et al., 2019). This effect was also observed when either propionate (674.9 mM) or butyrate (567.5 mM) was provided, however, it was completely abolished in *Ffar3*$^{-/-}$ mice without FFAR3/GPR41 receptor (Shimizu et al., 2019). Acetate is a ligand for fatty acid receptors, FFAR3/GPR41 and FFAR2/GPR43, which are

differentially expressed in the intestine, spleen, adipose tissues, pancreas, and other tissues (Mishra et al., 2020). These receptors modulate signaling pathways involved in immune regulation, gut hormonal synthesis and epithelial integrity, and neuronal functions (Hernandez et al., 2019; Mishra et al., 2020; Moffett et al., 2020).

Although the overall importance of lactate and acetate to health modulatory outcomes with fermented FV consumption are not fully known, these compounds are commonly present in levels in those foods to make an impact. Lactate concentrations in lacto-fermented FV are in the mM range (Gaudioso et al., 2022; Gustaw et al., 2021; Kao & Lin, 2023); quantities on par with the half maximal effective concentration (EC_{50}) for HCA1/GPR81 receptor activation (5 – 20 mM lactate) (Koh et al., 2016). Concentrations of acetate in some lacto-fermented vegetables (Kiczorowski et al., 2022) (60 to 80 mM) are well above the levels needed to activate the FFAR2/GPR43 receptor (250 to 500 µM acetate) (Koh et al., 2016). Hence, it is plausible that fermented FVs serve as a dietary source of lactate and acetate at sufficient concentrations to confer benefits.

Phenolic compounds

Phenolic compounds are a major group of plant secondary metabolites (Rodríguez et al., 2009) and consist of more than 8,000 molecules that are categorized into flavonoids, phenolic acids, stilbenes, lignans, or curcuminoids, based on their structures (Septembre-Malaterre et al., 2018). This diverse group of functional phytochemicals is associated with reducing oxidative stress (Cosme et al., 2020; Matsumura et al., 2023; Rice-Evans et al., 1997; Zhao et al., 2021) and are often abundant in lacto-fermented FVs (Benatrehina et al., 2018; Septembre-Malaterre et al., 2018).

Lacto-fermentation generally increases the total phenolic content and the antioxidant capacity of FVs. The levels and types of phenolic compound vary by the type of FV, strains used for fermentation, and processing conditions (**Table 1**; Leonard et al., 2021; Zhao et al., 2021). To this regard, the effects of processing conditions on phenolic compounds in table olives have been extensively studied (Charoenprasert & Mitchell, 2012; D'Antuono et al., 2018; Johnson & Mitchell, 2018; Perpetuini et al., 2020; Pino et al., 2018). The total phenolic content, antioxidant capacity, and specific phenolic compound concentrations have also been characterized for thirty different types of commercial lacto-fermented FV pickles, where the total phenolic content ranged from 4.82 ± 0.02 to 235.19 ± 1.06 μg gallic acid equivalent (GAE)/mg and the antioxidant activity (in terms of half maximal inhibitory concentration, IC_{50}) ranged from 504.86 ± 29.22 IC_{50} μg/ml to 2.94 ± 0.03 IC_{50} μg/ml (Ciniviz & Yildiz, 2020). The predominant phenolic acids and their quantities in these thirty lacto-fermented FVs differed depending on the phenolic compound profiles of the FV matrices before fermentation (Ciniviz & Yildiz, 2020). The top six most abundant phenolic acids were gallic acid (hot pepper pickle pulp, 248.81 ± 0.01 mg/kg), syringic acid (cauliflower pickle juice, 1066.85 ± 4.29 mg/L), vanillic acid (carrot pickle juice, 31.81 ± 2.67 mg/L), caffeic acid (hot pepper pickle juice, 74.61 ± 3.28 mg/L), chlorogenic acid (greengage plum pickle pulp, 233.15 ± 2.56 mg/kg), and sinapic acid (sweet long green pepper pickle juice, 236.32 ± 1.79 mg/L) (Ciniviz & Yildiz, 2020).

Elevation of the total phenolic content and antioxidant capacity during lacto-fermentation can occur from the biotransformation of the parent phenolic compound into derivatives or due to the increased release of phenolic compounds from the plant matrices into the juices or extracts (Gaur & Gänzle, 2023; Yang et al., 2023). For example, reduction in caffeic and *p*-coumaric acids corresponded to significantly increased concentrations of their derivatives, dihydrocaffeic

acid and phloretic acid, after cherry juice fermentation (Filannino et al., 2015). *L. plantarum* (Muñoz et al., 2024) and *L. mesenteroides* (Filannino et al., 2018; Rodríguez et al., 2009) can metabolize and transform phenolic compounds through strain-specific tannase, decarboxylase, glycosyl hydrolases, or reductase activities. These enzymatic capacities benefit the LAB which perform them in FV fermentations. *L. mesenteroides* and *Weissella* spp. can increase their cellular NAD^+/NADH ratio and intracellular ATP levels through the reduction of hydroxycinnamic, protocatechuic, caffeic acids, giving them a metabolic advantage in FV-ferment environments (Filannino et al., 2015; Filannino et al., 2018; Filannino et al., 2014). Lactobacilli can consume sugar moieties released from flavonoids by endogenous glycosyl hydrolases (Gaur & Gänzle, 2023). Additionally, conversion of some phenolic acids, like hydroxycinnamic acids, can reduce their antimicrobial activity (Filannino et al., 2018; Rodríguez et al., 2009; Sánchez-Maldonado et al., 2011).

Increased phenolic content and antioxidant capacity of lacto-fermented FVs has been linked to beneficial effects *in vitro* and *in vivo* (Lin et al., 2016; Matsumura et al., 2023; Shahidi & Yeo, 2018). Antioxidant activity in the polyphenol extract from table olives was five-fold higher after lacto-fermentation and this corresponded to an ~8-fold higher capacity to reduce induced intracellular ROS levels in Caco-2 cells (D'Antuono et al., 2018). Increased total phenolic and flavonoid contents of lacto-fermented dragon fruit-kiwi juice increased tolerance to oxidative and heat stresses and life span of *Caenorhabditis elegans* (Tang et al., 2022).

The most abundant polyphenol in lacto-fermented table olives, hydroxytyrosol (258.2 ± 12.1 – 1393.3 ± 38 ug/g of FW, D'Antuono et al., 2018), was found to have protective qualities against metabolic (Pastor et al., 2021), cardiovascular (D'Angelo et al., 2020), and neurological (Chen et al., 2021) disorders. However, these studies have focused on the individual phenolic

compound without accounting for the potential additional effect of the fermented FV matrix. More *in vitro*, animal, and controlled human intervention studies with lacto-fermented FVs, from which the total phenolic compound diversity is known, are needed to fully understand the bioactive potential of these compounds in consumed fermented FVs.

Amino acid derivatives (PLA, ILA, GABA)

Phenyl-lactic acid (PLA)

3-Phenyllactic acid (PLA), also known as 2-hydroxy-3-phenylpropanoic acid and β-phenyl-lactic acid, is an organic acid and aromatic amino acid derivative from phenylalanine catabolism with two chiral isomers, L-PLA and D-PLA (Mu et al., 2012). PLA is of increasing interest because of its roles in regulating immune cell function (Kim et al., 2019; Peters et al., 2019) and lipid metabolism (Lee et al., 2023; Shelton et al., 2023; Valan Arasu et al., 2013). It is also of considerable industrial interest for its antifungal properties (Crowley et al., 2013; Rajanikar et al., 2021).

PLA has been detected in lacto-fermented carrots (~20 µg/mL; Kasperek et al., 2024), fermented bamboo shoots (10 µg/g; Kimura et al., 2020), kimchi (12 – 21.1 µg/ml; Jung et al., 2019), and sauerkraut (90 to 460 µM; Peters et al., 2019) and 20 – 30 µg/mL; Kasperek et al., 2024). The PLA in these ferments is likely produced by LAB since PLA-producing strains have been isolated from fermented foods (Chaudhari & Gokhale, 2016; Crowley et al., 2013; Peters et al., 2019; Zhang et al., 2022; Zhou et al., 2018). PLA biosynthetic capacity and enantiomer ratio is strain-dependent and PLA quantities produced by LAB in culture medium ranged from a low of 0.02 ± 0.01 mM (*L. fermentum* ITM6E and ITM18C from sourdough, in MRS; Valerio et al., 2004) to a high of 5.2 ± 0.5 mM (*L. plantarum* 1081 from pea, in MRS without acetate;

Rajanikar et al., 2021) with enantiomer ratios of racemic mixture (1:1), fully D-PLA (Chaudhari & Gokhale, 2016), or 11:1 L-PLA to D-PLA (*L. plantarum* CXG9 from Chinese fermented vegetable; Zhang et al., 2022). Higher concentrations of PLA were found in kimchi to which PLA-producing strains were added (Jung et al., 2019).

Genetic and biochemical analyses have also been undertaken to elucidate the pathway(s) and enzymes via which PLA is made by LAB (Mu et al., 2012; Rajanikar et al., 2021). In LAB, phenylalanine is transaminated to phenylpyruvic acid (PPA) via aromatic aminotransferase (AAT) and then PPA is reduced to PLA by lactate dehydrogenases, regenerating NAD^+ (Kimura et al., 2020; Mu et al., 2012; Rajanikar et al., 2021). Genes encoding the key enzymes for PLA biosynthesis have been identified in *L. plantarum* strains S1, YM-4-3, AY01, 5-2, and LY-78 via genomic and transcriptomic analyses (Sun et al., 2019; Wu et al., 2020). Deletion of *aat*, encoding for the aminotransferase Aat in *P. acidilactici* FAM18098, prevented the catabolism of phenylalanine and the synthesis of PLA in culture supernatants (Wenger et al., 2023). Lactate dehydrogenases, encoded by *ldhL* in *L. plantarum* IMAU10124 (Zhang et al., 2014) and SK002 (Jia et al., 2010) and in *P. pentosaceus* ATCC 25745 (Yu et al., 2012), and *L. rossiae* DSM 15814 (Luo et al., 2020) were noted to be capable of converting PPA to PLA through heterologous expression in *E. coli* and subsequent enzymatic analysis. PLA production in *L. plantarum* L3, involving aminotransferase (AraT) and lactate dehydrogenase (Ldh), was noted to be regulated by the S-ribosomal homocysteine lyase/autoinducer-2 (LuxS/AI-2) quorum sensing (QS) system, where the disruption of the QS system with furanone significantly reduced the supernatant signal molecule AI-2 levels, proteins levels of AraT, Ldh, and LuxS, cell density, and PLA content (Chai et al., 2023).

PLA is an activating ligand of the human hydroxycarboxylic acids receptor 3 (HCA3), particularly D-PLA (EC_{50} of 0.15 µM) has approximately 35-fold higher affinity for this receptor than L-PLA (EC_{50} of 5.2 µM) (Peters et al., 2019). D-PLA at 1 µM activated HCA3 and induced migratory responses in human monocytes (Peters et al., 2019). Volunteers consuming sauerkraut had significantly higher levels of plasma (reaching 0.9 µM 2 h post-prandial) and urine (reaching 9.1 µM 2 h post-prandial), suggesting that LAB-produced D-PLA in sauerkraut can reach sufficient levels in the plasma after ingestion to activate HCA3 (Peters et al., 2019; Peters et al., 2020). Other studies on LAB from other environments have shown the potential significance of PLA produced in lacto-fermented foods. PLA isolated after growth of *L. plantarum* KCC-10, an isolate from forage silage (Valan Arasu et al., 2013), increased adipocyte glucose uptake and differentiation through PPAR-γ2 signaling in murine 3T3-L1 preadipocytes (Ilavenil et al., 2015). Another study found that intestinal *Ligilactobacillus murinus,* and by association, PLA made by that microbe, increased the abundance of PPAR-γ in mice small intestinal epithelium, thereby regulating lipid metabolism and protecting against HF-induced obesity early in life (Shelton et al., 2023). These findings show that PLA in fermented FV foods can reach the human digestive tract and may result in effects on intestinal, immune, and other cell types.

Indole-3-lactate (ILA)

Indole-3-lactic acid (ILA) is an indole metabolite derived from tryptophan catabolism (Li et al., 2021). ILA is of particular interest because of its beneficial impact on the immune system (Xia et al., 2023), intestinal homeostasis (Henrick et al., 2021; Zhang et al., 2023), and liver metabolism (Li et al., 2021). ILA activated human HCA3 with 100-fold higher potency than the endogenous agonist 3-hydroxyoctanoate (3-HO) (Peters et al., 2019). ILA also activates the aryl

hydrocarbon receptor (AHR), which is a transcription factor regulating different aspects of cell cycle and proliferation, xenobiotic detoxification responses, development and modulate mucosal barrier function and inflammatory responses in a cell type-, developmental stage-, and ligand-specific manner (Hubbard et al., 2015; Kim et al., 2022; Larigot et al., 2018; Li et al., 2021).

ILA has been detected in lacto-fermented FVs in concentrations ranging from 0.005 to 0.06 µg/g in fermented bamboo shoots (Kimura et al., 2020) and between 0.1 to 2.8 µg/ml in sauerkraut, fermented carrots, fermented beets, kimchi, and pickles, with the highest levels found in fermented carrots and kimchi (Kasperek et al., 2024). Like PLA, the presence of ILA in lacto-fermented FVs is likely the result of LAB metabolism. The pathway of ILA synthesis involves the transamination of tryptophan to indole-3-pyruvate (IPyA) using aromatic amino acid aminotransferase (ArAT) followed by reducing IPyA to ILA by lactate dehydrogenase (Li et al., 2021) or D-2-hydroxyacid dehydrogenase (*fldH*) (T. Pan et al., 2023). This pathway appears to be common among LAB since examination of the tryptophan catabolism pathway genes in 148 LAB strains from 6 genera and 13 species found that ArAT was present in 98% of the strains examined and most of those LAB contained multiple ArAT genes (T. Pan et al., 2023). In fermented FVs, mixed vegetable juice inoculated with *C. allii* WiKim39 or *L. lactis* WiKim0124 contained ILA and ILA at concentrations ≥ 0.63 mM inhibited lipid accumulation in human mesenchymal stem cells (Lee et al., 2023). A freeze-dried powder of the fermented vegetable juice reduced weight gain and liver fat accumulation in DIO mice (Lee et al., 2023). ILA levels, along with other organic acids (lactate, 3-phenyllactate, β-hydroxybutyrate, γ-aminobutyrate), in a mixed vegetable fermentation containing *L. plantarum* PMO 08 were significantly increased compared to before the start of the fermentation (Kim et al., 2019). Fermented mixed vegetables

had improved antioxidative and anti-inflammatory effects in murine macrophages compared to the raw ingredients (Kim et al., 2019).

Studies of ILA and ILA-producing strains have provided insights into its health promoting effects in the intestine during immune and intestinal development. Metabolomics analysis showed that infants fed *Bifidobacterium infantis* EVC001 had elevated fecal ILA levels (Henrick et al., 2021). The ILA levels were associate with reduced intestinal Th2 and Th17 cytokines and upregulated immunoregulatory factor galectin-1 in T cells, suggesting that increased ILA may protect infants from inflammation (Henrick et al., 2021). The positive impact of *Bifidobacterium* supplementation associated elevation of fecal ILA on early life immune function development was also supported by a longitudinal observational study of twenty-five Danish infants and ILA's capacity to activate AHR *in vitro* (Laursen et al., 2021).

These findings along with other *in vitro* studies highlight the relevance of ILA produced by the gut microbiota or by ingested probiotic strains. Cellular signaling involved in the immunomodulatory (Cervantes-Barragan et al., 2017; Xia et al., 2023; Zhang et al., 2023) and cell differentiation (Meng et al., 2020; Wong et al., 2020) effects of ILA include the AHR, HCA3, Nrf2, Ras/ERK pathways (Ehrlich et al., 2020; Laursen et al., 2021; Q. Zhou et al., 2022). Additional studies quantifying ILA levels in lacto-fermented FVs and subsequent bioavailability and dietary intervention studies are imperative to determine whether lacto-fermented FVs can be a dietary means of increasing the intake of ILA or LAB strains that can synthesize ILA in the intestines.

γ-Aminobutyric acid (GABA)

γ-Aminobutyric acid (GABA) is a non-proteinogenic amino acid formed from L-glutamate by glutamate decarboxylase (GAD) and the co-factor pyridoxal-5-phosphate (PLP) (Sahab et al., 2020; Watanabe et al., 2002). It is an inhibitory neurotransmitter that maintains the balance between excitation and inhibition of neuronal signaling in the central nervous system (Auteri et al., 2015; Watanabe et al., 2002). GABA receptor interactions modulate gastrointestinal motility, secretions, and immune tolerance to food antigens and commensal microbes (Auteri et al., 2015). GABA is of growing interest due to its association with multiple beneficial physiological functions (Auteri et al., 2015; Watanabe et al., 2002), including anti-hypertensive, anti-diabetic, and immune modulatory effects (Ngo & Vo, 2019; Sahab et al., 2020).

GABA is found in various plant, animal, and microbial sources and many fermented foods (Sahab et al., 2020). GABA content in fresh fruits and vegetables, such as grapes, spinach, and tomatoes, can range from 0.15 to 2 mg/g (Heli et al., 2022; Hou et al., 2023). Lacto-fermentation of fresh FVs can elevate GABA content. GABA was found in high quantities (150 ± 44 mg/L) in fermented cucumbers (Moore et al., 2021). In Chinese northeastern sauerkrauts fermented for 30 days in three different households, GABA content increased to between 5 to 200 mg/L (Yang, Hu, Xiu, Jiang, Yang, Saren, et al., 2020; Yang, Hu, Xiu, Jiang, Yang, Sarengaowa, et al., 2020). Fermentation of a vegetable juice containing cabbage, broccoli, carrot, and beetroot resulted in GABA levels of 5 mg/ml, nearly twice as much as in the juice prior to the fermentation step (M. Lee et al., 2021).

Although GABA can be produced by many LAB species through the glutamate decarboxylase (GAD) enzyme (Yogeswara et al., 2020), *L. brevis* (Kim & Kim, 2012) and *L.*

plantarum (Chen et al., 2015; Iorizzo et al., 2023; S. J. Lee et al., 2021; Sahab et al., 2020) are best known for their GABA biosynthetic capacities. GABA is produced from the conversion of glutamine to glutamate, consuming intracellular protons and releasing CO_2. This enzymatic activity provides a mechanism for LAB to maintain intracellular pH in acidic environments (Cui et al., 2020; Das et al., 2022; S. J. Lee et al., 2021). To this regard, inoculation of pasteurized mulberry juice with either *Levilactobacillus brevis* F064A or litchi juice with *L. brevis* LBG-29 elevated GABA levels by at least 12-fold, reaching 3 to 3.31 g/L after 48 h of fermentation (Jin et al., 2023; Kanklai et al., 2020). In another study, *L. plantarum* strains were found to produce up to 80.5 g GABA/L during fermentation of either sterilized tomato juice, red seaweed juice, or litchi juice (Iorizzo et al., 2023). Lastly, co-inoculation of *L. brevis* YSJ3 and *L. plantarum* JLSC2-6 onto cauliflower stems resulted in significantly higher GABA concentrations (35 mg/L) compared to cauliflower stems with *L. brevis* YSJ3 (30 mg/L) or to which no additional bacteria were added (22 mg/L) (Zhang et al., 2024).

Studies examining the bioavailability and activity GABA in lacto-fermented FVs have yet to be performed. However, human studies have investigated the effects of oral GABA intake on stress, hypertension, and immune responses. A systemic review of fourteen RCTs examining the effects of oral GABA intake as a part of GABA-enriched natural products (e.g., tea and rice) or as a supplement on stress, anxiety, sleep, and related parameters found low to moderate level of support for the stress-reduction and stress-protective benefits of GABA intake, with an efficacious dose range of 2 to 100 mg (Hepsomali et al., 2020). In a 12-week placebo-controlled, single-blind RCT, the systolic and diastolic blood pressure of patients with mild hypertension was significantly reduced in subjects consuming a fermented milk containing approximately 10 mg GABA/100 ml milk (Inoue et al., 2003). Furthermore, animal and *in vitro* studies have noted

the neuroprotective, anti-hypertensive, anti-diabetic, satiety-inducing, and anti-inflammatory effects of GABA. These studies included the use of extracts of GABA-enriched (fermented) foods (Hou et al., 2023; Nakamura et al., 2022; Ngo & Vo, 2019; Oketch-Rabah et al., 2021). Investigation of GABA levels in lacto-fermented FVs and effects *in situ* are expected to lead to a better understanding of the contributions of this compound to the overall impact of these foods on human health.

Bacteriocins

Bacteriocins are peptides synthesized by bacteria and archaea that are best understood for their antimicrobial effects (Darbandi et al., 2022). LAB synthesize diverse bacteriocins (Alvarez-Sieiro et al., 2016; Pérez-Ramos et al., 2021), many of which are relatively small cationic peptides (20 to 70 amino acids) that impair cell membrane integrity of target bacterial cells through mechanisms involving membrane-bound docking receptors (Diep et al., 2009; Negash & Tsehai, 2020). LAB and their bacteriocins have primarily been studied as bio-preservatives to control food spoilage microorganisms and foodborne pathogens (Lahiri et al., 2022; Mokoena et al., 2021; Perez et al., 2022). However, bacteriocins may also have effects on human and animal cells as evidenced by studies performed *in vitro* (Meijerink et al., 2010; Molujin et al., 2022; van Hemert et al., 2010) and in animal models (Huang et al., 2021; Yin et al., 2018), showing anti-diarrheal (Hu et al., 2018), anti-obesogenic (Heeney et al., 2019), anti-inflammatory (Yin et al., 2018), anti-carcinogenic (De Giani et al., 2019) effects.

Bacteriocin-producing LAB strains and their bacteriocins were isolated from lacto-fermented durian pulp (“tempoyak”) (Grosu-Tudor et al., 2014; Kormin et al., 2001), artisanal fermented vegetables (Rwubuzizi et al., 2023), and kimchi (S. J. Lee et al., 2021). The use of

bacteriocin (kimchicin GJ7)-producing *L. citreum* GJ7 strain in kimchi fermentation prevented the over-ripening of kimchi after 125 days of storage and improved the kimchi sensorial quality and shelf life (Chang & Chang, 2010). In that study, after 20 days of storage at -1 °C, the kimchi juice filtrate from *L. citreum* GJ7-added starter kimchi showed higher antimicrobial activity than the filtrate from non-starter kimchi, verifying the retention of antimicrobial activity after the fermentation period (Chang & Chang, 2010).

The determination of bacteriocin concentrations in foods and throughout food processing remains a challenge due to the complexity of the food matrices. Quantification of nisin variants and their stability in cow milk (Ko et al., 2015), fruit-flavored yogurt drinks (Wee et al., 2024), and cheeses (Schneider et al., 2011) have been performed using LC-MS/MS. However, the concentrations of other bacteriocins in lacto-fermented FVs and other foods remain difficult to quantify. Confirming the presence, concentrations, and stability of bacteriocins in fermented FVs is a critical first step in correlating the beneficial effects to these bacteriocins and their producing strains after food consumption. While the *in vitro* and *in vivo* studies highlight the promise of applying LAB and their derived bacteriocins for their host directed benefits beyond their antimicrobial activity, further development of more sensitive analytical methods is required to start applying bacteriocins in fermented FVs.

Vitamins, minerals, glucosinolates, and lipids

Raw fruits and vegetables are significant sources of vitamin C and vitamin A (Kiczorowski et al., 2022; Paramithiotis et al., 2022; Septembre-Malaterre et al., 2018). By comparison, vitamin C (ascorbic acid) content of FVs was found to be lower after lacto-fermentation (Kiczorowski et al., 2022; Knez et al., 2023; Szutowska, 2020). Fermentation-

induced changes in vitamin A and β-carotenoids (vitamin A precursors) depend on substrate and fermentation conditions (Mapelli-Brahm et al., 2020). For instance, spontaneous fermentation reduced vitamin A and carotene levels in broccoli, cucumber, and pepper, but elevated their concentrations in carrot and red beet (Kiczorowski et al., 2022). Pasteurized carrot juices fermented by *L. gasseri* strains, DSM 20604 or DSM 20077, had 5 or 10-fold higher carotenoids content compared to unfermented juices (Xu et al., 2020). Additionally, lacto-fermentation of FVs are generally known to increase the concentrations of group B vitamins (e.g., vitamin B9/folate, vitamin B2/riboflavin, vitamin B12/cobalamin) and vitamin K (phylloquinone and menaquinones) compared to the raw ingredients through strain-specific biosynthesis of these vitamins by LAB species such as *L. plantarum* (Jägerstad et al., 2004; Thompson et al., 2020), *L. lactis*, *Leuconostoc* spp. (Abdul Hakim et al., 2023; Knez et al., 2023; Paramithiotis et al., 2022; Szutowska, 2020; Walther & Schmid, 2017).

Raw fruits and vegetables are also significant sources of minerals, including macronutrients like phosphorus, potassium, magnesium, sodium, and calcium as well as micronutrients like iron, selenium, copper, manganese, and zinc (Kiczorowski et al., 2022; Paramithiotis et al., 2022; Septembre-Malaterre et al., 2018). The mineral contents (e.g., potassium, calcium, iron, magnesium, zinc) in FVs are generally found to be lower after lacto-fermentation compared to the raw ingredients (Kiczorowski et al., 2022; Knez et al., 2023), except for sodium, which is elevated after fermentation and is associated with the addition of salt during fermentation (Kiczorowski et al., 2022). Despite the reduction in mineral content, it was noted that bioavailability can be improved due to the microbial production of phytases and the subsequent breakdown of anti-nutritional factors, like phytic acid (phytates), which form insoluble and non-digestible complexes with divalent cations (Mukherjee et al., 2024; Samtiya et

al., 2021). Additionally, the inclusion of a mixture of lacto-fermented vegetables in the diet of healthy volunteers (n = 17) four consecutive days was associated with increased iron absorption and the authors noted this could be potentially due to the increased levels of hydrated ferric iron (Fe^{3+}) after lactic acid fermentation (Scheers et al., 2016).

Glucosinolates are a group of plant secondary metabolites that can be found in cruciferous vegetables, including broccoli, cabbage, and cauliflower (Prieto et al., 2019), which are frequently the substrates of lactic acid fermentation (e.g., sauerkraut, kimchi). While the transformation and breakdown process of glucosinolates begins with the enzymatic activity of plant-associated myrosinases, glucosinolate degradation is enhanced by LAB during fermentation. The types and concentrations of these break down products are related to the glucosinolate concentration and type in the raw matrices and the capacity of the strains that drive the fermentation (Mullaney et al., 2013; Palani et al., 2016). Glucobrassicin (31.95 ± 3.76 µmol/100 g FW) and sinigrin (24.21 ± 0.11 µmol/100 g FW) were found to be the most abundant glucosinolates in shredded cabbage (Ciska & Pathak, 2004). For example, during cabbage fermentation, glucobrassicin and sinigrin, the most abundant glucosinolates in cabbage, were broken down into the glucobrassicin derivatives, ascorbigen, indole-3-carbinol, and indole-3-acetonitrile and sinigrin modified to allyl isothiocyanate, allyl cyanide, and 1-cyano-2,3-epithiopropane (Ciska et al., 2021; Ciska & Pathak, 2004; Mullaney et al., 2013; Šalić & Šamec, 2022). These products of glucosinolate decomposition have been associated with anti-carcinogenic (e.g., isothiocyanates, sulforaphane, ascorbigen) (Hallmann et al., 2017; Higdon et al., 2007; Mitsiogianni et al., 2019; Quirante-Moya et al., 2020; Wagner & Rimbach, 2009), antioxidant, and anti-inflammatory properties (Palani et al., 2016; Shahbazi et al., 2021).

Lastly, fatty acids in lacto-fermented FV are of interest for their potential health modulatory properties. Total fat levels were found to be reduced in lacto-fermented beetroot and red cabbage compared to the ingredients (Knez et al., 2023) while the fat contents in broccoli, cucumber, and pepper were elevated after fermentation (≥ 1.4-fold) (Kiczorowski et al., 2022). Fermentation of sterilized mixed vegetable juices with *L. plantarum* HY7712 or HY7725, *L. helveticus* HY7801, or *Bifidobacterium animalis* ssp. *lactis* HY8002 resulted in distinct lipidomic profiles, generally increasing triacylglycerol, phosphatidylglycerol, phosphatidylinositol, phosphatidylethanolamine, and phosphatidylserine levels compared to unfermented juices (Chung et al., 2020). Fermentation of avocado fruit puree with *L. plantarum* AVEF17 resulted in lower levels of oleic and linoleic acids and elevated levels of their mono-, di-, and tri-hydroxy-octadecenoic acid derivatives compared to before fermentation (Filannino et al., 2020). Lipolytic capacity was also noted during fermentation of walnut by strains of *L. plantarum*, *Weissella cibaria*, *Leuconostoc mesenteroides*, *Enterococcus faecalis*, or other lactobacilli, where levels of free fatty acids (linoleic, α-linolenic, palmitic, and oleic acids) and their hydroxylated or epoxidized derivatives, diacylglycerols and monoacylglycerols (from triacylglycerol hydrolysis) were generally higher after fermentation (Fiorino et al., 2023). While the lipid contents of lacto-fermented FVs and their bioconversion into bioactive fatty acids by LAB driving the fermentation require further investigation, studies involving some hydroxyl- and oxo-fatty acids and mono-unsaturated fatty acids (MUFA; e.g., oleic acid), such as found in table olives, have noted their immunomodulatory, anti-inflammatory, and intestinal barrier protective properties (Hira et al., 2018; Hosomi et al., 2020) and positive effects on cardiovascular health (Rocha et al., 2020).

Health benefits associated with the ingestion of live microbes as part of the food matrix

Health benefits associated with the ingestion of commensal (non-harmful) live microbes is an area of research that is receiving new attention (Marco et al., 2020). Unlike probiotics, food-associated live microbes are a more inclusive group of microorganisms for which the strain or the species may not be known. Fermented FVs produced without a pasteurization step can be sources of live (viable) microbe intake. These foods, such as sauerkraut, kimchi, and table olives, contain between 10^3 to 10^8 CFU/ml or CFU/g of LAB and other bacteria and fungi and will vary depending on the type of ferment, sampling period, and other factors (Rezac et al., 2018; Thierry et al., 2023).

Associations of live microbe intake and health were made after categorizing the foods in the National Health and Nutrition Examination Survey (NHANES) (2001-2018) dietary recall database to contain either low ($<10^4$ CFU/g), moderate (10^4 to 10^7 CFU/g), or high ($>10^7$ CFU/g) numbers of microbes (Marco et al., 2022). This analysis revealed that FVs typically contain < 10^6 CFU/g, but after accounting for the amounts (approximately 40 g per day) consumed on average, are two of the top three food groups providing live microbes to our diets along with fermented dairy (Marco et al., 2022). Regression analyses found that each 100-g increase in foods with medium to high levels of live microbes was associated with reduced systolic blood pressure, plasma glucose, C-reactive protein, insulin, and triglyceride levels and increased HDL (Hill et al., 2023). Intake of foods with live microbes was also associated with improved anthropometric measurements, like reducing waist circumference and BMI (Hill et al., 2023). A similar result was found when fermented foods were selectively examined, but significance was lost for C-reactive protein and plasma glucose levels (Hill et al., 2023). Other studies are similarly finding benefits with dietary live microbe intake, including lower prevalence of

cardiovascular disease (e.g., stroke and heart attack) and improved cardiovascular health (Han & Wang, 2022; Wang et al., 2024) and better cognitive performance (Lv et al., 2024), among other outcomes (Fan et al., 2024; Huo et al., 2023; Tang et al., 2024; Zhou et al., 2024).

Indications that the microbes in lacto-fermented FVs can affect health are evident from studies of bacteria isolated from kimchi (H. J. Kim et al., 2023; Lee et al., 2011), fermented cucumber (Anandharaj et al., 2015), and other fermented vegetables (Liu et al., 2021). Studies examining the potential benefits of bacteria from lacto-fermented vegetable products, like sauerkraut, table olives, Vietnamese fermented radish leaves, have been reviewed (Torres et al., 2020). Obese mice fed cactus pear juice inoculated with *L. plantarum* S-811 gained less weight and exhibited better glucose tolerance compared to mice fed *L. plantarum* S-811 alone (Verón et al., 2019). For kimchi, *Latilactobacillus sakei* WIKIM31 (S. S. Park et al., 2021) and *L. plantarum* strains DSR M2 and DSR 920 (J. Lee et al., 2018) exerted anti-inflammatory and anti-obesogenic effects in DIO mice. Similarly, *Leuconostoc kimchii* GJ2 showed the capacity to reduce serum and liver cholesterol levels in rats fed a high cholesterol diet (Lee et al., 2008). Furthermore, administration of kimchi supplemented with *L. kimchii* GJ2 exerted improved cholesterol-lowering effects (e.g., lower LDL-cholesterol and liver total cholesterol) compared to kimchi without this starter culture strain in rats fed a high-fat and high-cholesterol diet (Jo et al., 2015).

Although the microbes associated with fermented FVs are not equivalent to probiotics, they may still produce effector compounds that modulate host immune and epithelial responses (Sanders et al., 2018). These shared traits may allow the yet-to-be clinically proven fermented food-derived strains to grant some of the same health benefits (Sanders et al., 2018). Studies on the supplementation (e.g., using the food matrix as a delivery method) or the production of

fermented FV products via verified probiotic strains and the clinical confirmation of their health promoting properties are still scarce. To this regard, the "probiotic fermented foods" category requires additional evidence for the well-characterized probiotic strain and its derived health benefit obtained from randomized and controlled intervention studies (Marco et al., 2021). These clinical studies should verify the safety of the probiotic strain in the food matrix and that it can reach and sustain sufficient viable cell counts until the end of the product shelf-life to exert the claimed health benefits. Also, for the development of these probiotic fermented FVs, more investigations on metabolic activity, bioactive molecule production, and niche establishment in specific food matrices, processing conditions, and the final product sensory qualities are needed to ensure the feasibility of these applications.

Health risks and food safety issues with lacto-fermented FVs

While fermentation improves the shelf life and safety of the fresh FVs through the LAB production of organic acids, bacteriocins, and other antimicrobial compounds, lacto-fermented FV are not devoid of health and safety risks. The use of salt brine and the consumption of high salt lacto-FV ferments, such as kimchi, have also garnered some concerns in its potential association with hypertension and gastric cancer. However, analysis of the data collected from the Korea National Health and Nutrition Examination Survey (n = 20,114 Korean adults) (Song & Lee, 2014) and from a 12-year follow up study of the Korean Genome and Epidemiology Study (n = 5,932 participants) (Song et al., 2017) found no association between intake of salt-fermented vegetables like kimchi with increased risk of hypertension. The association of salt brine fermented vegetables (e.g., kimchi) with gastric cancer risk has been inconsistent (J. H. Kim et al., 2023). Multiple interacting factors can influence the potential risk of gastric cancer

associated with salt fermented vegetable intake in Korean population, including genetic disposition (Nan et al., 2005), the type of kimchi ferment (Kim et al., 2002), dietary intake of other high sodium-contributing food dishes (e.g., soups) (Wu et al., 2021). A case-control study found that the intake of moderate level of pickled vegetables, like kimchi, tended to lower the risk of gastric cancer (Kwak et al., 2021).

Several outbreaks of pathogenic *Escherichia coli* (e.g., STEC O157, ETEC) in kimchi have been noted in South Korea (Cho et al., 2013; Shin et al., 2016), Japan (Nishikawa et al., 1995; Ozeki et al., 2003) and Canada (Smith et al., 2023). The major microbial safety concerns with fermented FVs arises from the poor quality of starting ingredients, improper handling and sanitation of equipment, or contamination during fermentation or processing, leading to the growth of spoilage or pathogenic microorganisms. Although these cases have been linked to ferments from retail and food supply chains, there is a scarcity of foodborne illness reporting and tracking for home fermentations, which will encounter more variations during preparation, fermentation, and storage. While there is a myriad of information available online related to small-scale production methods (recipes) and in-person classes around the country, there is a concern for the accuracy of that information since the authors of most of these materials were not trained food safety specialists or microbiologists (Ramos et al., 2022).

Conclusions and next steps

Lacto-fermented FVs are staples of diets around the world. They are relatively easy to make, requiring no highly technical specialized equipment, and only a few ingredients, but result in expanding dietary diversity and contribute to food sustainability by reducing FV waste through their preservation properties. Although the microbial composition is generally

understood for many of these foods, there remain fundamental questions about microbial community dynamics in many traditional lacto-FV ferments. Application of metagenomics, transcriptomics, and other methods (Lee et al., 2017; Leech et al., 2020; Walsh et al., 2023; Wuyts et al., 2020) will be useful for untangling the complex interactions that occur during the fermentation process. For those foods that have been studied for effects on health, they were found to have positive impacts towards metabolic (e.g., anti-obesogenic, anti-diabetic) and immune (e.g., anti-inflammatory) functions and the reduction of oxidative stress. Many of these benefits can be attributed to synergistic effects of the exceptional nutritive characteristics of the FV matrices and phytochemicals, the microbially-derived bioactive metabolites, and the beneficial live microbes that associate with the raw material to produce the fermented products. With this expanding interest in lacto-fermented FVs and its production at home and in production facilities, it is also crucial to follow the appropriate practices to ensure the microbial food safety of the final products during starting ingredient selection, handling and sanitation, and processing.

Ultimately, RCTs investigating lacto-fermented FVs and their consumption in different populations and patient groups are needed. While the studies discussed in this review provide enticing support for health promoting properties of whole fermented FVs and their bioactive constituents, they also highlight the need to quantify the levels of these bioactive compounds and microbes and their bioavailability during GI transit and to determine whether the ingested quantities and duration are sufficient to exert the proposed physiological benefits. Furthermore, more investigations are necessary to uncover the underlying mechanisms of how fermented FV consumption leads to health benefits, such as improvements in the symptoms of inflammatory conditions and metabolic disorders. Future research is also needed to distinguish the relative

importance of different components of fermented FVs, whether live microbes, microbe-derived bioactive molecules, plant-derived nutrients, or other aspects, on the overall benefits.

With the increased bioactive compound content and live microbe numbers in lacto-ferments, it is feasible for fermented FV products to serve as a dietary source of these bioactive constituents and other nutrients as well as beneficial LAB and non-LAB live microbes. To further augment the nutritional and bioactive qualities of these ferments, more research is needed to develop and apply specific starter culture strains with the capacities to synthesize these desirable bioactive compounds during fermentation in the FV environments. Strain development has already started, for example, with the autochthonous LAB strains isolated from fermented cabbage with high GABA (Chen et al., 2015; Kim & Kim, 2012) or PLA (Sun et al., 2019; Zhang et al., 2022) production in culture medium, which will require additional investigation of their production capacity during FV fermentation.

Moreover, selected LAB strains may also be utilized to supplement bioactive molecules that are not enriched during spontaneous fermentation of FVs, for instance, in the case of conjugated linoleic acids (CLA). CLA isomers are commonly found in meat and dairy products (Shokryzadan et al., 2017), however, their levels in these foods are lower than the effective dose needed for exerting anti-obesogenic, cardio-protective, and anti-diabetic effects (Fuke & Nornberg, 2017). With additional ingredient and fermentation condition optimization, LAB strains with the required enzymatic pathways may be applied to enrich CLA in fermented FVs. Finally, careful selection of starter cultures can also be applied to reduce the production of undesirable compounds, like biogenic amines, during fermentation.

Increasing consumer demand for lacto-fermented FVs (Manskar & Raskin, 2020) propelled an elevated prevalence of home food fermentations (Holmes, 2020; Prince, 2018;

Snyder, 2020) and the expansion of small-scale processors in the food industry, especially since the COVID-19 pandemic (Ramos et al., 2022). Analyses of large cohort and longitudinal data are needed to understand the association of gastric cancer risk and the intake of various lacto-ferments in different populations. There is a recognized need for the development of reliable, experimentally tested, and "approved" fermentation recipes and practices for different types and mixtures of FV substrates, providing centralized guidance and resources for making lacto-fermented FVs at home and by small-scale producers (Ramos et al., 2022). Furthermore, lacto-ferment recipe development can assess the impact of lower salt content on the microbial dynamic and safety of the ferments. These fermentation practices and Good Manufacturing Practices (GMPs) need to be carefully performed before and throughout fermentation, whether at home or in the production facilities, to prevent the introduction of foodborne pathogens and spoilage microbes and to ensure the microbiological food safety of these lacto-ferments.

Together, while more development in the starter culture selection, product flavor and sensory testing, and shelf stability assessment (e.g., viable bacterial counts, bioactive compound stability) as well as experimentally tested recipes and fermentation resources are needed, consuming safely prepared lacto-fermented FVs does serve as a promising strategy to improve and sustain human health and well-being.

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Table 1.1. Phenolic compounds, flavonoids, and antioxidant capacities in lacto-fermented FVs.

Type of ferment	Strains	TPC/TFC/AC[a] after fermentation	Specific compounds after fermentation	Bioactivity	Reference
Fermented Fruit Juices					
Jujube-wolfberry juice	*L. plantarum* LP9-2	TPC ↑[b] TFC ↑	Gallic acid ↑ Rutin ↑	ND	(Zhao et al., 2023)
Goji berry juice	*Lacticaseibacillus paracasei* E10 *L. paracasei* JN-1 *L. paracasei* JN-8 *L. plantarum* M *L. rhamnosus* LGG		Chlorogenic acid (E10) ↑ (M) ↓ Rutin (M, JN-1, JN-8, LGG) ↓ *p*-Coumaric acid (M) ↓	ND	(Duan et al., 2023)
Dragon fruit-kiwi juice	*L. mesenteroides* DR125 *Streptococcus thermophilus* DR124	TPC and TFC	ND	Increased life span, oxidative and heat stress tolerance in *C. elegans*	(Tang et al., 2022)
Cherry juice	*L. plantarum* spp.	ND	Caffeic acid ↓ Dihydrocaffeic acid ↑ *p*-Coumaric acid ↓ Phloretic acid ↑	ND	(Filannino et al., 2015)
Fermented Fruits					

Guava fruit	*L. plantarum* NCIM 2912	TPC ↑	ND	ND	(Bhat et al., 2015)
Jujube puree	*Streptococcus thermophilus* CICC 6220	ND	Catechin ↑ Epicatechin ↑ *p*-Coumaric acid ↑ Caffeic acid ↑	ND	(X. Pan et al., 2023)
Jussara fruit pulp	*Bifidobacterium dentium* ATCC 27534 *B. lactis* CCT 7503 *L. acidophilus* ATCC 4356 *L. delbrueckii* ATCC 4796 *L. fermentum* ATCC 15442 *L. paracasei* ATCC 335 *L. plantarum* ATCC 10012 *L. rhamnosus* ATCC 7469 *L. brevis* ATCC 367	AC ↑	ND	ND	(Braga et al., 2018)
Table olives	None	TPC and AC ↑	Oleuropein ↓ Hydroxytyrosol ↑	Reduced intracellular ROS levels (Caco-2 cells)	(D'Antuono et al., 2018) (Charoenprasert & Mitchell, 2012)
Fermented Vegetables and Juices					
Curly kale juice	None	TPC and AC ↑	ND	ND	(Szutowska et al., 2020)
Red, yellow, green bell peppers	*L. plantarum* ATCC 4080	TPC and AC ↑	ND	ND	(Janiszewska-Turak et al., 2022)
White cabbage sauerkraut	None	TPC and AC ↑	ND	ND	(Ciska et al., 2005)

Red cabbage sauerkraut	*L. plantarum* ATCC 8014 (LP) *L. acidophilus* NCFM (LA) Controls	TPC and AC	4-hydroxybenzoic acid Caffeic acid Sinapic acid	ND	(Hunaefi et al., 2013)
Broccoli puree	*L. reuteri* FUA3168 *L. plantarum* C2 *L. plantarum* C5 *L. fermentum* FUA3165	ND	Chlorogenic acid (FUA3168) ↓ Quinic acid (C2, C5, FUA3165, FUA3168) ↓	ND	(Filannino et al., 2015)
Mixed vegetable juice	*Companilactobacillus allii* WiKim39 *Lactococcus lactis* WiKim0124	TPC (WiKim39 and WiKim0124) ↑ TFC (WiKim39) ↑ TFC (WiKim0124) ↓ AC (WiKim3 and WiKim01249) ↑	ND	ND	(M. Lee et al., 2021)

[a] Total phenolic content (TPC), total flavonoid content (TFC), antioxidant capacity (AC)

[b] An arrow indicates significant increases or decreases in TPC, TFC or AC. The lack of an arrow indicates TPC, TFC, and/or AC were measured but statistical tests were not applied.

GABA: γ-aminobutyric acid; PLA: phenyl-lactic acid; ILA: indole-3-lactic acid.

ND: Not determined

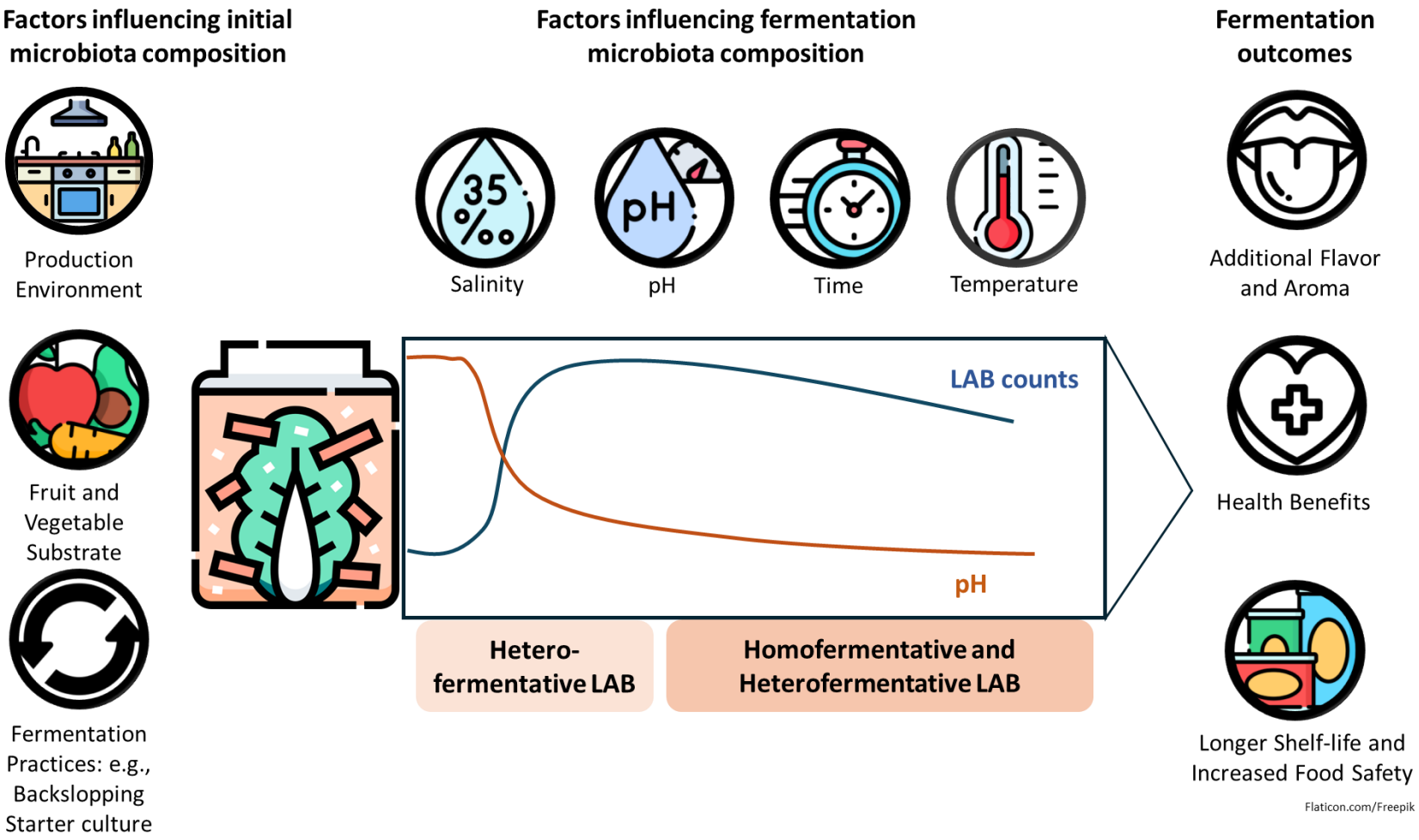


Figure 1.1. Factors influencing microbiota composition of vegetables and fruits and their lacto-fermentation.

Initially fruits and vegetables microbiota include members of Pseudomonadota and yeasts. As the lacto-fermentation progresses, a microbial succession occurs from heterofermentative lactic acid bacteria (LAB) to homofermentative LAB.

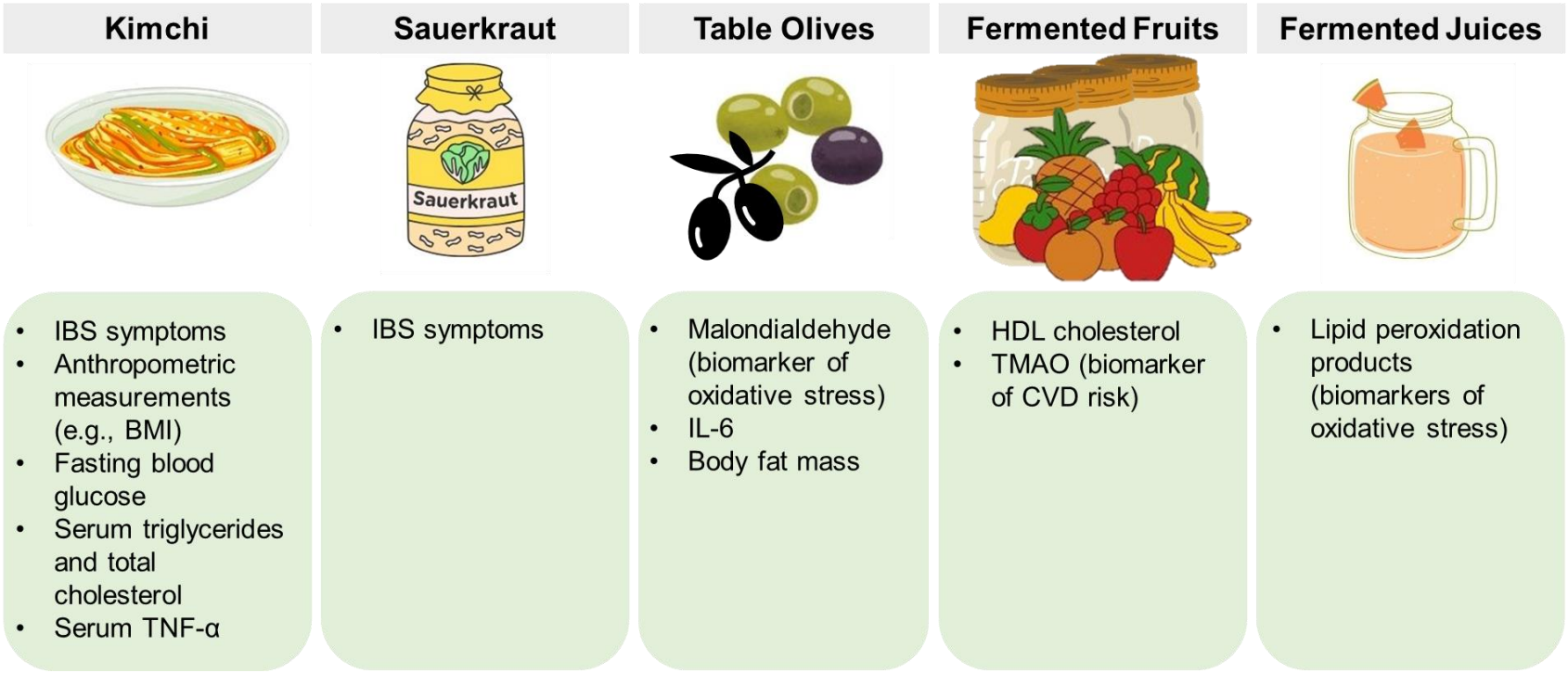


Figure 1.2. Health outcomes measured for fermented FVs in at least one RCT.

Improvements in these outcomes were found in at least one study. IBS: Irritable Bowel Syndrome. BMI: body mass index. HDL: high density lipoprotein. TMAO: trimethylamine-N-oxide. CVD: cardiovascular disease.

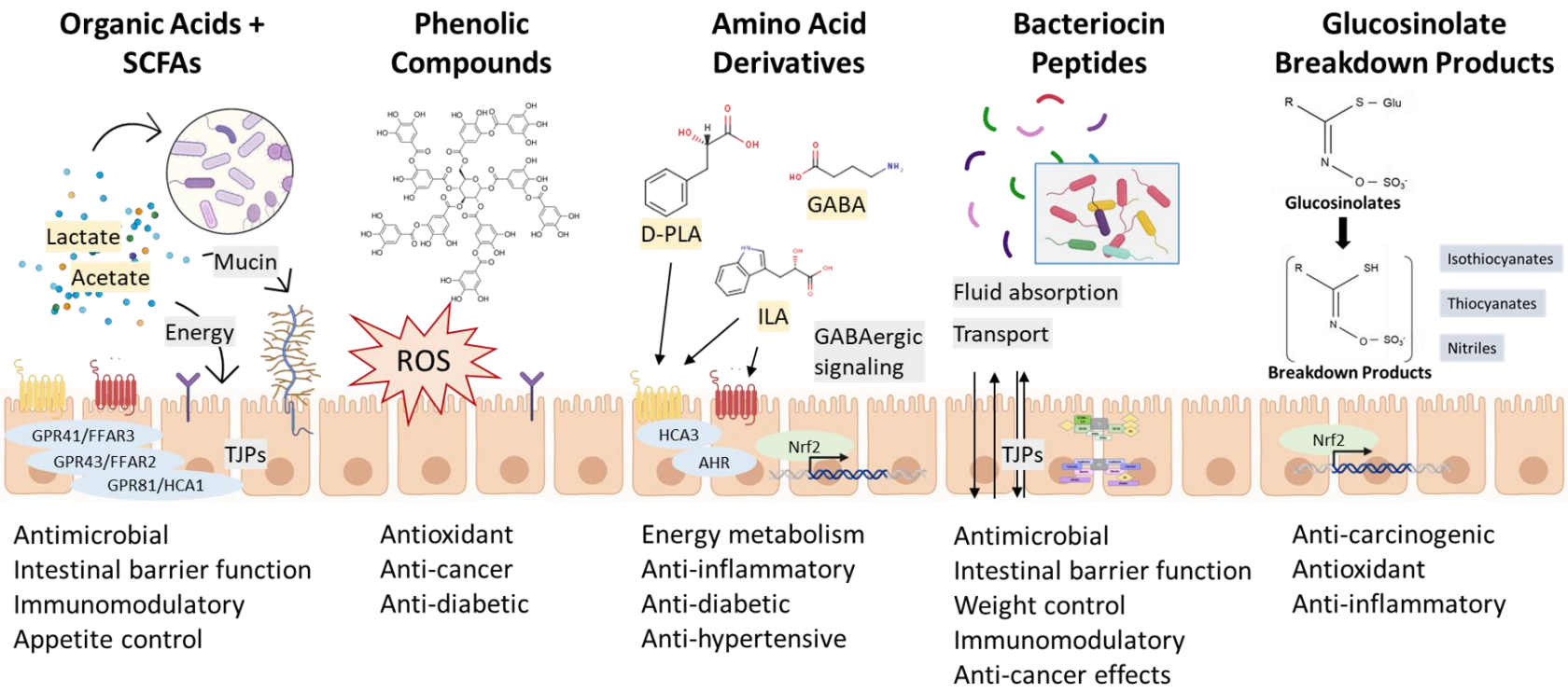


Figure 1.3. Overview of potential health promoting properties exhibited by bioactive compounds from lacto-fermented fruits and vegetables.

SCFAs: short chain fatty acids. GPR: G protein coupled receptor. FFAR: free fatty acid receptor. TJPs: tight junction proteins. ROS: reactive oxygen species. GABA: γ-aminobutyric acid. D-PLA: D-phenyllactate. ILA: indole-3-lactate. HCA: hydroxycarboxylic acid receptor. AHR: aryl hydrocarbon receptor. Nrf2: NF-E2-related factor 2. Components of the figure from BioRender.com.

Chapter 2. Metabolites in fermented cabbage protect against pro-inflammatory cytokine induced intestinal barrier disruption

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Fermented Food, Probiotic, Intestinal Barrier Function, ILA, PLA, GABA

Abstract

Fermented vegetables, such as fermented cabbage (sauerkraut), have garnered growing interest for their associations with a myriad of health benefits. However, the mechanistic details underlying the outcomes of consuming the whole ferments require further investigation. Therefore, this study aimed to examine the intestinal bioactivity of laboratory scale and commercial sauerkraut homogenates in polarized Caco-2 monolayers perturbed by pro-inflammatory cytokines (IFN-γ and TNF-α). Functional trans-epithelial electrical resistance (TER) and paracellular permeability to fluorescein isothiocyanate (FITC)-dextran measurements of Caco-2 monolayers revealed that fermented cabbage, but not cabbage, alleviated the near 50% reduction in TER and 3-fold higher permeability to FITC induced by IFN-γ and TNF-α. Furthermore, to assess the feasibility of using an allochthonous, probiotic LAB strain for cabbage fermentation, *Lactiplantibacillus plantarum* NCIMB8826R (LP8826R) (5×10^7 cells/g) was added to some of the cabbage fermentations. LP8826R numbers reached 86.50 ± 25.43% of the total lactic acid bacteria (LAB) numbers in the ferments with 72 h, indicating that LP8826R was able to thrive in that environment. Metabolomic analyses of the (fermented) cabbage homogenates uncovered distinct metabolite profiles between cabbage, laboratory scale, and commercial ferments and are enriched in bioactive compounds like D-phenyl-lactate (D-PLA), indole-3-lactate (ILA), γ-aminobutyric acid (GABA), 2-hydroxyisocarpoic acid (HICA), 3,4-dihydroxyhydrocinnimic acid (DHCA). Three selected fermentation-derived sauerkraut metabolites, lactate, D-PLA, and ILA, protected Caco-2 monolayers against IFN-γ and TNF-α induced damage. These results suggest that LAB-derived metabolites during sauerkraut fermentation likely exert a synergistic effect to contribute to the overall intestinal bioactivity of the whole ferment.

Introduction

Fermented vegetables have been staples of the human diet for thousands of years (Medina-Pradas et al., 2017). Besides imparting distinct organoleptic characteristics to the foods, the process of fermentation improves the preservation and safety of easily perishable and highly nutritive raw vegetables through microbial production of organic acids, bacteriocins, alcohols, and other antimicrobial fermentation end products (Marco et al., 2021; Ross et al., 2002). Recently, human and model animal studies have shown that fermented vegetables can benefit human health (Garnas, 2023; Nielsen et al., 2018; Raak et al., 2014; Shahbazi et al., 2021; Song et al., 2023). However, the mechanistic details underpinning these outcomes remain to be determined.

Fermented cabbage, here referred to as sauerkraut, is a popular fermented vegetable widely consumed in the U.S. and across the world (Di Cagno et al., 2016). It is made by mixing shredded cabbage with 2-3% (w/w) sodium chloride and incubating the mixture with minimal exposure to ambient oxygen levels at room temperature for two to three weeks (Medina-Pradas et al., 2017). During a cabbage fermentation, the lack of oxygen and presence of salt selects for the growth of lactic acid bacteria (LAB). LAB encompass a group of phylogenetically related, Gram-positive microorganisms that are traditionally grouped together based on their capacity to utilize sugar and produce lactic acid as the only or one of the main (> 50%) end products of their energy conservation metabolism, respectively (George et al., 2018; Wang et al., 2021; Zheng et al., 2020). Culture-dependent and -independent analyses have shown that the dominant LAB in sauerkraut change over time, with heterofermentative species, such as *Leuconostoc mesenteroides* and *Weissella* spp. starting the fermentation, followed by *Lactiplantibacillus plantarum* and *Levilactobacillus brevis* for the later stage of the fermentation (Einson et al.,

2018; Gaudioso et al., 2022; Medina-Pradas et al., 2017; Plengvidhya et al., 2007; Xiong et al., 2012; Yang, Hu, Xiu, Jiang, Yang, Saren, et al., 2020; Zabat et al., 2018). LAB transform the sugars and other nutrients in the cabbage matrix into lactic acid/lactate, acetic acid/acetate, mannitol, CO_2, as the primary end products of heterofermentative and homofermentative metabolism (Juana Frias, 2017; Septembre-Malaterre et al., 2018).

Cabbage fermentation by LAB changes the bioactive properties of the cabbage foods by in increasing phenolic compound concentrations (polyphenols, phenolic acids) (Hunaefi et al., 2013; Kaulmann et al., 2014), carotenoids (Kaulmann et al., 2014), glucosinolate breakdown products (ascorbigen, indole-3-carbinol, isothiocyanates) (Krajka-Kuźniak et al., 2011; Martinez-Villaluenga et al., 2012), and other bioactive metabolites (Siddeeg et al., 2022), including phenyl-lactic acid (PLA; 90 to 460 µM; Peters et al., 2019; 20 – 30 µg/mL; Kasperek et al., 2024; Peters et al., 2020), indole-3-lactic acid (ILA; 0.1 – 2.8 µg/ml; Kasperek et al., 2024), and γ-aminobutyric acid (GABA; 43.5 – 200 mg/L; Yang, Hu, Xiu, Jiang, Yang, Saren, et al., 2020; Yang, Hu, Xiu, Jiang, Yang, Sarengaowa, et al., 2020). Together, these bioactive constituents contribute to the associated immunomodulatory (Huang et al., 2020; Siddeeg et al., 2022; Zubaidah et al., 2020), antioxidant (Krajka-Kuźniak et al., 2011; Xu et al., 2022), and anti-carcinogenic (Juana Frias, 2017; Raak et al., 2014) properties of sauerkraut, as investigated by *in vitro* and *in vivo* studies. The capacity of sauerkraut to improve symptoms of irritable bowel syndrome (IBS) was studied in a randomized, double-blinded intervention pilot study (Nielsen et al., 2018). This study found that both pasteurized and unpasteurized sauerkraut significantly improved IBS symptoms ($p < 0.04$) and led to changes in the fecal microbiota of the patients, suggesting that the observed beneficial effect may be attributed metabolites present in the sauerkraut beyond the live microbiota (Nielsen et al., 2018).

To better understand the potential of fermented cabbage to impact intestinal function, we evaluated the potential of cell-free preparations of cabbage and commercial and laboratory-scale sauerkraut fermentations to protect against pro-inflammatory cytokine-induced damage in a polarized model with Caco-2 cells. Some laboratory fermentations also incorporated the use of a probiotic strain of *L. plantarum* NCIMB8826R (LP8826R). This strain was shown to induce intestinal epithelial repair (Crakes et al., 2019; Hirao et al., 2014) and exert anti-inflammatory (Yin et al., 2018) and anti-obesogenic (Heeney et al., 2018) effects *in vivo*. To begin to understand the differences between cabbage and fermented cabbage, metabolites were quantified using targeted and untargeted metabolomics. Based on those findings, lactate, D-PLA, and ILA, were investigated for barrier-protective capacities in the Caco-2 model.

Materials and Methods

Bacterial strain and growth conditions

The isolation of the rifampicin-resistant mutant *L. plantarum* NCIMB8826R (LP8826R) was described previously (Yin et al., 2018) and the strain has been maintained in glycerol at -80 °C. LP8826R was routinely grown at 37 °C in a commercial preparation of lactobacilli de Man, Rogosa and Sharpe (MRS) medium (Becton-Dickinson, Franklin Lakes, NJ, USA). When appropriate, 50 µg/ml rifampicin (Thermo Fisher Scientific, Waltham, MA, USA) was added to the media for enumeration. For inoculation into sauerkraut fermentations, LP8826R was incubated in MRS for 24 h at 37 °C. Cells were collected by centrifugation at 9,000 x g for 3 min at room temperature, washed twice in sterile phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4*7H_2O$, 1.4 mM KH_2PO_4, pH 7.2; Fisher Scientific, Waltham, MA, USA), and then suspended in sterile PBS.

Laboratory-scale cabbage fermentations

Green cabbages (*Brassica oleracea* var. capitata), purchased from local grocery stores, were shredded using a grater, mixed by hand with 2% canning salt (w/w), and spooned into 28 oz WhirlPak bags with 2% (w/w) brine. The mixtures were divided into six containers and incubated at 22 °C. After 3 days, LP8826R in PBS was added to half of the containers (n =3) in 1.75 to 2 ml to reach 5 x 10^7 CFU/g. The same volume of PBS was added to the other laboratory ferments. The experiment was performed twice, once with a total fermentation time of 14 days and another for 21 days. Brine pH (SevenEasy pH meter (Mettler-Toledo, Columbus, OH, USA) and salinity (SPER Scientific, Scottsdale, AZ, USA) were measured for the first seven days and then after 14 and 21 days.

Commercial sauerkraut

Containers (n = 6) of commercial sauerkraut from the same brand were purchased from local supermarkets between December 2021 and October 2022 (**Table 1**). They were stored at 4 °C and sampled within 3 days of purchase.

Intestinal epithelial cell culture maintenance

The human intestinal Caco-2 cells ATCC HTB-37, purchased from the American Type Cell Culture (Rockville, MD, USA), were grown in DMEM as previously described (Hubatsch et al., 2007). Cell culture reagents, Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), sodium pyruvate (100X), nonessential amino acids (100X), GlutaMax (100X), penicillin/streptomycin 10,000 U/ml, Trypsin-EDTA (0.25%, 1X), and sterile Dulbecco's

phosphate buffer saline (D-PBS, pH 7.4, no calcium, no magnesium), were obtained from Gibco (Life Technologies, Carlsbad, CA, USA). Briefly, the Caco-2 cells were incubated in DMEM containing 20% FBS, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 2 mM GlutaMax, and 1% v/v penicillin/streptomycin at 37 °C in 10% CO_2, split using Trypsin-EDTA, and passaged in 75 cm^2 culture flasks (Fisher Scientific, Waltham, MA, USA) until they were ready for seeding.

Bacterial enumeration and cell-free preparation of (fermented) cabbage homogenates

Sauerkraut solids (about 10 g) and brine (1 ml) were homogenized in a filtered WhirlPak bag with a rubber mallet. The sauerkraut homogenate was serially diluted in PBS and plated on MRS + 25 µg/ml natamycin agar for LAB enumeration and on MRS + 25 µg/ml natamycin + 50 µg/ml rifampicin agar for LP8826R enumeration.

For cell culture experiments, the homogenate was centrifuged at 10,000 x g for 10 min at 4 °C to remove debris. The homogenate supernatant was pH adjusted to 7.4 using 1M or 6M sodium hydroxide (NaOH) and then passed through a 0.22 µm surfactant free cellulose acetate (SFCA) filter (Globe Scientific, Mahwah, NJ, USA). To first establish a suitable range of fermented cabbage homogenate for assessment on Caco-2 cell monolayers, Caco-2 cell culture medium (DMEM) was adjusted to contain 1, 10, or 50% (v/v) of the fermented cabbage homogenate supernatants. A 2.91% (w/v) solution of NaCl was made to match the salinity of commercial sauerkraut homogenate after pH adjustment (29.1 ppt) and was used to examine the impact of salt on the Caco-2 cell monolayers. The homogenate supernatants and the salt solution were then diluted in DMEM containing supplements (1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 2 mM GlutaMax) and stored at -80 °C.

Lactate, ILA, and D-PLA preparation for cell culture

The three metabolites selected for cell culture experiments, DL-lactic acid (W261106-1KG-K), DL-indole-3-lactic acid (ILA; I5508-250MG-A), D-(+)-3-Phenyllactic acid (D-PLA; 376906-5G), were obtained from Sigma-Aldrich (St. Louis, MO, USA). A 10X solution, based on the final concentration intended for use in the cell culture experiment, was prepared for each metabolite in water (lactate, D-PLA) or 0.052% (v/v) DMSO (ILA) and a mixture of all three metabolites. The 10X solutions of D-PLA and ILA were sonicated for 10 min at 40 kHz in a Branson 8510 Ultrasonic Cleaner bath (Branson Ultrasonics Corp., Danbury, CT, USA) to fully dissolve the respective metabolite. The solutions were then pH adjusted to 7.40 ± 0.04 with NaOH and diluted in DMEM containing supplements (1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 2 mM GlutaMax) to 50 mM lactate, 60 µM D-PLA, 25 µM ILA in 0.0052% (v/v) DMSO, and a mixture of all three compounds at the indicated concentrations, sterile filtered through 0.22 µm SFCA filter, and stored at -80 °C.

Intestinal epithelial monolayer cytokine stimulation

To form a monolayer, Caco-2 cells between passages 35 and 36 were seeded onto polycarbonate membranes in Transwell inserts (0.33 cm^2 surface area, 6.5 mm, 0.4 µm pore size, 24 wells; Corning, NY, USA) at a density of 1 x 10^4 cells/cm^2. After incubation for at least 21 days, the cells were fully differentiated and formed polarized monolayers with intercellular tight junction structures. Trans-epithelial electrical resistance (TER) was measured after 15 min equilibration at room temperature with an epithelial voltohmmeter (World Precision Instruments,

Sarasota, FL, USA) equipped with a STX-2 “chopstick” electrode. Caco-2 monolayers with an initial TER of more than 250 Ohm·cm^2 were used for the subsequent experiments.

Prior to the application of either the (fermented) cabbage homogenates or compounds, TER values were measured. Cabbage homogenates (10% v/v in DMEM), 50 mM lactate, 25 µM ILA, 60 µM D-PLA, a mixture of the three in DMEM containing supplements, or sterile water or 0.0052% DMSO in DMEM containing supplements were applied only to the apical side of the monolayers. After 3 h incubation at 37 °C, 100 ng/ml IFN-γ (R&D Systems, Minneapolis, MN, USA) was added to the basolateral compartment. The medium (DMEM with supplements) of the basolateral compartment was replaced 21 h later and supplemented with 10 ng/ml TNF-α (R&D Systems, Minneapolis, MN, USA). TER values of each monolayer were measured right before TNF-α addition and 24, 32, and 48 h after TNF-α stimulation.

Paracellular permeability assay

Paracellular permeability was determined by the flux of fluorescein isothiocyanate-dextran (FITC; 4 kDa; Sigma-Aldrich, St. Louis, MO, USA) through differentiated Caco-2 monolayers as previously described (Zhai et al. 2019). After 48 h stimulation with TNF-α, the apical side contents were replaced with DMEM with supplements containing FITC (2 mg/ml). After 30, 60, 90, and 120 min, FITC concentration on the basolateral side was quantified by measuring the fluorescence intensity (485 nm excitation and 530 nm emission) in a microplate reader (Synergy-2, BioTek, Winooski, VT, USA). The concentration of FITC was calculated using a standard curve of FITC in DMEM with supplements. The apparent permeability coefficient, P_{app} (cm/s), was determined using the following equation: P_{app} = [dQ/dt] x [1 / (A x

C_0)], where dQ/dt is the quantity of FITC transported per second (ng/sec), A is the surface area of the filter (cm^2), and C_0 is the initial FITC concentration in DMEM on the apical side (ng/ml).

ELISA quantification of IL-8

After 48 h stimulation with TNF-α, spent media collected from the basolateral compartment was used for the quantification of interleukin 8 (IL-8) via ELISA (Life Technologies, Carlsbad, CA, USA). All assays were performed in technical duplicates and according to the manufacturer's instructions.

RNA extraction and reverse-transcription quantitative PCR (RT-qPCR)

After 48 h exposure to TNF-α, total RNA from the Caco-2 monolayers was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and purified using the TRIzol-chloroform protocol. RNA was quantified on the NanoDrop 2000c (Thermo Fisher, Waltham, MA, USA). Quality was assessed on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and found to be intact (RIN 8.8 - 9.8). RNA was reverse transcribed to cDNA using the High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's protocol. RT-qPCR was performed using the Fast SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) on a 7500 Fast Real-time PCR system (Applied Biosystems, Carlsbad, CA, USA). The primers used for RT-qPCR are listed in **Table 2**. Amplification was initiated at 95 °C for 20 sec and was followed by 40 cycles of 95 °C for 3 sec and 60 °C for 30 sec. Primer specificity was assessed using melting curves following the amplification stage. All reactions were performed in duplicates and reactions lacking RT controls for each sample were included to check for DNA contamination. Data were

normalized using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) with *GAPDH* as the housekeeping gene and untreated cells as the reference condition.

Metabolomic analysis of cell-free (fermented) cabbage homogenates

Homogenates from the laboratory fermentations and commercial sauerkraut were prepared for gas chromatography-time of flight mass spectrometry (GC-TOF/MS) and reverse-phase liquid chromatography high resolution tandem mass spectrometry (RP-LC-HRMS/MS) at the West Coast Metabolomics Center (WCMC) at UC Davis (https://metabolomics.ucdavis.edu/) as described previously (Fiehn, 2016; Folz et al., 2023). For GC-TOF/MS, homogenates were extracted using 1mL of 3:3:2 acetonitrile (ACN):isopropyl alcohol (IPA):water (H_2O) (v/v/v). Half of the sample was dried to completeness and then derivatized using 10 µl of 40 mg/ml of methoxyamine in pyridine. They are shaken at 30 °C for 1.5 h, then 91 µl of MSTFA + FAMEs were added to each sample and shaken at 37 °C for 0.5 h to finish derivatization. Samples are then vialed, capped, and injected onto the instrument. For RP-LC-HRMS/MS, samples were extracted using 1 ml of 80:20 methanol (MeOH):H_2O. Samples were vortexed and centrifuged and 450 µl of the supernatant was dried for analysis. Dried samples were resuspended with 100 µl of a solution 75:25 H_2O:ACN containing internal standards (CUDA, D3-L-Carnitine, Val-Tyr-Val, D4-Daidzein, D9-Reserpine, and D5-Hippuric Acid). Samples were then vortexed for 10 seconds, sonicated for 5 minutes at room temperature, and then centrifuged for 2 minutes at 16,000 x g. 60 µl supernatant from each sample was transferred into a LC-MS vial. 30 µl supernatant from each sample was then transferred into an Eppendorf tube and vortexed for use as a pool.

GC-TOF/MS analysis was performed on an Agilent 7890A gas chromatograph (Agilent, Böblingen, Germany) coupled to a Leco Pegasus IV TOF mass spectrometer (Leco, St. Joseph, MO, USA). 0.5 µl of derivatized sample is injected using a splitless method onto a RESTEK RTX-5SIL MS column with an Intergra-Guard at 275C with a helium flow of 1 ml/min. The GC oven is set to hold at 50 °C for 1 min then ramp to 20 °C/min to 330 °C and then hold for 5 min. The transfer line is set to 280 °C while the EI ion source is set to 250 °C. The Mass spec parameters collect data from 85m/z to 500m/z at an acquisition rate of 17 spectra/sec. Raw untargeted GC-TOF/MS data files were preprocessed by Leco ChromaTOF software and further analyzed using the BinBase pipeline (Skogerson et al., 2011) for identifying metabolites by retention index matching and mass spectra alignment. The method was designed for untargeted and targeted primary metabolite detection and quantification, namely for carbohydrates and sugar phosphates, amino acids, hydroxyl acids, free fatty acids, purines, pyrimidines, and aromatics (Fiehn 2016).

RP-LC-HRMS/MS was performed using a Vanquish LC (Thermo Scientific, Waltham, MA, USA) using a Waters Acquity Premier BEH C18 1.7 µm, 2.1 x 50 mm column, coupled to a Q-Exactive HF^+ orbital ion trap mass spectrometer (Thermo Scientific) with positive and negative ion electrospray ionization (ESI) modes with the following parameters: mass range 80−1200 m/z; full scan MS1 mass resolving power 60,000, data-dependent MSMS (dd-MSMS) 2 scans per cycle (4 scans per cycle for pooled MSMS injections), normalized collision energy at 20%, 30%, and 40%, dd-MSMS mass resolving power 15,000. MS-DIAL v. 4.92 (Tsugawa et al., 2020) was used to process untargeted LC-MS/MS data with retention times and MS/MS spectra matching compared to the MassBank of North America (Horai et al., 2010). Using this method, flavonoids, anthocyanins, polyphenols, alkaloids, coumarins, terpene- and polyphenol-

glycosides, kaempferols, flavanones, cinnamates, and related compounds were detected. The information on data acquisition, processing, and raw data normalization can be found in the document shared by the WCMC: https://www.metabolomicsworkbench.org/data/file_retrieve_and_download.php?FILENAME=Data_Dictionary_Fiehn_laboratory_GCTOF_MS_primary_metabolism_10-15-2013_general.pdf (**Supplemental File 1**).

Peak height was reported for all untargeted analyses. Untargeted metabolite data were normalized using the mTIC method by calculating the sum of all peak heights for all identified metabolites (excluding the unknowns) in each sample while targeted data were normalized to the amount of sample injected onto the column. GC-TOF/MS data were provided by WCMC as a table containing identified BinBase name, peak heights, retention index, m/z ratio, PubChem ID, KEGG ID, and InChl Key values. LC-MS/MS data were provided by WCMC as a table containing identified metabolite name, MS-DIAL identifier, peak heights, m/z ratio, ESI mode, InChI key and retention time. A complete list of detectable metabolites using these methodologies can be found at https://metabolomics.ucdavis.edu/metabolites (**Supplemental File 2**).

Statistical analysis

Data were analyzed using GraphPad Prism 10 software for Windows (GraphPad Software, Inc., La Jolla, CA, USA), unless stated otherwise. The cabbage fermentation (pH, cell counts), Caco-2 cell culture (TER, FITC, IL-8, RT-qPCR), and targeted GC-MS data were reported as mean ± standard deviation (SD) and analyzed by ANOVA with post hoc test for comparing multiple treatments. Peak height datasets (**Supplemental Files 3** and **4**, first tab

"Peak Heights") were uploaded to the MetaboAnalyst 6.0 web-based platform for downstream analysis (Pang et al., 2024; Xia & Wishart, 2011). Before statistical analysis, features (metabolites) with > 50% missing values were removed, and the remaining missing values were replaced using LoDs method (1/5 of the minimum positive value of each variable). Furthermore, features with > 10% inter-quantile range (IQR) variance were filtered out. For the GC-TOF/MS data, the peak heights were 1og 10 transformed prior to further analysis. For the RP-LC-HRMS/MS data, the peak heights were normalized to the sum of the peak heights of seven internal standards (**Supplemental File 5**) for each sample and then subjected to log 10 transformation. Principal component analysis (PCA) was applied to evaluate the compositions of the six different (fermented) cabbage homogenates. To identify metabolites significantly elevated or reduced after fermentation, the relative peak heights of each fermented homogenates were compared to cabbage homogenates (D0) using Wilcoxon rank-sum test (FDR of 0.05) (**Supplemental Files 3** and **4**). Heatmaps of the top 35 most significantly changed metabolites were generated via hierarchical cluster analysis (Euclidean distance measure, Ward clustering method). PERMANOVA and pairwise Adonis tests were performed using the LMSstat package (https://github.com/CHKim5/LMSstat/blob/master/README.md) and the pairwiseAdonis R package (Martinez Arbizu, 2020), respectively, in R 4.3.0 (R Core Team, 2023).

Results

LP8826R dominates and modifies cabbage fermentations

According to culturable bacterial cell numbers on MRS agar, a nutritionally replete medium used to enrich LAB (De Man et al., 1960), there were initially $4.80 \times 10^3 \pm 3.52 \times 10^3$ CFU/ml in the cabbage homogenate prior to incubation. This number increased to $1.55 \times 10^9 \pm$

8.22 x 10^8 CFU/ml after 2 days of incubation at 22 °C (**Fig 1A**). The changed cell numbers coincided a reduction in pH from pH 6.13 ± 0.13 to pH 4.00 ± 0.08 during that time (**Fig. 1A** and **1C**). Subsequently, bacterial numbers declined to approximately 1 x 10^8 CFU/ml by day 4 and were sustained at that level for at least 10 days. On day 21 of the laboratory scale fermentation (LSF), 4.11 x 10^7 ± 6.60 x 10^7 CFU/ml homogenate was reached (**Fig. 1A**). Brine pH continued to gradually decrease after day 2 of the fermentation, reaching pH of 3.61 ± 0.03 and 3.56 ± 0.12 on day 14 and 21, respectively (**Fig. 1C**). Brine salinity was similar between day 14 (19.90 ± 1.57 parts per thousand (ppt)) and day 21 (19.50 ± 0.17 ppt).

For laboratory scale cabbage fermentations to which LP8826R was added (LSF + LP8826R), at a level of 5 x 10^7 CFU/g ferment, the numbers of that strain increased on average 5-fold after inoculation on day 3 from 6.33 x 10^7 ± 2.34 x 10^7 CFU/ml homogenate to 3.11 x 10^8 ± 2.25 x 10^8 CFU/ml on day 6, reaching 86.50 ± 25.43% of the total LAB numbers in the ferments and were maintained until day 21 (**Fig. 1A** and **1B**). Addition of the LP8826R strain briefly elevated the total culturable LAB numbers in cabbage fermentations from 7.62 x 10^8 ± 4.19 x 10^8 CFU/ml homogenate before addition to 1.42 x 10^9 ± 4.50 x 10^8 CFU/ml homogenate after strain addition on day 3 (**Fig. 1A**). The total culturable LAB numbers then stabilized to approximately 4 x 10^8 CFU/ml on day 4, maintained until day 14 of the fermentation (5.21 x 10^8 ± 1.14 x 10^8 CFU/ml homogenate), and eventually significantly reduced by more than 2 log to 1.05 x 10^6 ± 6.52 x 10^5 CFU/ml on day 21 of the fermentation (p = 0.0206; two-way ANOVA mixed effects analysis with Tukey's multiple comparisons test) (**Fig. 1A**). After the addition of LP8826 strain, brine pH of these ferments gradually decreased from pH of 3.74 ± 0.02 on day 3 to 3.02 ± 0.02 on day 21 (**Fig. 1C**).

Comparing to laboratory scale ferments without LP8826R (LSF), the total culturable LAB numbers in these (LSF + LP8826R) ferments were significantly higher on day 4 (p = 0.0320), day 5 (p = 0.0374), day 6 (p = 0.0298), and day 14 ($p < 0.0001$; two-way ANOVA mixed effects analysis with Tukey's multiple comparisons test) of the fermentation (**Fig. 1A**). After 21 day of fermentation, the total culturable LAB numbers between these two laboratory scale ferments were not significantly different (p = 0.4015) (**Fig. 1A**). Brine pH of the laboratory scale ferments with LP8826R was significantly lower than ferments without this strain starting on day 6 of the fermentation ($p < 0.05$, two-way ANOVA with Tukey's multiple comparisons test and mixed effects analysis) (**Fig. 1C**). The drastic reduction in culturable LAB numbers between day 14 and day 21 observed in laboratory scale ferments with LP8826R may have been the result of the significantly reduced pH in those fermentations. The addition of L8826R did not result in changes to salinity on day 14 (20.53 ± 1.76 ppt) and 21 (20.17 ± 0.45 ppt) compared to laboratory scale ferments without this strain.

Commercial sauerkraut contained $3.44 \times 10^6 \pm 4.55 \times 10^6$ CFU/ml homogenate according to growth on MRS agar (**Table 1**). These numbers were not significantly different than found for the laboratory scale ferments on day 14 (p = 0.4269) and day 21 (p = 0.3913; one-way ANOVA with Tukey's multiple comparisons test) (**Fig. 1A**). However, the total culturable LAB numbers in commercial ferments were significantly lower compared to laboratory scale ferments with LP8826R on day 14 ($p < 0.0001$) but not day 21 (p = 0.9957) (**Fig. 1A**). Homogenate pH of commercial sauerkraut (pH 3.34 ± 0.08) was lower compared to day 14 (p = 0.0001) and day 21 (p = 0.0092; one-way ANOVA with Tukey's multiple comparisons test) of the laboratory scale ferments (LSF) (**Fig. 1C** and **Table 2**). On the other hand, homogenate pH of commercial sauerkraut (pH 3.34 ± 0.08) was higher compared to day 14 (p = 0.0026) and day 21 (p = 0.0012;

one-way ANOVA with Tukey's multiple comparisons test) of the laboratory scale ferments with LP8826R (LSF + LP8826R) (**Fig. 1C** and **Table 1**). The brine salinity of commercial sauerkraut was 26.73 ± 1.01 ppt and significantly higher compared to the laboratory ferments without and with LP8826R (one-way ANOVA with Tukey's multiple comparisons test, $p < 0.002$) (**Table 1**).

Cabbage ferments prevent cytokine-induced intestinal barrier disruption

We aimed to determine whether (fermented) cabbage homogenates can alleviate barrier integrity disruption in Caco-2 monolayers challenged with pro-inflammatory cytokines. Firstly, basolateral incubation with IFN-γ for 21 h followed by TNF-α resulted in significant reductions of TER compared to untreated controls within 24 h after application that were sustained for the remaining 24 h (**Fig. 2A**; $p < 0.0001$). This difference corresponded with a significantly increased transfer of FITC from the apical to the basolateral compartment compared to the untreated controls (**Fig. 2B**; P_{app} coefficient; $p < 0.0001$), confirming that the cytokines disrupted barrier integrity.

Secondly, to establish a suitable range of fermented cabbage homogenate for assessment on Caco-2 cell monolayers, Caco-2 cell culture medium (DMEM) was adjusted to contain 1, 10, or 50% (v/v) of filtered (0.2 µm), pH-adjusted (pH 7.4) fermented cabbage homogenates. Exposure to either 1 or 10% (v/v) of the homogenates did not significantly change TER ($p > 0.345$, two-way ANOVA with Tukey's multiple comparisons test) or permeability to FITC ($p > 0.992$, one-way ANOVA with Tukey's multiple comparisons test) compared to the untreated controls (**Fig. S1**). However, TER was negatively affected when 50% (v/v) was applied, resulting in an average 10-fold reduction within 24 h and elevated permeability to FITC ($p < 0.05$; one-way ANOVA with Tukey's multiple comparisons test) (**Fig. S1A** and **S1B**). This

change was not a result of diluting the DMEM culture medium since applying an equal quantity of water in the untreated controls did not result in TER reductions (**Fig. S1A**) or increases in paracellular permeability to FITC (**Fig. S1B**). Because there were no significant differences in TER and FITC permeability for the Caco-2 cell monolayers treated with either 1 or 10% v/v of the fermented cabbage homogenate in DMEM (**Fig. S1A** and **S1B**), 10% (v/v) of the pH-adjusted and filtered cabbage and sauerkraut homogenates were investigated further.

Thirdly, because NaCl is used to ferment cabbage, we also determined the impact of salt on intestinal barrier integrity. A 2.91% (w/v) solution of NaCl made to match the salinity of commercial sauerkraut homogenate after pH adjustment (29.1 ppt). Caco-2 cell monolayers exposed to DMEM adjusted to contain 10% (v/v) of this salt solution exhibited no significant differences in TER or permeability to FITC compared to untreated or cytokines, IFN-γ and TNF-α, treated monolayers (**Fig. S2A** and **S2B**).

Lastly, Caco-2 cell monolayers treated with cabbage homogenates (cabbage in brine from day 0 of the fermentation) showed reductions in TER over the 48h period and were undistinguishable from the cytokine-treated monolayers 32h after TNF-α application (**Fig. S3**). This is supported by the significant increase in Caco-2 FITC permeability compared to untreated monolayers ($p < 0.0007$) (**Fig. 2B**). On the other hand, IFN-γ and TNF-α induced reductions in TER did not occur when the monolayers were incubated with the laboratory scale sauerkraut homogenate (10% v/v) either with or without LP8826R (**Fig. 2A** and **Fig. S3**). This was found for samples collected either after 7- or 14-day incubation ($p \geq 0.2008$ compared to untreated; two-way ANOVA with Tukey's multiple comparisons test) (**Fig. 2A** and **Fig. S3**). A similar trend in TER (was observed for monolayers treated with 10% v/v commercial sauerkraut homogenate ($p = 0.6781$ compared to untreated; two-way ANOVA with Tukey's multiple

comparisons test) (**Fig. 2A** and **Fig. S3**). By 48h following TNF-α addition, all five groups of Caco-2 cell monolayers treated with sauerkraut homogenates exhibited significantly higher TER ($p < 0.007$) and significantly lower permeability to FITC ($p < 0.0292$) than the IFN-γ /TNF-α controls, suggesting a barrier protective property of the sauerkraut homogenates (**Fig. 2A** and **2B**). Together, these results indicated that sauerkraut homogenates, but not cabbage homogenate in brine, had the capacity to protect against inflammation-induced intestinal barrier damage.

Fermented cabbage variably altered IL-8 secretion and expression of barrier-modulatory genes

Concentrations of IL-8, a pro-inflammatory chemokine and an indicator of inflammatory responses in epithelial cells (Donato et al., 2010; Jobin et al., 1999; Sonnier et al., 2010), were significantly higher in the basolateral medium of Caco-2 cell monolayers exposed to IFN-γ and TNF-α (153.61 ± 28.83 pg/ml) compared to the untreated monolayers (2.27 ± 0.73 pg/ml) ($p < 0.0001$) (**Fig. 3**). Reductions of secreted IL-8 were not found for the laboratory scale fermented cabbage homogenates, with or without LP8826R, compared to the controls (**Fig. 3**). Conversely, significant reductions in IL-8 were found for the monolayers treated with commercial sauerkraut homogenates (111.79 ± 22.10 pg/ml, $p = 0.0137$), albeit the quantities were still higher than the controls not exposed to the cytokines (**Fig. 3**). Similarly, significantly reduced levels were also found for the cabbage and brine mixture (day 0; 110.54 ± 21.49 pg/ml) compared to controls exposed to cytokines ($p = 0.0106$; one-way ANOVA with Tukey's multiple comparisons test) (**Fig. 3**).

To investigate the effects of the fermented cabbage on Caco-2 expression of genes responsive to IFN-γ and TNF-α and modulate barrier function, RT-qPCR was performed to

quantify levels of the TNF receptor (*TNFR2*; Ma et al., 2004; Wang et al., 2006), myosin light chain kinase (*MLCK*; Donato et al., 2010; Ma et al., 2004; Suzuki, 2013; Ye et al., 2006), and *CLDN2*, encoding for the pore-associated tight junction protein Claudin-2 (Tanaka et al., 2017). Transcript levels of *TNFR2* and *CLDN2* were approximately 2-fold higher with IFN-γ and TNF-α exposure compared to the controls. Surprisingly, *MLCK* expression was slightly reduced by cytokine challenge (**Fig. S4**). Application of fermented cabbage homogenates prior to IFN-γ and TNF-α did not significantly alter the expression levels of *TNFR2*, *MLCK*, or *CLDN2*, with the exception for the LP8826R-inoculated cabbage ferments, which had similar *MLCK* gene expression as found for the controls (**Fig. S4**).

Cabbage, laboratory scale fermented, and commercial sauerkraut homogenates have distinct metabolomes

To investigate whether soluble metabolites in fermented cabbage differed compared to raw cabbage, we applied untargeted GC-TOF/MS and RP-LC-HRMS/MS metabolomics. GC-TOF/MS detected 583 metabolites in (fermented) cabbage homogenates and 147 were identified (**Supplemental File 3**). Principal component analysis (PCA) showed that the GC-TOF/MS identified metabolome profiles differed significantly between (fermented) cabbage homogenates (PERMANOVA $p = 0.001$, $R_2 = 0.95305$). The first two principal components accounted for 68.7% (45.7% for PC 1 and 23% for PC2) of the total variance (**Fig. 4A**). Metabolites of homogenized cabbage and brine (D0) clustered separately from the sauerkraut homogenates ($p_{adj} \leq 0.06$, pairwise Adonis test) (**Fig. 4A**). The commercially prepared ferments were also distinct from the laboratory scale fermented sauerkrauts ($p_{adj} = 0.045$, pairwise Adonis test) (**Fig. 4A**). Among the laboratory scale ferments, metabolites from those with LP8826R were initially

similar to those prepared without adding this exogenous strain (p_{adj} = 0.210 compared to LSF on day 7), but were then different by day 14 (p_{adj} = 0.045 between with and without LP8826R) and resembled the commercial sauerkraut (p_{adj} = 0.075, pairwise Adonis test) (**Fig. 4A**).

A total of 59 metabolites in the GC-TOF/MS analysis were significantly increased in all ferments compared to the raw cabbage homogenates (Wilcoxon rank-sum test, $p < 0.05$ and fold change ≥ 2) (**Supplemental File 3**). These compounds mainly encompassed fermentation end products (e.g., lactate, butane-2,3-diol), sugar and sugar alcohols (e.g., sorbitol, mannitol, trehalose, isomaltose), amino acids and derivatives (e.g., PLA, ILA, phenylalanine), nucleobases (e.g., uracil, cytosine). Conversely, the relative abundances of 49 metabolites were significantly reduced in all ferments compared to the raw cabbage homogenates, including core metabolism intermediates (e.g., pyruvic acid, citric acid, alpha-ketoglutarate), sugars and sugar acids (e.g., glucose, fructose, mannose, raffinose, sucrose), amino acids (e.g., aspartic acid, asparagine, glutamic acid, serine) (Wilcoxon rank-sum test, $p < 0.05$; **Supplemental File 3**, **Fig. S6**, and **Fig. 5A**). Cabbage and brine homogenates were positively correlated and distinguished from fermented homogenates by the peak heights of sucrose, raffinose and to a lesser extent, the levels of mannose and fructose (**Fig. S5A**). On the other hand, the separation of the cabbage ferments could be attributed to the peak heights of fermentation end products (e.g., sorbitol, lactic acid, mannitol, and butane-2,3-diol) as well as potential bioactive compounds (e.g., PLA, trehalose, and ILA) (**Fig. 5A**, **Fig. S5A**, and **Fig. S6**). Commercial sauerkraut homogenates were further distinguished from laboratory scale sauerkraut homogenates with LP8826R by the peak heights of putrescine and glucose-6-phosphate (**Fig. 5A**, **Fig. S5A**, and **Fig. S6**).

RP-LC-HRMS/MS detected 6,778 metabolites in (fermented) cabbage homogenates and 333 metabolites were identified. Similarly, the composition and peak heights of the 333 RP-LC-

HRMS/MS identified metabolites were significantly different between the (fermented) cabbage homogenate types analyzed according to PCA (PERMANOVA, $p = 0.001$, $R_2 = 0.80297$). The first two principal components accounted for 81.6% (63.6% for PC1 and 18% for PC2) of the total variance (**Fig. 4B**). RP-LC-HRMS/MS identified metabolites from cabbage and brine (D0) homogenates clustered separately from fermented cabbage homogenates ($p_{adj} \leq 0.06$, pairwise Adonis test) (**Fig. 4B**). The composition and abundance of these metabolites in the laboratory scale ferments were similar across time points. The commercial sauerkraut was distinct from laboratory scale sauerkraut ferments ($p_{adj} < 0.05$, pairwise Adonis test), except for the ferments with added LP8826R at day 14 ($p_{adj} = 0.075$, pairwise Adonis test) (**Fig. 4B**). Hence, the metabolite profiles show that fermentation changes the metabolomes and the addition of LP8826R results in a metabolome like that found in the commercial sauerkraut (**Fig. 4B**).

A total of 254 compounds detected and identified by RP-LC-HRMS/MS were significantly increased in all the fermented cabbage homogenates compared to the raw cabbage (Wilcoxon rank-sum test, $p < 0.05$ and fold change ≥ 2) (**Supplemental File 4**). These compounds mainly consisted of small di- and tri-peptides (144 out of 254 compounds; 56.7%) (e.g., phe-phe), various phenolic and heterocyclic compounds (e.g., gentisic acid, 3,4-dihydroxyhydrocinnamic acid), amino acids and their derivatives (e.g., PLA, ILA), fatty acids (e.g., mevalonic acid), sugar alcohols (e.g., mannitol, sorbitol), and vitamins (e.g., vitamin C, vitamin B5) (**Supplemental File 4** and **Fig. S7**). On the other hand, the relative abundances of 49 compounds in the ferments were significantly lower than cabbage and brine homogenates, encompassing mostly of sugars (e.g., fructose, sucrose), peptides, amino acids and their derivatives (e.g., glutamine, arginine), fatty acids (Wilcoxon rank-sum test, $p < 0.05$; **Supplemental File 4**). Cabbage and brine homogenates were distinguished from ferments by the

lower relative abundances of fatty acids, (9Z)-5,8,11-trihydroxyoctadec-9-enoic acid, (10e, 15Z)-9,12,13-trihydroxyoctadeca-10,15-dienoic acid, and 3-[4-methoxy-2-[(2s,3r,4s,6r)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxyphenyl]prop-2-enoic acid, as well as epigallocatechin (**Fig. 5B**, **Fig. S5B**, and **Supplemental File 4**). Conversely, the relative levels of 12,13-dihydroxy-9Z-octadecenoic acid, 3,4-dihydroxyhydrocinnamic acid, ILA, and PLA separated commercial sauerkrauts and those with LP8826R at day 14 compared to the other laboratory scale ferments (**Fig. 5B**, **Fig. S5B**, **Fig. S7**, and **Supplemental File 4**).

Fermented cabbage metabolomes are enriched with diverse bioactive compounds

From the untargeted metabolomics data sets, we narrowed down a list of candidate metabolites for further bioactivity testing based on whether they are statistically significant and are at 1.5-fold or higher levels compared to cabbage and brine (day 0); exhibited a fermentation time dependent increase (e.g., higher levels in day 14 vs. day 7); were relatively more abundant in the metabolomes (peak heights $\geq 10^3$); and have previous known studies of bioactivity. From the GC-TOF/MS and RP-LC-HRMS/MS datasets, lactate, trehalose, 2-hydroxyisocaproic acid, PLA, ILA, glutamic acid, methionine sulfoxide, and 3,4-dihydroxyhydrocinnimic acid were selected as candidates. From these metabolites, ILA and D-PLA were selected for further verification with targeted metabolomics and bioactivity assessment. Additionally, GABA, which was significantly higher in day 7 sauerkraut homogenates compared to cabbage (day 0), and identified only in the untargeted RP-LC-HRMS/MS dataset, was included for its potential anti-hypertensive, anti-obesogenic, and immunomodulatory bioactivity, as noted in previous studies (Hou et al., 2023; Inoue et al., 2003; Nakamura et al., 2022; Ngo & Vo, 2019; Oketch-Rabah et al., 2021; Patterson et al., 2019).

To verify the untargeted metabolomics and gain estimates of total quantities of known bioactive compounds in the ferments, we applied targeted GC-TOF/MS to measure ILA, D-PLA, and GABA. Cabbage and brine (D0, before fermentation) homogenates contained around 8.50 ± 0.00 µg/ml ILA. Interestingly, the levels of this compound increase by approximately 1 µg/ml in the commercial sauerkraut and laboratory scale ferments containing LP8826R (approximately 10 µg/ml or 50µM) (**Fig. 6**). D-PLA was found in approximately the same concentrations in the raw cabbage as ILA (6.10 ± 0.11 µg/ml) but were increased in all ferments, reaching approximately 2-fold higher in the commercial and LP8826R-inoculated ferments (approximately 20 µg/ml or 120 µM) (**Fig. 6**). Finally, GABA concentrations more than doubled after fermentation compared to the raw cabbage homogenate (D0) ($p < 0.0001$) (**Fig. 6**). However, there were no significant differences in GABA concentrations between time points and laboratory and commercial ferments (**Fig. 6**).

Lactate, ILA, and D-PLA protect Caco-2 cell monolayers from cytokine-induced damage

Moreover, the potential barrier protective effects of lactate, ILA, and D-PLA were assessed in Caco-2 cell monolayers. Along with targeted metabolomics estimation of ILA and D-PLA levels, lactate concentrations for the Caco-2 cell bioactivity assay was noted to range between 2.5 – 15 g/L in sauerkraut (Gaudioso et al., 2022; Viander et al., 2003; Yang, Hu, Xiu, Jiang, Yang, Saren, et al., 2020; Yang, Hu, Xiu, Jiang, Yang, Sarengaowa, et al., 2020), which is approximately 100 mM (~10 g/L). Since the barrier protective effect of sauerkraut homogenates were examined at 10% (v/v) homogenate and accounting for variations among biological replicates and between sauerkraut groups, the target concentrations of the individual metabolites

were determined to be 50 mM lactate, 60µM D-PLA, 25µM ILA, and a mixture of these three compounds.

A loss in TER was not observed when the Caco-2 cells were exposed to apical applications of 50 mM lactate, 60 µM D-PLA, or 25 µM ILA. Apical treatment with these metabolites individually and in a mixture alleviated a cytokine-induced TER reduction at 32 h after TNF-α addition ($p < 0.01$, two-way ANOVA with Tukey's multiple comparisons test) (**Fig. S8**), while this effect on TER was not observed 24 or 48 h after TNF-α addition (**Fig. S8** and **Fig. 7A**). ILA and mixture of the three compounds had the greatest effect and TER was not significantly reduced compared to untreated cell monolayers at 32 h after TNF-α application (**Fig. S8**). Paracellular permeability was also affected by the metabolite additions such that permeability to FITC-dextran was significantly reduced by at least 2-fold on average when lactate, D-PLA, ILA, and metabolite mixture were applied ($p \leq 0.002$, one-way ANOVA with Tukey's multiple comparisons test) and were comparable to the untreated cells ($p > 0.4997$) (**Fig. 7B**). These functional results emphasized the capacity of these individual metabolites at concentrations found in sauerkraut homogenates to protect Caco-2 cell monolayers from inflammation-induced permeability.

Discussion

Lacto-fermented fruits and vegetables (FV), like fermented cabbage (e.g., sauerkraut), have garnered extensive interests for their potential positive influences on health outcomes. These properties can likely be attributed to the combined actions of substrate phytochemicals, beneficial live microbes (e.g., LAB) that perform the fermentation, and the bioactive compounds produced during fermentation, although the mechanistic details underlying the contribution and

interactions of these individual components in lacto-fermented FV foods require further investigation. To understand the potential bioactivity distinguishing fermented cabbage from its ingredients, we examined the prophylactic capacity of cabbage, laboratory scale and commercial cabbage ferments to alleviate inflammation induced intestinal permeabilization, which is often associated with metabolic and inflammatory disorders. To that regard, this study revealed that cell-free homogenates of commercial and laboratory scale cabbage ferments, but not raw cabbage, protected against proinflammatory cytokine-induced intestinal barrier disruption in a polarized model with Caco-2 cell monolayers. The differences in barrier protection may be explained by the distinct metabolome profiles and enrichment of gut barrier protective compounds. The retention of intestinal barrier protection between the selected fermentation-derived metabolites, lactate, D-PLA, and ILA, individually and in combination, suggested that the barrier protective bioactivity of sauerkraut homogenates likely involved the synergistic effect of multiple metabolites produced by the microbiota during fermentation.

Apical treatment with fermented cabbage homogenates alleviated the increased barrier permeability induced by sequential exposure to IFN-γ and TNF-α, as quantified through functional TER and FITC measurements. Fermented cabbage homogenates, especially those from laboratory scale ferments with LP8826R and commercial ferments, ameliorated the cytokine-induced TER reduction by approximately 40% throughout the 48 h after basolateral TNF-α application while cabbage homogenate only prevented TER reduction until 24 h after TNF-α application. A similar trend of impact on cytokine-induced TER reduction in this Caco-2 monolayer model was also observed for ultra-high temperature milk fermented by *Lacticaseibacillus paracasei* BL23, which resulted in a recovery of approximately 20% in TER in treated monolayers compared to cytokine-challenged monolayers without treatment (Zhai et

al., 2019). Moreover, cytokine challenge elevated the paracellular permeability to FITC by more than 2-fold in Caco-2 monolayers and apical treatment with fermented cabbage, but not cabbage, prevented this increase in permeability. This trend was also observed in fermented milk treated Caco-2 monolayers after cytokine challenge (Zhai et al., 2019). In polarized Caco-2 monolayers without pro-inflammatory cytokine challenge, a 24-h incubation with sauerkraut brine from two artisanal producers did not significantly affect barrier integrity (TER) compared to untreated monolayers, indicating that sauerkraut brine did not have a detrimental effect to Caco-2 monolayers (Gaudioso et al., 2022). Treatment with a freeze-dried powder of fermented *Portulaca oleracea* L. (purslane) puree juice, but not raw or chemically acidified purslane puree juice powders, alleviated part of the IL-1β-induced TER reduction in Caco-2 monolayers (Di Cagno et al., 2019). In Caco-2 monolayers challenged with TNF-α and IL-1β, barrier protective property, quantified with TER and permeability to Lucifer Yellow, was observed for sour cherry anthocyanin extract (Le Phuong Nguyen et al., 2018).

Surprisingly, we did not observe elevated *MLCK* transcript levels in Caco-2 monolayers 48 h after TNF-α application. TNF-α was known to induce an increase in *MLCK* mRNA levels in Caco-2 cells up to 4 h after addition (Ma et al., 2005), while higher MLCK protein levels in TNF-α-treated Caco-2 cells can be observed up to 48 h post cytokine challenge (Ma et al., 2005; Wang et al., 2005), suggesting that an earlier time point would be more likely for observing the different *MLCK* mRNA levels in response to treatment of (fermented) cabbage homogenates. Furthermore, treatment with cabbage and commercial ferments, but not laboratory scale ferments, prior to cytokine challenge reduced basolateral IL-8 production by approximately 1.5-fold compared to monolayers challenged with cytokines in the polarized Caco-2 monolayers 48 h after TNF-α addition. A similar magnitude of reduction in basolateral IL-8 production was also

seen in Caco-2 monolayers treated with fermented milk prior to cytokine challenge (Zhai et al., 2019). Application of fermented purslane juice powder, but not the powders of raw or chemically acidified purslane juice, lowered apical IL-8 production by approximately 3-fold compared to cytokine control in Caco-2 cells 24 h after IL-1β challenge (Di Cagno et al., 2019). Treatment of Caco-2 cells with pomegranate peel extracts (Mastrogiovanni et al., 2019) or sour cherry anthocyanin extracts (Le Phuong Nguyen et al., 2018) led to approximately 2 or 4-fold lower apical IL-8 quantities after a 24 h challenge with a combination of TNF-α and IL-1β or TNF-α, respectively. The observed distinction in IL-8 levels between laboratory scale ferments and the cabbage and commercial ferments in our study may be attributed to the different composition of fermentation-derived metabolites present in these cell-free (fermented) cabbage homogenates, likely contributing to the overall intestinal barrier protective effect through multiple signaling pathways that may not involve a change in IL-8 secretion.

GC-TOF/MS and RP-LC-HRMS/MS metabolomics showed that fermentation resulted in significant changes to the cabbage metabolome. This was reported previously (Gaudioso et al., 2022; Hu et al., 2021; Yang, Hu, Xiu, Jiang, Yang, Saren, et al., 2020; Yang, Hu, Xiu, Jiang, Yang, Sarengaowa, et al., 2020). Cabbage and fermented cabbage homogenates have distinct composition and abundances of the metabolites identified by GC-TOF/MS, including carbohydrates, amino acids and derivatives, hydroxyl acids, free fatty acids. Compared to the cabbage and brine, the top 10 significantly increased (at least 10-fold) metabolites consisted of fermentation end products (e.g., lactic acid, butane-2,3-diol), sugar alcohols (e.g., sorbitol, mannitol, erythritol), amino acid derivatives (e.g., PLA, HICA), and other compounds (e.g., trehalose, isomaltose, cytosine). RP-LC-HRMS/MS identified compounds that are significantly increased by fermentation, other than numerous di-peptides and those also identified by GC-

TOF/MS (e.g., lactate, PLA, ILA, sugar alcohols, HICA), also included fatty acids (e.g., mevalonic acid, 12,13-dihydroxy-9Z-octadecenoic acid) and other organic acids (e.g., 4-hydroxyphenyllactic acid, 3,4-dihydroxyhydrocinnamic acid). On the contrary, the top 10 significantly lowered (less than 0.2-fold) metabolites during fermentation encompassed sugars and derivatives (e.g., sucrose, raffinose, mannose, fructose, galactinol, gluconic acid) and metabolites involved in fermentative metabolism (e.g., citric acid, malic acid, fumaric acid, glucose-6-phosphate). Other than the sugars and sugar derivatives, large numbers of di-peptides, and amino acids, RP-LC-HRMS/MS also found that fermentation lowered the levels of fatty acids (e.g., 9,10-dihydrojasmonic acid, (9Z)-5,8,11-trihydroxyoctadec-9-enoic acid), epigallocatechin, and iberin. These trends during cabbage fermentation were also observed for artisanal sauerkrauts (3% salt w/w; 13 – 22 °C; 35 days) (Gaudioso et al., 2022), home fermented spontaneous (Yang, Hu, Xiu, Jiang, Yang, Saren, et al., 2020) or laboratory scale Chinese northeast sauerkrauts with starter cultures (1% w/w salt; 18 – 20 °C; 30 days) (Hu et al., 2021; Yang, Hu, Xiu, Jiang, Yang, Sarengaowa, et al., 2020).

We did not detect acetic acid, butyric acid, and propionic acid in our laboratory scale and commercial ferments while these organic acids were enriched after 35 days of fermentation in the artisanal sauerkrauts, quantified using proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) (Gaudioso et al., 2022). The relative abundances of tyrosine, phenylalanine, and leucine were significantly increased after fermentation in our study, and this was also noted in artisanal sauerkraut (Gaudioso et al., 2022) and to a smaller degree, in Chinese northeast sauerkrauts, which also exhibited increases in aspartate, glutamate, glycine, and proline after 30 days of fermentation (Yang, Hu, Xiu, Jiang, Yang, Saren, et al., 2020). Laboratory scale ferments were separated from other ferments with higher levels of aspartic acid, ribose, uric acid, and uracil.

PCA biplot suggested that the abundances of butane-2,3-diol, sorbitol, lactic acid, and mannitol were associated with laboratory scale sauerkraut with LP8826R, which was consistent with the finding that there was a correlation between relative abundance of *Lactiplantibacillus* and the concentrations of lactic acid, acetic acid, phenylalanine, and tyrosine in 35-day fermented artisanal sauerkrauts (Gaudioso et al., 2022). Sorbitol and mannitol are isomers, differing only by the orientation of a hydroxyl group (Bhatt et al., 2013). Utilization of microbial community starters, with high ratios of *Leuconostoc*, *L. plantarum*, and *Levilactobacillus brevis*, elevated sorbitol levels in kimchi after a 50-day fermentation compared to baseline (Park et al., 2019). Moreover, the levels of putrescine, and to a small extent, tyrosine, seem to be associated with commercial sauerkrauts, as noted in the biplots. Putrescine relative abundances were comparable among the laboratory scale ferments and cabbage homogenates regardless of the addition of LP8826R and were significantly lower than that found in commercial sauerkrauts. The higher levels of putrescine in commercial sauerkraut compared to laboratory scale ferments is likely in part due to the longer storage times at 4 °C, which was found to increase putrescine levels in sauerkraut (Peñas et al., 2010).

Lactate, PLA, and ILA are three of the metabolites that were significantly elevated after cabbage fermentation, as noted in the untargeted GC-TOF/MS and RP-LC-HRMS/MS analyses. Targeted GC-TOF/MS quantification confirmed the presence of PLA and ILA in the (fermented) cabbage homogenates, which reached approximately 20 µg/ml or 120 µM and approximately 10 µg/ml or 50µM, respectively. The ferment type-dependent trends in PLA and ILA levels indicated that LP8826R likely had a greater capacity to produce PLA and ILA compared to the autochthonous LAB that fermented the laboratory scale sauerkraut and a similar capacity for their production compared to the LAB in commercial sauerkraut. While lactic acid/lactate levels

were not quantified in this study, untargeted metabolomics analyses showed that its levels were elevated by more than 60-fold after fermentation. Lactate concentrations in fermented cabbage can reach 26 - 167 mM (Gaudioso et al., 2022; Viander et al., 2003; Yang, Hu, Xiu, Jiang, Yang, Saren, et al., 2020).

In vitro and *in vivo* animal model studies have found that the administration of or microbiota-associated lactate (Iraporda et al., 2015; Lee et al., 2018; Li et al., 2024; Yu et al., 2021; Zhou et al., 2022), D-PLA (Lee et al., 2024; Shelton et al., 2023), and ILA (Henrick et al., 2021; Zhang et al., 2023) exhibited anti-inflammatory and intestinal barrier protective properties. These bioactive properties were noted to involve signaling through HCA1/GPR81 (lactate; Li et al., 2024), HCA3 (D-PLA and ILA; Peters et al., 2019), PPAR-γ (PLA; Shelton et al., 2023), the aryl hydrocarbon receptor (AHR) transcription factor (ILA; Cervantes-Barragan et al., 2017; Cui et al., 2023; Ehrlich et al., 2020; Meng et al., 2020), and the nuclear factor erythroid 2-related factor 2 (Nrf2) (ILA; Cui et al., 2023; Ehrlich et al., 2020; Zhang et al., 2023). The half maximal effective concentration (EC_{50}) of lactate to activate the HCA1/GPR81 receptor is between 5 to 20 mM (Koh et al., 2016) while the EC_{50} for HCA3 receptor activation by PLA and ILA is in the µM range (0.15 µM for D-PLA, 5.2 µM for L-PLA, and 0.18 µM for ILA) (Peters et al., 2019). Depending on the bioavailability and stability of these compounds during GI transit as well as sauerkraut product differences (e.g., strains, conditions, time), the concentrations of D-PLA (approximately 120 µM) and ILA (approximately 50 µM) present in the laboratory scale and commercial sauerkraut homogenates, prepared from 10 g of solids and 1 ml of brine (an estimated serving size of 10 g sauerkraut), are likely sufficient to induce receptor activation.

Treatment of the Caco-2 monolayers with these three metabolites individually and as a mixture, especially for ILA and the mixture, protected against cytokine-induced intestinal barrier

permeabilization, indicating the contribution of the fermentation-derived sauerkraut metabolome to the bioactivity of the whole ferment. Treatment with 100 mM lactate was noted to reduce *CCL20* transcription in IL-1β-stimulated Caco-2 cells (Iraporda et al., 2015). In colon epithelial cells isolated from wild-type and GPR81$^{-/-}$ mice, lactate (1 to 10 mM) prevented the TNF-α-induced activation of NF-κB pathway and the resulting reduction in TJP levels, a barrier protective effect that requires the presence of GPR81 (Li et al., 2024). Exposure to 5 mM D-PLA was found to activate PPAR-γ in mouse intestinal organoids and reduced lipid secretion in Caco-2 monolayers (Shelton et al., 2023). The barrier protective activity of ILA, quantified by TER, has been shown in Caco-2 monolayers co-incubation with 500 µM ILA and LPS and this protection was abrogated by AHR antagonist (Cui et al., 2023). Although the compound concentrations, cell type, and source of inflammatory perturbation may differ, these findings are consistent with the observed bioactivity of the three metabolites enriched in cabbage ferments in Caco-2 monolayer model of this study while also suggest putative signaling pathways that may target different stages of TNF-α induced intestinal barrier disruption.

To determine whether LP8826R can remain viable during cabbage fermentation, we inoculated LP8826R (5 x 10^7 CFU/g, constituting 8.58 ± 4.57% of the total LAB numbers) to a subset of the laboratory scale ferments on day 3 of the fermentation, at the start of the heterofermentative to homofermentative LAB microbial succession that often occur during cabbage fermentation (Plengvidhya et al., 2007; Yang, Hu, Xiu, Jiang, Yang, Saren, et al., 2020; Zabat et al., 2018). The exogenous LP8826R dominated the total culturable LAB population 3 days after its addition (by day 6 of the fermentation), leading to elevated total LAB numbers and lower brine pH in the LP8826R-inoculated ferments compared to the control ferments without LP8826R. The near one log higher total culturable LAB numbers between days 7 and 14 and

reduced pH (day 21) of ferments inoculated with starter culture strain compared to ferments without an added strain were also observed during the fermentation of Chinese northeastern sauerkraut (1% w/w salt; 18 – 20 °C for 30 d) using *L. plantarum* (Lpl) strain (10^7 CFU/ml) add on day 0, though in the case of northeastern sauerkrauts, the addition of the starter culture strain resulted in a less drastic reduction in pH on day 23 (without vs. with Lpl: pH 3.52 vs. pH 3.41) (Yang, Hu, Xiu, Jiang, Yang, Sarengaowa, et al., 2020) compared to the laboratory scale ferments in this study (without vs. with LP8826R: pH 3.56 vs. pH 3.02). These results along with the rapid increase in culturable LAB numbers and drop in brine pH after 2 days of fermentation supported the successful fermentation of the cabbage under laboratory scale and showed that the human originated LP8826R strain was able to adapt and thrive in a vegetable fermentation environment.

Further investigations should elucidate the specific signaling pathways via which these compounds consumed in mixtures in fermented cabbage can alter intestinal epithelial cell function. Verifying the potential health-promoting properties of these fermentation derived metabolites can also support the screening and development of starter culture strains, including well-established probiotic strains, with desired bioactive compound biosynthetic capacity and the ability to remain viable in these vegetable fermentation environments during fermentation and long-term storage. Ultimately, human studies are required to determine the intake recommendation of sauerkraut that will yield physiologically relevant impact in the target populations.

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Table 2.1. Parameters of commercial sauerkraut sampled.

Commercial Sauerkraut	LOT #	Besst By Date	Sampling Date	Brine Salinity (ppt)	Homogenate pH	Total LAB (CFU/ml)
1	ND	ND	Dec 08, 2021	ND	3.37	ND
2	ND	ND	Nov 12, 2021	ND	3.20	ND
3	2022234	012623	Sep 28, 2022	26.7	3.37	4.00×10^6
4	2022209	122922	Sep 28, 2022	27.5	3.30	9.00×10^4
5	2022234	012623	Sep 28, 2022	27.4	3.36	9.67×10^6
6	2022166	111922	Oct 09, 2022	25.3	3.42	8.00×10^1

ND: not determined.

Table 2.2. Primer sequences for qRT-PCR.

Gene	Primer sequences	Product (bp)	Efficiency (%)	Reference
GAPDH	F-5'-TCCCATCACCATCTTCCAG-3'	145	103.21	Rollenhagen et al., 2014
	R-5'-GATGACCCTTTTGGCTCC-3'			Jaramillo-Ferrada et al., 2011
TNFR2	F-5'-TGGCATTTACACCCTACGC-3'	143	96.99	Zhai et al., 2019
	R-5'-CACGGTGTCCGAGGTCTT-3'			
MLCK	F-5'-GCTTGGTCAGCCTGTTGTT-3'	135	103.09	Zhai et al., 2019
	R-5'-CCTTCTTTGACCACAACTCG-3'			
CLDN2	F-5'-TGTGACATCTATAGCACCCTTC-3'	168	104.29	Zhai et al., 2019
	R-5'-TACCGCCACTCTGTCTTTG-3'			

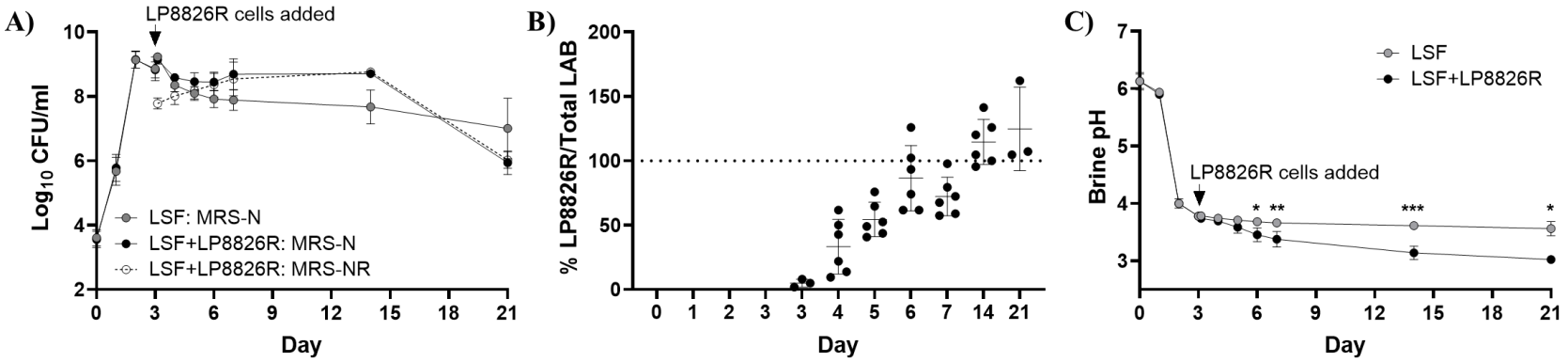


Figure 2.1. LAB numbers and brine pH in laboratory-scale ferments (LSF).

(**A**) Culturable LP8826R (open circles) and total LAB in LSF (gray circles) and LSF with LP8826R (black circles). LAB were enumerated on MRS with natamycin (25 µg/ml) (MRS-N) in LSF with LP8826R (black circles) and without added LP8826R (gray circles). LP8826R was enumerated on MRS containing natamycin (25 µg/ml) and rifampicin (50 µg/ml) (MRS-NR; open circles). Rifampicin resistant bacteria were not detected in the LSF lacking LP8826R additions (below 3.33 x 10^1 CFU/ml limit). (**B**) The percentage of LP8826R to total LAB numbers, and (**C**) brine pH over time. The mean ± SD is shown for 6 replicates. To determine differences in brine pH, two-way mixed effect ANOVA with Tukey's multiple comparisons test was performed (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

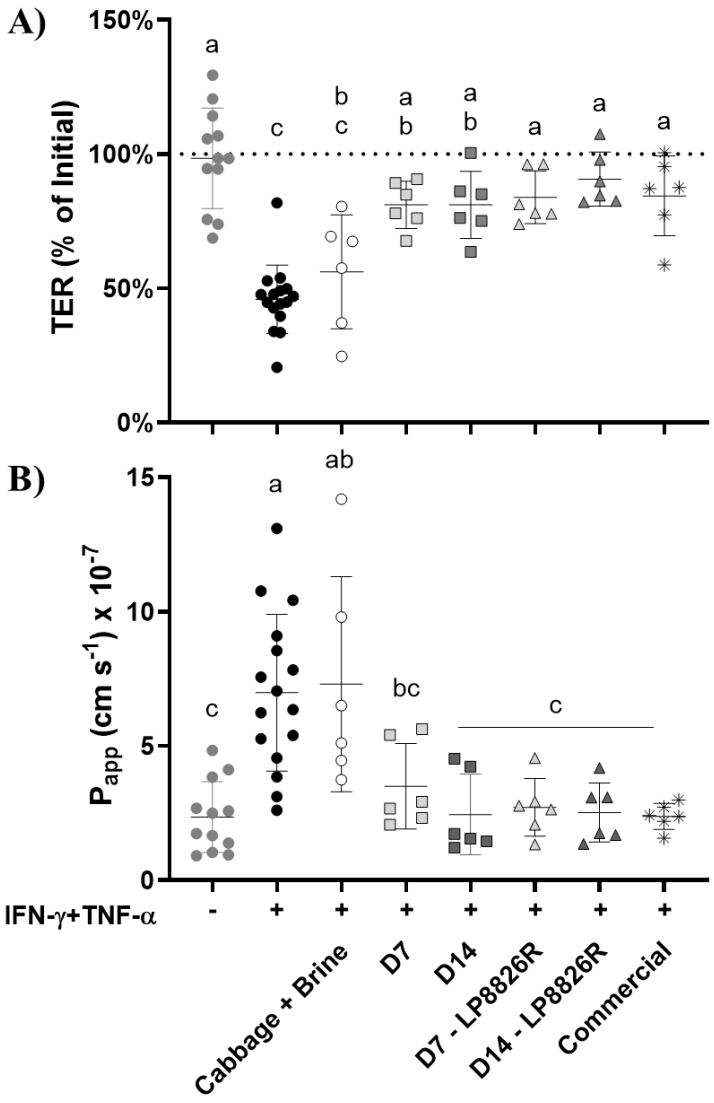


Figure 2.2. Effect of (fermented) cabbage homogenates on intestinal barrier permeability of cytokine-perturbed Caco-2 monolayers.

(**A**) Trans-epithelial electrical resistance (TER) of Caco-2 monolayers 48 h after basolateral IFN-γ and TNF-α sequential additions. Values are normalized to TER immediately prior to when TNF-α was added. (**B**) Apparent permeability coefficient (P_{app}) to FITC-dextran (4kD) of Caco-2 monolayers at the 48 h time point. D7 and D14 were collected on days 7 and 14, respectively, from LSF either with or without the LP8826R additions. Replicates included Caco-2 monolayers

not exposed to IFN-γ and TNF-α (n = 12), controls to which the cytokines were applied (n = 16), and those exposed to the cytokines and (fermented) cabbage (n = 6). The mean ± SD is shown for four independent experiments. The letters indicate significant differences based on one-way ANOVA with Tukey's multiple comparisons test.

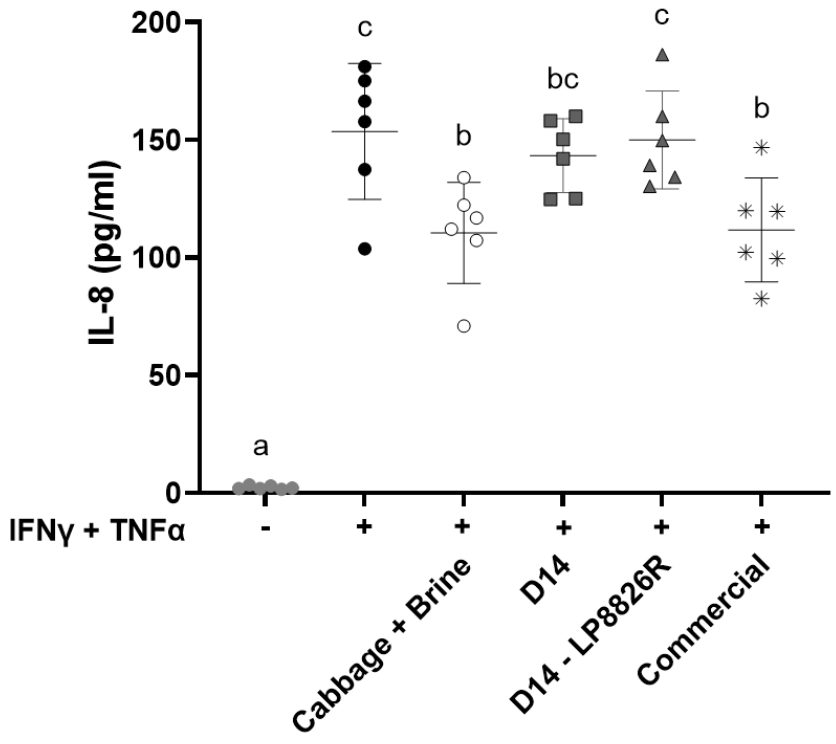
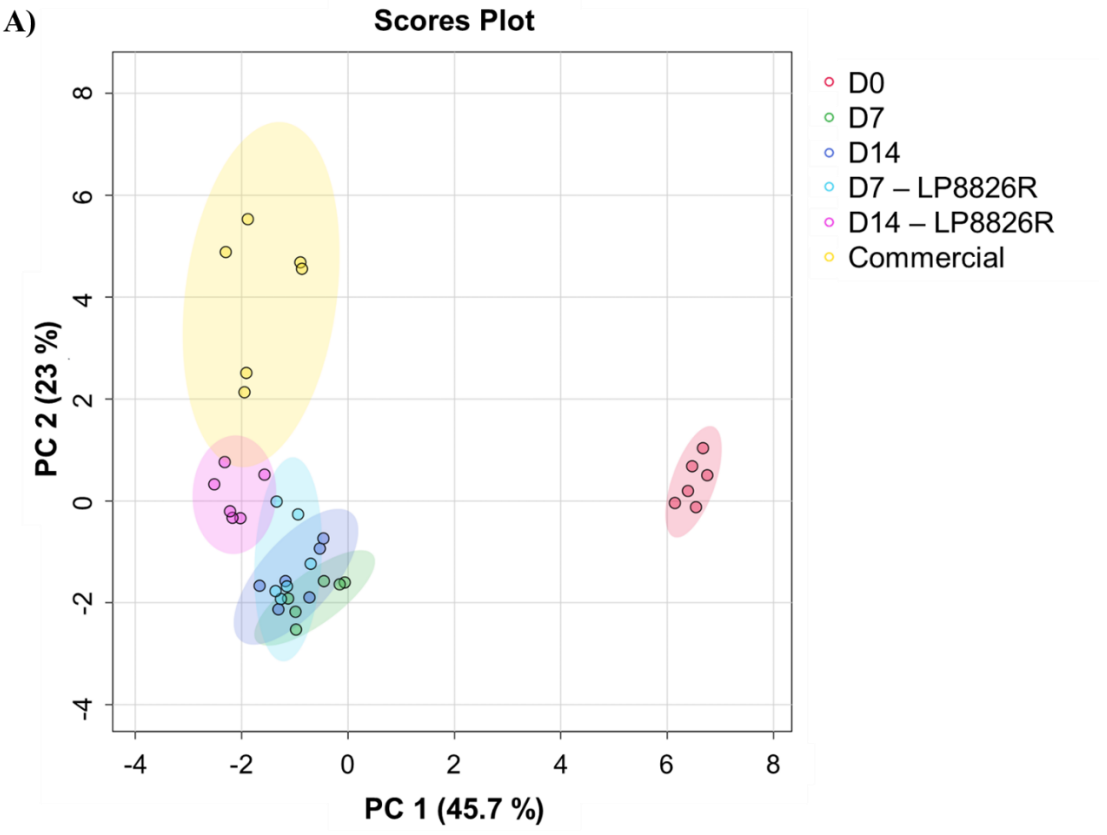


Figure 2.3. Basolateral IL-8 production by Caco-2 monolayers after sequential exposure to (100 ng/ml) IFN-γ and (10 ng/ml) TNF-α as quantified via ELISA.

DMEM was collected from the basolateral side of Caco-2 monolayers 48 h after sequential application of IFN-γ and TNF-α. D14 and D14 – LP8826R were collected on day 14 from LSF either with or without LP8826R addition. The mean ± SD is shown for selected basolateral spent media from four independent experiments (n = 6). Letters indicate significant differences based on one-way ANOVA with Tukey's multiple comparisons test.

A)


Scores Plot
PC 2 (23 %)
PC 1 (45.7 %)
D0
D7
D14
D7 – LP8826R
D14 – LP8826R
Commercial

B)

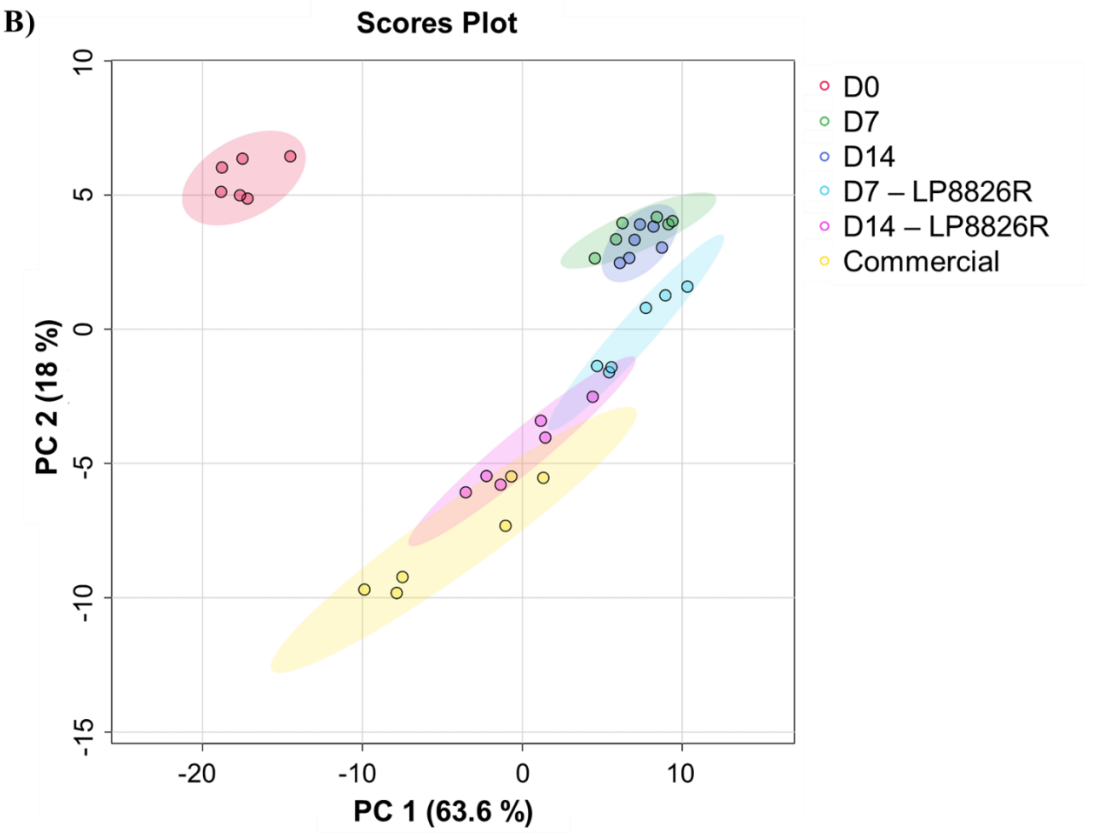
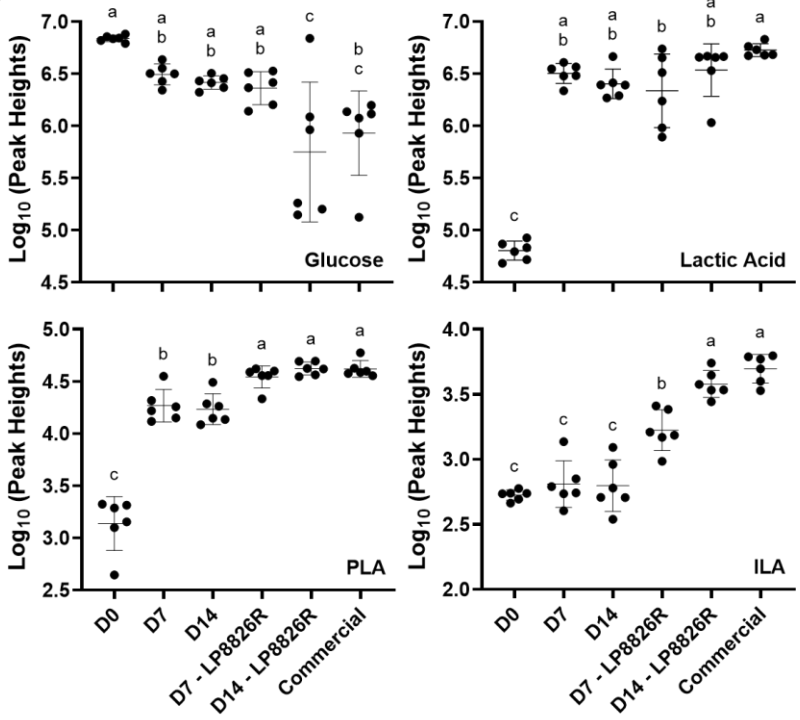

Scores Plot
PC 2 (18 %)
PC 1 (63.6 %)
D0
D7
D14
D7 – LP8826R
D14 – LP8826R
Commercial

Figure 2.4. Principal component analysis (PCA) of identified metabolites from (A) GC-TOF/MS (147 metabolites) and (B) RP-LC-HRMS/MS (333 metabolites) analyses.

PCA plots investigated the differences in metabolites among cabbage and fermented cabbage homogenates. Six replicates were tested for each condition. D0: cabbage and brine from day 0; D7/D7 – LP8826R: laboratory scale ferments with and without LP8826R from day 7; D14/D14 – LP8826R: laboratory scale ferments with or without LP8826R from day 14.

A)


Log10 (Peak Heights)
Glucose
Lactic Acid
PLA
ILA
D0
D7
D14
D7 - LP8826R
D14 - LP8826R
Commercial

B)

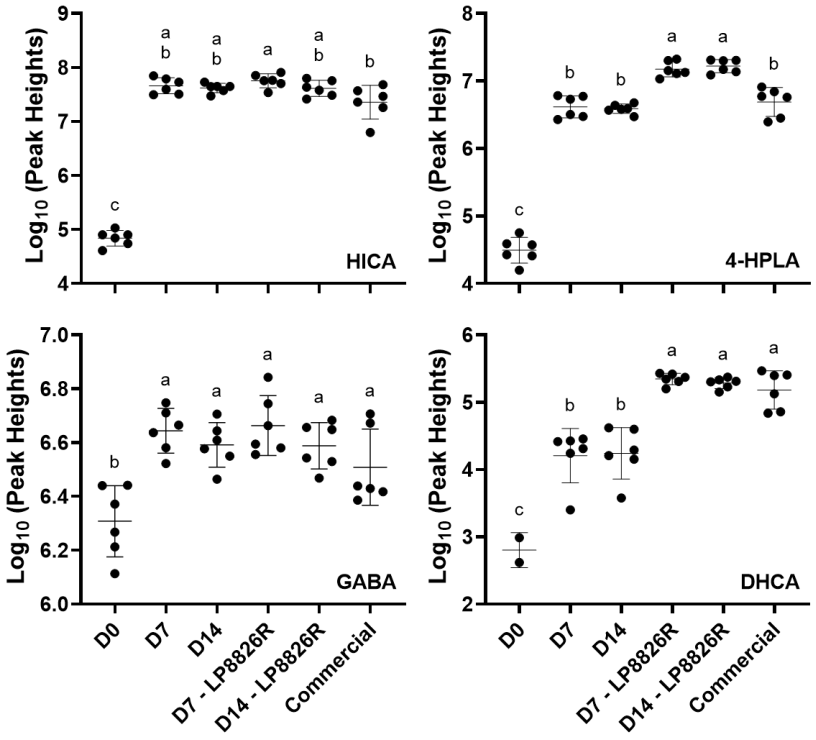

Log10 (Peak Heights)
HICA
4-HPLA
GABA
DHCA
D0
D7
D14
D7 - LP8826R
D14 - LP8826R
Commercial

Figure 2.5. Selected metabolites significantly affected by fermentation of cabbage from untargeted metabolomics analysis with (A) GC-TOF/MS or (B) RP-LC-HRMS/MS.

Mean ± SD for n = 6 replicates. D0: cabbage and brine from day 0; D7/D7 – LP8826R: laboratory scale ferments with and without LP8826R from day 7; D14/D14 – LP8826R: laboratory scale ferments with or without LP8826R from day 14. Mean values not sharing letter are significantly different based on one-way ANOVA with Tukey's multiple comparisons test. PLA: phenyl-lactic acid. ILA: indole-3-lactate. HICA: 2-hydroxyisocaproic acid. 4-HPLA: 4-hydroxyphenyllactic acid. GABA: γ-aminobutyric acid. DHCA: 3,4-hydroxylhydrocinnamic acid.

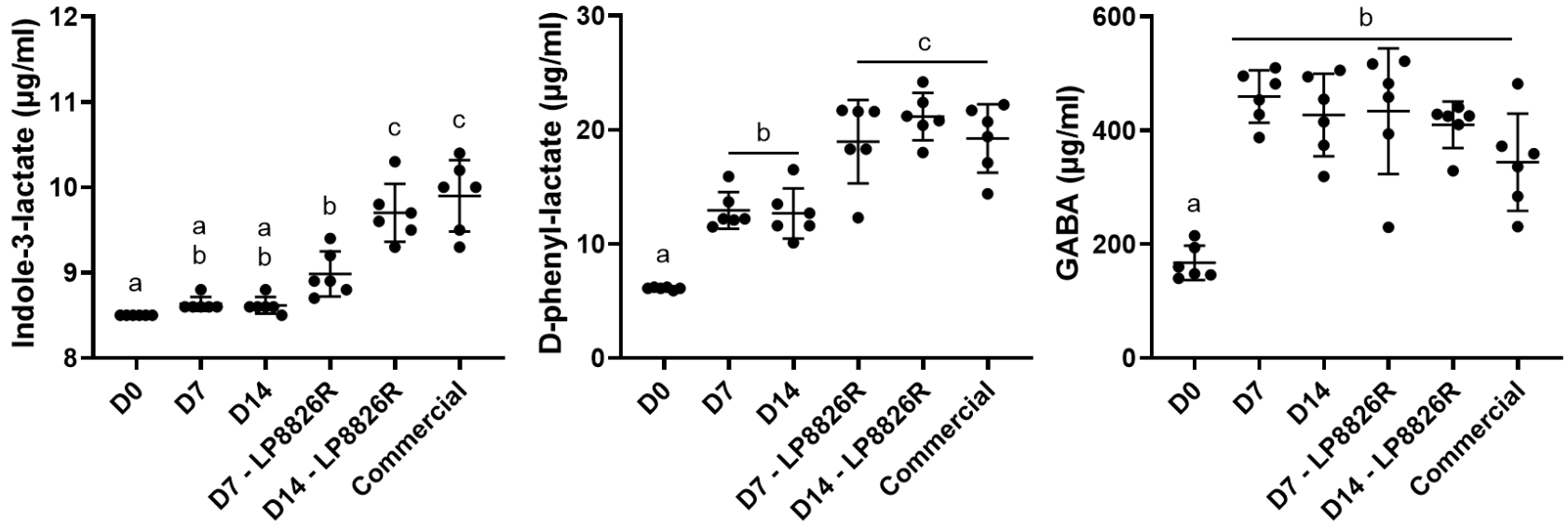


Figure 2.6. Targeted GC-TOF/MS metabolomics quantification of ILA, D-PLA, and GABA in cabbage, laboratory scale, and commercial sauerkraut ferments.

Mean ± SD for n = 6 replicates per condition. D0: cabbage and brine from day 0; D7/D7 – LP8826R: laboratory scale ferments with and without LP8826R from day 7; D14/D14 – LP8826R: laboratory scale ferments with or without LP8826R from day 14. Mean values not sharing letter are significantly different based on one-way ANOVA with Tukey's multiple comparisons test.

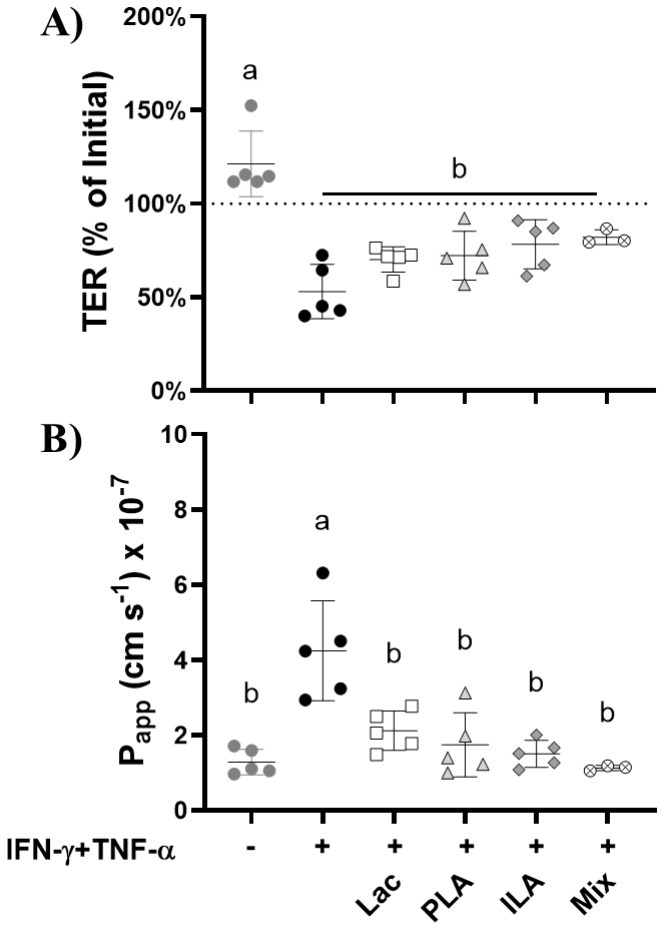


Figure 2.7. Effect of lactate, PLA, ILA, and their mixture on intestinal barrier permeability of cytokine-perturbed Caco-2 monolayers.

(**A**) Trans-epithelial electrical resistance (TER) of Caco-2 monolayers after IFN-γ and TNF-α sequential additions. TER values were normalized to TER measured immediately prior to TNF-α addition. (**B**) Apparent permeability coefficient (P_{app}) to FITC-dextran (4kD) of Caco-2 monolayers measured at the 48 h time point. NT: non-treated (n = 5); CK: cytokine only control (n = 5); Lac: 50 mM lactate (n = 5); PLA: 60 µM D-phenyl-lactate (n = 5); ILA: 25 µM indole-3-lactate (n = 5); Mix: a mixture of the three metabolites (n = 3). Mean ± SD. Mean values not sharing any letter are significantly different based on (TER) two-way or (FITC) one-way ANOVA with Tukey's multiple comparisons test.

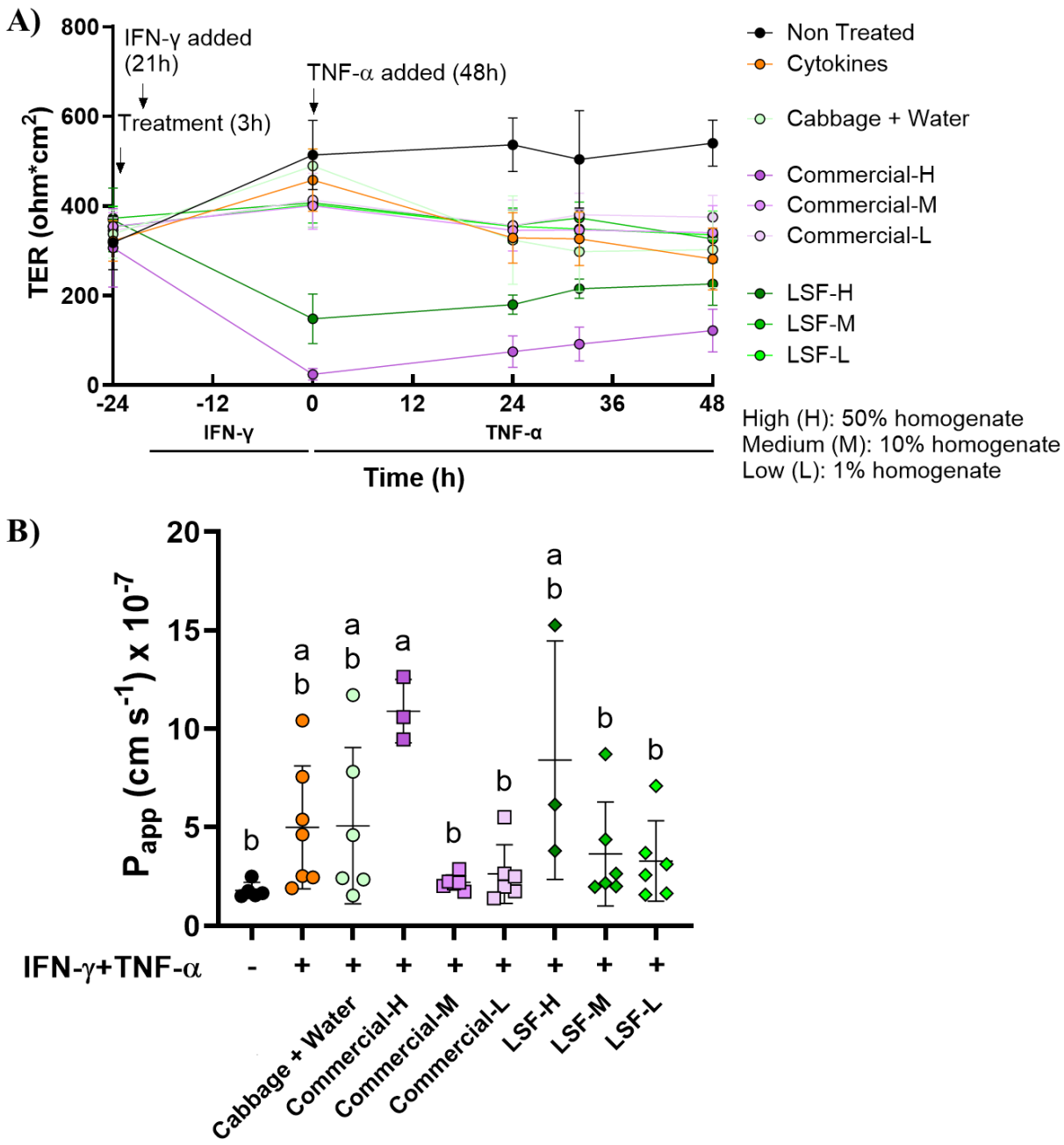


Figure S1. Pilot experiment showing barrier integrity of Caco-2 cell monolayers after exposure to different concentrations of (fermented) cabbage homogenates.

(**A**) Trans-epithelial electrical resistance (TER) of Caco-2 monolayers throughout the treatment time course, including apical pre-treatment with different (v/v) of sauerkraut homogenates for 3 h and sequential basolateral exposure to (100 ng/ml) INF-γ for 21 h and (10 ng/ml) TNF-α for

another 48 h. (**B**) Apparent permeability coefficient (P_{app}) to FITC quantification for Caco-2 monolayers. Homogenates were prepared from laboratory scale ferments (LSF), commercial cabbage ferments, or cabbage and water at (H) 50, (M) 10, or (L) 1% (v/v) concentrations in DMEM medium. Replicates included Caco-2 monolayers not exposed to IFN-γ and TNF-α (n = 5), controls to which the cytokines were applied (n = 7), and those exposed to the cytokines and (fermented) cabbage (at least n = 3). The mean ± SD is shown for two independent experiments. The letters indicate significant differences based on one-way ANOVA with Tukey's multiple comparisons test.

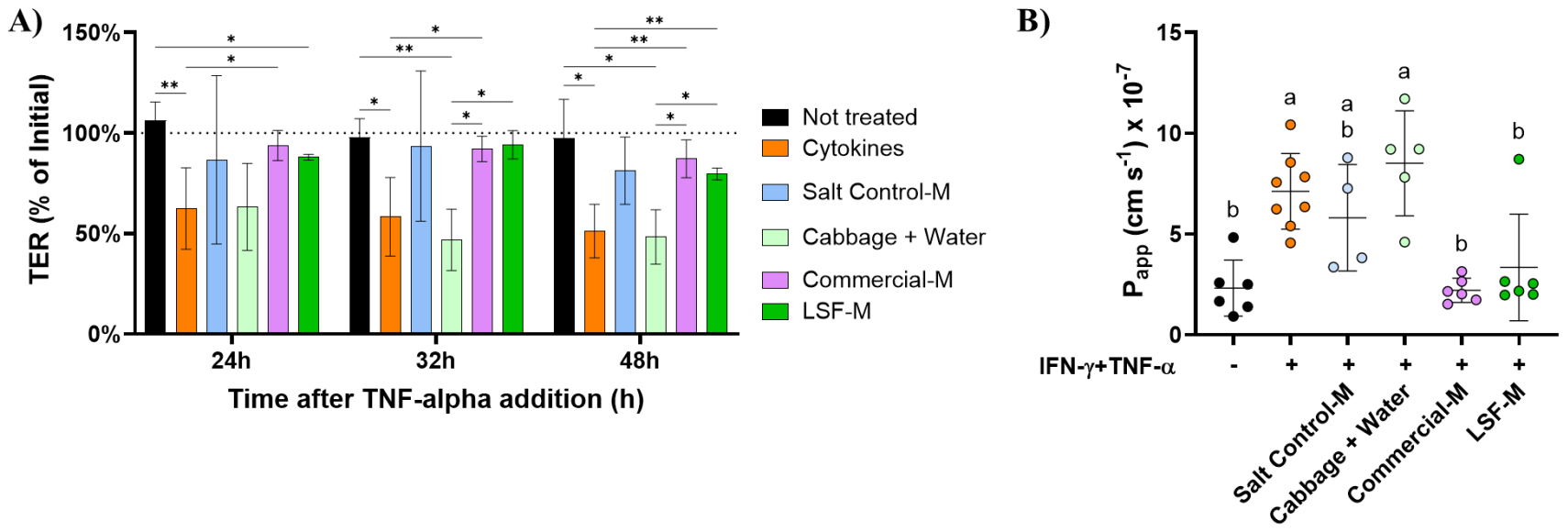


Figure S2. Effect of salt control on intestinal barrier permeability of cytokine-perturbed Caco-2 monolayers.

(**A**) Trans-epithelial electrical resistance (TER) of Caco-2 monolayers 24, 32, and 48 h after basolateral TNF-α (10 ng/ml) addition. Values are normalized to TER immediately prior to when TNF-α was added. (**B**) Permeability to FITC for Caco-2 monolayers treated 10% (v/v) (M) homogenates. Replicates included Caco-2 monolayers not exposed to IFN-γ and TNF-α (n = 5), controls to which the cytokines were applied (n = 7), and those exposed to the cytokines and salt control (0.291% salinity) or (fermented) cabbage (at least n = 3). The mean ± SD is shown for two independent experiments. The asterisks and letters indicate significant differences based on two-way or one-way ANOVA with Tukey's multiple comparisons test, respectively. LSF: laboratory scale ferment.

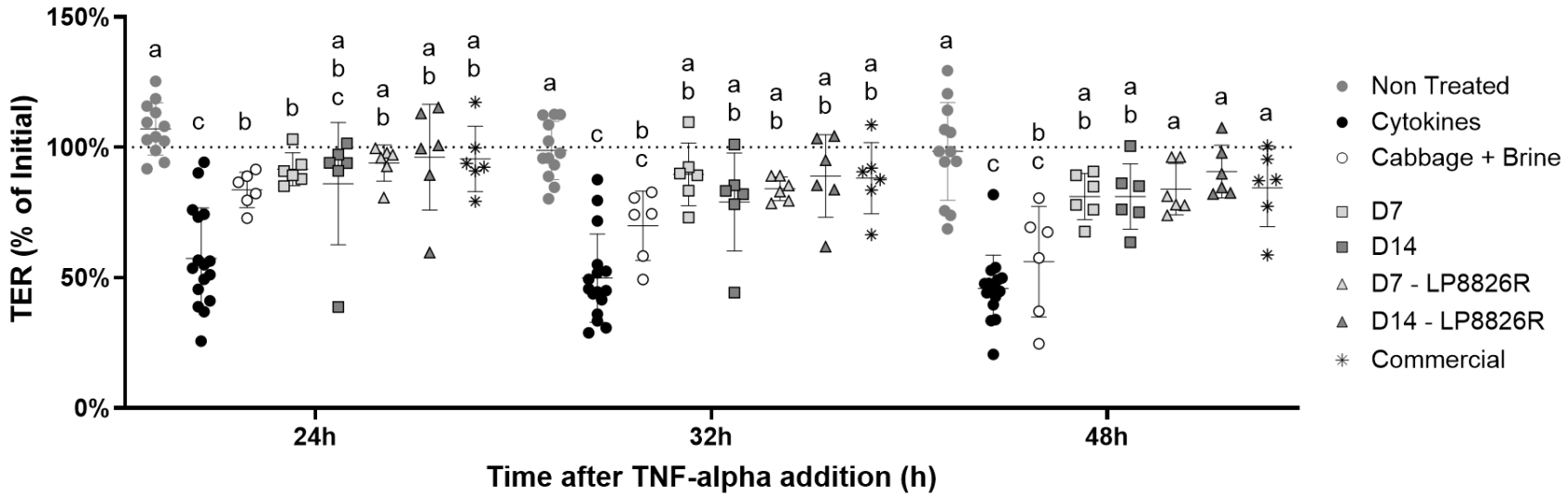


Figure S3. Effect of (fermented) cabbage homogenates on intestinal barrier permeability of cytokine-perturbed Caco-2 monolayers.

Trans-epithelial electrical resistance (TER) of Caco-2 monolayers 24, 32, and 48 h after basolateral TNF-α (10 ng/ml) addition. Values are normalized to TER immediately prior to when TNF-α was added. D7 and D14 were collected on days 7 and 14, respectively, from LSF either with or without the LP8826R additions. Replicates included Caco-2 monolayers not exposed to IFN-γ and TNF-α (n = 12), controls to which the cytokines were applied (n = 16), and those exposed to the cytokines and (fermented) cabbage (n = 6). The mean ± SD is shown for four independent experiments. The letters indicate significant differences based on two-way ANOVA with Tukey's multiple comparisons test.

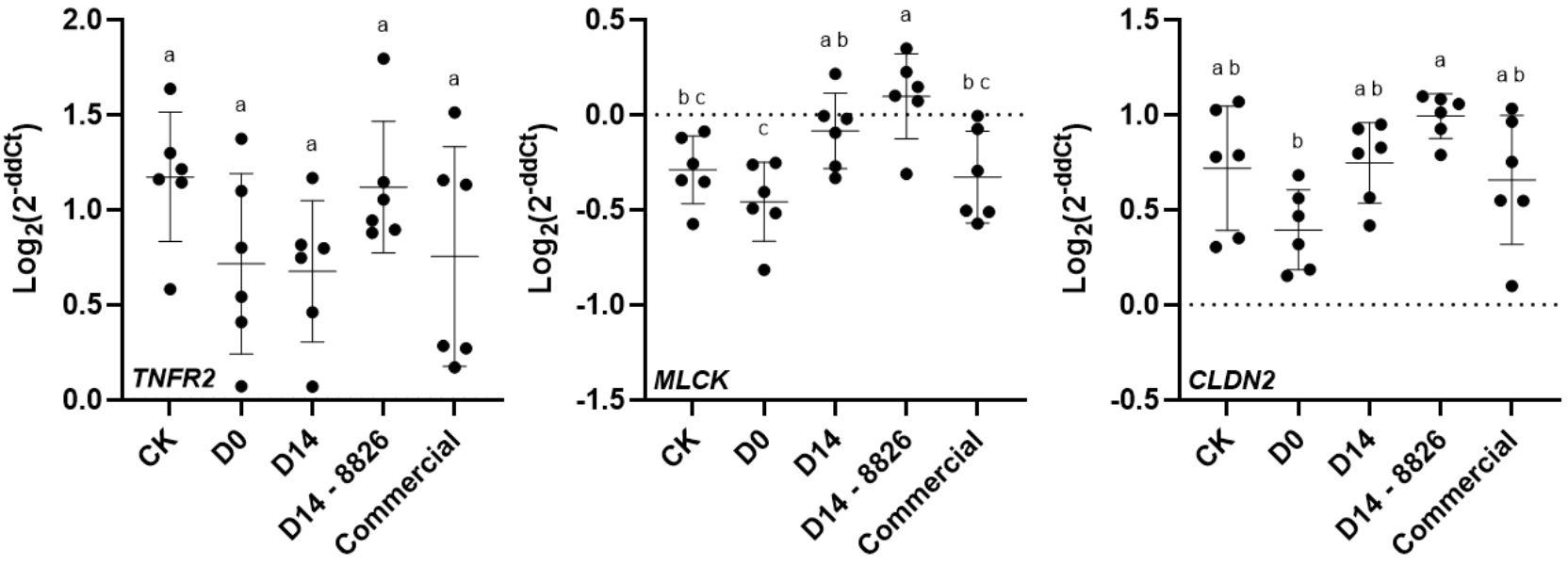
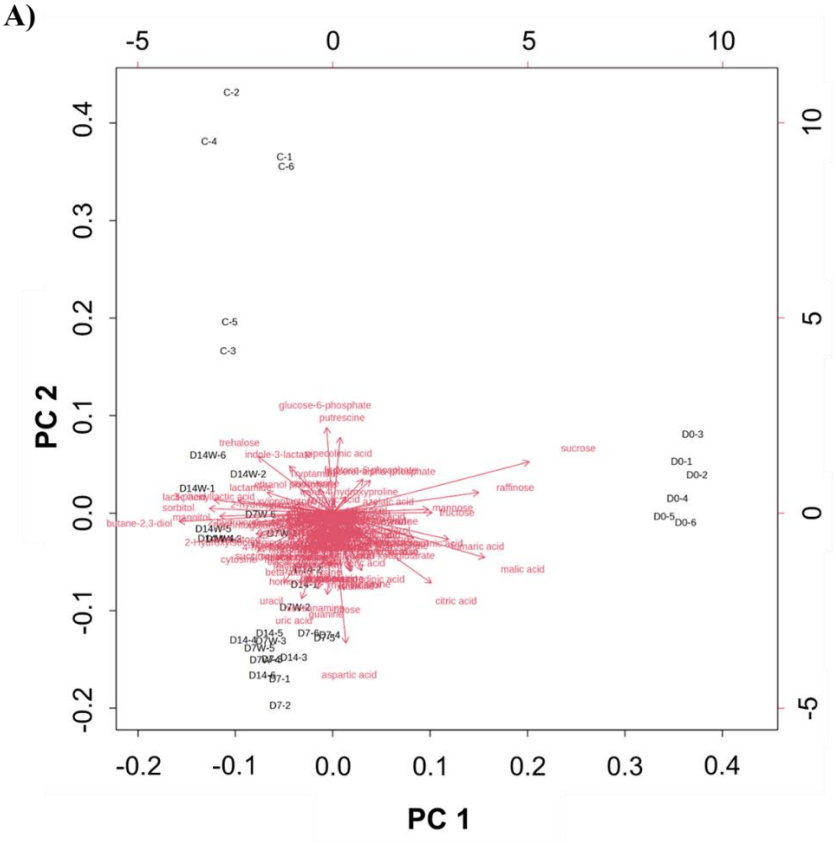


Figure S4. Transcript levels of *TNFR2*, *MLCK*, and *CLDN2* in Caco-2 monolayers after sequential exposure to IFN-γ and TNF-α.

The results were normalized to Caco-2 monolayers not exposed to IFN-γ and TNF-α using the $2^{-\Delta\Delta Ct}$ method with GAPDH as the housekeeping gene. Replicates included Caco-2 monolayers exposed to the cytokines (CK) and those exposed to the cytokines and (fermented) cabbage. The mean ± SD is shown for n = 6 replicates. The mean values that do not share any letters were significantly different based on Kruskal-Wallis with Dunn's multiple comparisons test.

A)


PC 1
PC 2
glucose-6-phosphate
putrescine
trehalose
indole-3-lactate
pipecolinic acid
sucrose
raffinose
malic acid
citric acid
aspartic acid
uric acid
mannose
fructose
sorbitol
mannitol
butane-2,3-diol
D0-1
D0-2
D0-3
D0-4
D0-5
D0-6
D14W-1
D14W-2
D14W-3
D14W-4
D14W-5
D14W-6
D7W-1
D7W-2
D7W-3
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C-1
C-2
C-3
C-4
C-5
C-6

B)

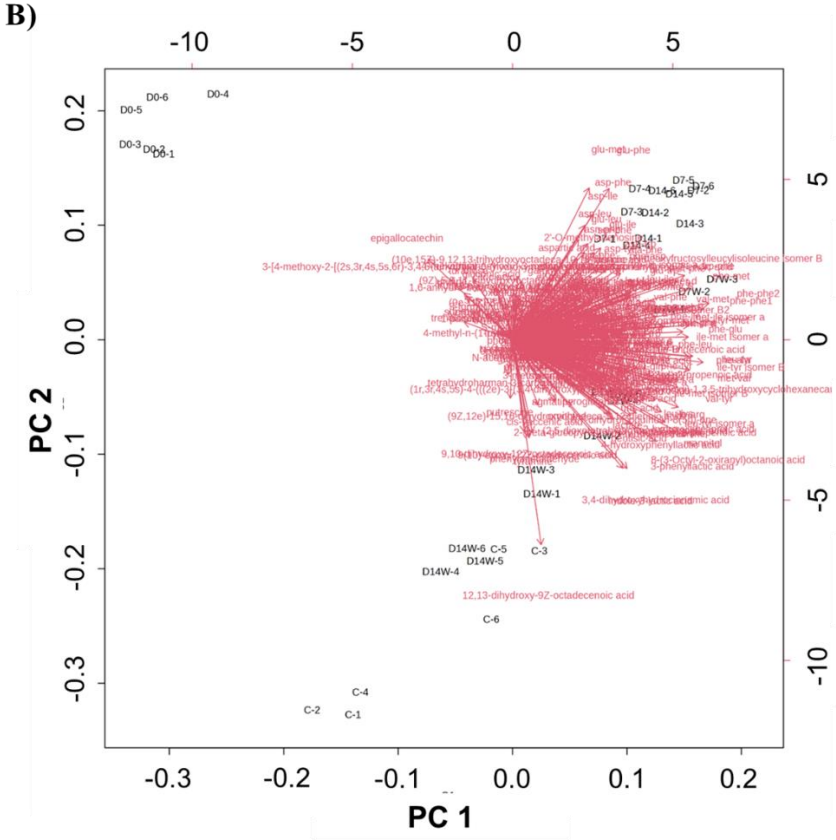

PC 1
PC 2
glu-met-glu-phe
asp-phe
epigallocatechin
12,13-dihydroxy-9Z-octadecenoic acid
D0-1
D0-2
D0-3
D0-4
D0-5
D0-6
D14W-1
D14W-2
D14W-3
D14W-4
D14W-5
D14W-6
D7W-1
D7W-2
D7W-3
D7W-4
D7W-5
D7W-6
C-1
C-2
C-3
C-4
C-5
C-6

Figure S5. Principal component analysis (PCA) biplots showing correlations between metabolites and (fermented) cabbage homogenates.

Metabolites and homogenates that are spatially in the same direction along an axis suggest positive correlations; metabolites and homogenates located in different directions indicate no correlations. The strength of the correlation is shown by the increasing distance from the origin of the plot.

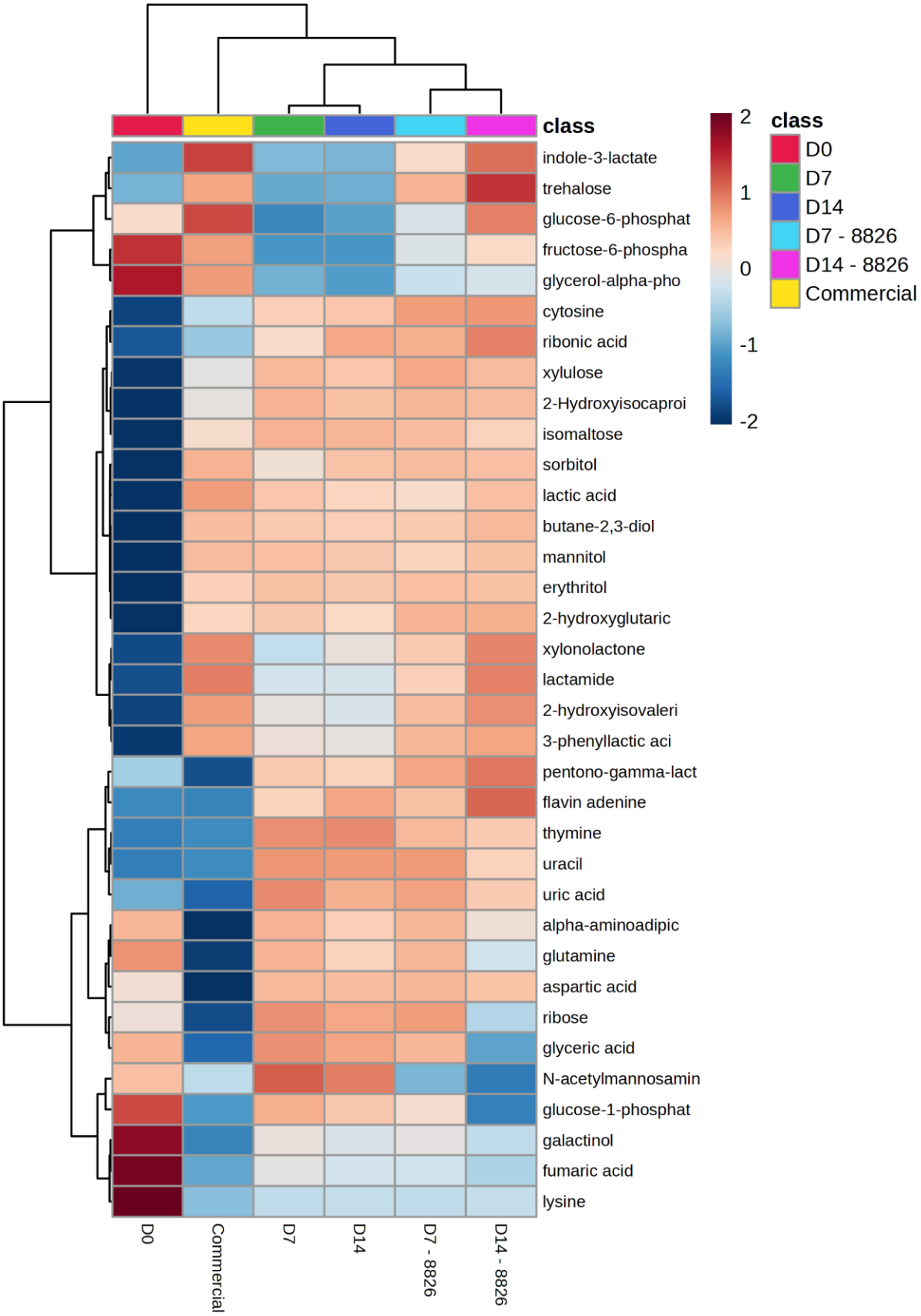

class
indole-3-lactate
trehalose
glucose-6-phosphat
fructose-6-phospha
glycerol-alpha-pho
cytosine
ribonic acid
xylulose
2-Hydroxyisocaproi
isomaltose
sorbitol
lactic acid
butane-2,3-diol
mannitol
erythritol
2-hydroxyglutaric
xylonolactone
lactamide
2-hydroxyisovaleri
3-phenyllactic aci
pentono-gamma-lact
flavin adenine
thymine
uracil
uric acid
alpha-aminoadipic
glutamine
aspartic acid
ribose
glyceric acid
N-acetylmannosamin
glucose-1-phosphat
galactinol
fumaric acid
lysine
D0
Commercial
D7
D14
D7 - 8826
D14 - 8826
2
1
0
-1
-2
class
D0
D7
D14
D7 - 8826
D14 - 8826
Commercial

Figure S6. Heatmaps of the relative peak heights of the top 35 metabolites detected by GC-TOF/MS that distinguish between different (fermented) cabbage homogenates.

Visualization of the compounds were created using normalized data and the following parameters: standardization (Autoscale metabolites features between -2 and 2: mean-centered and divided by the standard deviation of each variable), Distance measure (Euclidean), Clustering method (Ward), top 30 features determined by one-way ANOVA. Blue color represented lower relative peak heights while red color represented higher relative peak heights.

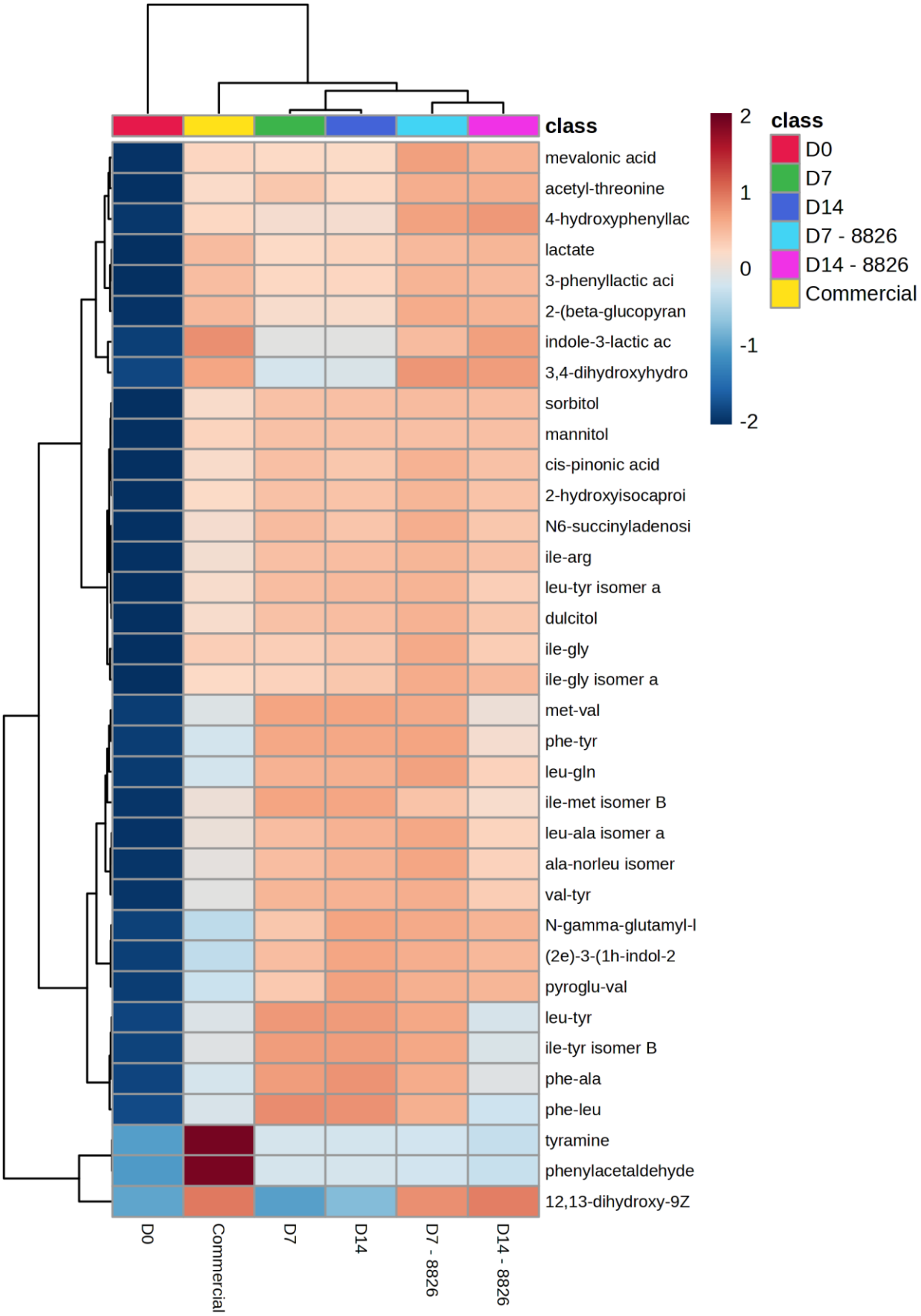

class
mevalonic acid
acetyl-threonine
4-hydroxyphenyllac
lactate
3-phenyllactic aci
2-(beta-glucopyran
indole-3-lactic ac
3,4-dihydroxyhydro
sorbitol
mannitol
cis-pinonic acid
2-hydroxyisocaproi
N6-succinyladenosi
ile-arg
leu-tyr isomer a
dulcitol
ile-gly
ile-gly isomer a
met-val
phe-tyr
leu-gln
ile-met isomer B
leu-ala isomer a
ala-norleu isomer
val-tyr
N-gamma-glutamyl-l
(2e)-3-(1h-indol-2
pyroglu-val
leu-tyr
ile-tyr isomer B
phe-ala
phe-leu
tyramine
phenylacetaldehyde
12,13-dihydroxy-9Z
D0
Commercial
D7
D14
D7 - 8826
D14 - 8826
2
1
0
-1
-2
class
D0
D7
D14
D7 - 8826
D14 - 8826
Commercial

Figure S7. Heatmaps of the relative peak heights of the top 35 metabolites detected by RP-LC-HRMS/MS that distinguish between different (fermented) cabbage homogenates. Visualization of the compounds were created using normalized data and the following parameters: standardization (Autoscale metabolites features between -2 and 2: mean-centered and divided by the standard deviation of each variable), Distance measure (Euclidean), Clustering method (Ward), top 30 features determined by one-way ANOVA. Blue color represented lower relative peak heights while red color represented higher relative peak heights.

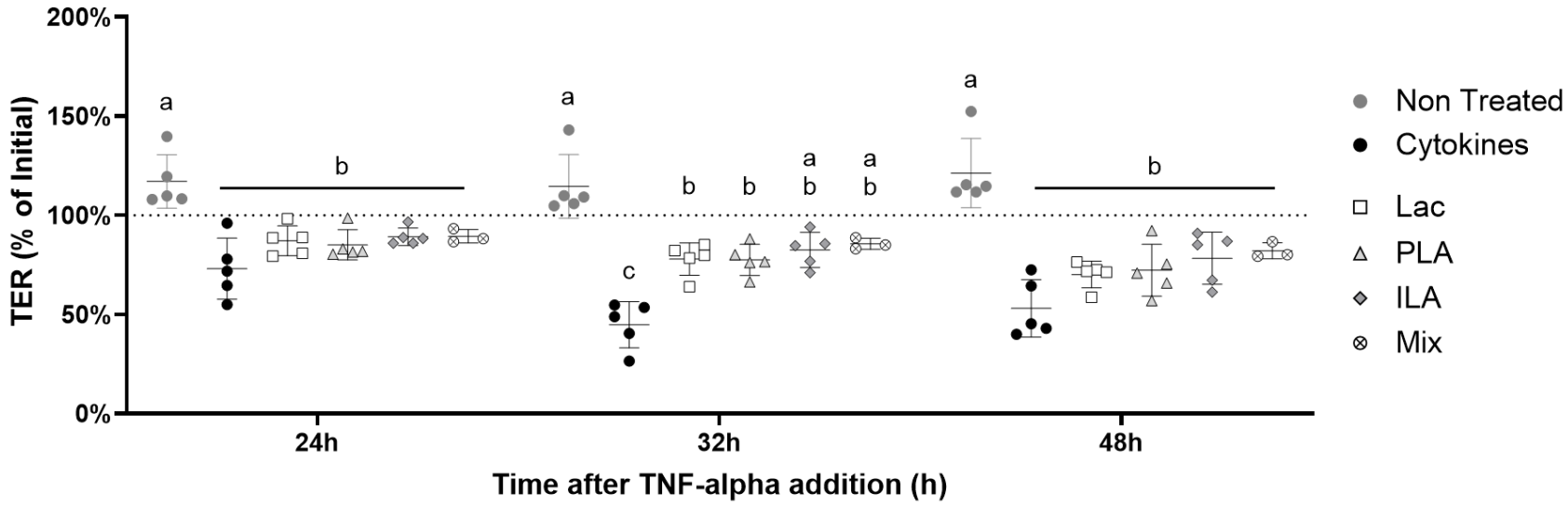


Figure S8. Effect of lactate, PLA, ILA, and their mixture on intestinal barrier permeability of cytokine-perturbed Caco-2 monolayers.

Trans-epithelial electrical resistance (TER) of Caco-2 monolayers 24, 32, and 48 h after basolateral TNF-α (10 ng/ml) addition. Values are normalized to TER immediately prior to when TNF-α was added. Replicates included Caco-2 monolayers not exposed to IFN-γ and TNF-α (Non Treated) (n = 5), controls to which the cytokines were applied (Cytokines) (n = 5), and those exposed to the cytokines and metabolites, 50 mM lactate (Lac), 60 µM D-phenyl-lactate (PLA), 25 µM indole-3-lactate (ILA), or a mixture of the three (Mix) (at least n = 3). The mean ± SD is shown. The letters indicate significant differences based on two-way ANOVA with Tukey's multiple comparisons test.

Supplemental Files are submitted separately on ProQuest ETD

Supplemental File 1. WCMC Fiehn Laboratory - Information on data acquisition, processing, and raw data normalization.

Supplemental File 2. List of metabolites detected by WCMC platforms (as of August 2024).

Supplemental File 3. Metabolites identified by GC-TOF/MS: peak heights, fold changes of ferments compared to day 0.

Supplemental File 4. Metabolites identified by RP-LC-HRMS/MS: peak heights, fold changes of ferments compared to day 0.

Supplemental File 5. Peak heights of internal standards used in RP-LC-HRMS/MS.

Chapter 3. Intestinal delivery of encapsulated bacteriocin peptides in cross-linked alginate microcapsules

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Key words: Microencapsulation, Bioactive peptides, Bacteriocin, Digestion, Enteric delivery, Alginate encapsulates, Peanut butter

Abstract

Oral delivery of larger bioactive peptides (> 20 amino acids) to the small intestine remains a challenge due to their sensitivity to proteolytic degradation and chemical denaturation during gastrointestinal transit. In this study, we investigated the capacity of crosslinked alginate microcapsules (CLAMs) formed by spray drying to protect Plantaricin EF (PlnEF) (C-EF) in gastric conditions and to dissolve and release PlnEF in the small intestine. PlnEF is an unmodified, two-peptide (PlnE: 33 amino acids; PlnF: 34 amino acids) bacteriocin produced by *Lactiplantibacillus plantarum* with antimicrobial and gut barrier protective properties. After 2 h incubation in simulated gastric fluid (SGF) (pH 1.5), 43.39% ± 8.27% intact PlnEF was liberated from the CLAMs encapsulates, as determined by an antimicrobial activity assay. Transfer of the undissolved fraction to simulated intestinal fluid (SIF) (pH 7) for another 2 h incubation resulted in an additional release of 16.13% ± 4.33%. No active PlnEF was found during SGF or sequential SIF incubations when pepsin (2,000 U/ml) was added to the SGF. To test PlnEF release in C-EF contained in a food matrix, C-EF was mixed in peanut butter (PB) (0.15 g C-EF in 1.5 g PB). A total of 12.52% ± 9.09% active PlnEF was detected after incubation of PB+C-EF in SGF without pepsin, whereas no activity was found when pepsin was included. Transfer of the remaining PB+C-EF fractions to SIF yielded the recovery of 46.67% ± 13.09% and 39.42% ± 11.53% active PlnEF in the SIF following exposure to SGF and to SGF with pepsin, respectively. Upon accounting for the undissolved fraction after SIF incubation, PlnEF was fully protected in the CLAMs-PB mixture and there was not a significant reduction in active PlnEF when pepsin was present. These results show that CLAMs alone do not guard PlnEF bacteriocin peptides from gastric conditions, however, mixing them in PB protected against proteolysis and improved intestinal release.

Introduction

Peptides are emerging as an important class of bioactive compounds with the potential to exert antimicrobial (Mahlapuu et al., 2016), metabolism-regulating (Akbarian et al., 2022; Recio et al., 2016), anti-oxidative (Guo et al., 2023; Krishnamoorthi et al., 2022; Tadesse & Emire, 2020), and other beneficial effects on human health (Akbarian et al., 2022; Daliri et al., 2017; Hayes & Tiwari, 2015; Pessione & Cirrincione, 2016). Bioactive peptides include very small peptides (2 – 20 amino acids, "encrypted"; (Daliri et al., 2017)) that are generated from the proteolysis of larger protein matrices, such as food and milk proteins (Daliri et al., 2017; Sánchez & Vázquez, 2017) or agricultural waste by-products, through enzymatic hydrolysis or microbial fermentation (Lemes et al., 2016; Pessione & Cirrincione, 2016; Raveschot et al., 2018). Other bioactive peptides have a larger size (> 20 amino acids) and are synthesized by bacteria (e.g., bacteriocins, nanocystin A) (Chikindas et al., 2018; Fernandes & Jobby, 2022) and eukaryotic organisms (e.g., insulin, oxytocin) (Wang et al., 2022).

Bacteriocins are gene-encoded, ribosomally-synthesized, antimicrobial peptides made by numerous bacteria and archaea (Darbandi et al., 2022). Hundreds of bacteriocins and bacteriocin-encoding gene clusters in Gram-positive lactic acid bacteria (LAB) have been identified via functional assays and genome mining studies (Hammami et al., 2010; Kassaa et al., 2019; Russell & Truman, 2020; Teng et al., 2022). These bacteriocins are generally larger cationic peptides (20 to 70 amino acids), with or without post-translational modifications, that exert potent antimicrobial activity in the picomolar range. The peptides frequently target relatives from the same or highly related species, but other microorganisms can also be negatively affected (Abdulhussain Kareem & Razavi, 2019; Alvarez-Sieiro et al., 2016; Cotter et al., 2005; Gillor et al., 2008). The bacteriocins nisin (34 amino acids, class Ia lantibiotic), pediocin PA-1 (44 amino

acids, class IIa), and enterocin AS-48 (70 amino acids, class Ib) and EJ97 (44 amino acids, class IIc) are used industrially for food preservation and contaminant suppression (Field et al., 2018; Sanchez-Hidalgo et al., 2003; Settanni & Corsetti, 2008; Yang et al., 2014). Besides antimicrobial activity, bacteriocins can also affect intestinal and immune cells (Hu et al., 2018; Teng et al., 2022; Fidanza et al., 2021). Bacteriocins are associated with the induction of cytokine production by regulating nuclear factor kappa-B (NF-κB), mitogen-activated protein kinase (MAPK), and Toll-like receptor (TLR) signaling pathways (Huang et al., 2021; Kindrachuk et al., 2013; O'Callaghan et al., 2012) and enhancing immune cell (neutrophils, macrophages, T cells) functions (Guryanova, 2023; Huang et al., 2021). Furthermore, certain bacteriocins were found to strengthen the intestinal barrier by increasing the expression and localization of epithelial tight junction proteins (Heeney et al., 2019; Huang et al., 2021; Wang et al., 2021). Bacteriocins therefore have great potential for use in foods and medicine (Zimina et al., 2020; Chandrakasan et al., 2019; Eghbal et al., 2022).

Despite the biotechnological potential of bacteriocins, they can be vulnerable to the acidic and proteolytic activities in the stomach (Bogh & Madsen, 2016; Chen & Li, 2012; McClements, 2018; Pei et al., 2022). One promising way to preserve their structural integrity in situ is by encapsulation, or the technology of packaging solid, liquid and gaseous cargo materials in small capsules that release their contents at controlled rates over extended periods of time (Champagne & Fustier, 2007). Depending on the encapsulation matrix (e.g., polysaccharides, liposomes, or protein-based) (Li et al., 2023), the peptides can be released at the intended location in the digestive tract (Champagne & Fustier, 2007; Chandrakasan et al., 2019; Tang et al., 2023). Thus far, nisin antimicrobial activity was shown to be retained after encapsulation in alginate (Ben Amara et al., 2017; Maresca et al., 2016), pectin (Ben Amara et al., 2017), and

calcium alginate-guar gum (Narsaiah et al., 2014). Nisin (da Silva Malheiros et al., 2010; Taylor et al., 2007; Were et al., 2004; Field et al., 2023), pediocin (Eghbal et al., 2022), and microcin J25 (Gomaa et al., 2017) activity was also retained after the liposome encapsulation process. Nisin fragments and intact nisin with antimicrobial activity were found in the mouse feces after the ingestion of nisin A encapsulated in resistant starch (high amylose corn starch) gel and dough matrices (Gough et al., 2018). These findings corresponded with significant changes in bacterial beta diversity of mouse fecal contents Additionally, dual coating liposome encapsulation was found to protect microcin J25 from *in vitro* sequential digestion in gastrointestinal conditions (Gomaa et al., 2017).

A recent development in industrially-scalable encapsulation is with cross-linked alginate microcapsules (CLAMs) formed by a spray-drying process (Jeoh et al. 2017 USPTO Patent US 9,700,519 B2; Jeoh et al. 2020 USPTO Patent US 10,610,492 B2). The CLAMs technology has been applied to protect and control delivery of sensitive bioactives, including enzymes (Santa-Maria et al., 2012), lipids (Strobel et al., 2016; Strobel et al., 2020), and plant-beneficial bacteria (Strobel et al., 2018; Kawakita et al., 2021) in biotechnology, food, and agricultural applications, respectively. The morphology, particle size, the extent of alginate cross-linking of similarly prepared CLAMs with and without other hydrophilic cargoes (e.g., 10, 150, 2000 kDa FITC-dextran and hyaluronic acid) have been extensively characterized (Strobel et al., 2019; Wong et al., 2020; Jeoh et al., 2021). CLAMS were effective at protecting corn oil and fish oil, emulsified lipophilic cargo, in simulated gastric conditions (< 25% release) (Strobel et al., 2016; Strobel et al., 2020). Therefore, we measured the effectiveness of CLAMs encapsulation for protection in simulated gastric fluid (SGF) and small intestinal fluid (SIF) release of the two-peptide bacteriocin, Plantaricin E (PlnE: 3.5 kDa, 33 amino acids); Plantaricin F (PlnF: 3.7 kDa, 34

amino acids). PlnEF is a class IIb two-peptide bacteriocin (Fernandes & Jobby, 2022) made by *Lactiplantibacillus plantarum* with potent antimicrobial activity (Moll et al., 1999; Tang et al., 2018; X. Zhang et al., 2016) and gut barrier modulatory effects (Heeney et al., 2019). Release of active (intact) PlnEF from CLAMs after sequential transfers in SGF, with and without 2,000 U/ml pepsin, and SIF conditions was measured using a radial diffusion assay with a PlnEF-sensitive strain. To understand factors affecting CLAMs effectiveness and investigate a potential food carrier matrix, CLAMs-PlnEF (C-EF) mixed into peanut butter (PB; PB+C-EF) was also tested. Peanut butter is an interesting CLAMs carrier vehicle because it is an energy- and nutrient-dense and shelf stable product that has been widely used as a component of Ready to Use Therapeutic Foods (RUTFs) for treatment of childhood malnutrition (Manary, 2006).

Materials and Methods

Bacterial strain and growth conditions

The PlnEF-sensitive indicator strain, *Lactiplantibacillus plantarum* NCIMB 700965 (LP965) was acquired from the National Collection of Industrial Food and Marine Bacteria (NCIMB) and maintained in glycerol at -80 °C. LP965 was routinely grown at 30 °C in a commercial preparation of lactobacilli De Man, Rogosa and Sharpe (MRS) medium (Becton-Dickinson, Franklin Lakes, NJ).

Plantaricin EF (PlnEF) peptides

The PlnEF bacteriocin peptides were originally isolated from *Lactiplantibacillus plantarum* C11 and the amino acid sequences were determined by peptide sequencing and mass spectrometry (Anderssen et al., 1998). In *L. plantarum*, the precursors of this two-peptide

bacteriocin are encoded by the *plnE and plnF* genes. For our study, PlnE (FNRGGYNFGKSVRHVVDAIGSVAGIRGILKSIR; 14.5 mg) and PlnF (VFHAYSARGVRNNYKSAVGPADWVISAVRGFIHG; 14.2 mg) were chemically synthesized by GenScript (Piscataway, NJ) and reached a purity of 98.2% (PlnE) and 99.8% (PlnF) as verified by HPLC analysis. The molecular weights of PlnE and PlnF are 3545.08 g/mol and 3703.15 g/mol, respectively.

Encapsulation of Plantaricin EF (PlnEF) in cross-linked alginate microcapsules (CLAMs)

Cross-linked alginate microcapsules (CLAMs) were prepared as previously described (Strobel et al., 2016). Briefly, CLAMs feed was prepared with final concentrations of 2% w/w sodium alginate (A1112, Sigma-Aldrich, St. Louis, MO), 0.2% w/w calcium phosphate dibasic salt (Sigma-Aldrich, St. Louis, MO) and 1% w/w succinic acid solution (titrated to pH 5.6 with ammonium hydroxide). The alginate with calcium salt solution was autoclaved before combining with filter sterilized succinic acid solution. To encapsulate PlnEF, equal ratios of lyophilized PlnE and PlnF were mixed with the CLAMs feed prior to spray drying to achieve a final concentration of 0.0004 g PlnEF/g microcapsule.

To produce dry microcapsules, the feed suspensions were spray-dried using a Buchi B290 laboratory spray-dryer (New Castle, DE) at an inlet temperature of 150 ^{o}C, 40 mm Hg nozzle pressure, 25-30% of maximum pump rate (6 mL/min), and maximum aspirator airflow rate. Spray-dried CLAMs-PlnEF (C-EF) and empty CLAMs (without PlnEF; C) powders were stored at -20 °C. Similarly produced CLAM powders with and without cargo have been extensively characterized, exhibiting median particle sizes of ~ 10 μm (range of ~1 (10^{th} percentile) to 15 μm (90^{th} percentile)) and extents of gelation of ~75 – 80% (Strobel et al., 2019;

Wong et al., 2020; Jeoh et al., 2021). Swelling characteristics of similarly prepared CLAMs in water were also previously characterized (Wong et al., 2020). For the simulated gastrointestinal digestion, 0.15 g of C-EF containing 60 µg of PlnEF, or 0.15 g of empty CLAMs were used with (PB+C-EF or PB+C) or without peanut butter (C-EF or C).

Preparation of peanut butter (PB) pellets containing CLAMs-PlnEF

Each gram of creamy PB (Jif™, The J. M. Smucker Company, Orrville, OH) contained 0.48 g of total fat (including 0.1 g saturated fat), 4.24 mg sodium, 0.24 g of total carbohydrate (including 0.06 g fiber, 0.09 g sugar), 0.21 g protein, 0.06 mg vitamin E, 0.52 mg calcium, 0.15 mg niacin, 0.03 mg iron, 5.6 mg potassium. Aliquots of 1.5 g PB, 1.5 g PB mixed with 0.15 g empty CLAMs (PB+C), and 1.5 g PB mixed with 0.15 g C-EF (PB+C-EF) were frozen at -80 °C until use.

CLAMs incubation in simulated gastric and small intestinal fluids

Simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared according to previous studies (Minekus et al., 2014; Strobel et al., 2016) without intestinal phase digestive enzymes (e.g., pancreatin) and bile salts. All reagents were purchased from Fisher Scientific International, Inc., Hampton, NH, unless stated otherwise stated. When specified, porcine gastric mucosal pepsin (P6887, Sigma Aldrich, St. Louis, MO) was added to SGF to reach a final concentration of 2,000 U/ml. These simulated digestive fluids were pH adjusted to 1.5 (SGF) or pH 7 (SIF) immediately prior to use. MilliQ water with a minimum resistivity of 18 MΩ-cm (Millipore, Billerica, MA) sterilized via autoclaving was used for all experiments.

For testing the CLAMs, 0.15 g C-EF, 1.5 g PB mixed with 0.15 g C-EF (PB+C-EF), or the corresponding empty CLAMs (C), PB alone, or PB+C controls was added to 20 ml SGF (pH 1.5) in a 50 ml conical tube. This constitutes a concentration of 3 ng PlnEF/µl SGF (60 µg/20 ml) for samples containing PlnEF, C-EF and PB+C-EF. The conical tube was incubated at 37 °C for 2 h under horizontal agitation at 50 rpm (0.0266 x g based on 1.9 cm orbit diameter, Thermo Scientific MaxQ 4450, Waltham, MA). After the SGF phase, the undissolved and undigested contents were collected using centrifugation at 8,118 x g for 5 min at 21 ºC. The supernatant containing any released PlnEF was collected. The pellet was then transferred to 20 ml SIF (pH 7) and incubated at 37 ºC for 2 h under horizontal agitation at 50 rpm (0.0266 x g based on 1.9 cm orbit diameter, Thermo Scientific MaxQ 4450, Waltham, MA). The incubation times were selected based on prior studies (Brodkorb et al., 2019; Minekus et al., 2014; Strobel et al., 2016) for the digestion of food components. Lastly, the suspension was centrifuged at 8,118 x g for 5 min at 21 ºC to collect the aqueous fraction. The pellet was then dissolved in 100 mM sodium citrate ($C_6H_9Na_3O_9$) with intermittent agitation by vortexing.

Radial diffusion assay to measure PlnEF peptide activity

Radial diffusion assays to measure PlnEF antimicrobial activity against the PlnEF sensitive strain, LP965 were performed as described previously (Deborah A. Steinberg, 1997) with several modifications. LP965 was grown in MRS to approximately 4.3 x 10^8 to 6.3 x 10^8 CFU/ml. The cells were collected by centrifugation at 3,000 x g for 5 minutes at 4 °C, washed once with 10 mM sodium phosphate buffer (pH 7.4, 77.4% sodium phosphate dibasic Na_2HPO_4 and 22.6% sodium phosphate monobasic NaH_2PO_4), and then collected again by centrifugation. Using a protocol modified from Steinberg and Lehrer (Deborah A. Steinberg, 1997),

approximately 4 x 10^6 CFU were mixed with 10 mL 1% w/v molten agarose (pH 7.4) (Fisher Scientific International, Inc., Hampton, NH) containing 10 mM sodium phosphate and 1% v/v MRS broth and allowed to solidify in a petri dish. Wells were then formed using a Pasteur pipette attached to a vacuum bleach trap. For testing, the wells were inoculated with two microliters (µL) of each sample, MilliQ water (negative control), PlnEF (positive control), SGF or SIF controls and the plates were incubated at 30 °C for 3 h. Next, an overlay agarose mixture (30% w/v MRS, 1% w/v agarose) was added on top of the underlay gel to provide additional nutrients for bacterial growth and the plates were then incubated for 48 h at 30 °C.

Standard curves were used to quantify the active fraction of PlnEF in the radial diffusion assay. The standard curve was prepared using PlnEF released from C-EF and PB+C-EF. Release was accomplished by incubation in 100 mM sodium citrate ($C_6H_9Na_3O_9$) for 10 min with intermittent mixing to fully dissociate the CLAMs. For CLAMs and PB mixtures, after incubation in 100 mM sodium citrate ($C_6H_9Na_3O_9$), the suspension was centrifuged at 8,118 x g for 5 min at 21 °C to pellet the PB and the supernatant was used in subsequent tests. The suspensions containing released PlnEF were serially diluted to a ratio of 1:1.5 in 100 mM sodium citrate ($C_6H_9Na_3O_9$) and the dilutions were added to the LP965 radial diffusion assay. The diameter of bacterial clearance around each well was measured with a ruler. The diameter of clearance around a well that contained only MilliQ water (negative control) was 1 mm. The resulting values of the diameter of clearance (mm) and the estimated PlnEF concentration (ng/µl) were plotted. The standard curves followed an exponential function, specifically, $y = 0.2722 * e^{0.3616x}$ for CLAMs-PlnEF (C-EF) and $y = 0.1706 * e^{0.6951x}$ for PB+CLAMs-PlnEF (PB+C-EF), where y represents the estimated PlnEF released (ng/µl) and x represents the diameter of clearance (mm). Each SGF and SIF sample was measured in triplicate, and data were collected

from at least three independent plates from two independent experiments. Representative radial diffusion assay images and standard curves are shown in **Fig. S1** and **Fig. S2**, respectively. The zones of clearance in the radial diffusion assays were quantifiable across a sensitive 7.5-fold range (0.4 – 3 ng/µl PlnEF). The standard deviation for the diameters of clearance (mm) from at least two independent experiments (n = 6 to 7) ranged from 0 – 0.68 mm.

Statistical analysis

Data were analyzed using GraphPad Prism 9 (GraphPad Software, Inc., San Diego, CA). Unless otherwise stated, data are presented as mean ± standard deviation (SD). Group means were compared by one-way ANOVA or Kruskal-Wallis followed by an appropriate post-hoc test. A p value < 0.05 was considered significant unless otherwise noted.

Results

CLAMs alone provide limited protection against PlnEF gastric release

The release profile of PlnEF peptides from CLAMs (C-EF) upon sequential exposure to simulated gastric fluid (SGF) and the simulated intestinal fluid (SIF) without the associated pepsin enzyme was investigated first. After 2 h incubation in SGF, 1.33 ± 0.13 ng active PlnEF /µl was detected in the supernatant, as determined by a radial diffusion assay with the PlnEF sensitive strain LP965 (**Fig. 1 and Table 1**). This quantity of PlnEF constituted 43.39% ± 8.27% of the 3 ng PlnEF /µl contained in the CLAMs tested. Incubation of the remaining undissolved C-EF in SIF for another 2 h resulted in the release of an additional 0.96 ± 0.05 ng PlnEF /µl, or 16.13% ± 4.33%, which is significantly lower than that released in the SGF (p = 0.0001) (**Fig. 1 and Table 1**). However, the CLAMs were not completely solubilized at that time and the

remaining insoluble fraction harbored 1.31 ± 0.17 ng active PlnEF /µl (40.48% ± 9.89% PlnEF), quantified after dissolution of the C-EF in 100 mM sodium citrate (**Fig. 1 and Table 1**). Those undissociated CLAMs may still be relevant in situ because extending incubation of CLAMs-PlnEF in SIF to 6 h led to near complete dissolution of C-EF and recovery of PlnEF (1.14 ± 0.10 ng PlnEF/µl total) in the released (aqueous) phase (**Fig. S3**). Notably, these quantities recovered in the different fractions accounted for approximately all of the PlnEF provided in the CLAMs cargo (103.0 ± 10.1 % recovery) (**Table 1**). In summary, the results show that a relatively high fraction of the PlnEF bacteriocin was released from CLAMs in the SGF and the remaining PlnEF remained intact and active in the small intestine conditions.

Mixture in a food matrix delayed PlnEF release

Incubation of PB+C-EF in SGF for 2 h resulted in a release of 0.57 ± 0.27 ng intact PlnEF /µl (12.52% ± 9.09%) (**Table 1 and Fig. 2**), an amount that is approximately 3.5-fold lower than found for C-EF in SGF. Another 1.33 ± 0.28 ng PlnEF /µl (46.67% ± 13.09%) (**Fig. 1 and Table 1**) was recovered in the soluble fraction after transfer of the undissolved PB+C-EF into SIF for another 2 h incubation. In contrast to C-EF, significantly greater amounts of PlnEF were released from the CLAMs in the small intestinal (SIF) in 2 hours, relative to the gastric (SGF) conditions ($p = 0.0002$) (**Fig. 1 and Fig. 2**). Notably, the PB+C-EF was not fully dissolved after the sequential (2 h) SGF and (2 h) SIF incubations, such that a high quantity of active PlnEF (1.22 ± 0.13 ng PlnEF /µl), constituting 40.80% ± 5.82% of total PlnEF tested, remained in the PB+C-EF fraction at that time (**Fig. 1, Table 1, and Fig. 2**). In total, the PlnEF in the CLAMs was fully accounted for in the different factions (119 ± 7.9 % recovery). Hence, PB slowed active PlnEF release from CLAMs in SGF, and active PlnEF quantities were significantly greater in the soluble and insoluble fractions in SIF.

Peanut butter protected CLAMs-PlnEF from pepsin

We next compared the capacity of CLAMs and PB to protect PlnEF against pepsin. Pepsin is a gastric protease able to digest PlnEF (Chaalel et al., 2015). After 2 h incubation of C-EF in SIF with pepsin (2,000 U/ml), no active PlnEF was found in either the soluble or insoluble fractions (**Fig. 1 and Table 1**). Active PlnEF was also not detected in either fraction after subsequent incubation in SIF (**Fig. 1**).

Similarly, PlnEF activity was not found in the supernatant after PB+C-EF was incubated in SGF with pepsin. However, intact PlnEF was recovered again when the undissolved PB+C-EF was incubated in SIF for 2 h. A total of 1.01 ± 0.25 ng PlnEF/µl, or 39.42% ± 11.53% of the total PlnEF tested, was detected in those suspensions, suggesting that the peptides remained intact in PB+C-EF, but not the C-EF matrix with pepsin exposure. The remaining PlnEF in the insoluble fraction was also active and was found to contain 1.58 ± 0.37 ng active PlnEF/µl, or 60.58% ± 11.53% of the total added (**Fig. 1 and Table 1**). Taken together, there was 85.8% ± 10.0% recovery of PlnEF from PB+C-EF when pepsin was included (**Table 1**). This quantity was not significantly different from the fraction of active PlnEF recovered from PB+C-EF when pepsin was excluded ($p > 0.99$; Kruskal-Wallis with Dunn's multiple comparisons test). Unlike CLAMs alone, the addition of PB provided protection against pepsin in SGF and supported intact PlnEF delivery to the SIF.

Discussion

CLAMs provided minimal protection for the peptide bacteriocin PlnEF in simulated gastric conditions. A high fraction (~40%) of PlnEF was released from the CLAMs in the SGF

and CLAMs offered no protection against pepsin. Mixing CLAMs with PB significantly improved the release profile in both SGF and SIF and guarded against pepsin proteolysis. A large fraction of CLAMs in PB-C-EF also remained intact after 2 h in SIF, suggesting that CLAMs could also potentially reach the large intestine. These findings show the limitations of CLAMs for bioactive peptide delivery to the digestive tract, but also indicate that the use of CLAMs as a carrier is improved when combined with food matrices like peanut butter.

CLAMs were previously found to shield corn oil from release in the gastric conditions (Strobel et al., 2016), such that 22% was released in simulated gastric fluid (pH 1.5) and 81% was released in simulated intestinal fluid (pH 7) after 2 h incubation. This difference in release profiles between corn oil and PlnEF is likely due to their macromolecular properties (McClements, 2018; Z. Zhang et al., 2016). The average particle sizes of the corn oil droplets ranged from 200 to 300 nm (Strobel et al., 2016), while the size of PlnEF (PlnE (3.5 kDa) and PlnF (3.7 kDa)) is around 2.6 nm (in diameter, assuming a globular structure of PlnEF complex) based on an equation associating protein mass (Dalton) to minimal radius R_m (nm) (Erickson, 2009). Because previous studies noted that the pore sizes of the calcium alginate hydrogels can range from 10 to 20 nm (Eghbal et al., 2022; Fundueanu et al., 1999), these pores may not be sufficient to fully retain the smaller PlnEF complexes. Furthermore, at pH 1.5, the alginate network forms insoluble acid gels due to the protonation of the carboxyl groups on the alginates, the reduction of repulsion, and the formation of hydrogen bonding between alginates under the acid pH (Doumeche et al., 2004; Strobel et al., 2016; Jeoh et al., 2021). Consequently, while PlnE (isoelectric point of 12.0) and PlnF (isoelectric point of 10.6) have six positively charged amino acid residues (Arg, Lys, His) at pH 1.5, the removal of negative charges from the alginates likely weakens any electrostatic interactions between PlnEF and the alginate matrix, resulting in

lower retention of the peptides when CLAMs-PlnEF was exposed to gastric conditions. Ionic interactions between PlnEF and the alginate matrix can also be weakened in a solution containing other ions, namely, the electrostatic screening effect (McClements, 2018). Together, the smaller theoretical particle size of PlnEF, the reduced ionic interactions, and the potential electrostatic screening effect from the salts in the SGF likely contributed to the increased release of PlnEF from CLAMs during the gastric phase.

There was no functional PlnEF recovered after incubating CLAMs-PlnEF in pepsin containing SGF. Stevanovic *et al*. (2020) (Dajic Stevanovic et al., 2020) noted that pepsin, with a hydrodynamic radius of around 3 nm (Gtari et al., 2017), can diffuse through the matrix structure of the calcium alginate hydrogels, where the pore sizes range from 10 – 20 nm (Eghbal et al., 2022; Fundueanu et al., 1999). Therefore, pepsin likely also diffused into the CLAMs microcapsules and degraded the PlnEF remaining in CLAMs during SGF incubation.

CLAMs-PlnEF mixed in PB showed improved retention in SGF (pH 1.5) and enhanced protection from pepsin. PB may serve as a hydrophobic layer to increase the overall hydrophobicity of the matrix and reduce the rate of diffusion of the aqueous solutions and molecules, such as pepsin, into the matrix. PB has been utilized as an effective delivery matrix for probiotics (Klu & Chen, 2016; Klu et al., 2014), small molecule drugs (Gonzales et al., 2014; Warren et al., 2021); and antibiotic (Tran et al., 2020). Incorporation of hydrophobic carrier materials, such as lipids and low melting point fats, has been shown to reduce the diffusion of water-soluble vitamin from protein-based encapsulation matrix (Egan et al., 2014), to improve protection for protein-polysaccharide matrix encapsulated bacteria during spray-drying and *in vitro* gastric digestion (Liu et al., 2016), and to increase the retention of phenolic compound encapsulated in polysaccharide materials (Gomes et al., 2010). The addition of hydrophobic latex

to a matrix of gelatin, alginate and succinic acid further reduced the release of peptides with similar molecular weights as PlnE (3.5 kDa) and PlnF (3.7 kDa), semaglutide (4113 Da), liraglutide (3751 Da), and GLP-1 (3298 Da), but not the smaller gonadorelin acetate (1242 Da) or oxytocin acetate (1007 Da) in SGF (Tang et al., 2023). The CLAMs encapsulation likely aided the release of PlnEF from PB by reducing the peptide surface contact and undesirable interactions with the protein and lipid components in the PB (Champagne & Fustier, 2007). Hence, future studies should investigate how CLAMs and other encapsulates are affected when consumed together with PB and other food matrices.

The current study was a first step in determining the feasibility of applying CLAMs encapsulation technology for the delivery of peptide cargo, highlighting the retention of bioactive peptide after the encapsulation and spray-drying process. While CLAMs were unable to guard PlnEF in gastric conditions with pepsin, the incorporation of C-EF in PB prior to exposure to gastric conditions protected the majority of the bacteriocin. One limitation of this study was that we did not compare the effects of incorporating PlnEF (no CLAMs) directly into PB. Therefore, future studies should investigate food-based approaches for bacteriocin delivery. Additionally, there may be opportunities to improve delivery of encapsulates in food matrices like peanut butter which not only may help shield peptides sensitive to proteolytic enzymes but also may be useful for increasing shelf-life stability (Silva et al., 2022; Mishra et al., 2024). Alternatively, complex coacervation via spray drying in a matrix consisting of protein and alginate (the CoCo process), showing promise for protection and controlled release of protein and peptides in fluids, could be explored (Tang et al., 2022; Tang et al., 2023). The findings ultimately should be verified in human and animal studies. Protecting protease-sensitive bioactive peptides from the harsh, enzyme-rich conditions of the gastrointestinal environment and releasing the peptides at the appropriate enteric location remain two of the major hurdles to

the oral delivery of therapeutic peptides. Because bacteriocins encompass diverse peptides with different sizes, charges, and protease sensitivities, the encapsulation and carrier delivery approach will need to be evaluated for each bacteriocin individually. However, enteric delivery systems such as CLAMs are crucial for realizing their full prophylactic and therapeutic potentials.

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Declaration of Interest

Maria Marco, Lei Wei, and Dana Wong declare no conflicts of interest. Tina Jeoh is a co-inventor on the original CLAMs patent (US9700519B2).

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Table 3.1. Estimated PlnEF release from simulated gastrointestinal conditions.

	Estimated PlnEF Release (ng/µl)[a,b]				% Recovery
	SGF	**SIF**	**Remainder**	**Sum**	
CLAMs-PlnEF (n = 7)	1.33 ± 0.13	0.96 ± 0.05	1.31 ± 0.17	3.60 ± 0.24	119.0 ± 7.9
CLAMs-PlnEF (pepsin) (n = 6)	BDL[c]	BDL[c]	BDL[c]	BDL[c]	BDL[c]
PB+C-EF (n = 6)	0.57 ± 0.27	1.33 ± 0.28	1.22 ± 0.13	3.12 ± 0.30	103.0 ± 10.1
PB+C-EF (pepsin) (n = 6)	BDL[c]	1.01 ± 0.25	1.58 ± 0.37	2.58 ± 0.30	85.8 ± 10.0

BDL = below detection level; PB = peanut butter; C-EF = CLAMs-PlnEF.

SGF: PlnEF released into the supernatant during 2h incubation in simulated gastric fluid, pH 1.5; SIF: PlnEF released into the supernatant during 2h incubation in simulated intestinal fluid, pH 7; Remainder: PlnEF remaining in the solid portion of the CLAMs or peanut butter + CLAMs after the sequential SGF and SIF incubation.

[a] Estimated total PlnEF release was calculated based on input PlnEF encapsulated at 0.0004 g PlnEF per g microcapsule to form CLAMS-PlnEF and the release assay volume of 20 ml.

[b] Mean ± Standard Deviation (SD)

[c] Detection limit: 1 mm diameter was the lower detection limit of the radial diffusion assay and considered to be no observable PlnEF antimicrobial activity, estimated to be 0.39 ng/µl PlnEF for CLAMs-PlnEF (C-EF) samples and 0.34 ng/µl PlnEF (for peanut butter + CLAMs-PlnEF (PB+C-EF) samples.

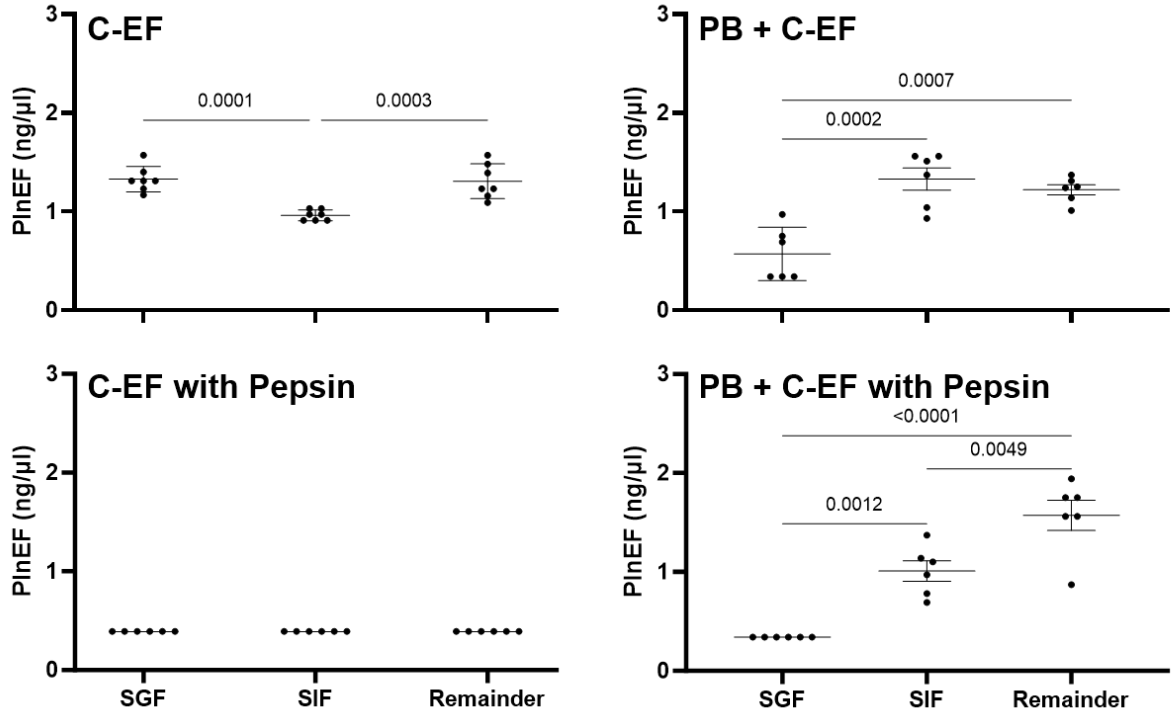


Figure 3.1. PlnEF release from CLAMs or peanut butter + CLAMs in simulated gastrointestinal fluids without or with pepsin.

SGF: PlnEF released into the supernatant during 2h of incubation in simulated gastric fluid, pH 1.5; with 2,000 U/ml pepsin when noted. SIF: PlnEF released into the supernatant during 2h of incubation in simulated intestinal fluid, pH 7; Remainder: PlnEF remaining in the solid portions of CLAMs or peanut butter + CLAMs after SIF incubation. PlnEF release from CLAMs-PlnEF (C-EF) and from peanut butter + CLAMs-PlnEF (PB+C-EF) estimated from standard curves of radial diffusion assays. Mean ± Standard Deviation (SD). One-way ANOVA with Tukey's multiple comparisons test, $p < 0.05$ shown. n = 3, from two independent experiments are shown.

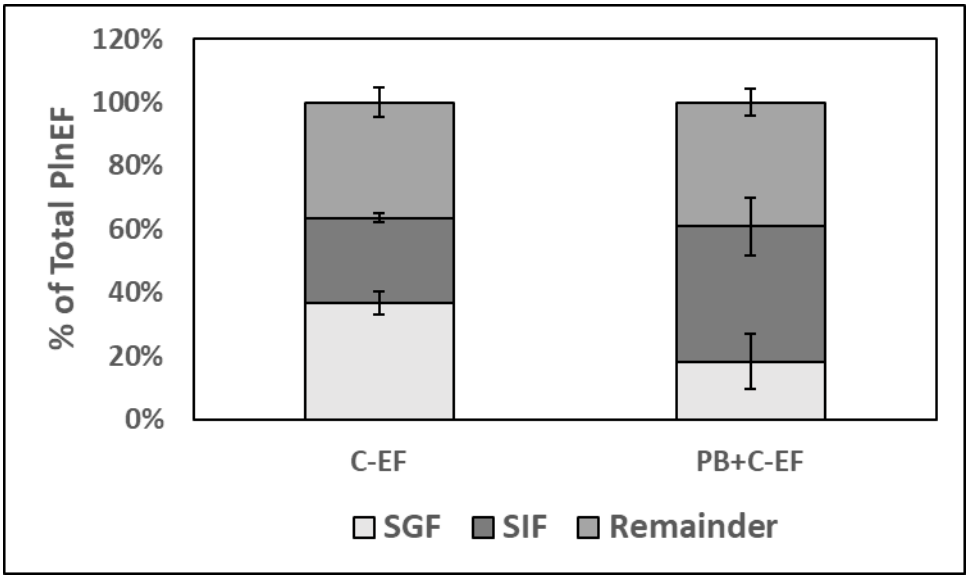


Figure 3.2. Percentage of PlnEF released into the supernatant during sequential SGF (without pepsin) and SIF incubation.

SGF: PlnEF released into the supernatant during 2h incubation in simulated gastric fluid, pH 1.5, without pepsin. SIF: PlnEF released into the supernatant during 2h incubation in simulated intestinal fluid, pH 7. Remainder: PlnEF remaining in the solid portions of CLAMs or peanut butter + CLAMs after SIF incubation. PlnEF release from CLAMs-PlnEF (C-EF) and from peanut butter + CLAMs-PlnEF (PB+C-EF) estimated from standard curves of radial diffusion assays. Mean ± Standard Deviation (SD).

Chapter 4. Bacteriocin plantaricin EF effects on human and porcine intestinal epithelial cell barrier function and intracellular ion levels

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Abstract

The intestinal epithelium has an essential role in nutrient uptake, immune development, and the separation of luminal contents (e.g., gut microbiota, antigens, toxins) and the sterile serosa. Disruptions of this intestinal epithelial barrier are associated with numerous metabolic and inflammatory disorders. This study assessed the potential intestinal barrier protective properties of plantaricin EF (PlnEF), a two-peptide bacteriocin produced by *Lactiplantibacillus plantarum* NCIMB8826 (LP8826R), and a variant of PlnEF (mEF) with a single amino acid change in PlnE-G9I with reduced antimicrobial activity, in polarized human Caco-2 monolayers challenged sequentially with basolateral IFN-γ and TNF-α. PlnEF (1000 nM) and mEF (500 nM) alleviated the TER reduction and lowered the paracellular permeability to fluorescein isothiocyanate-dextran (FITC; 4 kDa) by approximately 2.7-fold compared to cytokine-challenged Caco-2 monolayer controls. This barrier protective effect of PlnEF and mEF was not observed in polarized porcine IPEC-J2 monolayers challenged with cytokines. Exposure of non-polarized Caco-2 cells to PlnEF led to elevated transcript levels of genes encoding for tight junction proteins (TJPs), *OCLN* and *CLDN1*, as well as homologs of the bacterial CorC cysteine-β-synthase (CBS) domain (*CNNM4*, a Mg^{2+} efflux protein and *AMPKγ1*, the cellular energy sensor and regulator) hypothesized to be a potential PlnEF binding region. These results indicate that a PlnEF associated protective effect is likely linked to the modulation of numerous cellular pathways. Despite that PlnEF causes disruptions to ion gradients in sensitive bacterial cells, inductively coupled plasma mass spectrometry (ICP-MS) showed no significant changes in intracellular ion levels of Caco-2 cells after exposure to (500 nM) PlnEF. When tested on IPEC-J2, a small but significant ($p < 0.02$) reduction in intracellular Mg^{2+} and K^{+} was found. These findings show the putative prophylactic potential of PlnEF to prevent disruptions to intestinal

barrier function, but additional studies are needed to determine the specific cellular pathways affected by the bacteriocin and the significance of those changes in the digestive tract.

Introduction

The intestinal epithelium serves as a selectively permeable mucosal barrier that allows the absorption of fluids and nutrients while preventing the movement of pathogens, antigens, and other toxic compounds into the sterile serosal circulation of the host from the gut lumen (Lee et al., 2018; Ulluwishewa et al., 2011). Increased intestinal barrier permeability, or a leaky gut, has been associated with a variety of chronic inflammatory, autoimmune, and metabolic diseases like inflammatory bowel disease, type I diabetes, and obesity (Bischoff et al., 2014; Lee et al., 2018; Lee, 2015; Stolfi et al., 2022). The critical involvement of the intestinal epithelium in health and disease underlines the intestinal barrier integrity and functions as a promising target for therapeutics intervention in humans (Di Vincenzo et al., 2024; Gao et al., 2023; Odenwald & Turner, 2017) and swine (Moeser et al., 2017; Szabo et al., 2023; Tang et al., 2022). Given the importance of intestinal microorganisms in influencing intestinal barrier functions (Di Vincenzo et al., 2024; Ghosh et al., 2021; Schreiber et al., 2024), this may be achieved through the intake of microbes (e.g., probiotic supplementation) (Bodke & Jogdand, 2022; Gareau et al., 2010; Zheng et al., 2023) or microbial-derived bioactive compounds, such as those in fermented foods or postbiotics (Hill et al., 2023; van Baarlen et al., 2013; Yan & Polk, 2020) or as food or feed additives (Kim & Duarte, 2021; Pereira et al., 2022). Investigations into epithelial responses induced by specific strains and bioactive metabolites are still needed.

Lactic acid bacteria (LAB) are a phylogenetically related group of Gram-positive microorganisms that produce lactic acid as a sole or one of the main (> 50%) products of their homo-fermentative or hetero-fermentative energy conservation metabolism (Gänzle, 2015). They are intimately involved in the production of many fermented foods and beverages, contributing to their sensory properties, enhanced nutritional quality, and health-promoting effects through

transformation of substrates and secretion of bioactive compounds (Melini et al., 2019; Paramithiotis et al., 2022; Septembre-Malaterre et al., 2018). Moreover, many lactobacilli species have been investigated for their potential probiotic attributes, as such that lactobacilli (Eun et al., 2011; Gou et al., 2022; Huang et al., 2022; Kanmani & Kim, 2019; Seth et al., 2008) or lactobacilli-derived bioactive metabolites (Chugh & Kamal-Eldin, 2020; Heeney et al., 2018; van Baarlen et al., 2013) are able to improve symptoms of intestinal disorders or exert intestinal barrier protective effects in response to pro-inflammatory cytokines and other perturbations (e.g., hydrogen peroxide, LPS) through modulation of host signaling initiated by intestinal epithelial cells (Hegarty et al., 2016; Yin et al., 2018; Zhai et al., 2019).

Lactiplantibacillus plantarum is one of the LAB species essential for lactic acid fermentation of fruits and vegetables and strains of this species are well-established probiotics. Administration of *L. plantarum* NCIMB8826R (LP8826R) is associated with increased localization of tight junction proteins in the human duodenum (Karczewski et al., 2010), improved ileum intestinal epithelial repair (Crakes et al., 2019) and anti-inflammatory effects (Hirao et al., 2014) in Simian Immunodeficiency Virus (SIV)-infected rhesus macaques, and anti-obesogenic effects in mice with diet-induced obesity through mechanisms affecting tight junction protein levels in intestinal epithelial cells (Heeney et al., 2018).

LP8826R produces a two-peptide (class IIb) bacteriocin, Plantaricin EF (PlnEF) that disrupts the membrane integrity of the sensitive bacterial cells (Diep et al., 1996; Moll et al., 1999), potentially through a magnesium efflux protein, CorC (Heeney et al., 2019). The PlnEF bacteriocin peptides were originally isolated from *L. plantarum* C11 and the amino acid sequences were determined by peptide sequencing and mass spectrometry (Anderssen et al., 1998). In *L. plantarum*, the precursors of this two-peptide bacteriocin are encoded by the *plnE*

and *plnF* genes. Bacteriocins are well-known as ribosomally synthesized antimicrobial peptides that typically target bacteria from the same or highly related species in the picomolar to nanomolar range (Dobson et al., 2012), likely through receptor mediated binding interactions (Cotter, 2014). More recently, a growing number of studies revealed the potential of bacteriocins to influence host homeostasis, such as conferring diarrhea resistance in early weaned piglets (Hu et al., 2018), improving human intestinal cell barrier function (Miyauchi et al., 2012) and exerting anti-carcinogenic activity (Norouzi et al., 2018). To this regard, PlnEF biosynthetic capacity in LP8826R was associated with immunomodulatory capacity in immune cells (Meijerink et al., 2010; van Hemert et al., 2010) and in mice with chemically induced colitis (Yin et al., 2018). Furthermore, ingestion of LP8826R, but not the isogenic PlnEF-deficient mutant, led to reduced food intake and weight gain as well as increased levels of ileal tight junction protein, ZO-1, in diet induced obese mice (Heeney et al., 2018). However, the precise effects of PlnEF on the intestinal epithelial barrier remain to be determined.

We hypothesized that PlnEF signaling alters intracellular ion levels and tight junction protein expression to improve barrier function in intestinal epithelial cells. Herein, the capacity of a wild-type and mutant form of PlnEF to protect against pro-inflammatory cytokine-induced barrier permeability was assessed in differentiated and polarized human Caco-2 and porcine IPEC-J2 monolayers. Effects of PlnEF were assessed by quantifying transcript levels of genes encoding ion transporters and tight junction transmembrane proteins and quantification of intracellular ion levels after PlnEF treatment.

Materials and Methods

PlnEF and mutant peptides

PlnE (FNRGGYNFGKSVRHVVDAIGSVAGIRGILKSIR; 98.2% purity), PlnF (VFHAYSARGVRNNYKSAVGPADWVISAVRGFIHG; 99.8% purity), PlnE-G9I, a mutant of PlnE with a glycine to isoleucine mutation in position 9 (Ekblad et al., 2016), (FNRGGYNF**I**KSVRHVVDAIGSVAGIRGILKSIR; 98.7% purity), and PlnA (KSSAYSLQMGATAIKQVKKLFKKWGW; between 98 – 99% purity) were chemically synthesized by GenScript (Piscataway, NJ). PlnE, PlnE-G9I, PlnF, and PlnA peptides were re-constituted in sterile water to 100 µM. Aliquots were stored at -20 °C until use. Prior to application, PlnE or PlnE-G9I were mixed with PlnF in a one-to-one ratio.

Caco-2 cell culture

Human intestinal Caco-2 cells ATCC HTB-37 (American Type Cell Culture, Rockville, MD, USA), were grown in DMEM as previously described (Hubatsch et al., 2007). Cell culture reagents, Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), sodium pyruvate (100X), nonessential amino acids (100X), GlutaMax (100X), penicillin/streptomycin 10,000 U/ml, Trypsin-EDTA (0.25%, 1X), and sterile Dulbecco's phosphate buffer saline (D-PBS, pH 7.4, no calcium, no magnesium), were obtained from Gibco (Life Technologies, Carlsbad, CA, USA). Caco-2 cells were routinely incubated in DMEM, 20% fetal bovine serum (FBS), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 2 mM GlutaMax, and 1% v/v penicillin/streptomycin at 37 °C in 10% CO_2, split using Trypsin-EDTA (1X), and passaged in 75 cm^2 culture flasks (Fisher Scientific, Waltham, MA, USA) until ready for seeding.

IPEC-J2 cell culture

The porcine non-transformed intestinal IPEC-J2 cell line, originally derived from jejunal epithelia isolated from a neonatal, un-suckled piglet (Schierack et al., 2006), was provided by Dr. Peng Ji (Department of Nutrition, UC Davis, Davis, CA, USA). IPEC-J2 cells were grown in DMEM/F12 as previously described (Tang et al., 2018; Xiao et al., 2019; Zakrzewski et al., 2013). The cell culture reagents, Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12), fetal bovine serum (FBS), penicillin/streptomycin 10,000 U/ml, Trypsin-EDTA (0.25%, 1X), and sterile Dulbecco's phosphate buffer saline (D-PBS, pH 7.4, no calcium, no magnesium), were obtained from Gibco (Life Technologies, Carlsbad, CA, USA). Porcine serum (PS) was obtained from Millipore Sigma (Burlington, MA, USA). Briefly, the IPEC-J2 cells were incubated in DMEM/F12, 10% fetal bovine serum (FBS), and 1% v/v penicillin/streptomycin at 37 °C in 5% CO_2, split using Trypsin-EDTA (1X), and passaged in 75 cm^2 culture flasks (Fisher Scientific, Waltham, MA, USA) until ready for seeding.

PlnEF effects on undifferentiated Caco-2 and IPEC-J2 cells

For measuring the gene expression, protein levels, and intracellular ion level changes after exposure to PlnEF, mEF, or individual plantaricin peptides (Pln), Caco-2 cells (between passages 33 and 36) or IPEC-J2 cells (between passages 23 and 26) were seeded on polystyrene six-well tissue culture plates (9.6 cm^2 per well; Genesee Scientific, El Cajon, CA, USA) at a seeding density of 1 x 10^4 cells/cm^2 for Caco-2 cells or 3.36 x 10^4 cells/cm^2 for IPEC-J2 cells. After reaching 100% confluence (between 6 to 7 days after seeding for Caco-2 cells and between 5 to 6 after seeding for IPEC-J2 cells), cells were treated with DMEM or DMEM/F12 containing 500 or 1000 nM PlnEF or mutant PlnE-G9I + PlnF (mEF), or individual peptides (PlnE or PlnA), or an equal volume of sterile water (untreated control) and incubated for 1, 3, 6, 24, or 32 h.

PlnEF effects on gene expression and ion levels were also examined in Caco-2 cells 7 and 14 days after reaching confluence. At the end of each incubation periods, cells were harvested and stored at -80 °C until further analysis.

Caco-2 and IPEC-J2 monolayers

To form a monolayer, the Caco-2 cells (between passages 33 and 36) were seeded onto polycarbonate membranes in Transwell inserts (0.33 cm^2 surface area, 6.5 mm, 0.4 µm pore size, 24 wells; Corning, NY, USA) at a density of 1 x 10^4 cells/cm^2. After incubation for at least 21 days, the cells were fully differentiated and formed polarized monolayers with intercellular tight junction structures. Trans-epithelial electrical resistance (TER) was measured after 15 min equilibration at room temperature with an epithelial voltohmmeter (World Precision Instruments, Sarasota, FL, USA) equipped with a STX-2 "chopstick" electrode. Caco-2 monolayers with an initial TER of more than 250 Ohm·cm^2 were used for subsequent experiments.

For polarized IPEC-J2 monolayer experiments, seeding densities of (low; used for six-well non-polarized experiments) 3.33 x 10^4, (medium) 1.67 x 10^5, and (high) 8.33 x 10^5 (medium and high seeding density noted in Schierack et al., 2006) cells/cm^2 were tested. IPEC-J2 cells seeded with the low density did not form monolayers after 14 days of incubation (data not shown). Cells seeded at the medium seeding density did not reach the target TER threshold of 300 Ohm·cm^2 by day 14 after seeding. Therefore, the high seeding density of 8.33 x 10^5 cells/cm^2 was selected for subsequent polarized IPEC-J2 monolayers experiments.

Next, the IPEC-J2 cells (between passages 28 and 30) were seeded onto polycarbonate membranes in Transwell inserts (0.33 cm^2 surface area, 6.5 mm, 0.4 µm pore size, 24 wells; Corning, NY, USA) at a density of 8.33 x 10^5 cells/cm^2. After incubation for at least 14 days, the

cells were fully differentiated and formed polarized monolayers with intercellular tight junction structures. Trans-epithelial electrical resistance (TER) was measured after 15 min equilibration at room temperature with an epithelial voltohmmeter (World Precision Instruments, Sarasota, FL, USA) equipped with a STX-2 “chopstick” electrode. IPEC-J2 monolayers with an initial TER of more than 300 Ohm·cm^2 were used for the subsequent experiments.

PlnEF effects on IFN-γ and TNF-α mediated disruptions to Caco-2 and IPEC-2 cell monolayers

For Caco-2 cell monolayers, initial TER values were measured for each monolayer before adding DMEM (without FBS and phenol red) containing sterile water (the same volume as peptides) or DMEM containing 500 or 1000 nM PlnEF or mutant PlnE-G9I + PlnF (mEF) or individual PlnE or PlnF to the apical surface. The cells were incubated with these apical treatments for 3 h at 37 °C and then 100 ng/ml human IFN-γ (R&D Systems, Minneapolis, MN, USA) was added to the basolateral compartment. The DMEM of the basolateral compartment was replaced 21 h later and supplemented with 10 ng/ml human TNF-α (R&D Systems, Minneapolis, MN, USA). TER values of each monolayer were measured right before TNF-α addition and 24, 32, and 48 h after TNF-α stimulation.

For IPEC-J2 porcine monolayers, initial TER values were measured for each monolayer before adding DMEM/F12 (without FBS and phenol red) containing sterile water (the same volume as the peptides) or DMEM/F12 containing 500 or 1000 nM PlnEF to the apical surface. The cells were incubated with these apical treatments for 3 h at 37 °C and then 100 ng/ml porcine IFN-γ (R&D Systems, Minneapolis, MN, USA) was added to the basolateral compartment. The DMEM/F12 of the basolateral compartment was replaced 21 h later and

supplemented with 100 ng/ml porcine TNF-α (R&D Systems, Minneapolis, MN, USA). TER values of each monolayer were measured right before TNF-α addition and 24, 32, and 48 h after TNF-α stimulation.

Paracellular permeability assay

Paracellular permeability was determined by the flux of fluorescein isothiocyanate-dextran (4 kDa; FITC; Sigma-Aldrich, St. Louis, MO, USA) through differentiated Caco-2 or IPEC-J2 monolayers as previously described (Zhai et al., 2019). After 48 h stimulation with (human or porcine) TNF-α, the apical side contents were replaced with DMEM or DMEM/F12 (without FBS and phenol red) containing FD-4 (2 mg/ml). After 30, 60, 90, and 120 min, FD-4 concentration on the basolateral side was quantified by measuring the fluorescence intensity (485 nm excitation and 530 nm emission) in a microplate reader (Synergy-2, BioTek, Winooski, VT, USA). The concentration of FD-4 was calculated using a standard curve of FD-4 in DMEM or DMEM/F12 (without FBS and phenol red). The apparent permeability coefficient, P_{app} (cm/s), was determined using the following equation: $P_{app} = [dQ/dt] \times [1 / (A \times C_0)]$, where dQ/dt is the quantity of FD-4 transported per second (ng/sec), A is the surface area of the filter (cm^2), and C_0 is the initial FD-4 concentration in DMEM or DMEM/F12 on the apical side (ng/ml).

Position-Specific Iterative Basic Local Alignment Search Tool (PSI-BLAST) search for human homologs of CorC CBS-domain containing protein hits

Using the amino acid sequence of the *L. plantarum* NCIMB 700965 CorC CBS domain (Y216 – G351:

YLYMERAFEFNDKIAKDIMIDRTQLVVIDINDTVKQAIRVYLQKRFSRLPV

VANNDKDKILGYVYNYDLIRQGEVDDDIRVNKLLRNIITIPETTPIQAILQKMIDKQTPIV
V

VVDEYGGTSGIVTDKDIYEELFG) (Heeney et al., 2019) as a query, PSI-BLAST (Altschul et al., 1997; Bhagwat & Aravind, 2007) was used to search the non-redundant protein sequence (nr) database to identify human (taxid: 9606) protein homologs hits containing the CorC CBS domains. The default parameters were used, including expect threshold (10), matrix (BLOSUM62), and PSI-BLAST threshold (0.005). The protein hits obtained from three iterations of PSI-BLAST were compiled and the top targets were selected for further analysis.

Caco-2 RNA extraction and quantitative reverse-transcription PCR (qRT-PCR)

Total RNA from polarized Caco-2 monolayers after 48 h exposure to TNF-α or non-polarized Caco-2 cells after PlnEF treatment was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and purified using the TRIzol-chloroform protocol. RNA was quantified on the NanoDrop 2000c (Thermo Fisher, Waltham, MA, USA). Quality was assessed on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and found to be intact (RIN 8.8 - 9.8). RNA was reverse transcribed to cDNA using the High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's protocol. Quantitative reverse-transcription PCR (RT-qPCR) was performed using Fast SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) on a 7500 Fast Real-time PCR system (Applied Biosystems, Carlsbad, CA, USA). The primers used for RT-qPCR are listed in **Table 1**. Amplification was initiated at 95 °C for 20 seconds and was followed by 40 cycles of 95 °C for 3 seconds (denaturation) and 60 °C for 30 seconds (annealing and extension). Primer specificity was assessed using melting curve following the amplification stage. All

reactions were performed in duplicates and no reverse transcriptase (NRT) controls for each sample was included to check for DNA contamination and amplification in the prepared RNA. Data were normalized using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001) with *GAPDH* as the housekeeping gene and untreated cells as the reference condition.

Caco-2 protein extraction and Western blots

Caco-2 cells were rinsed with ice cold D-PBS, harvested with 18 cm Cell Scrapers (Biologix USA, Camarillo, CA, USA), and re-suspended in ice cold RIPA lysis buffer (Thermo Scientific, Waltham, MA, USA) with 1X protease and phosphatase inhibitor cocktail (Halt, Thermo Fisher Scientific, Waltham, MA, USA). Protein supernatants were collected after centrifugation of the cell lysates at 18,407 x g for 10 min at 4 °C. Total protein was quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) and 10 µg total protein was resolved by SDS polyacrylamide gels electrophoresis (SDS-PAGE) with stack gel (4.95% bis-acrylamide (VWR Life Sciences, Radnor, PA, USA), 62.5 mM Tris-HCl (Fisher Scientific, Waltham, MA, USA) buffer, pH 6.8, 0.1% SDS (VWR Life Sciences), 0.05% ammonium persulfate (APS; Bio-Rad Laboratories, Hercules, CA, USA), 0.2% TEMED (Bio-Rad Laboratories), in MilliQ water) and 12% resolving gel (12% bis-acrylamide, 0.39 M Tris-HCl, pH 8.8, 0.1% SDS, 0.1% APS, 0.04% TEMED, in MilliQ water). Separated protein bands were transferred onto PVDF membranes and blocked with blocking buffer containing 5% skim milk and 1X TBST (1X Tris-buffered saline, 0.1% Tween 20) for 1 h at 4 °C. Then, the membranes were probed overnight at 4 °C with agitation using the following primary antibodies: rabbit anti-ZO-1 (Invitrogen, 61-7300), rabbit anti-occludin (Thermo Fisher, 71-1500 polyclonal), rabbit anti-GAPDH (CST, 5174 D16H11 XP monoclonal, Cell Signaling

Technology, Danvers, MA, USA). Membranes were washed three times with 1X TBS-T buffer and incubated with HRP-conjugated anti-rabbit IgG (CST) secondary antibodies for 1 h at room temperature. Proteins were detected using LumiGLO Reagent and Peroxide (CST) chemiluminescent substrate kit and images were taken with iBright CL750 Imaging System (Thermo Fisher). Finally, band intensities were quantified using the ImageJ software (Schneider et al., 2012).

Inductively coupled plasma mass spectrometry (ICP-MS) quantification of intracellular ion levels in Caco-2 and IPEC-J2 cells

Caco-2 or IPEC-J2 cells were rinsed in 37 C D-PBS, trypsinized, washed in D-PBS, then pelleted by centrifugation at 124 x g at room temperature for 5 min (for Caco-2 cells) or at 300 x g at room temperature for 10 min (for IPEC-J2 cells). An aliquot of washed cells was used for cell counting via hemocytometer. ICP-MS quantification of intracellular ions in Caco-2 and IPEC-J2 cell pellets was performed at the UC Davis Interdisciplinary Center for Plasma Mass Spectrometry (https://icpms.ucdavis.edu/). Briefly, the cell pellets were digested by incubating at 95 °C for 45 min in a 60% concentrated trace-metal-grade HNO_3. After cooling to room temperatures, the digests were incubated at 95 °C for 5 min in 6% H_2O_2 followed by three additions (100, 150, and 200 µl) of 30% H_2O_2 in 5 min intervals and heating at 95 °C for another 2 h. The digests were then diluted with water to a final concentration of 10% HNO_3 and analyzed with an Agilent 7500C3 ICP-MS instrument (Agilent Technologies, Palo Alto, CA, USA) for simultaneous quantification of select ions (Na, P, K, Mg, Ca, Zn, Fe, Mn, Cu). The numbers of atoms per cell were calculated for the observed quantities of each ion (parts per billion [ng/ml]) normalized to cell numbers estimated by hemocytometers.

Statistical analysis

Unless otherwise stated, data were analyzed using GraphPad Prism 10 software for Windows (GraphPad Software, Inc., La Jolla, CA, USA), reported as mean ± standard deviation (SD), and analyzed by ANOVA with post hoc test for comparing multiple treatments.

Results

PlnEF increases transcript but not protein levels of Caco-2 tight junction proteins

The response of Caco-2 cells to 500 nM PlnEF exposure was measured over time in cells at the time of confluence and 7 and 14 days later. When PlnEF was applied to Caco-2 cells on the day of confluence, TJPs, *OCLN*, *ZO-1*, *CLDN1*, *CLDN2*, *CLDN3*, and *CLDN4*, were expressed at similar levels as controls (not exposed PlnEF) according to qRT-PCR when measured 1, 3, and 6 h later (**Fig. 1**). After approximately 24 h, transcript quantities for *OCLN*, *ZO-1*, *CLDN1*, *CLDN3*, but not *CLDN2*, were significantly increased compared initial levels (Unpaired t test, 24 h vs. 1h: $p = 0.0028$ for *OCLN*, $p = 0.0080$ for *ZO-1*, $p = 0.0001$ for *CLDN1*, $p = 0.0005$ for *CLDN3*) (**Fig. 1**). The TJP, *CLDN4* and inhibitor of nuclear factor kappa-B kinase subunit beta *IKK-β,* did not significantly increase when measured 24 h after PlnEF addition compared to the earliest time point (**Fig. 1**). Notably, these transcript level changes were not observed in Caco-2 cells exposed to PlnEF either 7 or 14 days after reaching confluence (**Fig. S1**).

To assess whether changes in gene transcript levels corresponded with protein levels in Caco-2 cells and if individual peptides PlnE or PlnF had any effects, Western blot analysis was applied to quantify OCLN and ZO-1 in cells collected after 24-h treatment with 500 nM PlnEF.

However, no change in either protein was found compared to Caco-2 cell controls (**Fig. 2**). Caco-2 cells exposed to the individual PlnE peptide had comparable ZO-1 levels as the untreated control (**Fig. 2**). Caco-2 cells treated with PlnA peptide, which is the inducer peptide for the activation of *pln* locus and PlnEF synthesis in *L. plantarum*, had a slightly higher trend of ZO-1 and a similar level of OCLN compared to the untreated cells (**Fig. 2**).

PlnEF increases expression of CorC CBS domain-containing gene homologs in Caco-2 cells

The cysteine-β-synthase (CBS) domain of CorC magnesium efflux protein, CorC, was identified as the putative receptor for PlnEF induced antimicrobial activity in the PlnEF-sensitive indicator strain *L. plantarum* NCIMB 700965 (LP965) (Heeney et al., 2019). Thus, we hypothesized that this CBS domain structural region may serve as a potential binding site for PlnEF. To find the potential target(s) of PlnEF in Caco-2, we used the amino acid sequences of the bacterial CorC CBS domain (Y216 – G351) as a query for PSI-BLAST searches of human homologs containing this structural region (**Fig. S2A**). Three iterations of PSI-BLAST returned proteins hits that were annotated to eleven protein isoforms, belonging to four major protein families, Cyclin M/CBS-pair domain divalent metal cation transport mediators (CNNM), AMP-activated protein kinase (AMPKγ1) subunits, inosine monophosphate dehydrogenase (IMPDH), and chloride voltage gated channels (CLC/CLCN). Specifically, the top four protein hits were CNNM4 (E value: 3E-10), CNNM2 (E value: 2E-7), AMPKγ1 (E value: 0.003), and CLC-5 (E value: 0.047) (**Fig. S2B**).

Thus, gene expression levels of Caco-2 *CNNM4, CNNM2,* AMPKγ1, and *CLC-5* were quantified by qRT-PCR. After exposure to PlnEF for 24 h, levels of *AMPKγ1*, *CNNM4,* and *CNNM2* were significantly increased compared to the controls and levels measured after 1 h

exposure (Unpaired t test, 1h vs. 24h, p = 0.0073 for *AMPKγ1*, p = 0.0154 for *CNNM4*, p = 0.0228 for *CNNM2*), reaching approximately 8-, 6-, and 4-fold increase on average (**Fig. 3**). This increase was not found for *CLC-5* (p = 0.1031) (**Fig. 3**). Next, to investigate whether the PlnEF-induced changes in gene expression was specific to these targets obtained from PSI-BLAST, we also quantified the expression of genes encoding for other isoforms and subunits of AMPK (AMPKα1, AMPKα2, AMPKγ2) and ion transporters (CLC-3, NHE3, and CFTR). PlnEF treatment also resulted in significant changes to the expression of these genes. There was an approximately 4-fold increase in transcript levels for five (*AMPKα1*, *AMPKα2*, *AMPKγ2*, *CLC-3*, *NHE3*) out of these six genes after 24 h compared to the 1 h exposure (Unpaired t test, 1h vs. 24h, $p < 0.05$). *CFTR* expression was also increased, although only by 2-fold (p = 0.0505) (**Fig. 4**). These results suggested that PlnEF treatment causes global changes to Caco-2 gene expression.

PlnEF ameliorates cytokine-induced barrier permeability in polarized Caco-2 monolayers

Assessment of the intestinal barrier protective effect of PlnEF was performed in Caco-2 monolayers challenged with pro-inflammatory cytokines. TNF-α and IFN-γ applied in sequence impaired barrier function in the Caco-2 monolayers. Specifically, it was shown that basolateral exposure of IFN-γ for 21 h followed by TNF-α led to a significant reduction of TER by 2.5-fold (54.23 ± 10.40 % of initial) compared to untreated monolayers (139.15 ± 21.34% of initial) after 48 h (p = 0.0002; two-way ANOVA with Tukey's multiple comparisons test) (**Fig. 5A**). This corresponded with a 5-fold higher P_{app} coefficient and the significantly increased transfer of FITC from the apical to the basolateral compartment compared to untreated controls (p = 0.0004;

one-way ANOVA with multiple comparisons test) (**Fig. 5B)**, indicating that the cytokines disrupted barrier integrity.

The reductions in TER 48 h after exposure to TNF-α were significantly reduced when the monolayers were treated with 1000 nM PlnEF for 3 h prior to IFN-γ addition, resulting in higher TER compared to cytokine controls ($p = 0.0077$; two-way ANOVA with Tukey's multiple comparisons test) (**Fig. 5A**). This trend was also observed in assessment of paracellular permeability such that where monolayers to which 1000 nM PlnEF was added exhibited an approximately 2.7-fold lower translocation of FITC compared to the cytokine-challenged monolayers ($p = 0.0024$; one-way ANOVA with Tukey's multiple comparisons test) and a similar level of permeability as untreated monolayers not exposed to cytokines ($p = 0.7036$) (**Fig. 5B**). This protection was limited to 1000 nM since 500 nM PlnEF, either measured by TER ($p = 0.2016$; two-way ANOVA with Tukey's multiple comparisons test) (**Fig. 5A**) or FITC permeability did not exhibit significant improvement compared to cytokine-challenged monolayers (**Fig. 5B**).

To determine whether apical PlnEF treatment and basolateral IFN-γ and TNF-α exposure resulted in sustained changes to Caco-2 gene expression, qRT-PCR was performed on cells collected 72 h after PlnEF application. To this regard, *TNFR2* transcripts levels were approximately 2-fold elevated compared to untreated monolayers (**Fig. 6**). Remarkably, PlnEF resulted in further increases in *TNFR2* gene expression levels, resulting in significantly higher gene expression level in 1000 nM PlnEF-treated monolayers compared to the cytokine-challenged controls ($p = 0.0204$) and those to which 500 nM PlnEF was applied ($p = 0.0179$; one-way ANOVA with Tukey's multiple comparisons test) (**Fig. 6**). *CLDN2* transcript levels were also higher in Caco-2 monolayers to which IFN-γ and TNF-α were applied (**Fig. 6**).

Expression levels were similarly elevated with either 500 or 1000 nM PlnEF addition (**Fig. 6**). The expression levels of other *CLDN* genes, *CNNM2* and *CNNM4,* and *AMPKγ1* were unchanged with PlnEF exposure. Together, these results revealed a dose dependent capacity of PlnEF to protect against inflammation-induced perturbation on the intestinal barrier integrity, potentially through signaling pathways modulating TJs.

mEF protected polarized Caco-2 monolayers challenged with cytokines

To determine if the barrier protective effect of PlnEF was limited to specific amino acid configurations, a mutant PlnE-G9I (mE) peptide with reduced helix-helix interactions with PlnF and thus resulting to more than 130-fold lower antimicrobial activity against the indicator strain, *Latilactobacillus curvatus* LTH 1174 (Ekblad et al., 2016), was tested on Caco-2 monolayers. It was found that 500 nM, but not 1000 nM, mEF alleviated reductions in TER following IFN-γ and TNF-α additions (54.23 ± 10.40% of initial; $p = 0.0193$, two-way ANOVA with Tukey's multiple comparisons test) (**Fig. 5A**). Consistent with the TER measurements, 500 nM, but not 1000 nM mEF, provided a nearly 4-fold reduction in permeability to FITC compared to the IFN-γ and TNF-α treated controls (**Fig. 5B**).

Development of the polarized porcine IPEC-J2 monolayer model

To assess the general capacity of PlnEF to confer barrier protection in other cell types, PlnEF effect was examined on polarized, porcine small intestinal cell IPEC-J2 monolayers. To develop the monolayers model and verify their sensitivity to porcine IFN-γ and TNF-α, several methodological developments were performed. Firstly, IPEC-J2 cell seeding density optimization was conducted using non-polarized cells. IPEC-J2 cells (between passages 25 and

28) were seeded in six-well tissue culture plates at densities of (low) 1.0 x 10^4 (as for Caco-2 cells), (medium) 3.33 x 10^4, or (high) 1.8 x 10^5 (Schierack et al., 2006) cells/cm^2 in DMEM/F12 containing 10% FBS or 10% PS, and 1% v/v penicillin/streptomycin. As shown in the representative images (**Fig. S3**), IPEC-J2 cells seeded at the high seeding density were overcrowded approximately 2 h after seeding while the low seeding density was not sufficient for proliferation (took 6 days to reach near 50% confluence). IPEC-J2 cells seeded at the medium density of 3.33 x 10^4 cells/cm^2 reached 100% confluence in 5 days or 9 days when grown in DMEM/F12 containing 10% FBS or 10% PS, respectively (**Fig. S4**). IPEC-J2 cells grown in medium containing 10% PS formed cell aggregates upon seeding, likely due to the transition from a cryo-preservation medium containing 10% FBS to a growth medium containing 10% PS (**Fig. S4**). Based on these tests, we selected DMEM/F12 containing 10% FBS as the medium condition and the medium seeding density for the non-polarized IPEC-J2 experiments.

Next, to determine which concentrations of porcine IFN-γ and TNF-α result in measurable increase in permeability, as quantified with TER and paracellular permeability to FITC-dextran (4kD; FITC), we examined the response of IPEC-J2 monolayers to the following combinations of porcine cytokines: 100 ng/ml IFN-γ and 10 ng/ml TNF- α; 1000 ng/ml IFN-γ and 20 ng/ml TNF-α; 100 ng/ml INF-γ and 100 ng/ml TNF-α; and 100 ng/ml IFN-γ and 500 ng/ml TNF-α. The 10-fold differences in IFN-γ did not result in additional TER reduction or increase in the translocation of FITC from apical to the basolateral compartment (data not shown). Among the four TNF-α concentrations examined, 100 ng/ml resulted in the greatest drop in TER over a 48-h period and the largest increase in permeability to FITC compared to monolayers without cytokine exposure. Thus, 100 ng/ml IFN-γ and 100 ng/ml TNF-α were selected for testing PlnEF.

PlnEF does not exert intestinal barrier protective effects on polarized porcine IPEC-J2 monolayers

Basolateral application of porcine IFN-γ (100 ng/ml) followed by porcine TNF-α (100 ng/ml) resulted in a gradual reduction of TER by approximately 2.6-, 3.2-, and 5.2-fold over the following 24 ($p = 0.0713$), 32 ($p = 0.0387$), and 48 h ($p = 0.0222$) compared to untreated monolayers (two-way ANOVA with Tukey's multiple comparisons test) (**Fig. 7A**). This increase in monolayer permeability was also reflected in the near 2-fold increase (P_{app} of 3.87 ± 0.77 cm · s^{-1} x 10^{-7}) in the apical-to-basolateral translocation of FITC compared to untreated monolayers (P_{app} of 2.03 ± 0.33 cm · s^{-1} x 10^{-7}; $p = 0.0086$, one-way ANOVA with Tukey's multiple comparisons test) (**Fig. 7B**), verifying that the cytokines disrupted barrier integrity.

In IPEC-J2 monolayers treated with 500 or 1000 nM PlnEF prior to cytokine addition, reductions in TER were still observed 24, 32, and 48 h after exposure to TNF-α, resulting in at least 2-fold lower TER compared to untreated monolayers ($p < 0.05$, two-way ANOVA with Tukey's multiple comparisons test), (**Fig. 7A**). This corresponded to the similar paracellular permeability to FITC in monolayers treated with 500 or 1000 nM PlnEF compared with the cytokine-challenged controls (500 nM: $p = 0.7799$; 1000 nM: $p = 0.8207$; one-way ANOVA with Tukey's multiple comparisons test) (**Fig. 7B**). Jointly, there results indicate that PlnEF did not alleviate the cytokine-induced barrier damage in IPEC-J2 cells.

Differential influence of PlnEF and mEF on the intracellular ion levels of non-polarized human Caco-2 cells and porcine IPEC-J2 cells

Because CBS domain-containing human homologs are involved in ion transport (*CNNM4*, *CNNM2*, *CLC-5*) and transcription of other ion transporters (*CLC-3*, *NHE3*) were influenced by PlnEF, we measured the intracellular ion levels of non-polarized Caco-2 cells (0, 7 and, 14 days post confluence) after incubation with 500 nM of either PlnEF or mEF. A 32-h incubation of Caco-2 cells at the point of confluence with 500 nM PlnEF led to reduced levels of K^+ ($p = 0.0263$) and Mg^{2+} ($p = 0.0366$) compared to the controls (**Fig. 8**). However, no significant changes were found in a separate experiment when either 500 nM PlnEF or the mEF was applied (**Fig. 9A**). Likewise, incubation with PlnEF or mEF for 1 h did not lead to significant changes in intracellular ion levels compared to untreated Caco-2 cells (**Fig. 10A**). For Caco-2 cells measured 7 days post-confluence, there was no change in intracellular ion levels when incubated with PlnEF (**Fig. 8**; **Fig. S5**).

Lastly, like found for non-polarized Caco-2 cells, there were no significant changes in IPEC-J2 intracellular Na^+, P^{3-}, K^+, Mg^{2+}, and Zn^{2+} levels after exposure to 500 nM PlnEF or mEF for 32 h compared to untreated cells (Unpaired t test: $p > 0.05$, compared to untreated) (**Fig. 9B**). Incubation with 500 nM PlnEF for 24 h also did not led to any changes in intracellular ion levels (**Fig. S6**). Yet, when the intracellular ion levels were measured after 1 h exposure, IPEC-J2 cells treated with 500 nM mEF, but not PlnEF, showed approximately 1.2-fold lower levels of K^+, Mg^{2+}, Zn^{2+}, and Mn^{2+} compared to untreated cells ($p < 0.05$, unpaired t test: compared to untreated) (**Fig. 10B**), suggesting potential differences in how intestinal epithelial cells of different origins may respond to PlnEF and mEF exposure.

Discussion

It was previously reported that PlnEF protected Caco-2 cell monolayers from IFN-γ and TNF-α induced barrier defects (Heeney et al., 2018). Therefore, in this study we aimed to investigate cellular responses to PlnEF and to assess whether the activity is PlnEF- and cell type-specific. The results showed that this barrier protective effect can potentially be attributed to the modulation of TJPs as shown by the elevation of tight junction protein transcript levels after PlnEF treatment in non-polarized Caco-2 cells. Moreover, PlnEF treatment induced a global transcript response, influencing the levels of gene encoding for bacterial CorC CBS domain containing homologs as well as other non-CBS homologs like some ion transport proteins. Along with PlnEF, a mutant PlnE-G9I + PlnF (mEF) combination, with reduced interactions between the individual peptides, also exerted barrier protective property in Caco-2 monolayers. In contrast to its protective effect in human Caco-2 monolayers, PlnEF did not alleviate cytokine induced barrier damage in porcine IPEC-J2 monolayers. Additionally, PlnEF did not affect the intracellular ion levels of non-polarized Caco-2 and IPEC-J2 cells. Yet, treatment of non-polarized IPEC-J2 cells with mEF lowered intracellular magnesium and potassium levels compared to untreated cells. These results highlighted the potential species- and host cell origin dependent effect of PlnEF and mEF on intestinal epithelial cells.

In non-polarized Caco-2 cells, a 24 h treatment of PlnEF led to higher gene expression of *OCLN*, *ZO-1*, *CLDN1*, *CLDN3* and did not change *CLDN2* transcript levels compared to controls. In polarized Caco-2 monolayers, PlnEF at 1000 nM alleviated some of the increased intestinal barrier permeability in monolayers challenged sequentially with basolateral IFN-γ and TNF-α. Exposure to 1000 nM PlnEF prior to sequential cytokine challenge led to higher *TNFR2* transcript levels in the monolayers compared to monolayers without apical treatment and where

500 nM PlnEF was applied. This is consistent with previous studies where IFN-γ priming increased *TNFR2* mRNA transcript and protein levels after 24 h and sensitized intestinal epithelial cells to TNF-α induced barrier integrity disruption (Ma et al., 2004; Wang et al., 2006). though the monolayers in our study were examined at a later time point. The subsequent induction of barrier permeability involves the activation of the transcriptional factors nuclear factor kappa β (NF-κβ) (Al-Sadi et al., 2016; Gao et al., 2023; Graham et al., 2006), re-distribution of ZO-1 and OCLN proteins, and elevated CLDN2 levels at TJs (Buckley & Turner, 2018; Capaldo & Nusrat, 2009; Schoultz & Keita, 2019; Shen et al., 2006). These changes in TJP gene expression were reflected in non-polarized Caco-2 cells after PlnEF treatment though there were no changes to the abundances of ZO-1 and OCLN proteins. However, in polarized Caco-2 monolayers challenged with pro-inflammatory cytokines, we did not observe significant changes in the transcript levels of TJPs (*CLDN1*, *CLDN2*, *CLDN3*, *OCLN*) 72 h after the application of apical PlnEF treatments. While PlnEF treatment may not induce drastic changes in gene expression or abundances of TJPs, it may still modulate TJ structural organization by affecting phosphorylation status (Dörfel et al., 2013; Nunbhakdi-Craig et al., 2002; Suzuki et al., 2009) and localization (Basuroy et al., 2005; Rao et al., 2002).

Besides measuring for changes in TJPs, qRT-PCR was applied to detect and quantify the levels of genes hypothesized to encode protein targets of PlnEF. PSI-BLAST search of the human homologs of bacterial CorC CBS domain revealed that CNNM4, CNNM2, and AMPKγ1 as the top three protein hits. Gene expression of *CNNM2/4* and *AMPKγ1* in PlnEF-treated non-polarized Caco-2 cells were significantly elevated. However, this PlnEF-induced effect on transcript levels was not observed in Caco-2 monolayers challenged with cytokines. Additionally, PlnEF treatment increased the transcript levels of non-CBS domain containing

homologs, other AMPK subunits and ion transporters (*NHE3*, *CFTR*) in non-polarized Caco-2 cells. This induction of additional targets along with the basolateral (CNNM2/4) and intracellular (AMPKγ1) locations of CBS domain containing homologs suggest that the observed PlnEF-induced effects in Caco-2 cells may not have involved the direct interaction of PlnEF and these proteins and is probably a more global response. Along with its central role as the cellular energy sensor and metabolism regulator, AMPK activation also mediated the process of tight junction assembly (Rowart et al., 2018; Tsukita et al., 2019). Furthermore, while we did find increased gene expression of *CNNM2/4* and *AMPKγ1* in PlnEF-treated Caco-2 cells, whether this elevation at the transcript levels will transfer to an increase in protein abundance and activation status remain to be discovered. The potential influence of AMPK activation in PlnEF-induced intestinal barrier protective capability in Caco-2 monolayers challenged with cytokines can be verified through specific activator (e.g., AICAR) and inhibitor (e.g., Compound C) as well as a targeted proteomics approach focusing on the phosphorylation status of AMPK and its downstream targets involved in TJ formation (e.g., PKCβ, CLCA1) (Miao et al., 2016; Rowart et al., 2018; Yang et al., 2013).

To determine if the barrier protective effect of PlnEF requires a particular structural conformation of PlnE and PlnF, we assessed the impact of mEF, a mutant PlnE-G9I (mE) peptide that reduced helix-helix interactions with PlnF, on Caco-2 monolayers challenged with cytokines. Treatment of a PlnE mutant peptide together with PlnF (mEF) at 500 nM also protected the Caco-2 monolayers against IFN-γ and TNF-α induced barrier disruption. Our prior study noted that application of individual peptides (500 nM), PlnE or PlnF, did not protect Caco-2 monolayers against cytokine-induced permeabilization (Heeney et al., 2018).

PlnEF can be detected using mass spectrometry in the apical spent media of Caco-2 monolayers 72 h after 1000 nM pre-treatment while the abundances of PlnEF in the basolateral spent media were near or below detection limit (data not shown). It is unclear whether PlnEF and mEF can translocate across the Caco-2 monolayers and what the underlying mechanism(s) may be involved. The migration of nisin A, plantaricin 423, and bacST4SA across the Caco-2 monolayers have been shown (Dreyer et al., 2019). Mass spectrometric identification of the interacting proteins of PlnEF and mEF after co-immuno-precipitation is needed to pinpoint the putative receptor(s) in Caco-2 cells.

PlnEF did not provide an intestinal barrier protective effect to porcine IPEC-J2 monolayers. Unlike Caco-2 cells that have a human origin and spontaneously differentiate (Garcia-Lorenzo et al., 2012; Natoli et al., 2011; Tremblay et al., 2006), IPEC-J2 cells are a non-transformed and non-tumorigenic cell line isolated from the jejunum of piglet (Vergauwen, 2015). IPEC-J2 cells were also shown to form tighter monolayer compared to colorectal adenocarcinoma derived Caco-2 cells (Pisal et al., 2008; Vergauwen, 2015) while other differences include reduced mucus layer thickness, the absence of anchorage independent growth, gene expression of cytokines and chemokines, glycosylation patterns, and differential responsiveness to hormones, cytokines, and LPS (Nossol et al., 2015; Vergauwen, 2015). The small intestinal origin of IPEC-J2 cells and the large intestinal origin of Caco-2 cells can also contribute to their differential response to bacterial metabolites, such as PlnEF. Metabolically, small intestinal enterocytes are known to utilize mainly glucose, glutamine, and glutamate for energy generation while for large intestinal colonocytes, microbiota-derived short chain fatty acids (SCFAs) serve as a major energy source (Blachier, 2023; Rath & Haller, 2022; Rath et al., 2018). Another distinction is the carcinoma origin of Caco-2 cells compared to non-transformed

IPEC-J2 cells. Bacteriocins have been noted to exert anti-proliferative effect against head and neck cancer (nisin), colorectal cancer (nisin, plantaricin, pediocin), liver cancer (enterocin), potentially through disrupting membrane integrity and inducing apoptosis pathways (Molujin et al., 2022; Wang et al., 2024). The majority of bacteriocins are cationic and amphipathic peptides, which may selectively interact with cancerous cells, which have elevated negative cell surface charge due to different glycosylation patterns and increase lactate secretion compared to healthy cells (Le et al., 2019; Rodrigues et al., 2019). For instance, plantaricin P1053 enhanced the viability of normal colon cells but reduced the viability of colon cancer cells (De Giani et al., 2019). While Caco-2 monolayers have spontaneously differentiated into enterocyte-like cells (Engle et al., 1998) prior to exposure to PlnEF, it is likely that the carcinoma origin of these cells can result in altered cell surface charges and properties. Together, these differences likely contributed to the differential responses of Caco-2 and IPEC-J2 monolayers after PlnEF treatment. Bioactive compounds, such as ethyl pyruvate (Dong et al., 2019), chitosan oligosaccharide (Shi et al., 2019), butyrate (Yan & Ajuwon, 2017), and 4-phenylbutyric acid (Jiang et al., 2021) exhibited protective properties against LPS-induced damage in IPEC-J2 monolayers. Furthermore, TGF-β1 (Xiao et al., 2017) and the viable or autoclaved cells of *Akkermansia muciniphila* (Luo et al., 2021) protected IPEC-J2 monolayers from TNF-α induced barrier integrity disruption. Additional optimization using these potential positive controls or other bacteriocins shown to support intestinal barrier function (e.g., gassericin A (Hu et al., 2018), Apb118 (Miyauchi et al., 2012)) in IPEC-J2 monolayers compared to Caco-2 monolayers are needed to understand the potential cell type specific influence of PlnEF.

Because of the transcript level changes in *CNNM4* and *CNNM2*, which are Mg^{2+} extrusion proteins, and other ion transporters, we assessed the effect on PlnEF on the

intracellular ion levels of non-polarized Caco-2 and IPEC-J2 cells. PlnEF treatment did not significantly affect the ion levels of non-polarized Caco-2 and IPEC-J2 cells as measured in bulk by ICP-MS. Application of mEF also did not result in changes in the intracellular ion levels of Caco-2 cells. However, treatment of non-polarized IPEC-J2 cells with mEF lowered the intracellular Mg^{2+} and K^{+} compared to untreated cells. These results suggested possible species- and host cell-type dependent effects of PlnEF and mEF on intestinal epithelial cells. CNNM4 and CNNM2 play important roles in maintaining intracellular Mg^{2+} levels through Mg^{2+}/Na^{+} exchange and Mg^{2+} (re)absorption across the intestinal epithelium (Funato & Miki, 2022; Gimenez-Mascarell et al., 2019; Yamazaki et al., 2013). Direct binding of phosphatase of regenerating liver (PRL) with CNNM4 prevented CNNM4-dependent Mg^{2+} efflux, resulting in increased intracellular Mg^{2+} and ATP levels (Funato et al., 2014). There may be a connection between elevated *CNNM2/4* expression and reduced intracellular Mg^{2+} concentrations, which can trigger the phosphorylation and activation of AMPK and a change in the energy metabolism of the Caco-2 cells. More in-depth analyses are needed to investigate this hypothesis. For instance, a more time-sensitive and dynamic method, such as time lapse fluorescence imaging using the Mg^{2+} specific mag-fura-2 indicator (Froschauer et al., 2004), may be more appropriate to provide real time quantification of intracellular Mg^{2+} changes upon PlnEF exposure in live cells.

In summary, study provided initial insights into the intestinal barrier protective effect of a LAB-derived biomolecule well-known for its antimicrobial activity, highlighting the enticing dual functions of this bioactive compound. Continued investigations are still required to uncover the intestinal epithelial cell receptor(s) and signaling pathway(s) activated upon exposure to PlnEF and mEF, which may include targets that modulate energy metabolism, differentiation,

and TJ assembly. This will likely necessitate more global omics and real time techniques such as proteomics (phosphorylation and total protein), RNA-seq (gene expression), and time lapse fluorescence microscopy (ion flux) combined with verification using deletion or down-regulation of candidate(s) *in vitro*. The molecular mechanisms identified will help to inform therapeutic targets for intestinal epithelial permeability in a strain- and biomolecule-specific manner as well as provide guidance for the use of probiotic strain and/or probiotic-derived bioactive compounds in improving human and livestock health.

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Table 4.1. Primers used for qRT-PCR.

Gene	Primer sequences	Product (bp)	Efficiency (%)	References
GAPDH	F-5'-TCC CAT CAC CAT CTT CCA G-3' R-5'-GAT GAC CCT TTT GGC TCC-3'	145	97.9	Zhai et al., 2019
ZO-1	F-5'-TCC AGT CCC TTA CCT TTC G-3' R-5'-CTT AAA GAA TCA GCT TGT GGT G-3'	123	91.5	Zhai et al., 2019
OCLN	F-5'-CCT TTT GTT TCA TTG CCG-3' R-5'-ATG CCC AGG ATA GCA CTC AC-3'	114	102.6	Zhai et al., 2019
CLDN1	F-5'-ACG AGG GGC TGT GGA TGT-3' R-5'-CAA CCA CCA TCA AGG CAC-3'	120	100.6	Zhai et al., 2019
CLDN2	F-5'-TGT GAC ATC TAT AGC ACC CTT C-3' R-5'-TAC CGC CAC TCT GTC TTT G-3'	168	104.3	Zhai et al., 2019
CLDN3	F-5'-CAT CGG CAG CAA CAT CAT-3' R-5'-AAG GTC CTG TGG CAG TGC-3'	127	96.2	Zhai et al., 2019
CLDN4	F-5'-GGC AGC AAC ATT GTC ACC-3' R-5'-TGC TGA TGA TGA CGA GGG-3'	154	101.9	Zhai et al., 2019
IkkB	F-5'-ATC CCC GAT AAG CCT GCC A-3' R-5'-CTT GGG CTC TTG AAG GAT ACA G-3'	131	ND	Liu et al., 2018
TNFR2	F-5'-TGG CAT TTA CAC CCT ACG C-3' R-5'-CAC GGT GTC CGA GGT CTT-3'	143	107.1	Zhai et al., 2019
CNNM4	F-5'-TCC TGG GCC AGT ACA TCT C-3' R-5'-GAA ACT GAG GGC TGT TCT CC-3'	125	105.7	Lameris et al., 2015
CNNM2	F-5'-GAC AGC CTC TCC AGG TGA AAA-3' R-5'-TGG TTA TTG CTG CTC CCC AG-3'	122	106.0	Lin et al., 2017
AMPKA1	F-5'-AGG AGA GCT ATT TGA TTA TAT CTG TAA GAA TG-3' R-5'-ACA CCA GAA AGG ATC TGT TGG AA-3'	86	100.6	Neurath et al., 2016
AMPKA2	F-5'-CGG CTC TTT CAG CAG ATT CTG T-3' R-5'-ATC GGC TAT CTT GGC ATT CAT G-3'	119	99.9	Neurath et al., 2016; Luo et al., 2009
AMPKG1	F-5'-TGG AAG CAG AGG TTC ACC GA-3' R-5'-TCT CCA CCT GTG AGC ACC AG-3'	105	96.1	Mahlapuu et al., 2004

AMPKG2	F-5'-CCA GAT GCA AGC CTC TTC GA-3' R-5'-GTG CAT TCC CAC TGA TAG GGT C-3'	90	104.4	Mahlapuu et al., 2004
CLC-3	F-5'-CAA TGG GCG CCT CCT TGG CA-3' R-5'-AGC GGG GTC TTG GTT TGC CG-3'	79	108.6	Ye et al., 2014
CLC-5	F-5'-CAG AGT GGA ATA GTT GGT C-3' R-5'-AGA GAT ACG GCA AGG AAG-3'	120	95.6	Ceol et al., 2012
NHE3	F-5'-ACC TGT TCG TCA GCA CCA C-3' R-5'-GCT CGC TCC TCT TCA CCT T-3'	77	99.9	Singh et al., 2012
CFTR	F-5'-GCT TCC TAT GAC CCG GAT AAC-3' R-5'-GGA GCA GTG TCC TCA CAA TAA-3'	93	100.5	DiFranco et al., 2017

ND: not determined.

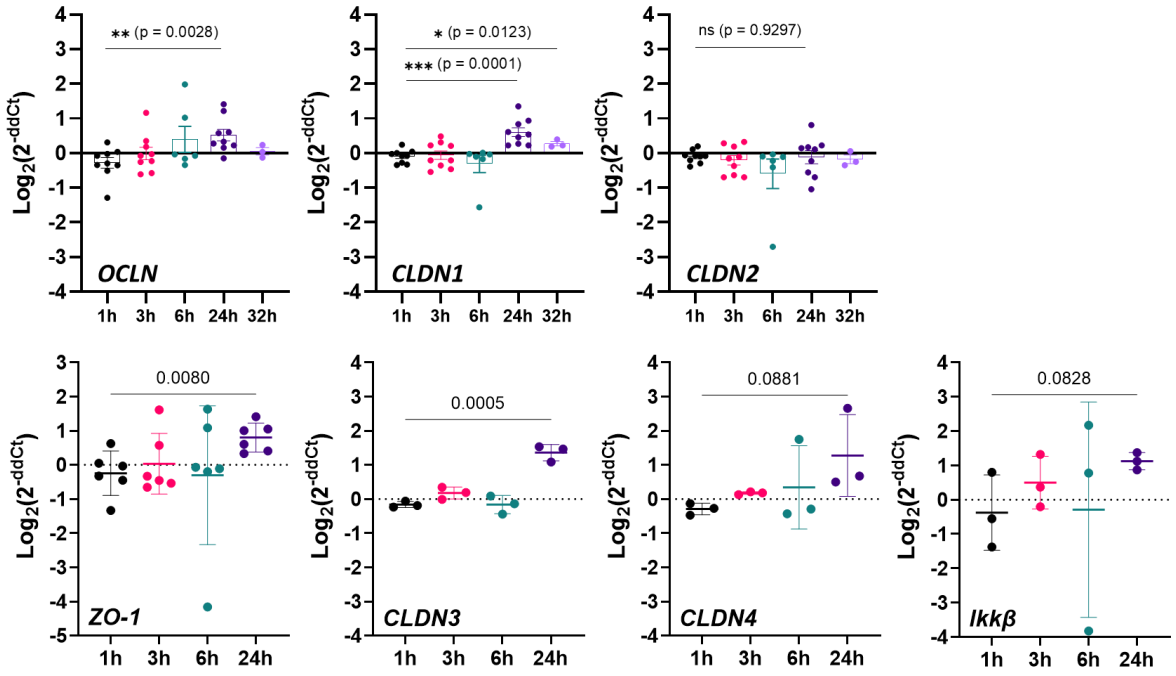


Figure 4.1. PlnEF treatment increased the transcript levels of genes encoding tight junction proteins (TJPs) but not NF-κB signaling-related factor.

Non-polarized Caco-2 cells at 0-days post confluence treated with 500 nM PlnEF or untreated (controls) for 1, 3, 6, 24, or 32 h. Mean ± SD; Unpaired t-test compared to 1h time point with at least triplicates per time point. The results were normalized using the $2^{-\Delta\Delta Ct}$ method with GAPDH as the housekeeping gene and the untreated cells (no PlnEF) as the control condition. *OCLN*, *CLDN1*, and *CLDN2* data were combined from three independent experiments.

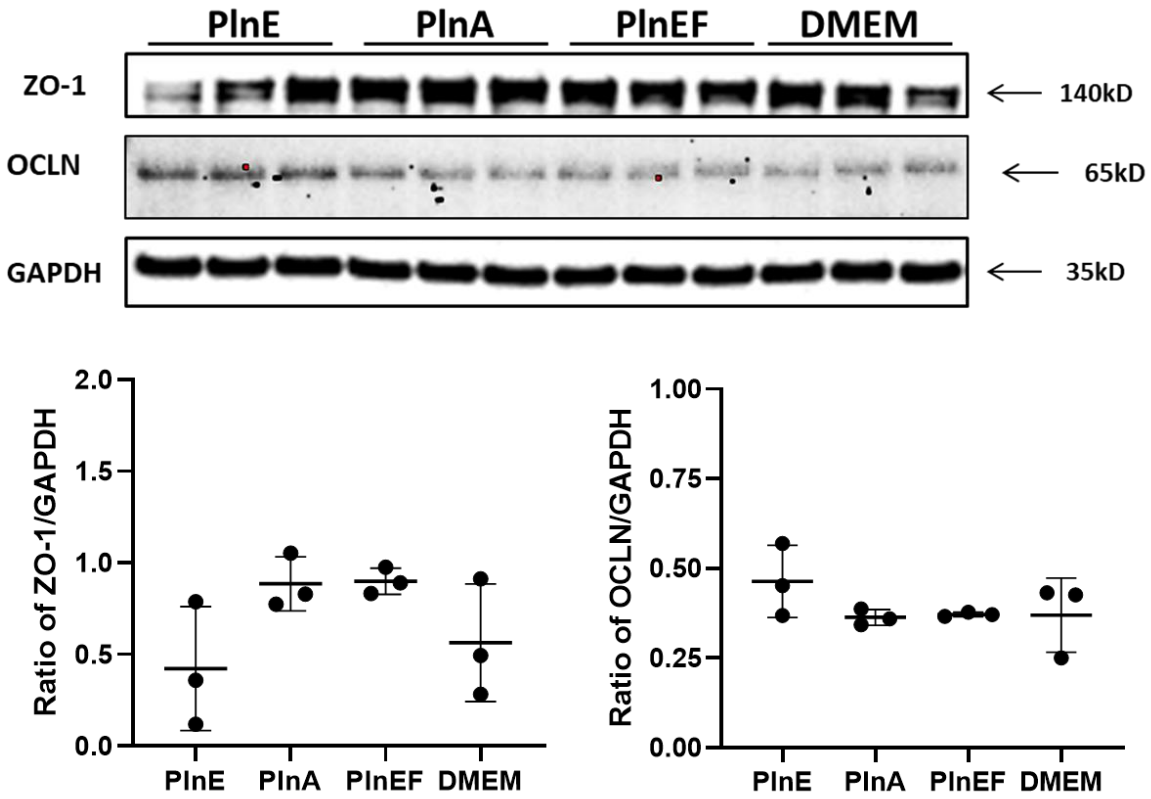


Figure 4.2. Plantaricin treatment did not affect ZO-1 and OCLN protein levels in non-polarized Caco-2 cell.

Non-polarized Caco-2 cells (0-days post confluence) treated with 500 nM PlnE, PlnA, PlnEF, or untreated (DMEM) for 24h. Band area of the protein of interest was quantified via ImageJ and normalized to that of the housekeeping protein GAPDH. Mean ± SD. One-way ANOVA with Tukey's multiple comparisons test, $p < 0.05$ shown, not significant.

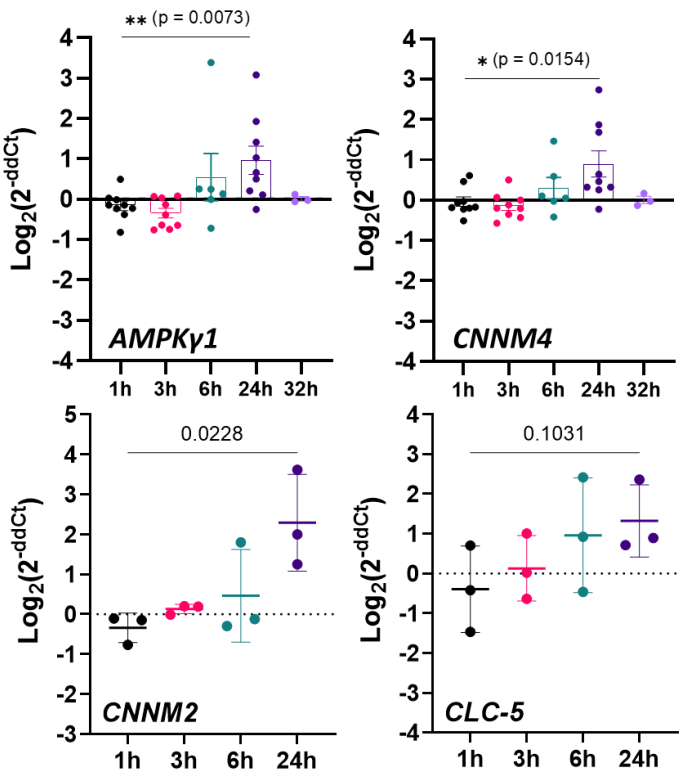


Figure 4.3. PlnEF treatment increased the transcript levels of genes encoding CBS-domain containing proteins.

Non-polarized Caco-2 cells at 0-days post confluence treated with 500 nM PlnEF or untreated for 1, 3, 6, 24, or 32h. Mean ± SD; at least triplicates for each time point. Unpaired t-test between 1h and 24h time points. The results were normalized using the $2^{-\Delta\Delta Ct}$ method with GAPDH as the housekeeping gene and the untreated cells (no PlnEF) as the control condition. *AMPKγ1* and *CNNM4* data were combined from three independent experiments.

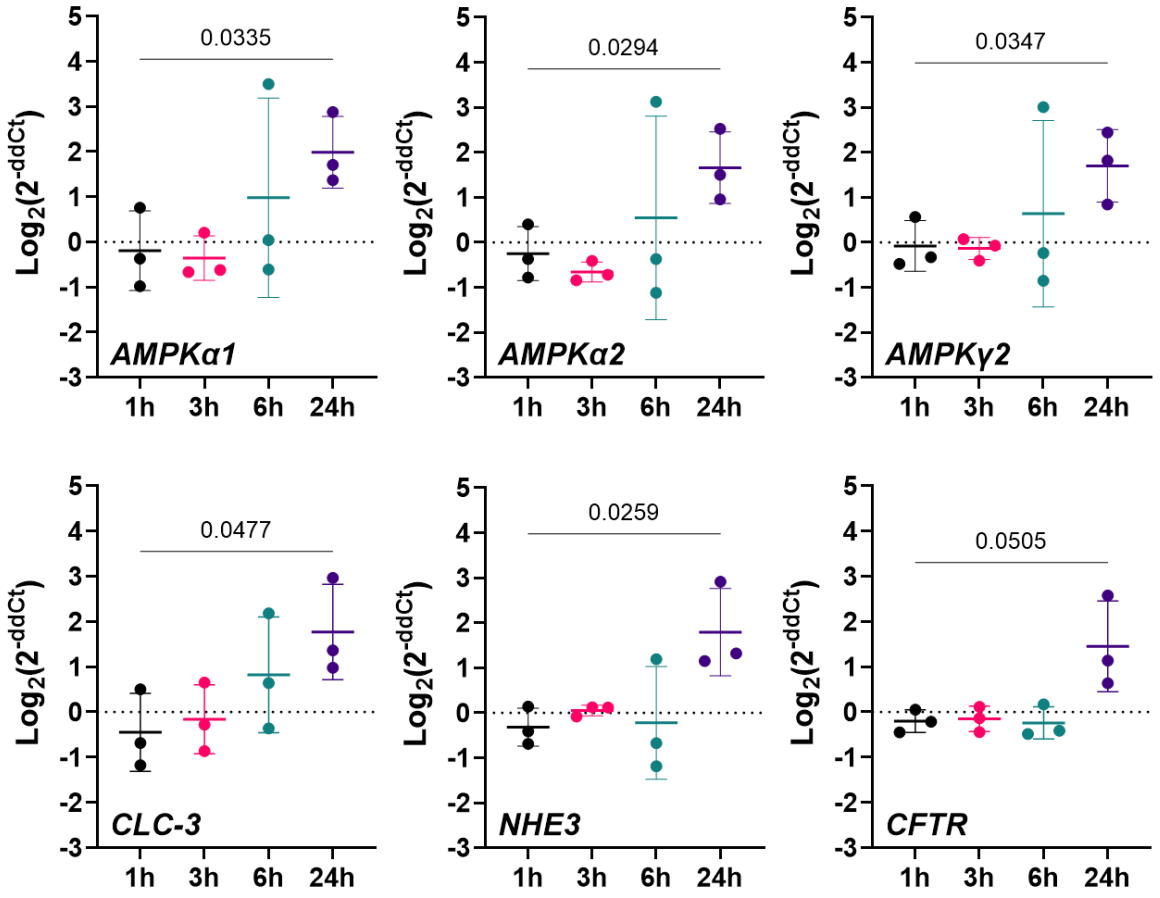
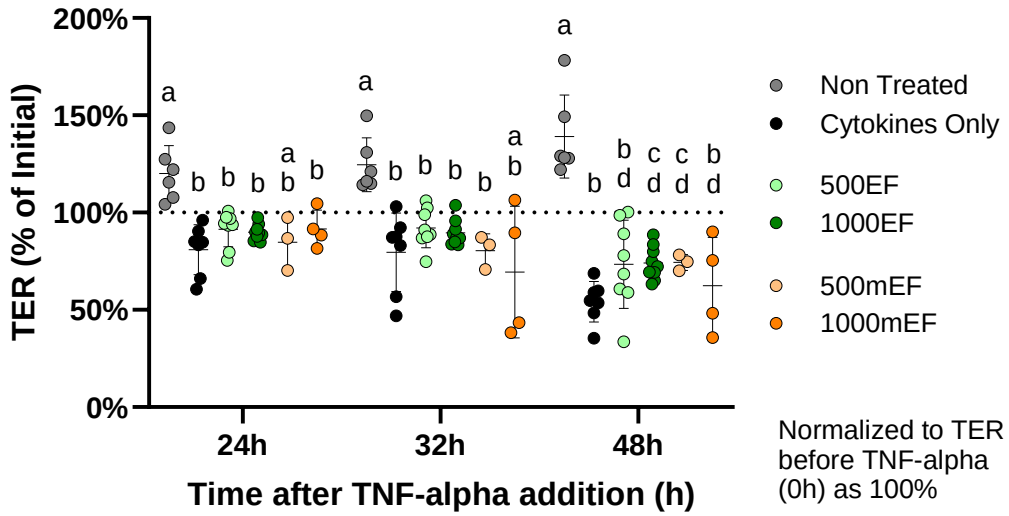


Figure 4.4. PlnEF treatment increased the transcript levels of genes encoding AMPK subunits and ion transporters.

Non-polarized Caco-2 cells treated with or without 500 nM PlnEF for 1, 3, 6, or 24h. The results were normalized using the $2^{-\Delta\Delta Ct}$ method with GAPDH as the housekeeping gene. Mean ± SD. Unpaired t test between 1 h and 24 h time points with triplicates for each time point.

A)



B)

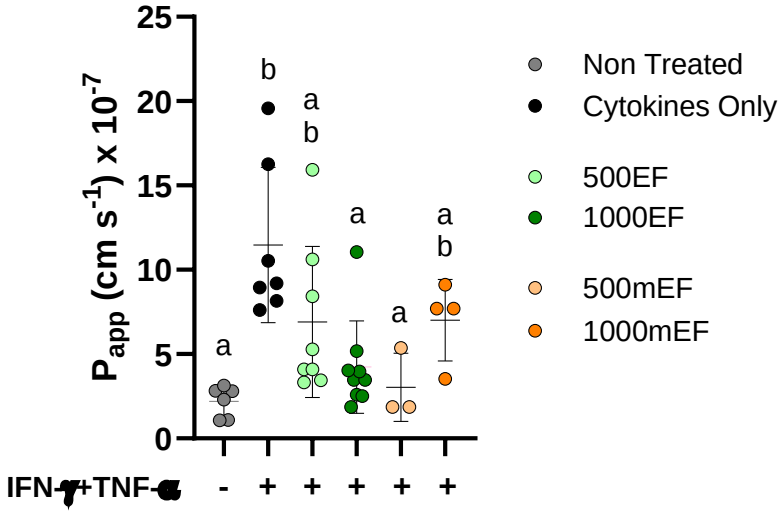


Figure 4.5. Bacteriocin PlnEF (1000 nM) and mutant PlnE-G9I + PlnF (500 nM) protected human Caco-2 intestinal epithelial cells from cytokine-induced barrier perturbation.

Caco-2 cell monolayers pre-treated with 500 or 1000 nM PlnEF or mutant PlnEF (mEF), then sequentially exposed to IFN-γ (100 ng/ml, 21h) and TNF-α (10 ng/ml, 48h). (**A**) Trans-epithelial electrical resistance (TER) of monolayers 24, 32, and 48h following TNF-α addition; (**B**) Apparent permeability coefficient (P_{app}) of 4kD FITC-dextran across monolayer. Mean values

not sharing any letter are significantly different based on two-way ANOVA (TER) or one-way ANOVA (FITC) with Tukey's multiple comparisons test; $p < 0.05$ shown. Data combined from two independent experiments, at least $n = 3$. Mean ± SD.

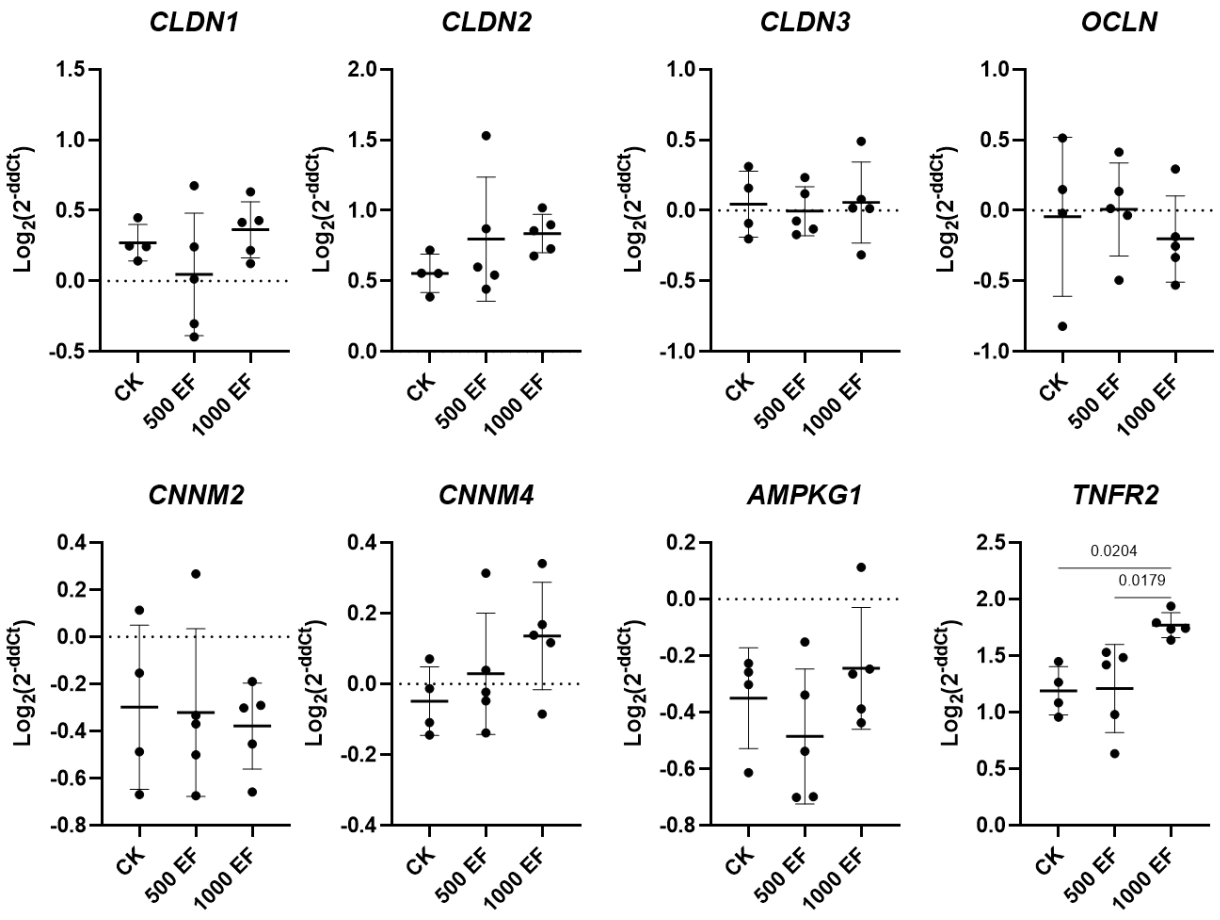
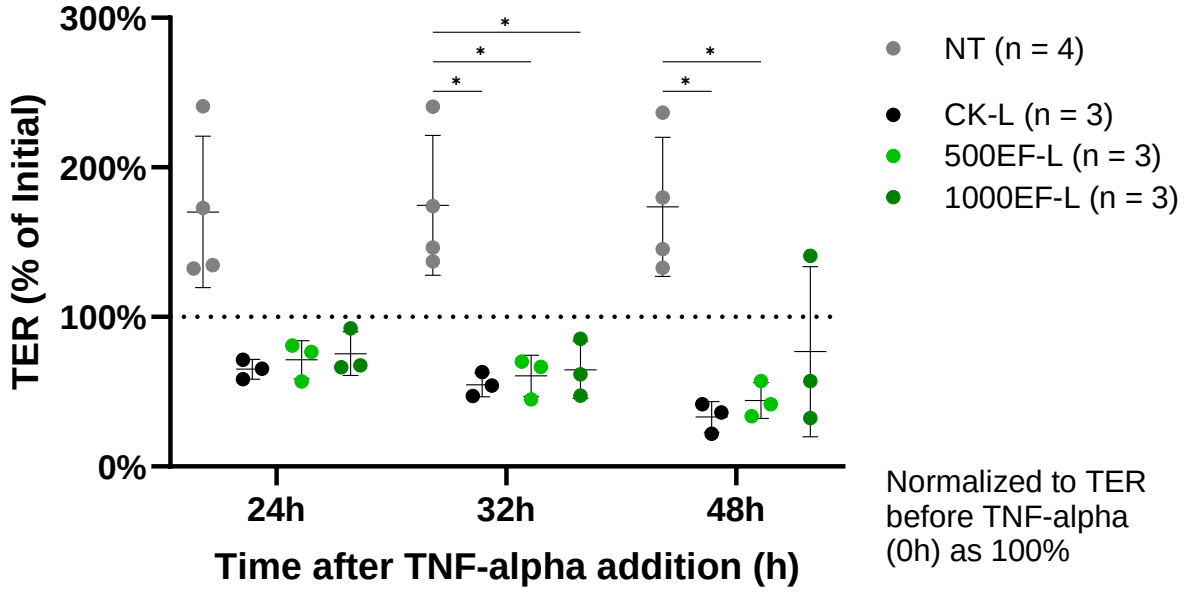


Figure 4.6. Transcript levels of genes encoding for tight junction proteins, CBS-domain containing proteins, and *TNFR2* in polarized Caco-2 cell monolayers challenged with cytokines.

Caco-2 monolayers were apically treated with 500 or 1000nM PlnEF and followed by sequential basolateral exposure to IFN-γ and TNF-α. The results were normalized using the $2^{-\Delta\Delta Ct}$ method with GAPDH as the housekeeping gene and the untreated cells (no PlnEF) as the control condition. Mean ± SD. One-way ANOVA with Tukey's multiple comparisons test, $p < 0.05$ shown.

A)



B)

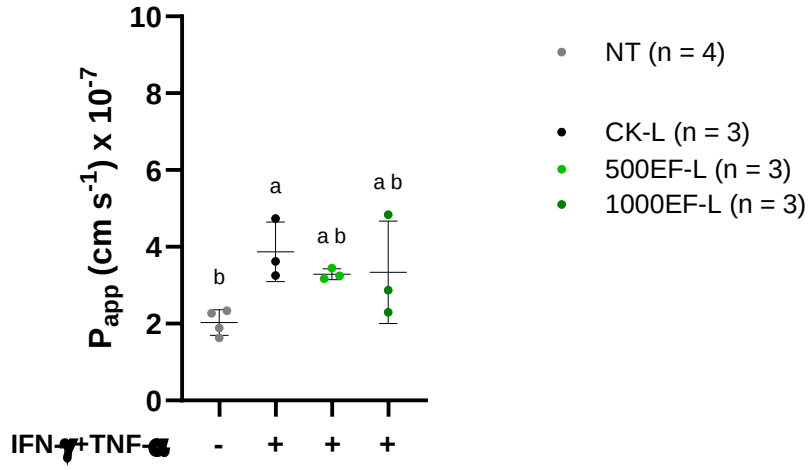


Figure 4.7. Bacteriocin PlnEF did not protect porcine IPEC-J2 intestinal epithelial cells from cytokines-induced barrier perturbation.

IPEC-J2 cell monolayers pre-treated with 500 or 1000 nM PlnEF, then sequentially exposed to IFN-γ (100 ng/ml, 21h) and TNF-α (100 ng/ml, 48h). (**A**) Trans-epithelial electrical resistance

(TER) of monolayers 24, 32, and 48h following TNF-α addition; (**B**) Apparent permeability coefficient (P_{app}) of 4kD FITC-dextran across monolayer. Mean values not sharing any letter are significantly different based on two-way ANOVA (TER) or one-way ANOVA (FITC) with Tukey's multiple comparisons test; $p < 0.05$ shown. At least $n = 3$. Mean ± SD.

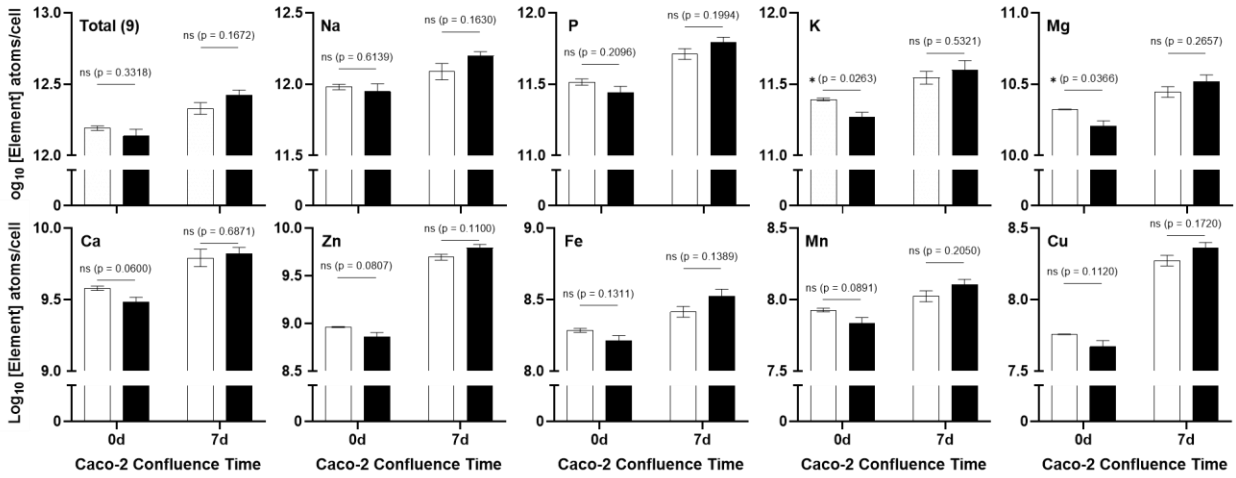
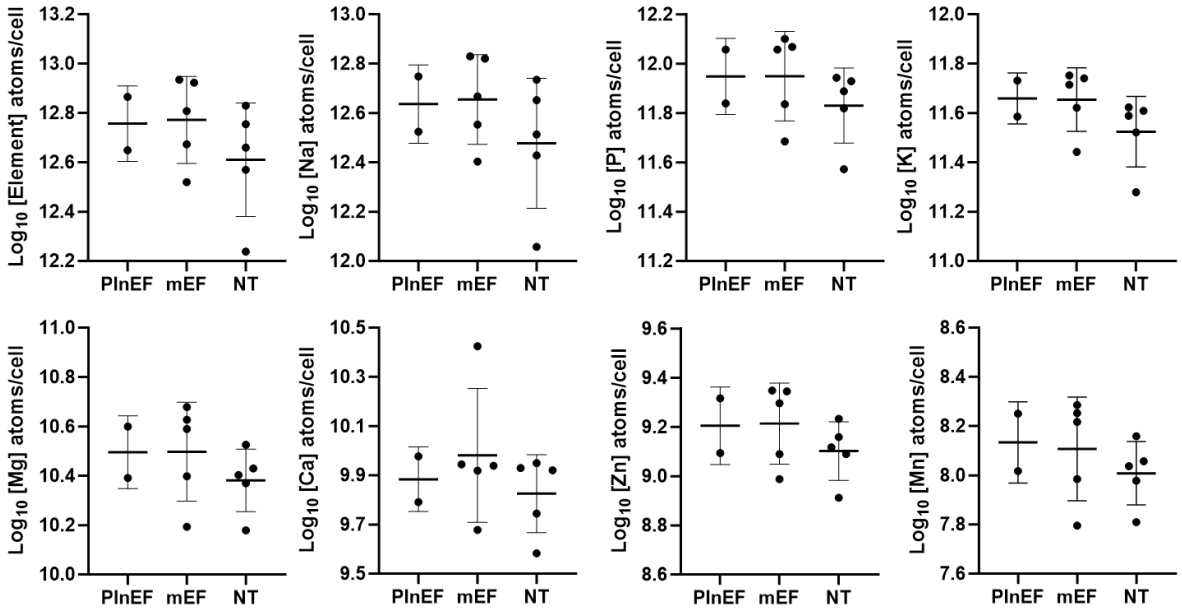


Figure 4.8. Intracellular cation levels in non-polarized Caco-2 cells treated with PlnEF.

Non-polarized Caco-2 cells at 0-days post confluence were treated with (black) or without (white) 500nM PlnEF for 32h exhibited significantly lower levels of K^+ and Mg^{2+} compared to untreated cells. PlnEF-treated Caco-2 cells at 7 days post confluence did not show significant changes in intracellular ion levels compared to untreated cells. Intracellular ion levels (in atoms) quantified by ICP-MS and normalized to cell numbers, atoms/cell. Mean ± SD. Unpaired t test between with and without PlnEF. At least triplicates per time point.

A)



B)

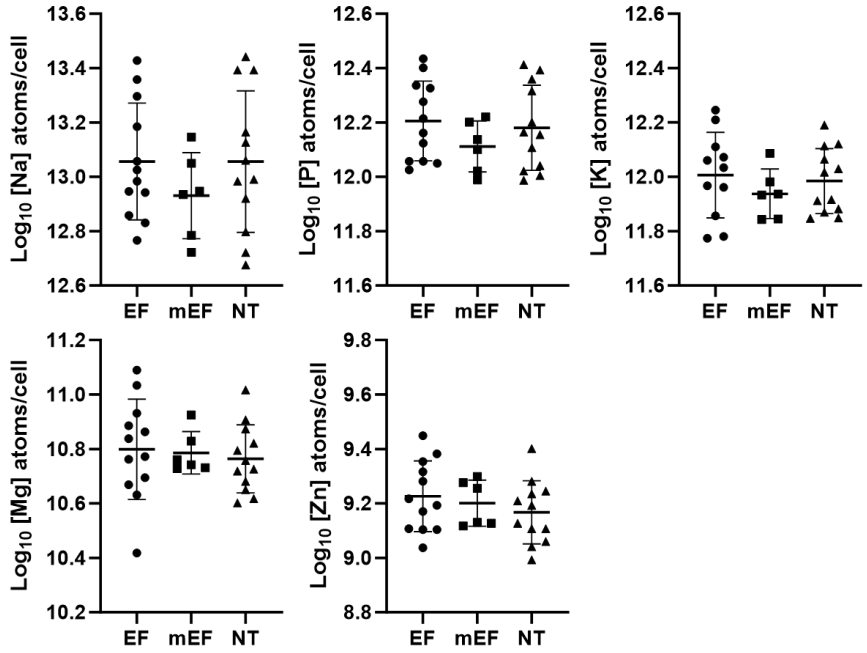
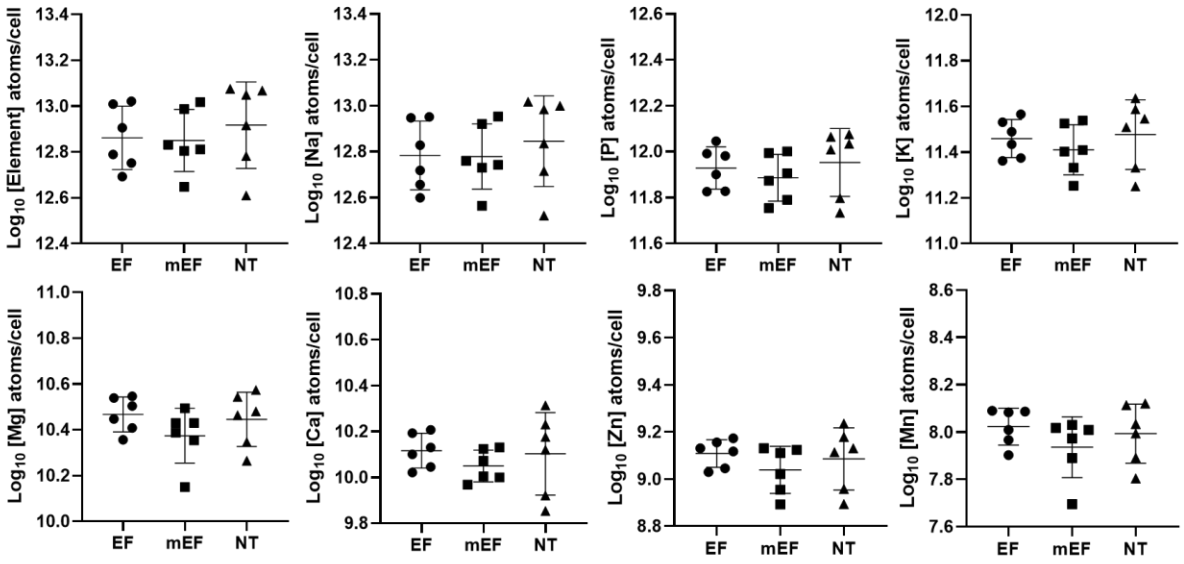


Figure 4.9. Intracellular cation levels in Caco-2 and IPEC-J2 cells after 32 h PlnEF exposure.

Non-polarized human Caco-2 (**A**) or porcine IPEC-J2 (**B**) cells (0-days post confluence) treated with 500nM PlnEF (n = 2), mutant PlnE-G9I/PlnF (mEF) (n = 5) or untreated (NT) (n = 5) for 32h. ICP-MS: normalized to cell count as atoms/cell. Mean ± SD. Unpaired t test (NT vs. PlnEF or mEF): not significant.

A)



B)

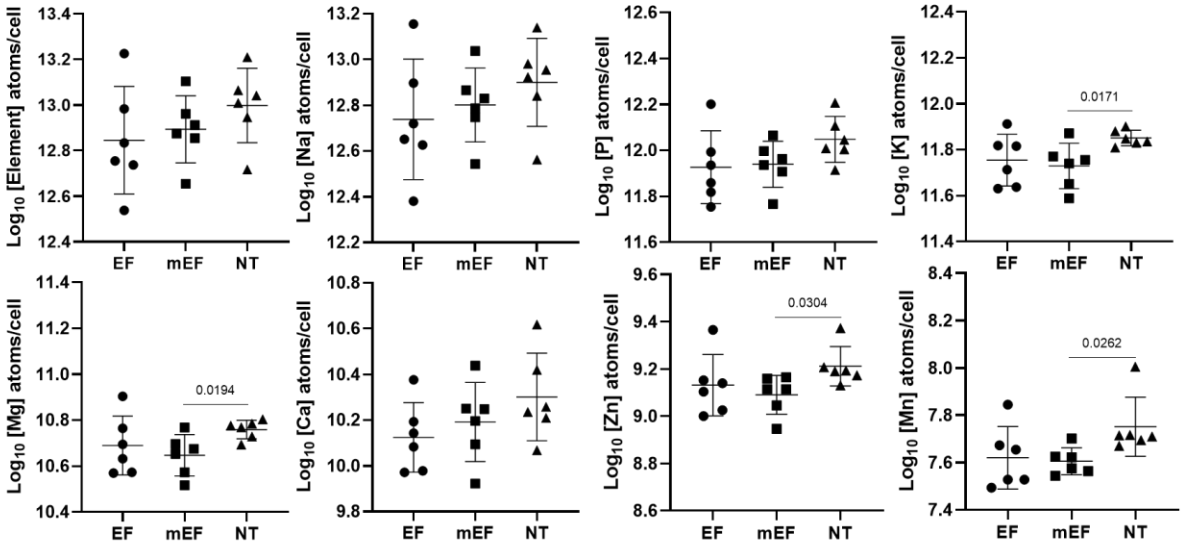


Figure 4.10. Intracellular cation levels in Caco-2 and IPEC-J2 cells after 1 h PlnEF exposure.

Non-polarized human Caco-2 (**A**) or porcine IPEC-J2 (**B**) cells at 0-days post confluence were treated with 500nM PlnEF, mutant PlnE-G9I/PlnF (mEF), or untreated (NT) for 1h. ICP-MS:

normalized to cell count as atoms/cell. Mean ± SD. Unpaired t test (NT vs. PlnEF or mEF): not significant.

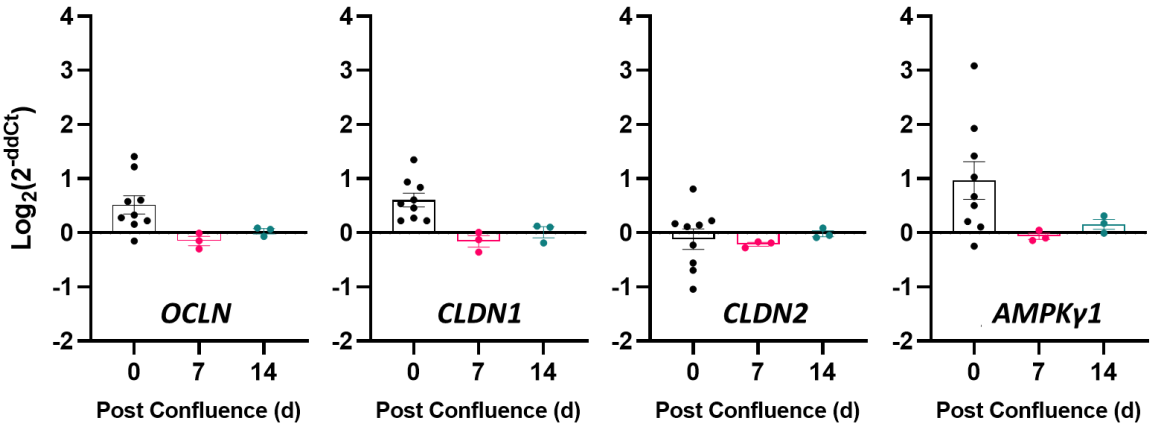


Figure S1. Influence of cell growth stage on PlnEF-induced gene expression changes.

Treatment with 500nM PlnEF for 24h led to the greatest gene expression change in 0 days post confluence, non-polarized Caco-2 cells compared to 7- or 14-days post confluence. The results were normalized using the $2^{-\Delta\Delta Ct}$ method with GAPDH as the housekeeping gene. Mean ± SD. At least triplicates for each time point.

A)

CorC CBS domain (residues 216 – 351) amino acid sequence used for PSI-BLAST

>LP965 CorC (Y216-G351)

YLYMERAFEFNDKIAKDIMIDRTQLVVIDINDTVKQAIRVYLQKRF

SRLPVVANNDKDKILGYVYNYDLIRQGEVDDDIRVNKLLRNIITIP

ETTPIQAILQKMIDKQTPIVVVVDEYGGTSGIVTDKDIYEELFG

B)

PSI-BLAST Protein Hits	E value
CNNM4	3E-10
CNNM2	2E-7
AMPKγ1	0.003
CLC-5	0.047
CLC-3	1.2
IMPDH	2.2

Figure S2. PSI-BLAST search using bacterial CorC CBS domain amino acid sequences as a query to find human homologs.

(**A**) Bacterial CorC CBS domain amino acid sequences between residues 216 and 351. (**B**) Top protein hits identified by three iterations of PSI-BLAST with E values.

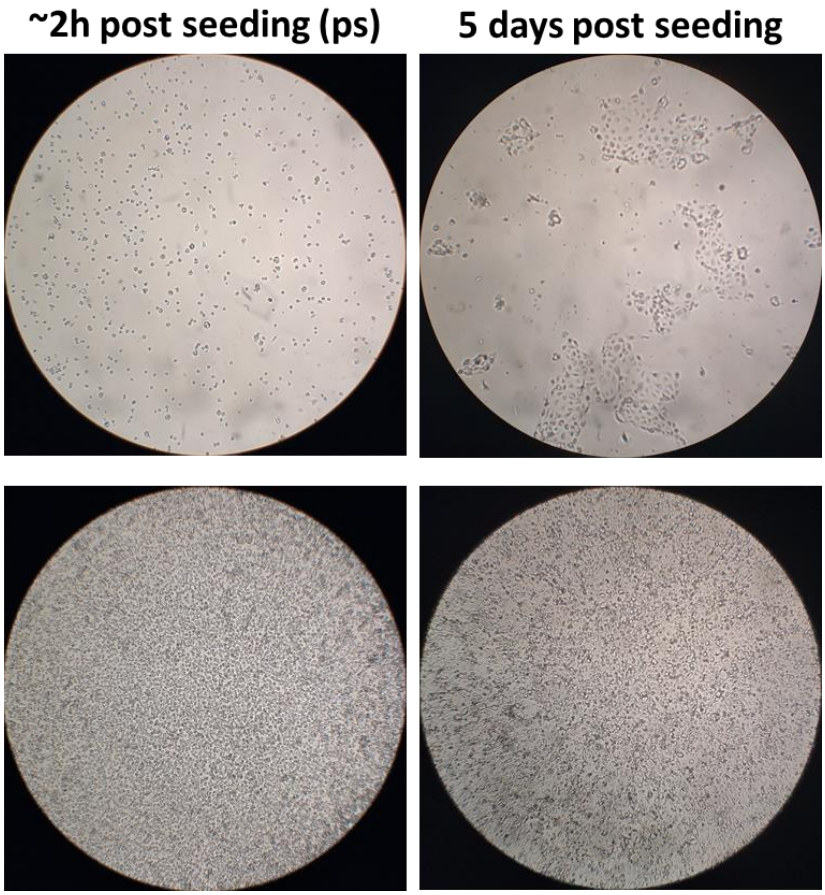


Figure S3. Representative images of IPEC-J2 cells seeded in six-well tissue culture plates at low and high densities.

Top: 1.0 x 10^4 cells/ cm^2; Bottom: 1.8 x 10^5 cells/cm^2. IPEC-J2 cells were grown in DMEM/F12 with 10% FBS and 1% v/v penicillin/streptomycin at 37 °C with 5% CO_2.

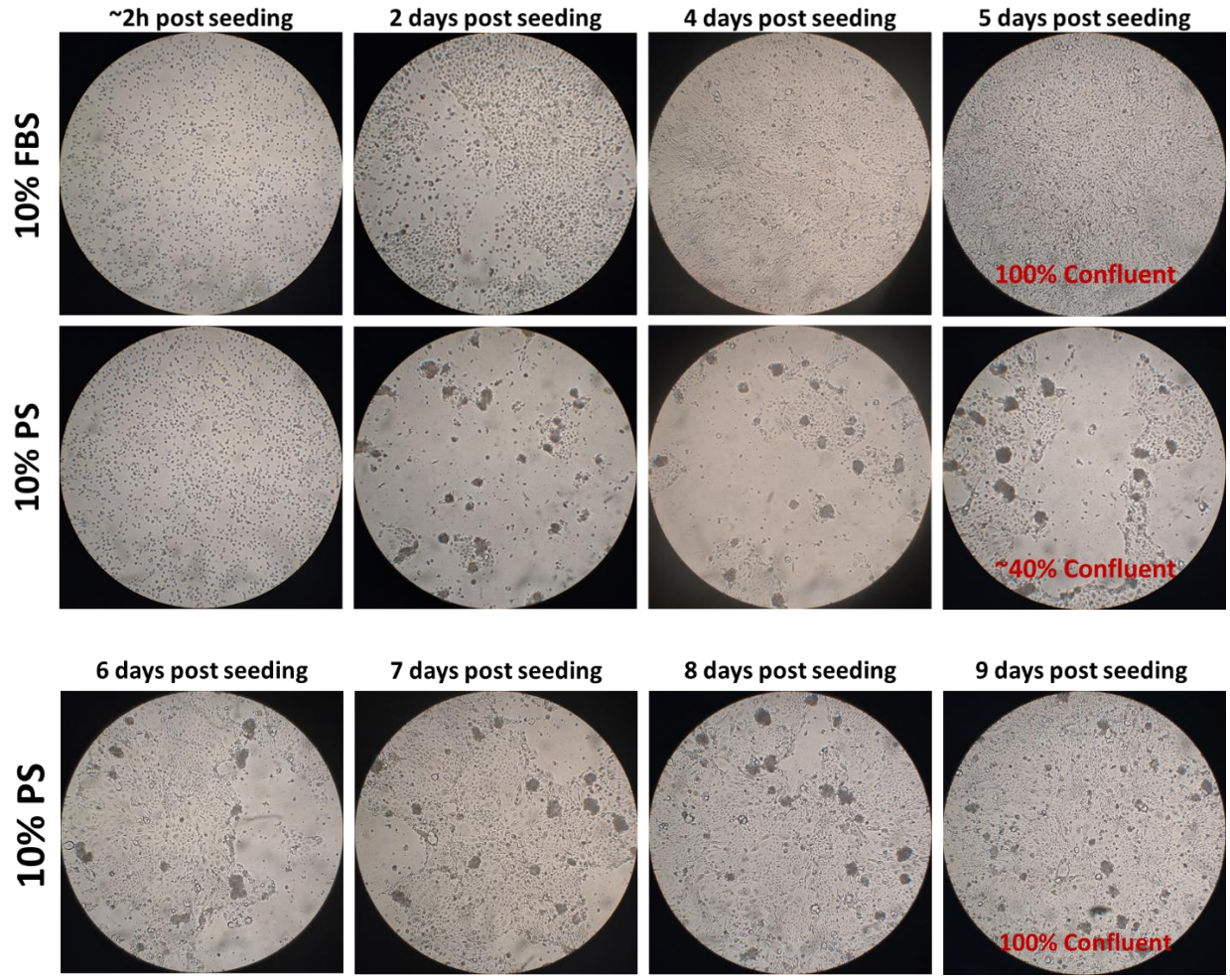


Figure S4. Representative images of IPEC-J2 cells grown on six-well tissue culture plates until 100% confluence.

Seeding density: 3.33 x 10^4 cells/cm^2. IPEC-J2 cells were grown in DMEM/F12 with 10% FBS or 10% porcine serum (PS) and 1% v/v penicillin/streptomycin at 37 °C with 5% CO_2.

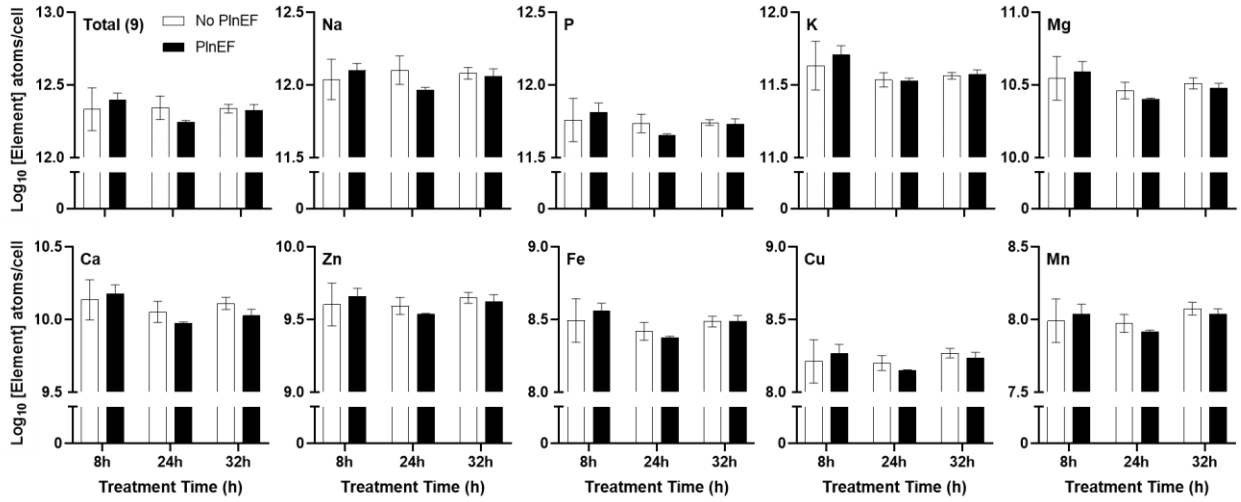


Figure S5. PlnEF did not affect intracellular ion levels in non-polarized Caco-2 cells.

Experiments was performed at 7 days after Caco-2 cell confluence. Cation concentrations (in atoms) quantified by ICP-MS and normalized to cell numbers, determined by hemocytometer. Mean ± SD. Triplicates for each time point. Unpaired t test between with and without PlnEF, not significant.

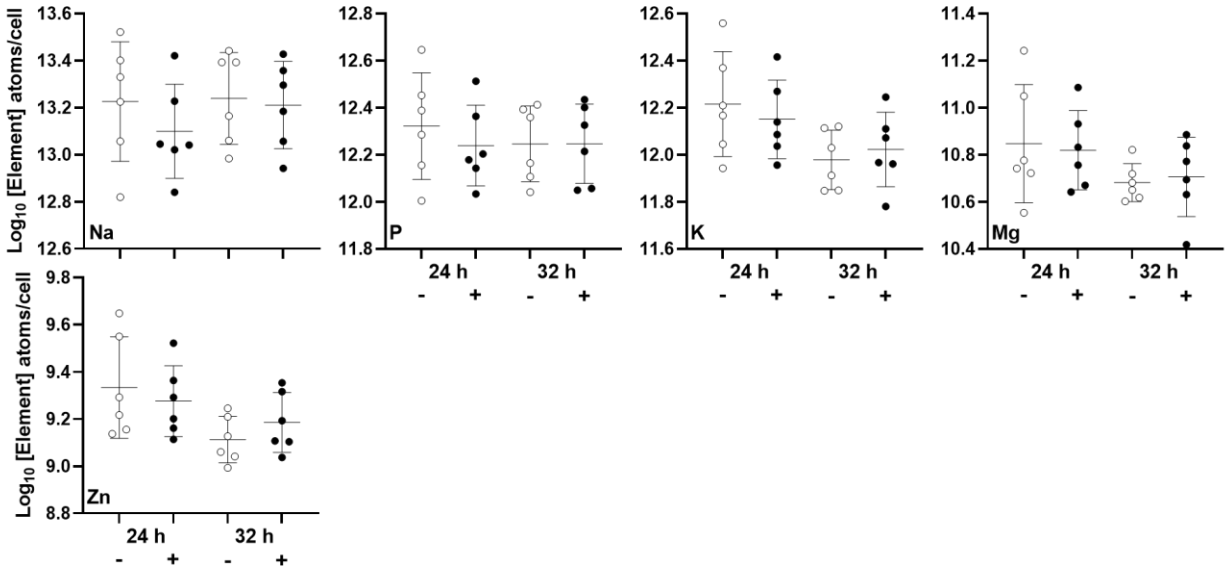


Figure S6. PlnEF did not affect intracellular ion levels in non-polarized IPEC-J2 cells.

Non-polarized IPEC-J2 cells at 0-day post confluence were treated with 500nM PlnEF or untreated for 24h or 32h (n = 6). ICP-MS: ion levels normalized to cell counts as atoms/cell. Mean ± SD. Unpaired t test (± PlnEF): not significant.

Chapter 5. Bacteriocin plantaricin EF receptor CorC interaction and impact on bacterial intracellular ion status

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Abstract

Bacteriocins are a diverse group of ribosomally synthesized antimicrobial peptides produced by both Gram-positive and Gram-negative bacteria and some archaea. Bacteriocin biosynthetic capacity contributes to intra- and inter-species interactions by targeting highly related or non-related species in a microbial community. Despite the discovery of bacteriocins over 100 years ago (Field et al., 2023), more mechanistic details underlying their modes of action and putative receptors in the target bacteria that establish the inhibition spectrum and potency of specific bacteriocins are still key areas for further investigation. The two-peptide bacteriocin, plantaricin EF (PlnEF), made by *Lactiplantibacillus plantarum* is a potent antimicrobial with inhibitory activity due to its capability to disrupt intracellular cation homeostasis. Exposure of the PlnEF-sensitive *L. plantarum* NCIMB700965 (LP965 WT) to PlnEF induced a dose-dependent increase in lag phase. By comparison, a spontaneous PlnEF-resistant mutant carrying a G334V point mutation in the magnesium efflux protein, CorC, LP965 MU, exposed to the same PlnEF concentrations exhibited a shortened lag phase. The mutant also contained more than 2-fold higher intracellular Mg^{2+} concentrations compared to the wild-type strain according to inductively coupled plasma mass spectrometry (ICP-MS), regardless of the presence or concentration of PlnEF. These results point to the connection between intracellular Mg^{2+} levels and PlnEF-induced membrane potential disruption, inviting further investigations of the potential direct binding interactions of PlnEF and CorC receptor or other partners in the target bacterial membrane as well as the roles of intracellular magnesium in the heterogenous cellular stress response in a bacterial population.

Introduction

Bacteriocins are a diverse group of ribosomally synthesized antimicrobial peptides produced by both Gram-positive and Gram-negative bacteria as well as some archaea with the capacity to target highly related (narrow spectrum) or non-related (broad spectrum) microbial species (Cotter et al., 2005; Gillor et al., 2008; Negash & Tsehai, 2020; Yang et al., 2014). Bacteriocins are categorized into different classes based on characteristics such as, size or length, structure, the presence of post-translational modifications, mode of action, inhibition potency (Cotter et al., 2005; Yang et al., 2014). Bacteriocin production may contribute to the ecological fitness of the producer strain, for instance, by facilitating the introduction of the strain into an established niche (e.g., *Escherichia coli* colicin, *Streptococcus mutans* mutacin 1140, *Lactobacillus salivarius* DPC6005 salivaricin P), inhibiting the growth of pathogens (e.g., *L. salivarius* UCC118 Abp118, *Pediococcus acidilactici* MM33 pediocin PA-1/AcH, *Lactococcus lactis* MM19 nisin Z), or engaging in interspecies cross talk as signaling peptides (e.g., *Lactiplantibacillus plantarum* DC400 PlnA and *Lactobacillus sanfranciscensis* DPPMA174) (Dobson et al., 2012; Gillor et al., 2008). Because of the expanding research on bacteriocins, their inhibition targets and potency, and applications (Cotter et al., 2005; Darbandi et al., 2022; Fernandes & Jobby, 2022; Gillor et al., 2008; Hegarty et al., 2016; Yang et al., 2014), more investigations of their mechanisms of action within a microbial community and with the host are needed in order to fully unlock their potentials as alternative antimicrobials and natural bio-preservatives in clinical and food production settings.

Gram-positive bacteria, particularly lactic acid bacteria (LAB), have been a major source of diverse bacteriocins (Fernandes & Jobby, 2022; Kassaa et al., 2019; Todorov, 2019). LAB play essential roles in the fermentation of many foods and beverages and many LAB genera are

Generally Recognized as Safe (GRAS) (Gänzle, 2022; Valentino et al., 2024; Wang et al., 2021). Different bacteriocins (including partially purified fractions) and bacteriocinogenic LAB strains have been isolated from various (fermented) food matrices, for example, nisin and lactococcin from *L. lactis* (raw milk cheeses), mesenterocins from *Leuconostoc mesenteroides* (water buffalo cheese), macedocin from *Streptococcus gallolyticus* subsp. *macedonicus* (Bulgarian-style yogurt) (Kassaa et al., 2019; Todorov, 2019). Among LAB bacteriocins included in the LABiocin database (517 bacteriocins at the time of the publication) (Kassaa et al., 2019), the old *Lactobacillus* genus, which has now been re-classified into 25 genera (Zheng et al., 2020), was found to be the most bacteriocinogenic LAB genus, followed by *Enterococcus* and *Streptococcus* genera.

Bacteriocins produced by LAB are usually small cationic peptides (20 to 70 amino acids) and exert their potent antimicrobial activity in the picomolar to nanomolar range against relatives from the same or highly related species (Diep et al., 2009; Dobson et al., 2012). These peptides generally disrupt the membrane integrity of target bacterial cells through mechanisms that involve membrane-bound docking receptors (Cotter, 2014; Diep et al., 2009). Bacteria that produce bacteriocins usually express the cognate immunity protein to protect themselves from the killing effects of the produced bacteriocins (Diep et al., 2007; Pérez-Ramos et al., 2021). The presence or absence of bacteriocin, target receptors and cognate immunity proteins in different strains of the same or highly related species contribute to the sensitivity or resistance to the specific bacteriocins.

Currently, only a small set of LAB bacteriocin target receptors have been identified (Cotter, 2014), including lipid II for nisin (class I lantibiotic) and lactococcin 972 (class II) (Martin & Breukink, 2007; Martínez et al., 2008), the subunits of the mannose

phosphotransferase (PTS) system for pediocin-like bacteriocins (class IIa) and lactococcins (class IId) (Diep et al., 2007; Kjos et al., 2011), maltose ABC transporter for the circular (class IIc) garvicin ML (Gabrielsen et al., 2012), Zn-dependent metallopeptidase for bacteriocin LsbB (class IId) (Uzelac et al., 2013), and an undecaprenyl pyrophosphate phosphatases (*uppP*) for lactococcin G (class IIb, unmodified, two-peptide) (Kjos et al., 2014). Molecular details of how bacteriocins initially interact with target bacterial cells and the subsequent changes leading to inhibition are crucial for elucidating the target range and potency and inform application strategies that are more tailored (e.g., specific pathogens with the receptors).

L. plantarum is a metabolically versatile and generalist LAB species with high intraspecific diversity that can be found in various environments (e.g., fermented foods and beverages and the digestive tracts of mammals and insects), and numerous strains of this species have been studied and commercially applied as probiotics (de Vries et al., 2006; Heeney et al., 2018; Million et al., 2012; Seddik et al., 2017). *L. plantarum* has also been investigated for its bacteriocin biosynthetic capacities. The mosaic *pln* (plantaricin) loci are responsible for bacteriocin plantaricin biosynthesis in *L. plantarum* (Diep et al., 2009). The *pln* locus encodes two main two-peptide (class IIb) bacteriocins, PlnEF and PlnJK, as well as other bacteriocins Plantaricin NC8 (class IIb) and bacteriocins related to pediocins (class IIa), along with the regulatory proteins, transporters, and immunity proteins (Choi et al., 2021; Diep et al., 2009; van Reenen et al., 1998). The organization and presence of different genes in these loci separate the *L. plantarum* species into three lineages, including those with conserved possession of more than one two-peptide bacteriocin (PlnEF and PlnJK) (lineage A), those that lost *pln* gene functions due to mobile genetic elements (lineage B), and strains in which one two-peptide bacteriocin (PlnEF) is conserved (lineage C) (Choi et al., 2021).

L. plantarum NCIMB8826 (LP8826R), originally isolated from human saliva, contains the *plnEFI* operon in its *pln* locus, encoding the two two-peptide bacteriocins (class IIb) Plantaricin EF (PlnEF) and Plantaricin JK (PlnJK), and their cognate immunity proteins, PlnI and PlnLR, respectively (Diep et al., 2009). PlnEF requires the complementary interaction of both peptides, PlnE and PlnF, in equal molar ratios for its antimicrobial activity (Anderssen et al., 1998; Ekblad et al., 2016; Hauge et al., 1999). PlnEF was found to form pores with high conductivity for monovalent cations but not anions in the target bacterial cell membrane and dissipate the transmembrane electrical potential and pH gradient (Moll et al., 1999; Zhang et al., 2016). A previous study identified the membrane-bound magnesium efflux protein, CorC, as the putative target receptor for PlnEF in the PlnEF-sensitive *L. plantarum* NCIMB700965 (LP965 WT) (Heeney et al., 2019). CorC from *Staphylococcus aureus* was found to be essential for its high Mg^{2+} tolerance and pathogenicity in the host (Armitano et al., 2016). Furthermore, examination of the LP965 WT genome and the genome of a spontaneous PlnEF-resistant mutant of this strain (LP965 MU) revealed that a single point mutation, G334V, in CorC conferred increased resistance to PlnEF (Heeney et al., 2019). This G334V mutation resides in a cysteine-β-synthase (CBS) domain and computational modeling suggested that this highly conserved region is a potential PlnEF binding site (Heeney et al., 2019). A recent crystal structure of *Thermus parvatiensis* CorC revealed that ATP binding to the CBS domain is needed for Mg^{+2} export (Huang et al., 2021). Interestingly, the exposure to high cation concentrations abrogated the G334V conferred resistance to PlnEF in LP965 MU (Heeney et al., 2019). The specific interactions between PlnEF and receptor CorC in bacteria targeted by PlnEF and the functions of CorC in maintaining cation homeostasis still need to be characterized.

The present study aimed to elucidate the details of how PlnEF exerts its antimicrobial effect against target bacterial cells and the role of the CorC G334V mutation in mediating the increased resistance to PlnEF. We hypothesized that PlnEF exerts its antimicrobial activity against sensitive bacteria by dysregulating intracellular cation homeostasis via its putative receptor CorC.

Materials and Methods

Bacterial strain and growth conditions

The PlnEF-sensitive indicator strain *L. plantarum* NCIMB 700965 (LP965 WT) was acquired from the National Collection of Industrial Food and Marine Bacteria (NCIMB; https://www.ncimb.com/culture-collection/) and maintained in 15% w/v glycerol at -80 °C. LP965 MU is a spontaneous PlnEF-resistant isolate (Heeney et al., 2019; **Table 1**). *Lacticaseibacillus paracasei* BL23 was from Dr. Vicente Monedero (Institute of Agricultural Chemistry and Food Technology, IATA-CSIC, Valencia, Spain; Acedo-Félix & Perez-Martinez, 2003; Mazé et al., 2010). Bacterial strains were routinely grown at 30 °C in lactobacilli De Man, Rogosa and Sharpe (MRS) medium (Becton-Dickinson, Franklin Lakes, NJ). Chloramphenicol (5 µg/ml; Research Products International RPI, Mount Prospect, IL, USA) was added when indicated. Chemically defined medium (CDM_L) was prepared as described in Aumiller et al., 2023 with and without magnesium addition.

Bacteriocins

PlnE (FNRGGYNFGKSVRHVVDAIGSVAGIRGILKSIR; 98.2% purity), PlnF (VFHAYSARGVRNNYKSAVGPADWVISAVRGFIHG; 99.8% purity), PlnE-G9I

(FNRGGYNF**I**KSVRHVVDAIGSVAGIRGILKSIR; 98.7% purity), and PlnF with N-terminus 5-FAM (fluorescent fluorescein) modification (5FAM-VFHAYSARGVRNNYKSAVGPADWVISAVRGFIHG; 98.5% purity) were chemically synthesized by GenScript (Piscataway, NJ). The Pln peptides were re-constituted in sterile water or 10% v/v DMSO (5FAM-PlnF) to a concentration of 100 µM and aliquots were stored at -20 °C. PlnE or PlnE-G9I were mixed with PlnF or FAM-PlnF in 1:1 ratio to form PlnEF (EF), PlnE-G9I + PlnF (mEF), PlnE + FAM-PlnF (EfF), or PlnE-G9I + FAM-PlnF (mEfF) prior to use. Nisin A was purchased from MP Biomedicals (Santa Ana, CA, USA). Nisin was re-constituted in 0.02 N hydrochloric acid (HCl; Fisher Scientific, Waltham, MA, USA) to a concentration of 1 mM and stored at -20 °C. Nisin was diluted to 25 µM in 0.02N HCl prior to use.

Bacterial growth curves

L. plantarum LP965 or *L. paracasei* BL23 variants (**Table 1**) were incubated in commercial MRS for 16 to 18 h at 30 °C, collected by centrifugation at 3,000 x g for 5 min, washed twice in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4) (pH 7.2), and then suspended in PBS (pH 7.2) to an optical density at 600 nm (OD600) of 0.1 or 1 as indicated. The cell suspensions were then distributed into 96-well microtiter plates (Thermo Fisher Scientific, Waltham, MA, USA) with wells containing MRS to reach a final OD600 of either 0.0024 or 0.024, as specified, and supplemented with the bacteriocin peptides. Controls included wells with MRS supplemented with equal volumes of water or 10% v/v DMSO instead of peptides and MRS with an equal volume of PBS instead of bacterial cells. To avoid edge effects in the microtiter plate, an antifog solution (0.1% Triton X-100 in 70% ethanol) was applied to the lid and the plates were sealed with Parafilm (Bemis,

Shirley, MA, USA). The plates were incubated in a Synergy 2 microplate reader (BioTek, Winooski, VT, USA) at 30 °C wherein growth was measured as changes in OD600 every 30 min for 48 h. These experiments were conducted at least twice for each condition. Assessment of LP965 WT and MU growth in MRS or CDM with or without supplemental magnesium, was performed in microtiter plates incubated at 30 °C for 72 h with OD600 measured every 15 min.

Radial diffusion assays

Radial diffusion assays to measure Pln activity were performed as described previously (Deborah A. Steinberg, 1997) with several modifications. Bacteria were grown in MRS to approximately 4.3 x 10^8 to 6.3 x 10^8 CFU/ml. BL23 variants transformed with pJIM2246, pJIMDH1, or pJIMDH2 (**Table 1**) were grown in MRS with 5 µg/ml chloramphenicol. The cells were collected by centrifugation at 3,000 x g for 5 minutes at 4 °C, washed once with 10 mM sodium phosphate buffer (pH 7.4, 77.4% w/v sodium phosphate dibasic Na_2HPO_4 and 22.6% w/v sodium phosphate monobasic NaH_2PO_4), and then collected again by centrifugation. Approximately 4 x 10^6 CFU were mixed with 10 mL 1% w/v molten agarose (pH 7.4) (Fisher Scientific International, Inc., Hampton, NH) containing 10 mM sodium phosphate and 1% v/v MRS broth and allowed to solidify in a petri dish. Wells were then formed using a Pasteur pipette attached to a vacuum bleach trap (Deborah A. Steinberg, 1997). The wells were inoculated with two microliters (µL) of the Pln peptides or either sterile water, 10% v/v DMSO, or 0.02 N HCl (negative controls) or 25 µM nisin (positive control), and the plates were incubated at 30 °C for 3 h. Next, an overlay agarose mixture (30% w/v MRS, 1% w/v agarose) was added on top of the underlay gel to provide nutrients for bacterial growth and the plates were

then incubated for 48 h at 30 °C. The diameters of the zones of clearance surrounding the wells in the radial diffusion assays were quantified via a ruler.

Single cell isolation and viability quantification

L. plantarum LP965 WT and MU were incubated in MRS under static conditions at 30 °C until stationary phase (13 – 28 h) before pelleting by centrifugation at 2,348 x g for 5 min at 21 °C. The cells were washed twice in PBS (pH 7.2), inoculated in MRS to reach a final OD600 of 0.05, and then incubated at 30 °C. PlnEF or an equal volume of sterile water was added during late exponential phase (OD_{600} 1.4 – 2.8), approximately 10 h, later to reach the final concentration of 100 nM (for LP965 WT) or 1000 nM (for LP965 MU). Two hours later, the cells were collected by centrifugation, washed once in PBS, and diluted to approximately 1 x 10^6 CFU/ml. Cell counts for LP965 WT and MU before and after 2 h PlnEF or water exposure were determined by bacterial enumeration on MRS agar. In parallel, single cells from this diluted suspension were dispensed into 96-well microtiter plates containing MRS via the B.SIGHT (CYTENA, Freiburg, Germany). A particle within a size range of 0 – 3 µm and a roundness range of 0.1 – 0.5 µm was considered a "single cell" and was dispensed into one well of the microtiter plate. Single cells were not dispensed into six of the 96 wells on each plate to check for environmental contamination. The 96-well microtiter plates were then incubated at 30 °C for 48 h and then each well was visually inspected for turbidity. For a subset, OD600 was monitored over 48 h at 15 min intervals using a spectrophotometer (Synergy 2, BioTek, Santa Clara, CA, USA).

CorC protein sequence alignments

LP965 WT and MU CorC protein sequences were obtained from the NCBI (Accession numbers CP023490 and CP026505, respectively) (Heeney et al., 2019) and used as a query in the tBLASTn (translated nucleotides basic local alignment search tool) tool (Gertz et al., 2006) to search against the whole genome shotgun contigs of 12 *L. plantarum* strains, 1B1, NCIMB8826 (LP8826R), AJ11, BGM37, EL11, WCFS1, 8.1, ATCC202195, B1.1, B1.3, K4, and WS1.1 (Yu et al., 2021) and *L. paracasei* BL23 (Mazé et al., 2010). Putative CorC or CorC-like protein sequences from these 15 strains were aligned with Clustal Omega 1. 2. 4. (Madeira et al., 2024; Sievers & Higgins, 2021). Furthermore, the DNA and translated protein sequences of CorC from LP965 WT (NCBI Accession Number CP023490.1) and LP8826 (NCBI Accession Number CP037961.1) were aligned with Serial Cloner 2.6.1. (Serial Basics 2013; http://serialbasics.free.fr/Serial_Cloner.html).

Inductively coupled plasma mass spectrometry (ICP-MS) quantification of intracellular ion levels

L. plantarum LP965 WT and MU were grown in MRS for 18 h at 30 °C then collected by centrifugation at 10,000 x g for 5 min, washed in PBS, and suspended in PBS or water to reach $1.46 \times 10^{10} \pm 8.70 \times 10^{9}$ CFU/ml prior to exposure to either 25 or 50 nM PlnEF for 40 min at 30 °C. Fractions of the suspensions were then plated on MRS agar for viable cell enumerations and prepared for ICP-MS analysis. For the latter, *L. plantarum* was collected by centrifugation at 10,000 x g for 3 min. The cell pellets were weighed and stored at -80 °C until use.

ICP-MS quantification of intracellular ions in bacterial cell pellets was performed at the UC Davis Interdisciplinary Center for Plasma Mass Spectrometry (https://icpms.ucdavis.edu/). Briefly, the cell pellets were digested by incubating at 95 °C for 45 min in a 60% concentrated

trace-metal-grade HNO_3. After cooling to room temperature, the digests were incubated at 95 °C for 5 min in 6% H_2O_2 followed by three additions (100, 150, and then 200 µl) of 30% H_2O_2 in 5 min intervals and heating at 95 °C for another 2 h. The digests were then diluted with water to a final concentration of 6% HNO_3 and analyzed with an Agilent 7500C3 ICP-MS instrument (Agilent Technologies, Palo Alto, CA, USA) for simultaneous quantification of select ions (sodium (Na^+), phosphorus (P^{3-}), potassium (K^+), magnesium (Mg^{2+}), calcium (Ca^{2+}), zinc (Zn^{2+}), manganese (Mn^{2+})). The numbers of atoms per cell were calculated for the observed quantities of each ion (parts per billion [ng/ml]) normalized to cell numbers estimated by viable plate counts on MRS agar.

Statistical analysis

Area under the curve (AUC) was used to examine the growth and survival of strains under different concentrations (Sprouffske & Wagner, 2016; Yu et al., 2021). The AUC was calculated with GraphPad Prism 9 (GraphPad Software, Inc., San Diego, CA, USA). Growth rates were calculated using non-linear regression function with exponential growth equation least squares fit of OD600 measurements within the exponential phase in GraphPad Prism 9. Unless otherwise stated, data are presented as mean ± standard deviation (SD). Group means were compared by one-way ANOVA followed by an appropriate post-hoc test or unpaired t test. A p value < 0.05 was considered significant unless otherwise noted.

Results

PlnEF increases lag phase in wild-type LP965 and not the LP965 CorC mutant

Growth of wild-type LP965 (WT) and the LP965 *corC* mutant (LP965 MU) were measured in MRS laboratory culture medium containing concentrations of PlnEF ranging from 25 to 250 nM **(Fig. 1**). As shown previously (Heeney et al., 2019), LP965 WT exhibited PlnEF dose-dependent reductions in growth starting at a concentration of 25 nM PlnEF ($p < 0.0001$, one-way ANOVA with Tukey's multiple comparisons test) (**Fig. 1A** and **1C**). LP965 WT exposed to 250 nM PlnEF did not recover growth within 24 h. This dose-dependent reduction of 24 h AUC in LP965 WT can be attributed to the extension of lag phase times since there were no significant differences in growth rates ($p = 0.9996$, one-way ANOVA with Tukey's multiple comparisons test).

LP965 MU also exhibited dose-dependent reductions when increasing concentrations of PlnEF. However, the antimicrobial effects were attenuated, and the lag phase time was decreased by at least 2-fold compared to the wild-type strain in 25, 50, and 100 nM PlnEF ($p < 0.0001$, one-way ANOVA with Tukey's multiple comparisons test) (**Fig. 1B** and **1C**). Growth of the mutant strain was also limited in 250 nM PlnEF within 24 h (**Fig. 1B** and **1C**). Reductions in AUC were due to the longer lag phase times in PlnEF since there was no significant change in growth rate ($p = 0.9996$, one-way ANOVA with Tukey's multiple comparisons test). A similar trend in 24 h AUC and growth rate in LP965 WT and MU incubated in MRS medium containing different concentrations of PlnEF was also observed when the initial OD600 was increased by 10-fold from 0.0024 to 0.024 (**Fig. S1**).

To begin to understand the necessity of combining PlnE and PlnF together on the observed antimicrobial effects, the two peptides were tested on *L. plantarum* individually in MRS broth (**Fig. 2A** and **2B**). As shown previously (Anderssen et al., 1998), the antimicrobial activity of PlnEF was greater than when either 25 or 50 nM PlnE or PlnF were applied separately

(**Fig. 2A** and **2B**). Neither PlnE nor PlnF resulted in longer lag phase times, affected growth rates (data not shown), or 24 h AUC values compared to the controls ($p \geq 0.9519$ for LP965 WT; $p \geq 0.1028$ for LP965 MU; one-way ANOVA with Tukey's multiple comparisons test) (**Fig. 2B**).

Single cell analysis shows PlnEF kills a fraction of cells in wild-type LP965 populations

Exposure of LP965 to PlnEF in broth medium resulted in extended lag phase times but did not completely inactivate the cells, as indicated by the delayed but still observable growth even when high amounts of PlnEF were added (**Fig. 1**). This is notable since, even at low (25 nM PlnEF) doses, there would have been approximately 3.41×10^7 more PlnEF molecules than individual LP965 cell at the time of inoculation (based on the estimated numbers at an OD600 of 0.0024). This amount should have been sufficient to kill all *L. plantarum* present. Therefore, to determine if the extended lag phase times were due to variations in *L. plantarum* sensitivity to PlnEF, single-celled analyses were performed.

To assess for heterogeneity in LP965 responses to PlnEF, the number of individual cells able to multiply after incubation in MRS or MRS containing 100 nM PlnEF were quantified. Analysis of individual cells grown for 2 h in MRS without PlnEF showed that only 85.7% ± 1.6% were viable (**Table 2**), indicating cell death either prior to or during single cell analysis. However, the number of viables cells decreased to 47.6% ± 1.3% when exposed to 100 nM PlnEF for 2 h (**Table 2**). Under those conditions, the number of LP965 WT cells decreased approximately 6-fold from $2.04 \times 10^9 \pm 3.34 \times 10^8$ CFU/ml before to $7.63 \times 10^8 \pm 1.58 \times 10^8$ CFU/ml after 2 h PlnEF exposure, as estimated by enumeration on MRS agar ($p = 0.0002$, two-way ANOVA with Sidak's multiple comparisons test) (**Table 2**). On the other hand, after 2 h exposure to a 10-fold higher PlnEF concentration, at 1000 nM, 39.3% ± 13.5% of LP965 MU

single cells recovered (**Table 2**). This emphasized the reduced sensitivity of LP965 MU compared to WT to PlnEF since the number of LP965 MU single cells that were viable after 2 h incubation in MRS without PlnEF, at 87.3% ± 1.8%, was comparable to that found for LP965 WT (**Table 2**).

Next, to measure the survival of individual *L. plantarum* cells in the presence of PlnEF after sorting, single cells, not previously exposed to PlnEF, were distributed into MRS medium containing PlnEF and incubated for 48 h. For single cells dispensed into MRS with 0.1, 25, or 100 nM PlnEF, 93.3%, 33.3%, and 13.3% of LP965 MU were able to grow, respectively. LP965 WT was more sensitive than LP965 MU, for which only 0%, 4.4%, and 80% of the single cells were able to multiply in the PlnEF-containing MRS during the 48-h incubation.

PlnEF peptides inhibit LP965 in an MRS agar radial diffusion assay

To assess whether PlnEF-mediated differences in lag phase between the two strains in broth culture result in observable inhibition in agar medium, radial diffusion assays were performed. As found for the growth curves, dose-dependent inhibition of the wild-type and mutant LP965 strains was observed (**Fig. 1D**). However, *L. plantarum* was less sensitive to PlnEF in the radial diffusion assays and no inhibition was found for either 25 or 50 nM PlnEF. Instead, changes in the zone of inhibition extended over a 1000-fold range between 50 and 50,000 nM. Notably, the LP965 WT and MU strains were similarly affected by PlnEF in the agar medium. Although WT LP965 was slightly more sensitive to 500 nM PlnEF ($p = 0.0004$, two-way ANOVA with Tukey's multiple comparisons test), no other significant differences were found (**Fig. 1D**).

Testing the PlnE and PlnF peptides separately in the agar diffusion assay showed that they are not as inhibitory compared to when the two are added together (PlnEF) (**Fig. 2C**). Interestingly, despite not inhibiting *L. plantarum* in MRS liquid medium, PlnE and PlnF inhibited the LP965 WT and MU strains at very high concentrations in agar radial diffusion assay (5 and 50 µM) (**Fig. 2C**). These effects appeared to be additive when combined, resulting in a zone of inhibition that was approximately 2-fold greater than when the peptides were applied separately (**Fig. 2C**).

A single amino acid change in PlnE attenuated PlnEF antimicrobial activity

Besides requiring the combination of PlnE and PlnF for antimicrobial activity against LP965, we tested the requirement for interactions between the two peptides. Mutation of the glycine residue at position 9 into isoleucine (G9I) in PlnE (mPlnE or mE) was found to reduce the antimicrobial activity of the two-peptide bacteriocin by more than 130-fold against a strain of *Latilactobacillus curvatus* by preventing helix-helix interactions (Ekblad et al., 2016). To investigate this possibility for LP965, the WT and MU strains were incubated in MRS broth with 25 or 50 nM mPlnE, PlnF, and the two together (**Fig. 3** and **Fig. S1**). Whereas dose-dependent reductions in growth were observed when PlnEF were present, the G9I mutation eliminated the PlnEF antimicrobial activity against both WT and MU strains ($p \geq 0.3996$, one-way ANOVA with Tukey's multiple comparisons test) (**Fig. 3B**). Remarkably, in the agar radial diffusion assay, mPlnE was inhibitory starting at 500 nM and resulted in zones of inhibition similar to when mPlnE and PlnF were combined ($p \geq 0.9845$, two-way ANOVA with Tukey's multiple comparisons test) (**Fig. 3C**), suggesting that the antimicrobial activity observed in mEF may be attributed largely to mE activity. However, they were still less effective than PlnEF since

exposure to mEF greater than 50 nM concentrations led to at least 1.5-fold lower diameters of clearance zone compared to PlnEF treated cells ($p < 0.0001$, two-way ANOVA with Tukey's multiple comparisons test) and did not result in differential levels of sensitivity between LP965 WT and MU ($p \geq 0.4662$) (**Fig. 3C**).

Fluorescent labeling PlnF blocked antimicrobial activity

To determine whether there were receptor-specific target interactions of PlnEF in sensitive bacteria, PlnF was synthesized with a N-terminus fluorescent fluorescein (5FAM-PlnF; fPlnF or fF) to allow for the visualization of Pln localization in sensitive bacteria. To verify that the 5FAM label did not interfere with the function of PlnEF, fPlnF was incubated with WT and MU LP965 in the presence and absence of PlnE (**Fig. 4**). However, the PlnE and fPlnF combination (EfF) at 25 or 50 nM did not impair LP965 growth (**Fig. 4B** and **Fig. S2**), suggesting that the N-terminus labeling likely detrimentally affected the interactions with PlnE and the resulting antimicrobial activity. Exposure to the individual peptides also did not result in LP965 inhibition compared to cells not exposed to bacteriocin ($p > 0.9999$, one-way ANOVA with Tukey's multiple comparisons test) (**Fig. 4B**).

LP965 MU has higher intracellular Mg^{2+} concentrations

To determine the influence of PlnEF on the intracellular ion homeostasis, ICP-MS was used to quantify the intracellular cation levels of LP965 WT and MU after 40 min incubation in PBS or water with either 25 or 50 nM PlnEF. Out of the seven cations quantified (sodium, phosphorus, potassium, manganese, magnesium, calcium, zinc), only Mg^{2+} concentrations were significantly changed (**Fig. 5**). Specifically, Mg^{2+} concentrations were higher in the LP965 MU

strain than the WT. The increase in Mg^{2+} was not PlnEF specific since there were approximately 2-fold higher Mg^{2+} concentrations in LP965 MU compared to LP965 WT ($p < 0.0135$, unpaired t test between WT and MU) (**Fig. 5**). A 40 min incubation in PBS reduced Na^{+}, P^{3-}, Mn^{2+}, and Zn^{2+} ion levels compared to 0 min in similar magnitude for both LP965 WT and MU ($p < 0.05$, unpaired t test between 0 min and 40 min) (**Fig. 5**). When LP965 WT and MU were incubated in water, the intracellular Na^{+} levels significantly decreased and K^{+}, Mn^{2+}, Mg^{2+}, and Ca^{2+} concentrations were significantly increased compared to PBS, highlighting that the cellular response to hypo-osmotic stress likely masked the more subtle changes associated with PlnEF treatment and incubation time (data not shown).

To test the hypothesis that elevated intracellular Mg^{2+} concentration in LP965 MU may give a growth advantage when incubating in conditions with low levels of Mg^{2+}, LP965 WT and MU were incubated in chemically defined medium (CDM) with and without Mg^{2+} (1mM magnesium chloride hexahydrate ($MgCl_2$) and 2.43 mM magnesium sulfate heptahydrate ($MgSO_4$)). Although the addition of Mg^{2+} improved LP965 strain growth, there were no significant differences between LP965 WT and MU strains (data not shown). In addition, to examine the potential differential responses of LP965 WT and MU against high metal ion stress, the capacity of LP965 WT and MU to grow in CDM containing increased cation levels was assessed. Incubation in CDM containing 10X or 50X higher concentrations of manganese, cobalt, calcium, zinc, and copper did not result in significant changes in growth between LP965 WT and MU (data not shown).

LP965 CorC is mutated in the PlnEF resistant LP965 strain but conserved across different *L. plantarum* strains

To begin to assess whether the findings for the LP965 CorC mutant may be relevant to other *L. plantarum* broadly, we compared amino acid sequences between WT and MU and for other *L. plantarum* strains. Amino acid alignments showed that valine (Val) at CorC residue 329 differed from the WT strain. Comparisons to 13 other *L. plantarum* strains showed a glycine (Gly) at that position. Otherwise, the CorC protein sequences were highly similar, with differences in only three positions, residue 53, 329, and 441 (**Fig. 6**). Notably, both LP965 WT and MU have serine (Ser) at residue 53 while the other 12 strains have alanine (Ala) at this position. Additionally, at CorC position 441, strains WCFS1, 1B1, LP8826, AJ11, BGM37, EL11 are predicted to contain arginine, whereas the others contain threonine (Thr). The latter is expected to lead to differences in size and charge at this residue position.

Furthermore, a tBLASTn search for CorC-like proteins in *L. paracasei* BL23 resulted in the identification of two transport proteins encoded by *ytaB* and *ysiA*, sharing 61% and 28% identities with LP CorC, respectively. Alignment of YtaB with CorC from LP965 WT, LP965 MU, and LP8826 showed 55 positions with gaps or non-conserved variations in amino acid sequences (**Fig. 7**). The N-terminus region between residue positions 40 and 55 and the C-terminus region after position 440 are the most variable, containing the least number of conserved residues between *L. plantarum* strains and BL23 (**Fig. 7**). BL23 YtaB also has a glycine (Gly) at position 329, which distinguished LP965 MU (329V) and other *L. plantarum* strains (329G).

Heterologous expression of CorC from LP965 WT and MU did not affect sensitivity to PlnEF in *Lacticaseibacillus paracasei* BL23

Heterologous expression of CorC from LP965 WT in BL23 was found to result in increased sensitivity to PlnEF compared the expression of LP965 MU CorC (Heeney et al., 2019). To verify this finding, growth of those BL23 variants (**Table 1**) containing *corC* from either WT or MU LP965 on a stably maintained plasmid was assessed in MRS broth and agar radial diffusion assays. BL23 variants showed lower sensitivity to PlnEF compared to LP965 WT in MRS broth and agar radial diffusion assays (data not shown). Unlike found by Heeney et al., 2019, BL23 variants were similarly inhibited by PlnEF, regardless of the presence of the LP965 *corC* gene as shown in MRS broth (**Fig. 8B** and **8C**) and agar radial diffusion (**Fig. 8A**) assays, regardless of the addition of chloramphenicol (**Fig. S3**). The findings suggest that BL23 variants are similarly sensitive to PlnEF and that inclusion of LP965 CorC does not increase that sensitivity.

Discussion

PlnEF is a potent bacteriocin that is active against members of the *L. plantarum* species as well as other Gram-positive bacteria (Tang et al., 2018) and yeast (Sharma & Srivastava, 2014). To begin to elucidate how the PlnEF peptides affect bacteria differently, this study investigated a sensitive strain of *L. plantarum* (LP965 WT) and a spontaneous PlnEF-resistant mutant (LP965 MU) with amino acid variation in CorC, a predicted Mg^{2+} efflux protein. Examination of growth curves, single cell measurements, and agar radial diffusion assays showed that the MU is more resistant and that PlnEF inhibition is greater than its individual peptides. Testing of a PlnE and PlnF variants along with expression of CorC in a heterologous host showed the challenges of developing approaches to assess bacteriocin-host interactions.

This study aimed to determine how PlnEF exerts its antimicrobial effects in PlnEF-sensitive wild-type LP965 and the role of the CorC G334V point mutation in conferring elevated resistance to PlnEF in LP965 MU. Comparisons between MRS broth and agar radial diffusion assays were used to investigate how PlnEF affects LP965 WT and MU. In MRS broth assay, LP965 WT exhibited a dose dependent increase in lag phase times upon exposure to PlnEF at concentrations between 25 and 250 nM while LP965 MU had a shorter lag phase compared to LP965 WT at each concentration. Due to limited sensitivity, the radial diffusion assay was not able to definitively distinguish the PlnEF inhibition between LP965 WT and MU at 25 and 50 nM PlnEF. Nevertheless, there was a weak trend of greater diameters of clearance zones for LP965 WT compared to MU at 50 nM PlnEF. At the higher concentration of 500 nM PlnEF applied in the agar radial diffusion assay, this differential sensitivity between LP965 WT and MU was also observed. Conversely, at the even higher concentrations of 5 and 50 μM in the agar radial diffusion assay, this CorC G334V mutation conferred distinction was no longer present and both LP965 WT and MU were equally inhibited. Single cell growth analysis revealed that the extended lag phase and the eventual recovery of growth after 24 h in MRS broth of wild-type LP965 (WT) can be linked to its heterogenous response to PlnEF exposure, which does not kill all the cells in the population.

CorC amino acid sequence alignment of 14 *L. plantarum* strains showed its high conservation, highlighting only three residue differences including the glycine to valine point mutation that led to reduced PlnEF sensitivity in LP965 MU. A tBLASTn search of *L. paracasei* BL23 with LP965 WT CorC amino acid sequence found YtaB as the protein with the highest sequence similarity of 61% to CorC. Alignment of YtaB and CorC amino acid sequences from LP965 WT and MU and LP8826 revealed low sequence conservation in the N- and C-terminal

regions. Unlike reported in a previous study (Heeney et al., 2019), although wild-type BL23 had reduced sensitivity to PlnEF compared to LP965 WT, the heterologous expression of *corC* from LP965 WT and MU in BL23 did not lead to differential sensitivity to PlnEF, as measured in MRS broth and agar radial diffusion assays.

PlnEF antimicrobial activity against LP965 WT and MU requires the helix-helix interactions and the presence of both PlnE and PlnF peptides. This is one-to-one ratio and interaction requirement is also observed for other class IIb two-peptide bacteriocins, like gassericin S and gassericin T (Kasuga et al., 2019), lactococcin G and enterocin 1071 (Oppegård et al., 2007), acidicin P (Xia et al., 2023), and plantaricin S and muricidin (Ekblad & Kristiansen, 2019). At concentrations 50 nM and below, individual PlnE or PlnF exhibited no inhibitory effects against LP965 WT and MU in MRS broth and agar diffusion assays. At concentrations 500 nM and higher tested in agar diffusion assay, PlnE had significantly higher inhibitory activity compared to PlnF. Furthermore, the PlnE and PlnF antimicrobial activities are not influenced by the CorC mutation, as they inhibited LP965 WT and MU equally. This differential inhibitory activity between the individual peptides of a two-peptide bacteriocin was also found for plantaricin S (Pls) and muricidin (Mur) in MRS broth, where Pls-α and Mur-β had approximately 10- and 1.5-fold higher inhibitory activity against the sensitive *Latilactobacillus sakei* NCFB 2714 strain compared to their corresponding partner peptide, Pls-β and Mur-α, respectively (Ekblad & Kristiansen, 2019). This effect appeared to be dependent on the target strain since this differential inhibition effect between the individual peptides of plantaricin S and muricidin was no longer present against the sensitive *Lactococcus lactis* IL1403 (Ekblad & Kristiansen, 2019). For gassericin T (GatAX), inhibitory activity was detected for one of its

individual peptides, GatA, which is approximately 30-fold lower compared to when the two peptides were combined (Kasuga et al., 2019).

PlnEF was known to induce the efflux of monovalent cations along with the dissipation of the transmembrane electrical potential and the pH gradient in LP965 WT at 60 nM concentration (Moll et al., 1999). PlnE and PlnF are unstructured under aqueous conditions, and a phospholipid bilayer with net negative charge is needed for the formation of amphiphilic alpha-helical structures between PlnE and PlnF that interact with the lipid membrane (Hauge et al., 1999; Nissen-Meyer et al., 2010). Scanning electron transmission electron microscopy analyses showed that exposure to 8 μM PlnEF for 30 min resulted in cell morphological changes and membrane damage in PlnEF-sensitive *L. plantarum* pl2 strain (Zhang et al., 2016). It is plausible to propose that PlnEF may directly interact with CorC at lower concentrations to initiate "docking" and subsequent disruption of membrane potential, which can be affected by the G334V mutation in CorC (receptor-dependent). However, at higher concentrations, pores formed by PlnEF and its interactions with the phospholipid bilayer can overcome this requirement for a "docking receptor" (receptor-independent) and result in comparable levels of inhibition in LP965 WT and MU.

Increased resistance to PlnEF shown by LP965 MU may be attributed to G334V-induced elevation of the intracellular Mg^{2+} levels, which may confer some degree of protection against PlnEF-induced damage of the membrane potential. Along with potassium, magnesium is one of the most abundant metal ions in living cells, representing 12% of the ion content of a cell (Galera-Laporta et al., 2021). Magnesium plays important roles as counterions and structure support for macromolecules, for example, chromosome condensation, folding of large RNA structures and catalytic centers of ribozymes (Galera-Laporta et al., 2021). Ribosomes were

found to contain more than 400 bound magnesium and potassium ions and these two ions stabilize the tertiary and quaternary structure of ribosomes and facilitate their proper functions (Akanuma et al., 2014; Galera-Laporta et al., 2021; Lee et al., 2019). Higher Mg^{2+} influx prevented transient increase in membrane potential (hyperpolarization) and promoted the growth and survival of *Bacillus subtilis* cells exposed to ribosome-targeting antibiotics (Lee et al., 2019). Additionally, magnesium coordination also stabilizes the ATP structures, forming the active ATP-Mg^{2+} complex and the principal energy source of the cell (Galera-Laporta et al., 2021). The loss of membrane potential induced by PlnEF disrupted the ATP production and resulted in reduced intracellular ATP concentrations in LP965 WT but not in LP965 MU (Heeney et al., 2019). The mechanistic connections between higher intracellular magnesium levels, ATP levels, and cellular response to membrane potential stress in LP965 need to be investigated.

Together, the results of this study provided a few directions for continued elucidation of the potential molecular mechanism(s) connecting PlnEF and CorC variants and the resulting inhibitory effect in LP965. One next step involves the identification of the direct binding partner of PlnEF in LP965 WT, which will either confirm the role of CorC as the receptor for PlnEF in sensitive bacteria or offer additional insights into the putative receptor recognition of PlnEF. Indirectly, the visualization of the localization of PlnEF may be pursued after optimizing the position of the fluorescent tag in PlnE or PlnF. The loss of inhibitory activity in PlnF was likely due to the inability of the N-terminus to interact with the phospholipid membrane by the steric hindrance of the fluorescein group (Kyriakou et al., 2016). Secondly, the impact of PlnEF on intracellular magnesium levels should be quantified via a more dynamic and real-time technique (e.g., time lapse fluorescent microscopy) with magnesium-specific fluorescent indicators (e.g., mag-fura 2) to visualize the heterogenous responses of LP965 WT and MU to PlnEF at the single

cell level. Specific dyes can also be applied to quantify the influence of PlnEF on the membrane potential ($DiSC_3$ dye), the pH gradient (BCECF indicator), and intracellular ATP levels. Thirdly, the construction of CorC efflux mutants or magnesium import protein mutants in LP965 or another PlnEF-sensitive strain is necessary to understand the role of different intracellular Mg^{2+} levels on sensitivity to PlnEF and other stressors.

These future investigations will contribute to a better understanding of bacteriocins' fundamental roles in modulating bacterial community structure, but also to more targeted application of bacteriocins as natural food preservatives by providing attributes that can help to distinguish different sensitivity levels of a strain towards a particular bacteriocin. This knowledge will support the application of this bacteriocin and/or the producer strains as a part of food/feed processing with implications for food safety and quality.

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Table 5.1. Strains used in this study.

	Description	**Reference**
Lactiplantibacillus plantarum NCIMB 700965 (LP965 WT)	PlnEF-sensitive strain	Sherwood, 1939
L. plantarum NCIMB 700965 MU (LP965 MU)	Spontaneous more PlnEF-resistant mutant, with G334V point mutation in CorC protein	Heeney et al., 2019
Lacticaseibacillus paracasei BL23 (BL23 WT)	PlnEF-resistance strain	Mazé et al., 2010
L. paracasei BL23 (BL23 + pJIM)	Transformed with pJIM2246 plasmid, a low copy cloning vector with Cm^R	Heeney et al., 2019
L. paracasei BL23 (BL23 + pJIMDH1)	Transformed with pJIM2246 plasmid carrying LP965 WT *corC*, Cm^R	Heeney et al., 2019
L. paracasei BL23 (BL23 + pJIMDH2)	Transformed with pJIM2246 plasmid carrying LP965 MU *corC*, Cm^R	Heeney et al., 2019

Table 5.2. Effect of PlnEF exposure on LP965 viability.
Cell density and viable cell counts for LP965 WT and MU before and after 2 h exposure to PlnEF or control (water) and the number of single cells that recovered growth after sorting[a].

	OD600		**CFU/ml**		**% Viable Cells[b]**	
LP965 WT cultured in MRS for 2 h						
	Untreated	100 nM PlnEF	Untreated	100 nM PlnEF	Untreated	100 nM PlnEF
Before	2.46 ± 0.30	2.53 ± 0.17	$1.02 \times 10^9 \pm 1.90 \times 10^8$	$1.36 \times 10^9 \pm 1.89 \times 10^8$		
After 2 h	5.49 ± 0.28	3.46 ± 0.51	$2.04 \times 10^9 \pm 3.34 \times 10^8$	$7.63 \times 10^8 \pm 1.58 \times 10^8$	85.7 ± 1.6	47.6 ± 1.3
LP965 MU cultured in MRS for 2 h						
	Untreated	1000 nM PlnEF	Untreated	1000 nM PlnEF	Untreated	1000 nM PlnEF
Before	1.57 ± 0.22	1.45 ± 0.09	$4.56 \times 10^8 \pm 6.94 \times 10^7$	$5.11 \times 10^8 \pm 1.68 \times 10^8$		
After 2 h	4.75 ± 0.38	1.14 ± 0.32	$1.38 \times 10^9 \pm 8.39 \times 10^7$	$1.02 \times 10^8 \pm 6.74 \times 10^7$	87.3 ± 1.8	39.3 ± 13.5

[a]Values shown as mean ± SD.

[b]Percentage of LP965 WT or MU single cells after sorting into wells of microtiter plates that were able to grow during 48 h incubation at 30 °C.

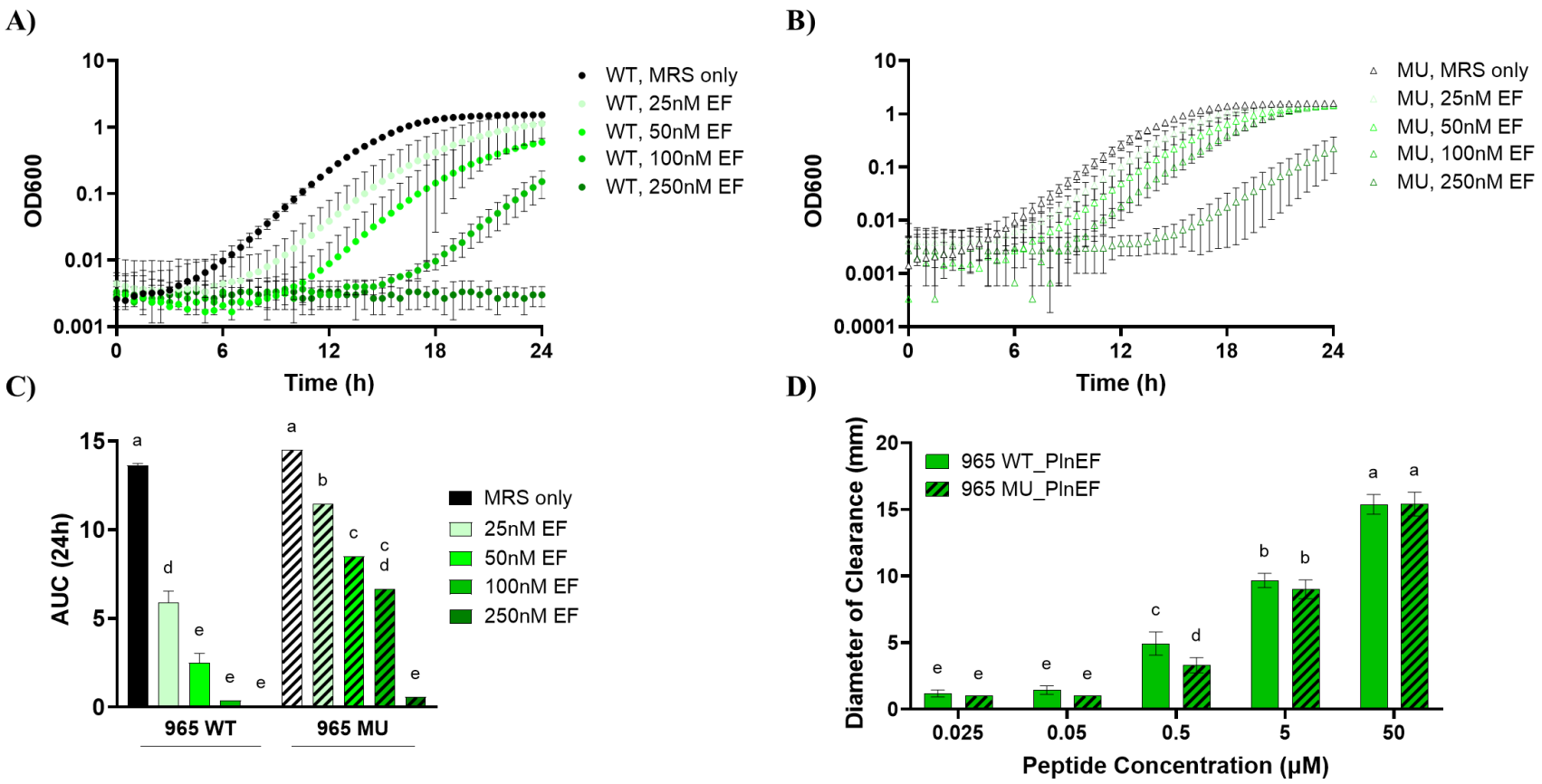


Figure 5.1. Antimicrobial activity of PlnEF against Lactiplantibacillus plantarum NCIMB700965 (LP965) WT and MU.

The growth of (**A**) LP965 WT (circles; solid colored bar) or (**B**) MU (open triangles; striped bar) exposed to different concentrations of PlnEF were assessed via growth curve assay performed at an initial OD600 of 0.0024. Sensitivity to PlnEF was assessed by (**C**) 24 h area under the curve (growth potential) of the growth assay and (**D**) diameters of the clearance zone in radial diffusion assay. Mean

± SD. Mean values not sharing any letters are significantly different based on one-way (24 h AUC) or two-way (diameter of clearance zone) ANOVA with Tukey's multiple comparisons test. Data combined from at least two independent experiments with technical triplicates each.

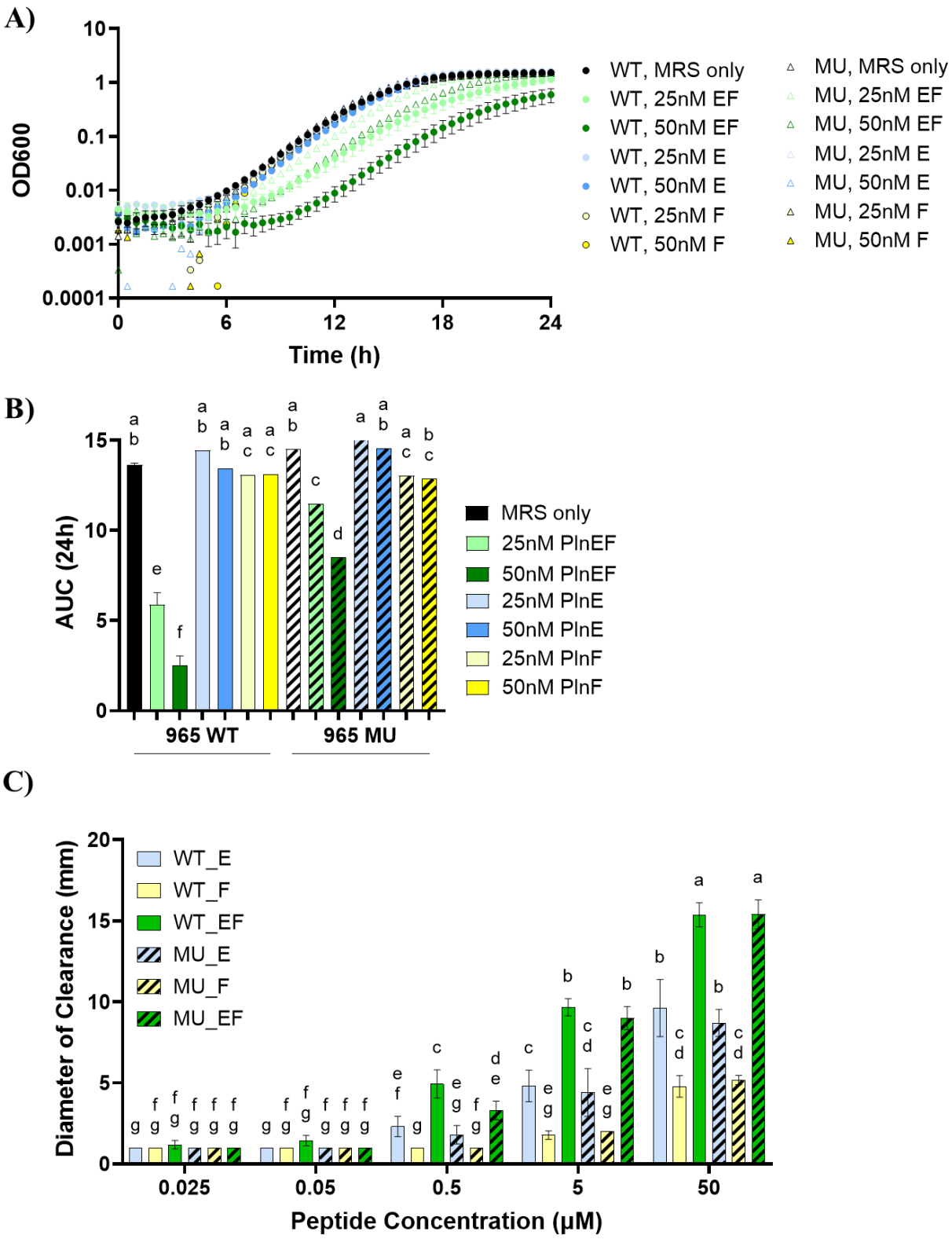

A)
OD600
10
1
0.1
0.01
0.001
0.0001
0
6
12
18
24
Time (h)
WT, MRS only
WT, 25nM EF
WT, 50nM EF
WT, 25nM E
WT, 50nM E
WT, 25nM F
WT, 50nM F
MU, MRS only
MU, 25nM EF
MU, 50nM EF
MU, 25nM E
MU, 50nM E
MU, 25nM F
MU, 50nM F
B)
AUC (24h)
15
10
5
0
965 WT
965 MU
MRS only
25nM PlnEF
50nM PlnEF
25nM PlnE
50nM PlnE
25nM PlnF
50nM PlnF
C)
Diameter of Clearance (mm)
20
15
10
5
0
0.025
0.05
0.5
5
50
Peptide Concentration (μM)
WT_E
WT_F
WT_EF
MU_E
MU_F
MU_EF

Figure 5.2. Antimicrobial activity of individual PlnE and PlnF compared to PlnEF against *Lactiplantibacillus plantarum* NCIMB700965 (LP965) WT and MU.

The growth of LP965 WT (circles; solid colored bar) or MU (open triangles; striped bar) exposed to different concentrations of PlnE, PlnF, or PlnEF were examined via (**A**) growth curve assay performed at an initial OD600 of 0.0024. Sensitivity to bacteriocin peptides was quantified by (**B**) 24 h area under the curve (growth potential) and (**C**) diameters of the clearance zone in radial diffusion assay. Mean ± SD. Mean values not sharing any letters are significantly different based on (24 h AUC) one-way or (diameter of clearance zone) two-way ANOVA with Tukey's multiple comparisons test. Data combined from at least two independent experiments with technical triplicates each.

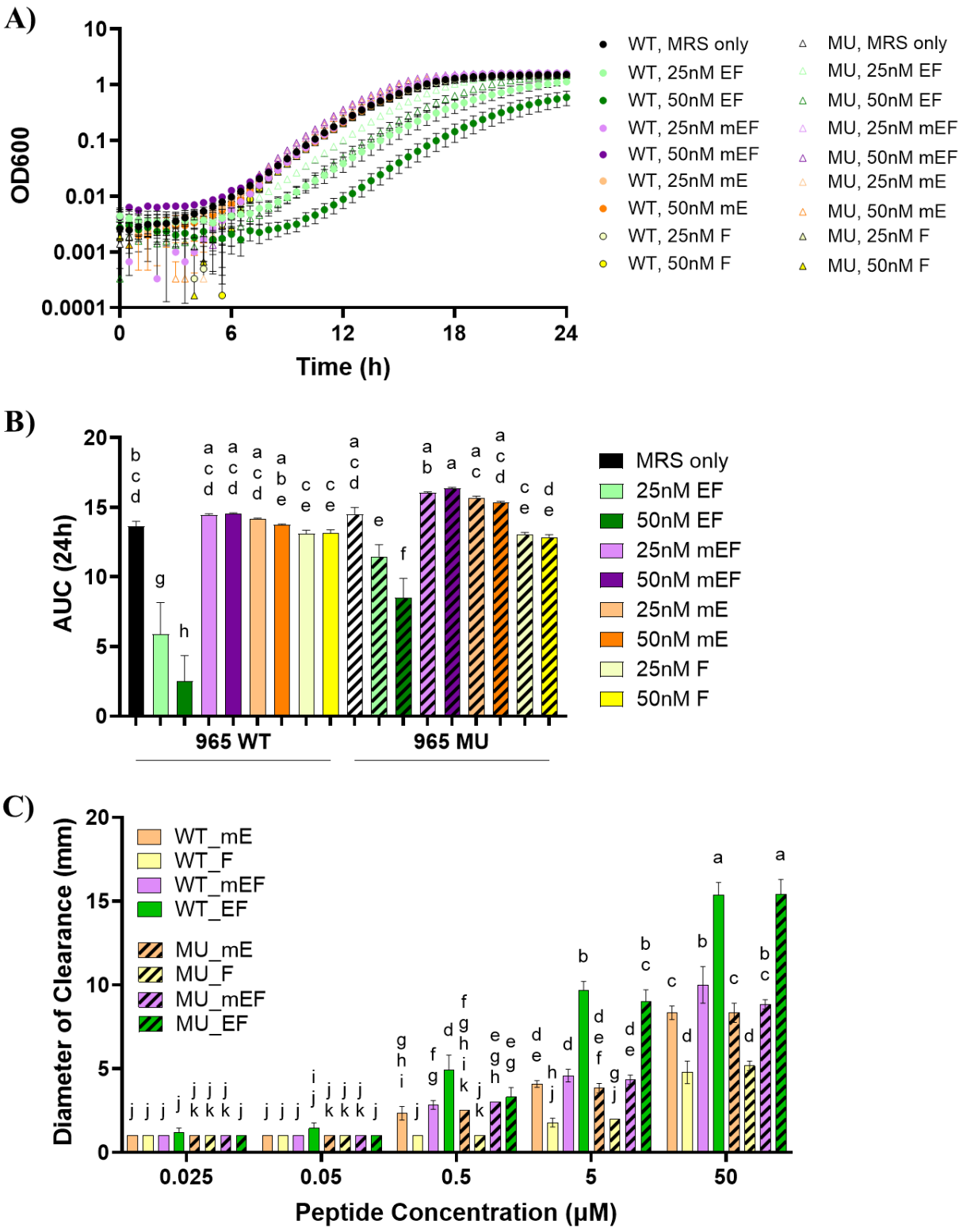

A)
OD600
Time (h)
WT, MRS only
WT, 25nM EF
WT, 50nM EF
WT, 25nM mEF
WT, 50nM mEF
WT, 25nM mE
WT, 50nM mE
WT, 25nM F
WT, 50nM F
MU, MRS only
MU, 25nM EF
MU, 50nM EF
MU, 25nM mEF
MU, 50nM mEF
MU, 25nM mE
MU, 50nM mE
MU, 25nM F
MU, 50nM F
B)
AUC (24h)
965 WT
965 MU
MRS only
25nM EF
50nM EF
25nM mEF
50nM mEF
25nM mE
50nM mE
25nM F
50nM F
C)
Diameter of Clearance (mm)
Peptide Concentration (µM)
WT_mE
WT_F
WT_mEF
WT_EF
MU_mE
MU_F
MU_mEF
MU_EF

Figure 5.3. Antimicrobial activity of mutant PlnE-G9I (mE), mE and PlnF (mEF) compared to PlnEF and PlnF against *Lactiplantibacillus plantarum* NCIMB700965 (LP965) WT and MU.

The growth of LP965 WT (circles; solid colored bar) or MU (open triangles; striped bar) exposed to different concentrations of PlnEF were examined in (**A**) growth curve assay performed at an initial OD600 of 0.0024. Sensitivity to bacteriocin peptides was quantified by (**B**) 24 h area under the curve (growth potential) and (**C**) diameters of the clearance zone in radial diffusion assay. Mean ± SD. Mean values not sharing any letters are significantly different based on (24 h AUC) one-way or (diameters of clearance zone) two-way ANOVA with Tukey's multiple comparisons test. Data combined from at least two independent experiments with technical triplicates each.

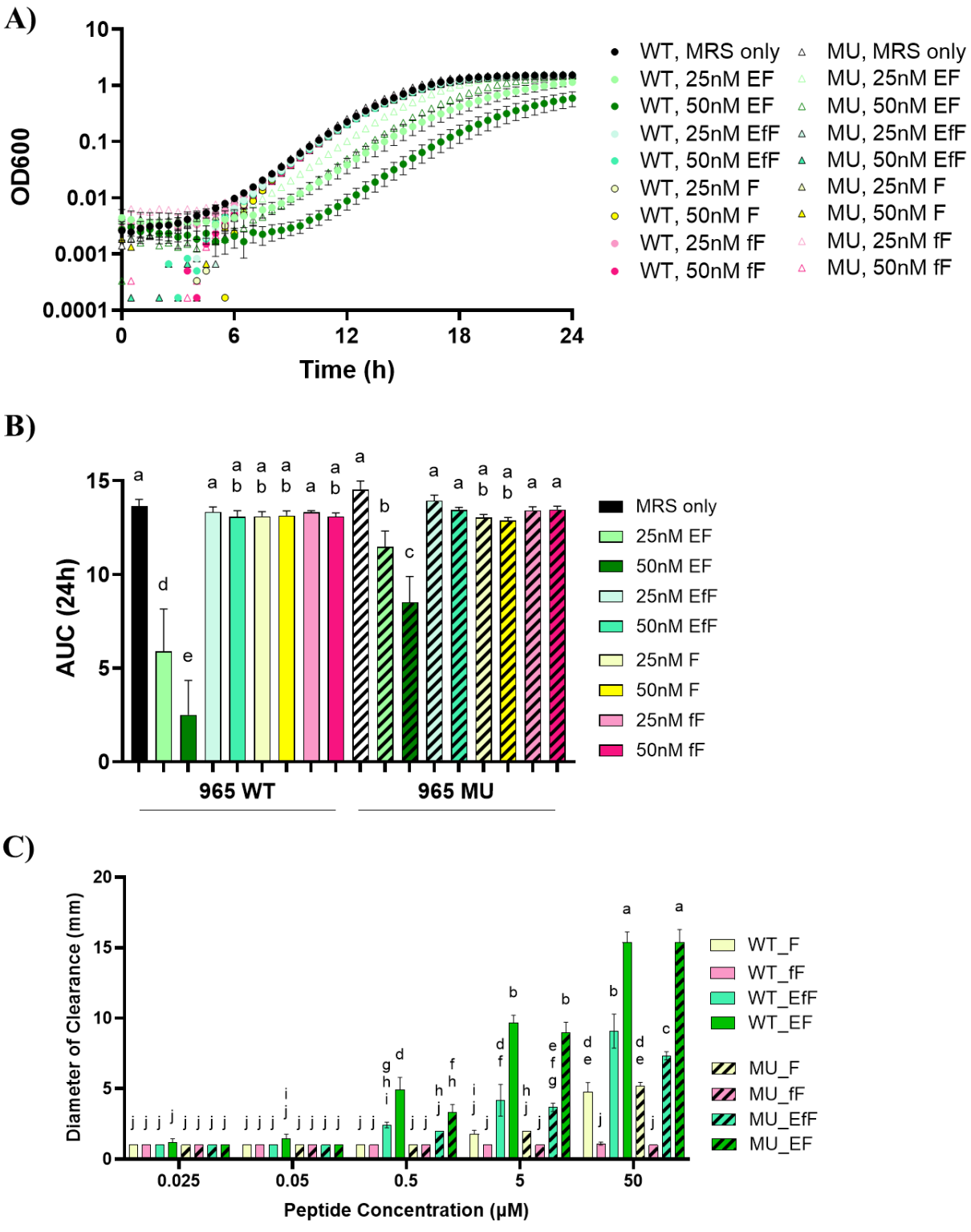

A)
OD600
Time (h)
WT, MRS only
WT, 25nM EF
WT, 50nM EF
WT, 25nM EfF
WT, 50nM EfF
WT, 25nM F
WT, 50nM F
WT, 25nM fF
WT, 50nM fF
MU, MRS only
MU, 25nM EF
MU, 50nM EF
MU, 25nM EfF
MU, 50nM EfF
MU, 25nM F
MU, 50nM F
MU, 25nM fF
MU, 50nM fF
B)
AUC (24h)
MRS only
25nM EF
50nM EF
25nM EfF
50nM EfF
25nM F
50nM F
25nM fF
50nM fF
965 WT
965 MU
C)
Diameter of Clearance (mm)
Peptide Concentration (µM)
WT_F
WT_fF
WT_EfF
WT_EF
MU_F
MU_fF
MU_EfF
MU_EF

Figure 5.4. Antimicrobial activity of fluorescently labeled PlnF (fF), PlnE and fF (EfF) compared to PlnEF and PlnF against *Lactiplantibacillus plantarum* NCIMB700965 (LP965) WT and MU.

The growth of LP965 WT (circles; solid colored bar) or MU (open triangles; striped bar) exposed to different concentrations of PlnEF were assessed via (**A**) growth curve assay performed with an initial OD600 of 0.0024. Sensitivity to bacteriocin peptides was quantified with (**B**) 24 h area under the curve (growth potential) in MRS broth and (**C**) diameters of the clearance zone in agar radial diffusion assay. Mean ± SD. Mean values not sharing any letters are significantly different based on (24 h AUC) one-way or (diameters) two-way ANOVA with Tukey's multiple comparisons test. Data combined from at least two independent experiments with technical triplicates each.

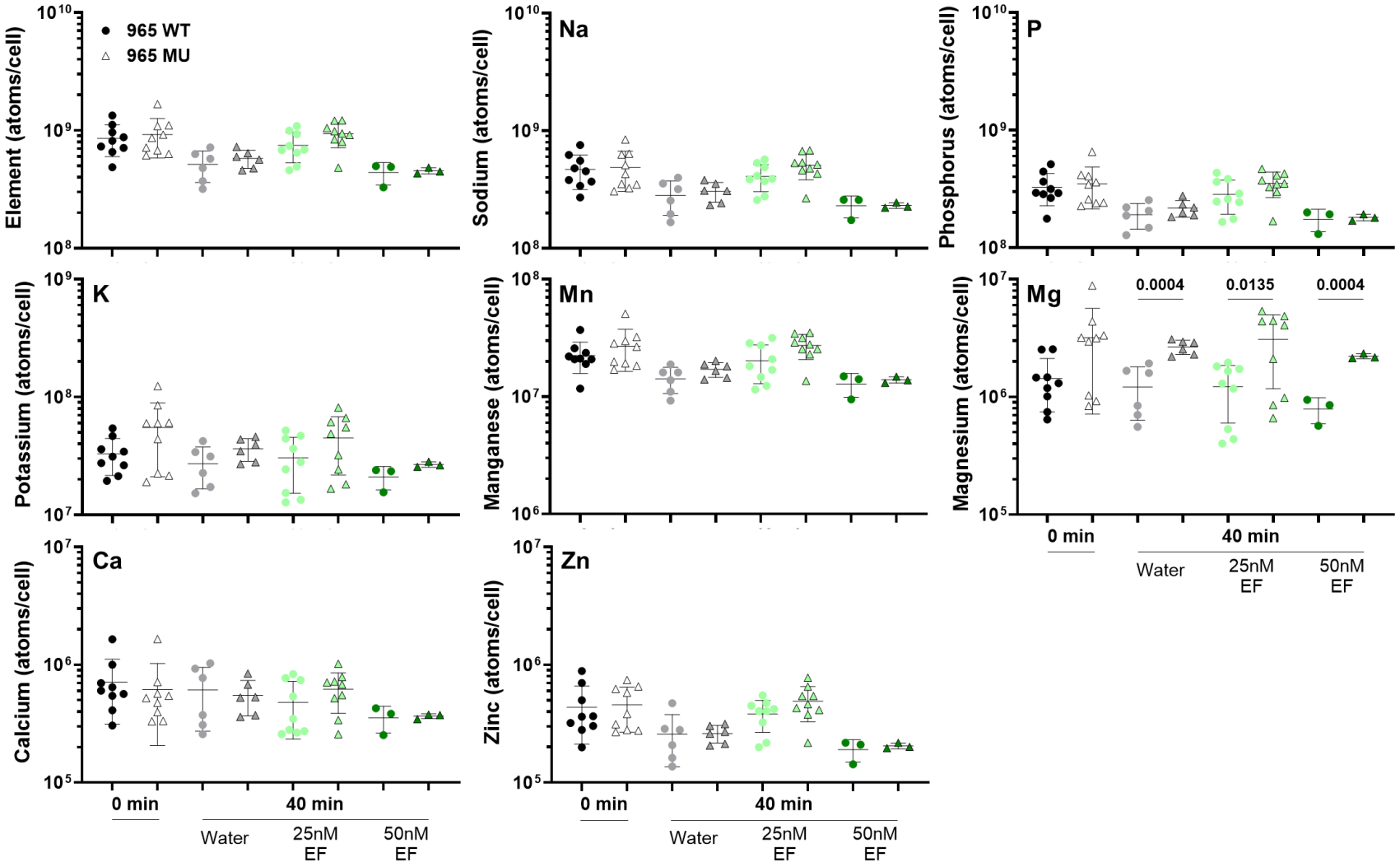


Figure 5.5. Intracellular ion levels of LP965 WT or MU incubated in PBS with or without exposure to PlnEF.

LP965 WT or MU before or after a 40 min incubation in PBS containing no PlnEF or 25 or 50 nM PlnEF. Intracellular ion levels (in atoms) quantified by ICP-MS and normalized to cell numbers estimated by viable plate counts, atoms/cell. Mean ± SD. Unpaired t test between WT and MU. At least triplicates per condition. Data combined from three independent experiments.

```
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965WT           MDSGQIILNLVIIVITFFVAAFFVACEFALVQTRPSALEEEQKKRDKPSKRISTSLHMVR 60
1B1             MDSGQIILNLVIIVITFFVAAFFVACEFALVQTRPSALEEEQKKRDKPSKRIATSLHMVR 60
8826            MDSGQIILNLVIIVITFFVAAFFVACEFALVQTRPSALEEEQKKRDKPSKRIATSLHMVR 60
AJ11            MDSGQIILNLVIIVITFFVAAFFVACEFALVQTRPSALEEEQKKRDKPSKRIATSLHMVR 60
BGM37           MDSGQIILNLVIIVITFFVAAFFVACEFALVQTRPSALEEEQKKRDKPSKRIATSLHMVR 60
EL11            MDSGQIILNLVIIVITFFVAAFFVACEFALVQTRPSALEEEQKKRDKPSKRIATSLHMVR 60
WCFS1           MDSGQIILNLVIIVITFFVAAFFVACEFALVQTRPSALEEEQKKRDKPSKRIATSLHMVR 60
8.1             MDSGQIILNLVIIVITFFVAAFFVACEFALVQTRPSALEEEQKKRDKPSKRIATSLHMVR 60
ATCC202195      MDSGQIILNLVIIVITFFVAAFFVACEFALVQTRPSALEEEQKKRDKPSKRIATSLHMVR 60
B1.1            MDSGQIILNLVIIVITFFVAAFFVACEFALVQTRPSALEEEQKKRDKPSKRIATSLHMVR 60
B1.3            MDSGQIILNLVIIVITFFVAAFFVACEFALVQTRPSALEEEQKKRDKPSKRIATSLHMVR 60
K4              MDSGQIILNLVIIVITFFVAAFFVACEFALVQTRPSALEEEQKKRDKPSKRIATSLHMVR 60
WS1.1           MDSGQIILNLVIIVITFFVAAFFVACEFALVQTRPSALEEEQKKRDKPSKRIATSLHMVR 60
                ************************************************:*******

965MU           NLNEYLSTTQVGVSLAGIILGWIGEQTTEHFVLDFLSLFNLSVSTAVLRGISIVIGVFLL 120
965WT           NLNEYLSTTQVGVSLAGIILGWIGEQTTEHFVLDFLSLFNLSVSTAVLRGISIVIGVFLL 120
1B1             NLNEYLSTTQVGVSLAGIILGWIGEQTTEHFVLDFLSLFNLSVSTAVLRGISIVIGVFLL 120
8826            NLNEYLSTTQVGVSLAGIILGWIGEQTTEHFVLDFLSLFNLSVSTAVLRGISIVIGVFLL 120
AJ11            NLNEYLSTTQVGVSLAGIILGWIGEQTTEHFVLDFLSLFNLSVSTAVLRGISIVIGVFLL 120
BGM37           NLNEYLSTTQVGVSLAGIILGWIGEQTTEHFVLDFLSLFNLSVSTAVLRGISIVIGVFLL 120
EL11            NLNEYLSTTQVGVSLAGIILGWIGEQTTEHFVLDFLSLFNLSVSTAVLRGISIVIGVFLL 120
WCFS1           NLNEYLSTTQVGVSLAGIILGWIGEQTTEHFVLDFLSLFNLSVSTAVLRGISIVIGVFLL 120
8.1             NLNEYLSTTQVGVSLAGIILGWIGEQTTEHFVLDFLSLFNLSVSTAVLRGISIVIGVFLL 120
ATCC202195      NLNEYLSTTQVGVSLAGIILGWIGEQTTEHFVLDFLSLFNLSVSTAVLRGISIVIGVFLL 120
B1.1            NLNEYLSTTQVGVSLAGIILGWIGEQTTEHFVLDFLSLFNLSVSTAVLRGISIVIGVFLL 120
B1.3            NLNEYLSTTQVGVSLAGIILGWIGEQTTEHFVLDFLSLFNLSVSTAVLRGISIVIGVFLL 120
K4              NLNEYLSTTQVGVSLAGIILGWIGEQTTEHFVLDFLSLFNLSVSTAVLRGISIVIGVFLL 120
WS1.1           NLNEYLSTTQVGVSLAGIILGWIGEQTTEHFVLDFLSLFNLSVSTAVLRGISIVIGVFLL 120
                ************************************************************

965MU           TYLEVVLTEIVPKNIAIDMPLKVMAVITTPLRWFHVMFYPFVWLLNVSSAGVVKMLGIPV 180
965WT           TYLEVVLTEIVPKNIAIDMPLKVMAVITTPLRWFHVMFYPFVWLLNVSSAGVVKMLGIPV 180
1B1             TYLEVVLTEIVPKNIAIDMPLKVMAVITTPLRWFHVMFYPFVWLLNVSSAGVVKMLGIPV 180
8826            TYLEVVLTEIVPKNIAIDMPLKVMAVITTPLRWFHVMFYPFVWLLNVSSAGVVKMLGIPV 180
AJ11            TYLEVVLTEIVPKNIAIDMPLKVMAVITTPLRWFHVMFYPFVWLLNVSSAGVVKMLGIPV 180
BGM37           TYLEVVLTEIVPKNIAIDMPLKVMAVITTPLRWFHVMFYPFVWLLNVSSAGVVKMLGIPV 180
EL11            TYLEVVLTEIVPKNIAIDMPLKVMAVITTPLRWFHVMFYPFVWLLNVSSAGVVKMLGIPV 180
WCFS1           TYLEVVLTEIVPKNIAIDMPLKVMAVITTPLRWFHVMFYPFVWLLNVSSAGVVKMLGIPV 180
8.1             TYLEVVLTEIVPKNIAIDMPLKVMAVITTPLRWFHVMFYPFVWLLNVSSAGVVKMLGIPV 180
ATCC202195      TYLEVVLTEIVPKNIAIDMPLKVMAVITTPLRWFHVMFYPFVWLLNVSSAGVVKMLGIPV 180
B1.1            TYLEVVLTEIVPKNIAIDMPLKVMAVITTPLRWFHVMFYPFVWLLNVSSAGVVKMLGIPV 180
B1.3            TYLEVVLTEIVPKNIAIDMPLKVMAVITTPLRWFHVMFYPFVWLLNVSSAGVVKMLGIPV 180
K4              TYLEVVLTEIVPKNIAIDMPLKVMAVITTPLRWFHVMFYPFVWLLNVSSAGVVKMLGIPV 180
WS1.1           TYLEVVLTEIVPKNIAIDMPLKVMAVITTPLRWFHVMFYPFVWLLNVSSAGVVKMLGIPV 180
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965WT           ANEDNDVYSQAEILNLSRNAVTGGELERNDYLYMERAFEFNDKIAKDIMIDRTQLVVIDI 240
1B1             ANEDNDVYSQAEILNLSRNAVTGGELERNDYLYMERAFEFNDKIAKDIMIDRTQLVVIDI 240
8826            ANEDNDVYSQAEILNLSRNAVTGGELERNDYLYMERAFEFNDKIAKDIMIDRTQLVVIDI 240
AJ11            ANEDNDVYSQAEILNLSRNAVTGGELERNDYLYMERAFEFNDKIAKDIMIDRTQLVVIDI 240
BGM37           ANEDNDVYSQAEILNLSRNAVTGGELERNDYLYMERAFEFNDKIAKDIMIDRTQLVVIDI 240
EL11            ANEDNDVYSQAEILNLSRNAVTGGELERNDYLYMERAFEFNDKIAKDIMIDRTQLVVIDI 240
WCFS1           ANEDNDVYSQAEILNLSRNAVTGGELERNDYLYMERAFEFNDKIAKDIMIDRTQLVVIDI 240
8.1             ANEDNDVYSQAEILNLSRNAVTGGELERNDYLYMERAFEFNDKIAKDIMIDRTQLVVIDI 240
ATCC202195      ANEDNDVYSQAEILNLSRNAVTGGELERNDYLYMERAFEFNDKIAKDIMIDRTQLVVIDI 240
B1.1            ANEDNDVYSQAEILNLSRNAVTGGELERNDYLYMERAFEFNDKIAKDIMIDRTQLVVIDI 240
B1.3            ANEDNDVYSQAEILNLSRNAVTGGELERNDYLYMERAFEFNDKIAKDIMIDRTQLVVIDI 240
K4              ANEDNDVYSQAEILNLSRNAVTGGELERNDYLYMERAFEFNDKIAKDIMIDRTQLVVIDI 240
WS1.1           ANEDNDVYSQAEILNLSRNAVTGGELERNDYLYMERAFEFNDKIAKDIMIDRTQLVVIDI 240
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965MU           NDTVKQAIRVYLQKRFSRLPVVANNDKDKILGYVYNYDLIRQGEVDDDIRVNKLLRNIIT 300
965WT           NDTVKQAIRVYLQKRFSRLPVVANNDKDKILGYVYNYDLIRQGEVDDDIRVNKLLRNIIT 300
1B1             NDTVKQAIRVYLQKRFSRLPVVANNDKDKILGYVYNYDLIRQGEVDDDIRVNKLLRNIIT 300
8826            NDTVKQAIRVYLQKRFSRLPVVANNDKDKILGYVYNYDLIRQGEVDDDIRVNKLLRNIIT 300
AJ11            NDTVKQAIRVYLQKRFSRLPVVANNDKDKILGYVYNYDLIRQGEVDDDIRVNKLLRNIIT 300
BGM37           NDTVKQAIRVYLQKRFSRLPVVANNDKDKILGYVYNYDLIRQGEVDDDIRVNKLLRNIIT 300
EL11            NDTVKQAIRVYLQKRFSRLPVVANNDKDKILGYVYNYDLIRQGEVDDDIRVNKLLRNIIT 300
WCFS1           NDTVKQAIRVYLQKRFSRLPVVANNDKDKILGYVYNYDLIRQGEVDDDIRVNKLLRNIIT 300
8.1             NDTVKQAIRVYLQKRFSRLPVVANNDKDKILGYVYNYDLIRQGEVDDDIRVNKLLRNIIT 300
ATCC202195      NDTVKQAIRVYLQKRFSRLPVVANNDKDKILGYVYNYDLIRQGEVDDDIRVNKLLRNIIT 300
B1.1            NDTVKQAIRVYLQKRFSRLPVVANNDKDKILGYVYNYDLIRQGEVDDDIRVNKLLRNIIT 300
B1.3            NDTVKQAIRVYLQKRFSRLPVVANNDKDKILGYVYNYDLIRQGEVDDDIRVNKLLRNIIT 300
K4              NDTVKQAIRVYLQKRFSRLPVVANNDKDKILGYVYNYDLIRQGEVDDDIRVNKLLRNIIT 300
WS1.1           NDTVKQAIRVYLQKRFSRLPVVANNDKDKILGYVYNYDLIRQGEVDDDIRVNKLLRNIIT 300
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965MU           IPETTPIQAILQKMIDKQTPIVVVVDEYVGTSGIVTDKDIYEELFGTVNDEIDNVSNEYI 360
965WT           IPETTPIQAILQKMIDKQTPIVVVVDEYGGTSGIVTDKDIYEELFGTVNDEIDNVSNEYI 360
1B1             IPETTPIQAILQKMIDKQTPIVVVVDEYGGTSGIVTDKDIYEELFGTVNDEIDNVSNEYI 360
8826            IPETTPIQAILQKMIDKQTPIVVVVDEYGGTSGIVTDKDIYEELFGTVNDEIDNVSNEYI 360
AJ11            IPETTPIQAILQKMIDKQTPIVVVVDEYGGTSGIVTDKDIYEELFGTVNDEIDNVSNEYI 360
BGM37           IPETTPIQAILQKMIDKQTPIVVVVDEYGGTSGIVTDKDIYEELFGTVNDEIDNVSNEYI 360
EL11            IPETTPIQAILQKMIDKQTPIVVVVDEYGGTSGIVTDKDIYEELFGTVNDEIDNVSNEYI 360
WCFS1           IPETTPIQAILQKMIDKQTPIVVVVDEYGGTSGIVTDKDIYEELFGTVNDEIDNVSNEYI 360
8.1             IPETTPIQAILQKMIDKQTPIVVVVDEYGGTSGIVTDKDIYEELFGTVNDEIDNVSNEYI 360
ATCC202195      IPETTPIQAILQKMIDKQTPIVVVVDEYGGTSGIVTDKDIYEELFGTVNDEIDNVSNEYI 360
B1.1            IPETTPIQAILQKMIDKQTPIVVVVDEYGGTSGIVTDKDIYEELFGTVNDEIDNVSNEYI 360
B1.3            IPETTPIQAILQKMIDKQTPIVVVVDEYGGTSGIVTDKDIYEELFGTVNDEIDNVSNEYI 360
K4              IPETTPIQAILQKMIDKQTPIVVVVDEYGGTSGIVTDKDIYEELFGTVNDEIDNVSNEYI 360
WS1.1           IPETTPIQAILQKMIDKQTPIVVVVDEYGGTSGIVTDKDIYEELFGTVNDEIDNVSNEYI 360
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965MU           EKQADGTYRISGKMTIYDFERYFKTDLQEFEDSDVVTLTGYILDNFPKIQAGDTIQIGNF 420
965WT           EKQADGTYRISGKMTIYDFERYFKTDLQEFEDSDVVTLTGYILDNFPKIQAGDTIQIGNF 420
1B1             EKQADGTYRISGKMTIYDFERYFKTDLQEFEDSDVVTLTGYILDNFPKIQAGDTIQIGNF 420
8826            EKQADGTYRISGKMTIYDFERYFKTDLQEFEDSDVVTLTGYILDNFPKIQAGDTIQIGNF 420
AJ11            EKQADGTYRISGKMTIYDFERYFKTDLQEFEDSDVVTLTGYILDNFPKIQAGDTIQIGNF 420
BGM37           EKQADGTYRISGKMTIYDFERYFKTDLQEFEDSDVVTLTGYILDNFPKIQAGDTIQIGNF 420
EL11            EKQADGTYRISGKMTIYDFERYFKTDLQEFEDSDVVTLTGYILDNFPKIQAGDTIQIGNF 420
WCFS1           EKQADGTYRISGKMTIYDFERYFKTDLQEFEDSDVVTLTGYILDNFPKIQAGDTIQIGNF 420
8.1             EKQADGTYRISGKMTIYDFERYFKTDLQEFEDSDVVTLTGYILDNFPKIQAGDTIQIGNF 420
ATCC202195      EKQADGTYRISGKMTIYDFERYFKTDLQEFEDSDVVTLTGYILDNFPKIQAGDTIQIGNF 420
B1.1            EKQADGTYRISGKMTIYDFERYFKTDLQEFEDSDVVTLTGYILDNFPKIQAGDTIQIGNF 420
B1.3            EKQADGTYRISGKMTIYDFERYFKTDLQEFEDSDVVTLTGYILDNFPKIQAGDTIQIGNF 420
K4              EKQADGTYRISGKMTIYDFERYFKTDLQEFEDSDVVTLTGYILDNFPKIQAGDTIQIGNF 420
WS1.1           EKQADGTYRISGKMTIYDFERYFKTDLQEFEDSDVVTLTGYILDNFPKIQAGDTIQIGNF 420
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965MU           NFKALDFENAYINWFEVSAQTDSTPDDHNDDQTQD        455
965WT           NFKALDFENAYINWFEVSAQTDSTPDDHNDDQTQD        455
1B1             NFKALDFENAYINWFEVSAQRDSTPDDHNDDQTQD        455
8826            NFKALDFENAYINWFEVSAQRDSTPDDHNDDQTQD        455
AJ11            NFKALDFENAYINWFEVSAQRDSTPDDHNDDQTQD        455
BGM37           NFKALDFENAYINWFEVSAQRDSTPDDHNDDQTQD        455
EL11            NFKALDFENAYINWFEVSAQRDSTPDDHNDDQTQD        455
WCFS1           NFKALDFENAYINWFEVSAQRDSTPDDHNDDQTQD        455
8.1             NFKALDFENAYINWFEVSAQTDSTPDDHNDDQTQD        455
ATCC202195      NFKALDFENAYINWFEVSAQTDSTPDDHNDDQTQD        455
B1.1            NFKALDFENAYINWFEVSAQTDSTPDDHNDDQTQD        455
B1.3            NFKALDFENAYINWFEVSAQTDSTPDDHNDDQTQD        455
K4              NFKALDFENAYINWFEVSAQTDSTPDDHNDDQTQD        455
WS1.1           NFKALDFENAYINWFEVSAQTDSTPDDHNDDQTQD        455
                ******************* **************
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Figure 5.6. Alignment of CorC protein sequences from 14 *L. plantarum* strains using Clustal Omega.

Amino acid residue positions not conserved among the 14 strains are in bold.

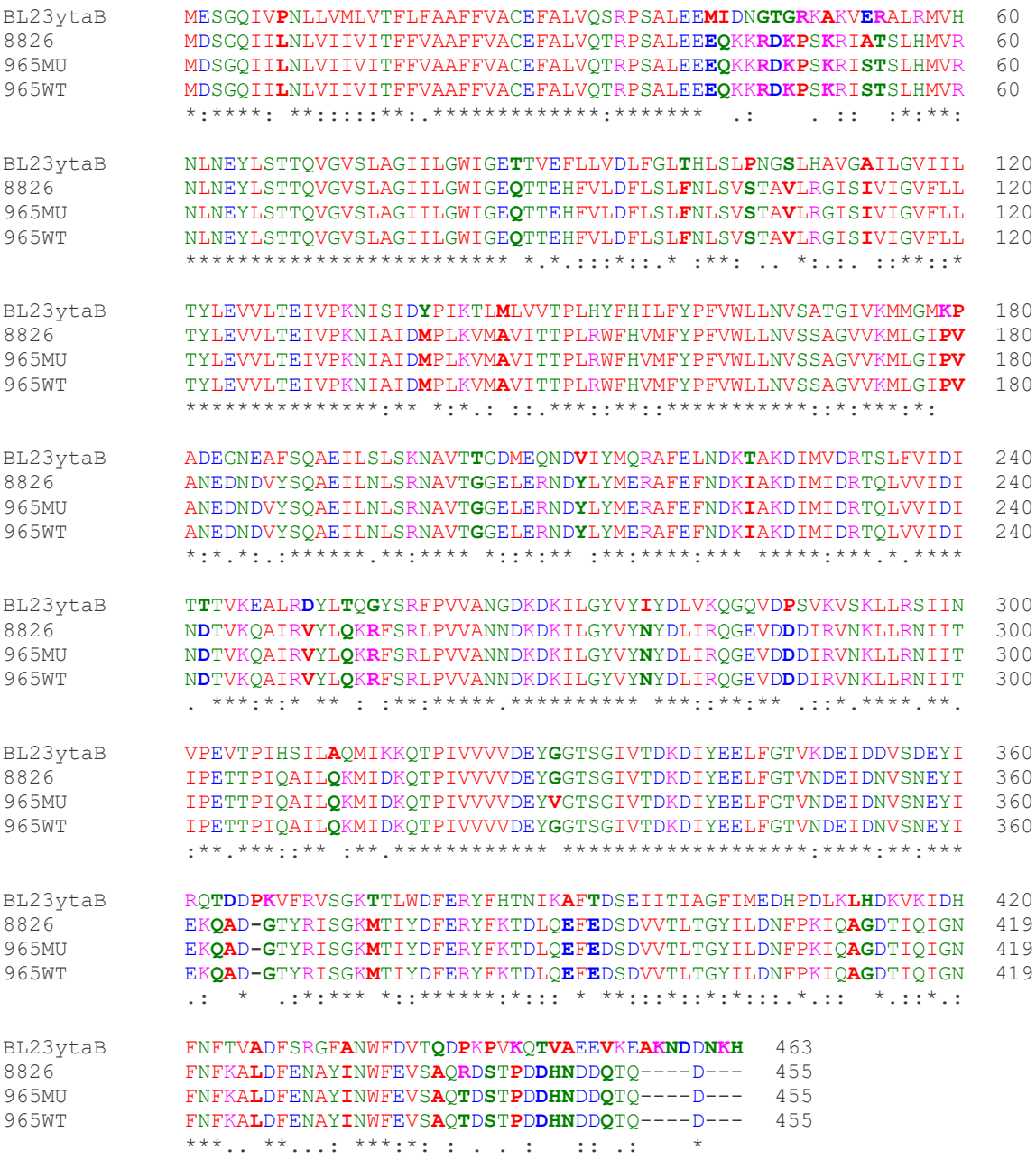


Figure 5.7. Alignment of CorC protein sequences from 3 *L. plantarum* strains and CorC-like protein from *L. paracasei* BL23 using Clustal Omega.

Amino acid residue positions not conserved among the strains are in bold. "*" indicates identical amino acid residues; ":" indicates residues that are strongly conserved in chemical properties; "." indicates residues that are weakly conserved in chemical properties.

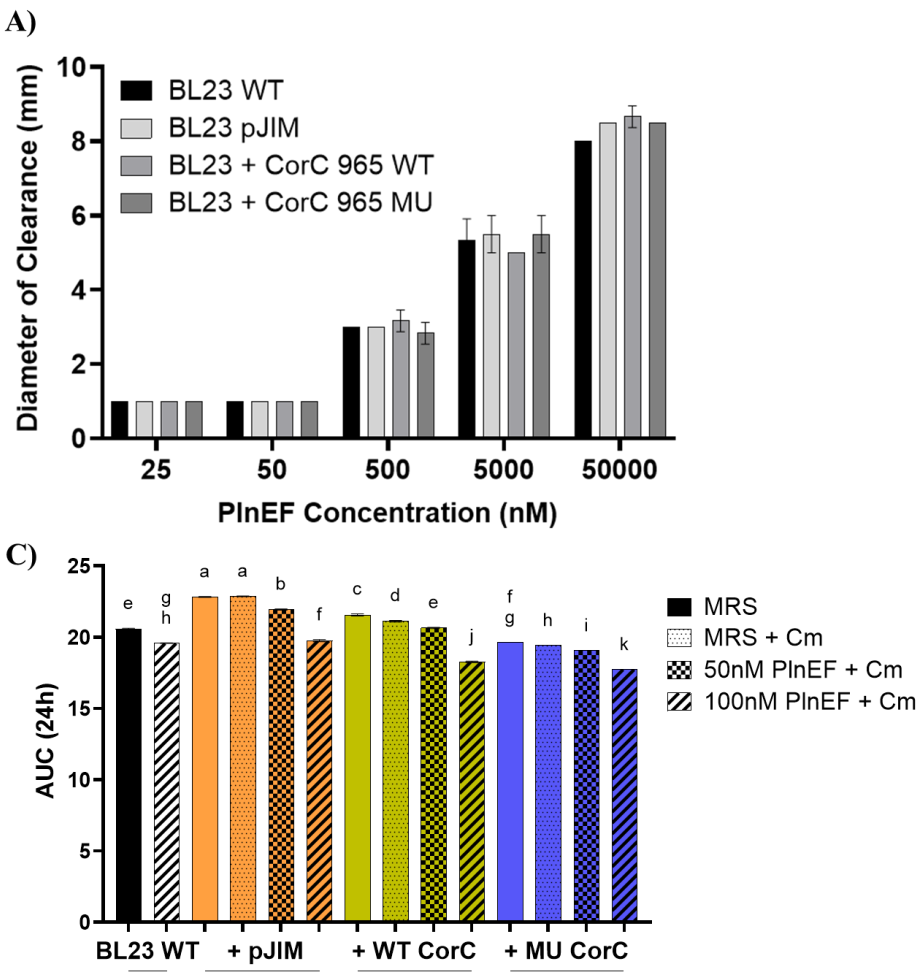


Figure 5.8. Antimicrobial activity of PlnEF against *L. paracasei* BL23 expressing plasmid-encoded CorC from LP965 WT or MU.

The growth of BL23 WT, BL23 with pJIM2246 vector, BL23 with LP965 WT CorC, and BL23 with LP965 MU CorC exposed to different PlnEF concentrations was assessed via (**A**) radial diffusion assay; (**B**) growth curve assay performed with an initial OD600 of 0.024 with 5 µg/ml chloramphenicol. Sensitivity to PlnEF in MRS broth was quantified with (**C**) 24 h AUC growth potential. Mean ± SD; n = 3. (24 h AUC) Mean values not sharing any letters are significantly different based on one-way ANOVA with Tukey's multiple comparisons test.

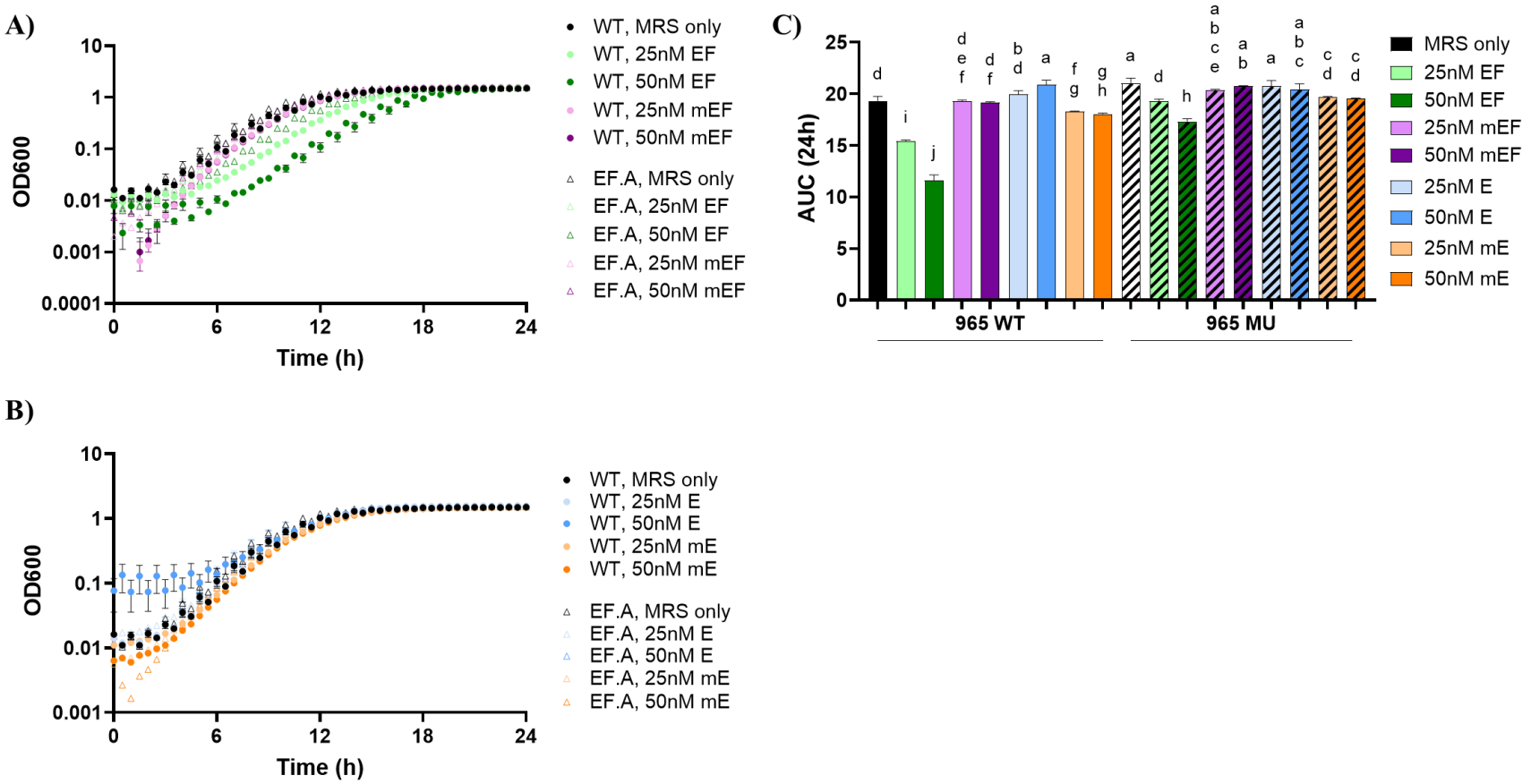


Figure S1. Antimicrobial activity of PlnEF, mEF, E, and mE against *Lactiplantibacillus plantarum* NCIMB700965 (LP965) WT and MU.

The growth of LP965 WT (circles; solid colored bar) or MU (open triangles; striped bar) exposed to different concentrations of plantaricins (Pln) were assessed via (**A**) and (**B**) growth curves assay performed at an initial OD600 of 0.024. Sensitivity to Pln was

quantified by (**C**) 24 h area under the curve (growth potential). Mean ± SD. (24 h AUC) Mean values not sharing any letters are significantly different based on one-way ANOVA with Tukey's multiple comparisons test. Data combined from at least two independent experiments with technical triplicates each.

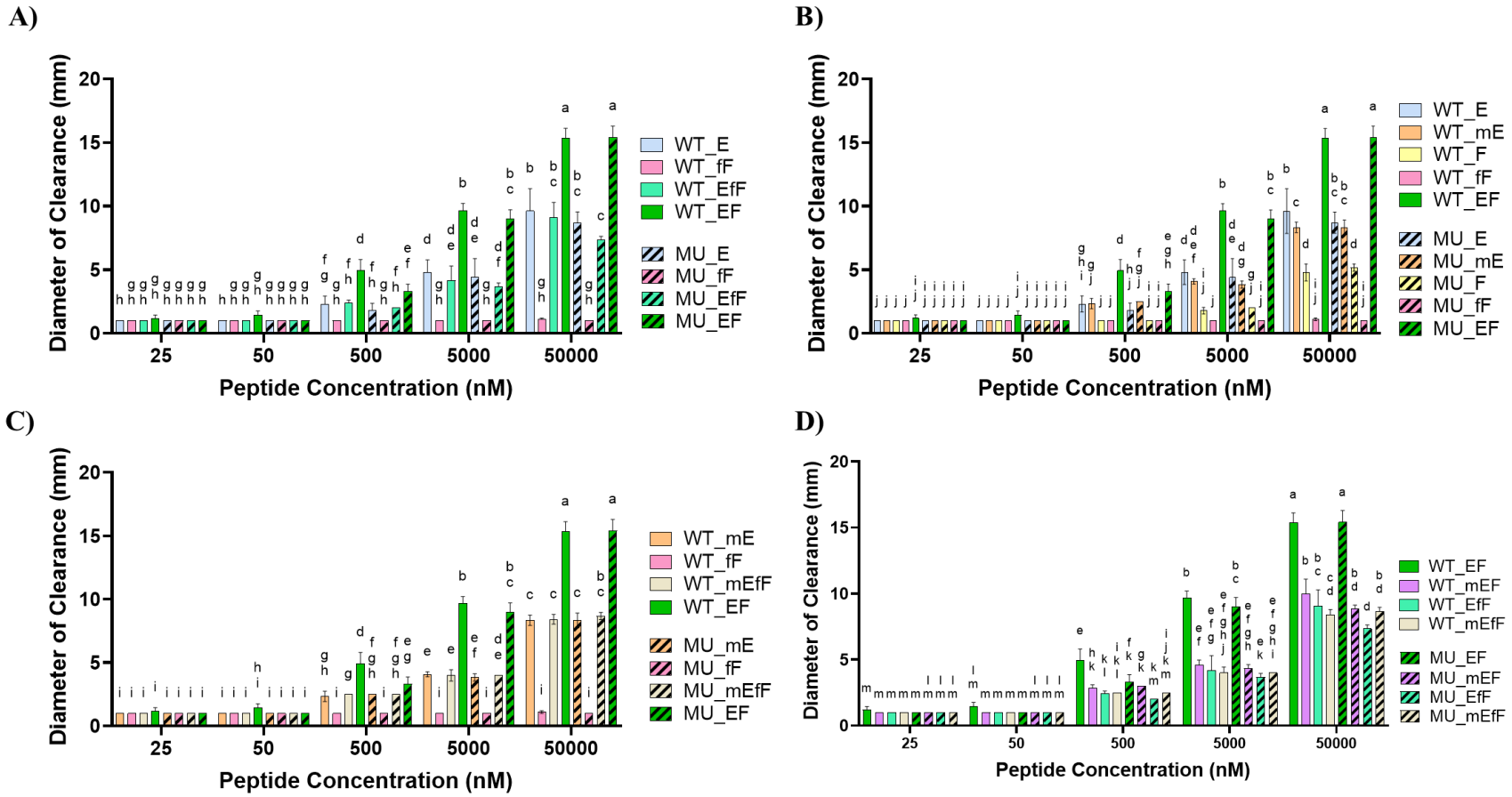


Figure S2. Diameters of clearance zone of LP965 WT or MU exposed to different Pln types and concentrations as assessed in radial diffusion assay.

Inhibition zone of PlnEF (EF) in LP965 WT or MU was used as a reference for comparing (**A**) PlnE, fluorescently labeled PlnF (fF), and their mixture (EfF); (**B**) PlnE, PlnE-G9I (mE), PlnF, and fluorescently labeled PlnF (fF); (**C**) PlnE-G9I, fluorescently labeled

PlnF, and their mixture (mEfF); and (**D**) three dual peptide combinations of PlnE-G9I + PlnF (mEF), EfF, and mEfF. Mean ± SD. Mean values not sharing any letters are significantly different based on two-way ANOVA with Tukey's multiple comparisons test. Data combined from at least two independent experiments with technical triplicates each.

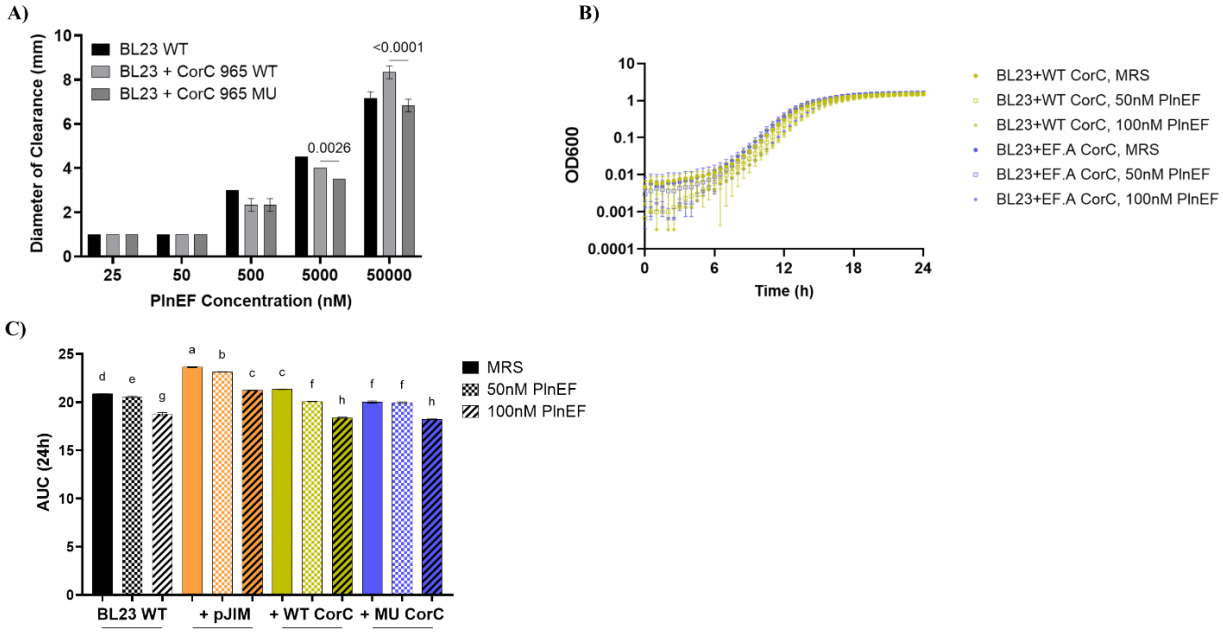


Figure S3. Antimicrobial activity of PlnEF against *L. paracasei* BL23 expressing plasmid-encoded CorC from LP965 WT or MU.

The growth of BL23 WT, BL23 with pJIM2246 vector, BL23 with LP965 WT CorC, and BL23 with LP965 MU CorC exposed to different PlnEF concentrations was assessed via (**A**) radial diffusion assay; (**B**) growth curve; and (**C**) 24 h AUC growth potential. Growth curve assay was performed at an initial OD600 of 0.024 without chloramphenicol. Mean ± SD; n = 3. (24 h AUC) Mean values not sharing any letters are significantly different based on one-way ANOVA with Tukey's multiple comparisons test.