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Degradation of food-derived opioid peptides by bifidobacteria

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RESEARCH ARTICLE

Abstract

Some food-derived opioid peptides have been reported to cause diseases, such as gastrointestinal inflammation, celiac disease, and mental disorders. *Bifidobacterium* is a major member of the dominant human gut microbiota, particularly in the gut of infants. In this study, we evaluated the potential of *Bifidobacterium* in the degradation of food-derived opioid peptides. All strains tested showed some level of dipeptidyl peptidase activity, which is thought to be involved in the degradation of food-derived opioid peptides. However, this activity was higher in bifidobacterial strains that are commonly found in the intestines of human infants, such as *Bifidobacterium longum* subsp. *longum*, *B. longum* subsp. *infantis*, *Bifidobacterium breve* and *Bifidobacterium bifidum*, than in those of other species, such as *Bifidobacterium animalis* and *Bifidobacterium pseudolongum*. In addition, some *B. longum* subsp. *infantis* and *B. bifidum* strains showed degradative activity in food-derived opioid peptides such as human and bovine milk-derived casomorphin-7 and wheat gluten-derived gliadorphin-7. A further screening of *B. bifidum* strains revealed some bifidobacterial strains that could degrade all three peptides. Our results revealed the potential of *Bifidobacterium* species in the degradation of food-derived opioid peptides, particularly for species commonly found in the intestine of infants. Selected strains of *B. longum* subsp. *infantis* and *B. bifidum* with high degradative capabilities can be used as probiotic microorganisms to eliminate food-derived opioid peptides and contribute to host health.

Keywords: *Bifidobacterium*, casomorphin, gliadorphin, food-derived opioid peptides

1. Introduction

Opioid peptides act as neuromodulators that modify the actions of other neurotransmitters and influence their release of various neurotransmitters. By altering the activity of their target neurons, they play a critical role in modulating various sensory, motivational, emotional, and cognitive functions (Froehlich, 1997). Based on their receptor-binding specificities, they have been classified into three types: endorphins, enkephalins, and dynorphins (Chen *et al.,* 1993a,b; Evans *et al.,* 1992; Kieffer *et al.,* 1992; Minami *et al.,* 1993). Food-derived peptides have also been reported to interact with opioid receptors (Bhat *et al.,* 2015; Yoshikawa, 2015). For example, casomorphin-7 (CM-7), which is derived from milk, and gliadorphin-7 (GD-7), which is derived from wheat gluten, activate the μ-opioid receptor (Graf *et al.,* 1986; Henschen *et al.,* 1979; Trivedi *et al.,* 2014).

Some food-derived peptides have beneficial health bioactivities (Harnedy and FitzGerald, 2012; Rutherfurd-Markwick, 2012); however, they have also been reported to cause adverse events. Bovine CM-7 (bCM-7), produced from A1 β-casein, has been reported to be a dominant causal trigger of type 1 diabetes (Chia *et al.,* 2017). Studies have revealed an elevated level of this peptide in the urine of autistic children (Sokolov *et al.,* 2014) and in the sera of infants with apnea that caused apparent life-threatening events (Wasilewska *et al.,* 2011). In addition, avoiding foods that contain these peptides can mitigate gastrointestinal inflammation (Jianqin *et al.,* 2016; Murray *et al.,* 2004) and celiac disease (Anania *et al.,* 2017; Brietzke *et al.,* 2017;

Posner and Bhimji, 2017), both of which are expected to occur in association with type 1 diabetes (Krzewska and Ben-Skowronek, 2016; Serena *et al.,* 2015).

Peptidase from bacterial cells, such as *Aspergillus oryzae* (Ehren *et al.*, 2009), *Lactobacillus sakei* (Sanz and Toldra, 2001) and *Lactococcus lactis* (Ustun and Ongen, 2012), have been reported to degrade food-derived opioid peptides. The administration of an *Aspergillus niger*-derived enzyme has been reported to cause a reduction in an immunodominant epitope by enhancing gluten digestion in the stomach of healthy volunteers (Salden *et al.,* 2015). Therefore, a bacterial strain that can degrade food-derived peptides could be useful to prevent diseases caused by these peptides.

Bifidobacteria were first isolated in 1899 by Henri Tissier (Poupard *et al.,* 1973; Tissier, 1899) and are currently classified into 47 taxa, including 38 species and 9 subspecies (Milani *et al.,* 2014; Parte, 2014; Stackebrandt *et al.,* 1997). Bifidobacteria are one of the dominant colonisers in the human gastrointestinal tract. They are usually found in breast-fed infants and are thought to be beneficial to their host (Arboleya *et al.,* 2016; O'Callaghan and Van Sinderen, 2016; Pannaraj *et al.,* 2017). Consequently, several studies have demonstrated that bifidobacteria have functional benefits to alleviate, prevent, and/or improve diseases, such as allergies (Enomoto *et al.,* 2014; Miraglia Del Giudice *et al.,* 2017), non-alcoholic fatty liver disease (Alisi *et al.,* 2014), type 2 diabetes (Ejtahed *et al.,* 2012; Panwar *et al.,* 2013), obesity (Ivey *et al.,* 2015; Minami *et al.,* 2015), and chronic intestinal diseases (Plaza-Diaz *et al.,* 2017; Tamaki *et al.,* 2016). Therefore, they are one of the most well-known bacteria used as probiotics; however, little is known about their peptidase activity or degradable capability in food-derived opioid peptides.

Our study aimed to examine the peptidase activity and degradative capability of bifidobacteria in food-derived opioid peptides. A total of 18 bifidobacterial strains were first assayed. We further evaluated more strains of *Bifidobacterium bifidum*, some of which showed a high activity at the first assay.

2. Materials and methods

Materials

The substrate for dipeptidyl aminopeptidase IV (H-Gly-Pro-AMC) and three food-derived opioid peptides, namely CM-7 from human milk (H-Tyr-Pro-Phe-Val-Glu-Pro-Ile-OH, hCM-7), CM-7 from bovine milk (H-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-OH, bCM-7), and GD-7 from wheat gluten (H-Tyr-Pro-Gln-Pro-Gln-Pro-Phe-OH, GD-7), were purchased from Bachem AG (Bubendorf, Switzerland). Formic acid was purchased from Wako Pure Chemical Co., Ltd. (Osaka, Japan). Acetonitrile (HPLC grade) was purchased from Kanto Chemical Co., Ltd. (Tokyo, Japan). Unless otherwise stated, all chemical reagents were of analytical grade.

Bacterial strains

Bifidobacterial strains used in this study are shown in Table 1. Strains were obtained from the Morinaga Culture Collection (MCC; Morinaga Milk Industry Co., Ltd., Zama, Japan) or purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA), the Japan Collection of Microorganisms (JCM; Wako, Japan), or the German Collection of Microorganisms (DSMZ; Braunschweig, Germany). All strains were individually cultured under anaerobic conditions in De Man, Rogosa and Sharpe (MRS) broth (Becton Dickinson, Franklin Lakes, NJ, USA) supplemented with 0.05% L-cysteine (Kanto Chemical Co., Ltd.) using an Anaero Pack (Mitsubishi Gas Chemical, Tokyo, Japan).

Dipeptidyl peptidase assay

All tested bifidobacterial strains were cultured at 37 °C for 16 h under anaerobic conditions in MRS broth. Bacterial cells were then harvested by centrifugation (high-speed centrifugal refrigerating machine HIMAC SCR20B (Hitachi Koki Co., Ltd., Tokyo, Japan))at 5,000×*g* (4 °C for 30 min) and washed twice with phosphate-buffered saline (PBS), Dulbecco's Formula (DS Pharma Biomedical Co., Ltd., Osaka, Japan). Subsequently, whole-cell pellets were suspended in PBS containing 0.05% L-cysteine (PBS-C). The optical density (at 600 nm) of each bacterial cell suspension was adjusted to the same value (OD_{600} = 0.2) using PBS-C. H-Gly-Pro-AMC (final concentration, 10 μM) was mixed well and incubated (37 °C) in a 96-well microplate (Thermo Scientific, Tokyo, Japan) under anaerobic conditions for 90 min. Finally, fluorescence was measured at excitation/emission wavelengths of 380/460 nm using an SH-9000 microplate reader (Colona Electric, Inc., Tokyo, Japan).

Degradation of food-derived opioid peptides

Bacterial cell suspensions were prepared using the same method as used for the dipeptidyl peptidase assay and mixed with food-derived opioid peptides (final concentration, 10 μM). The mixtures were then anaerobically incubated at 37 °C for 90 min. To measure the opioid peptides, we used the multiple reaction monitoring (MRM) as quantitative techniques (Croote and Quake, 2016; Fallahbaghery *et al.*, 2017). The remaining intact peptides in the filtered (pore size, 0.22 μm; Millipore, MA, USA) supernatants were quantified using liquid chromatography-tandem mass spectrometry (LC-MS/MS; TSQ Quantum Discovery Max, (Thermo Electron Corp., San Jose, CA, USA) by comparing their concentrations with those of the corresponding synthetic peptide standards. The LC-MS/MS system was

Table 1. *Bifidobacterium* species strains used in the study.

Species	Origin	Strain
B. bifidum	infant faeces	ATCC29521^T
	infant faeces	MCC1092
B. breve	intestine of infant	ATCC15700^T
	infant faeces	MCC1274 (B-3)
	infant faeces	MCC1851 (M-16V)
B. longum subsp. *infantis*	intestine of infant	ATCC15687^T
	intestine of infant	MCC1872 (M-63)
B. longum subsp. *longum*	intestine of adult	ATCC15707^T
	infant faeces	MCC5360 (BB536)
B. adolescentis	intestine of adult	ATCC15703^T
B. angulatum	human faeces	ATCC27535^T
B. dentium	dental caries	DSM20436^T
B. pseudocatenulatum	human faeces	ATCC27919^T
B. animalis subsp.*lactis*	yogurt	DSM10140^T
B. animalis subsp. *animalis*	rat faeces	ATCC25527^T
B. pseudolongum subsp. *globosum*	rumen	JCM5820^T
B. pseudolongum subsp. *pseudolongum*	swine faeces	ATCC25526^T
B. thermophilum	swine faeces	ATCC25525^T

equipped with electro spray ionization and running in positive mode. The LC-MS/MS spectrum (product ion data) of the precursor ion was evaluated to determine their final content. The *m/z* values of precursor and product ions were hCM-7, Q1/Q3, 864.5/636.30; bCM-7, Q1/Q3, 790.5/383.10; and GD-7, Q1/Q3, 876.5/263.00, respectively.

The degradation capability was calculated as 'hydrolysis %' using the following equation:

Hydrolysis % = 100 – (Peptide concentration of culture supernatant with bacterial cells) / (Peptide concentration of the control without bacterial cells) × 100

3. Results

Degradative capability of bifidobacteria with a dipeptide and food-derived opioid peptides

Synthetic substrates were utilised to assess the dipeptidyl peptidase (DPP) activity of 18 bifidobacteria strains (Table 1). As shown in Figure 1A, all strains showed DPP activity; however, no DPP activity was observed in the culture supernatants of any strain (data not shown). The activity was higher in strains of human infant-derived bifidobacteria, such as *Bifidobacterium longum* subsp. *longum*, *B. longum* subsp. *infantis*, *Bifidobacterium breve* and *B. bifidum*, than in those of other species, such as *Bifidobacterium animalis* and *Bifidobacterium pseudolongum*. In addition, some *B. longum* subsp. *infantis* and *B. bifidum* strains were capable of degrading food-derived opioid peptides, namely hCM-7 by MCC1872, ATCC29521^T, and MCC1982 (Figure 1B); bCM-7 by ATCC15687^T, MCC1872, and ATCC29521^T (Figure 1C); and GD-7 by ATCC15687^T, MCC1872, ATCC29521^T, and MCC1092 (Figure 1D).

Degradative capability of *Bifidobacterium bifidum* with a dipeptide and food-derived opioid peptides

A total of 29 *B. bifidum* strains were further evaluated for DPP activity (Figure 2A). Although the activity varied among the strains, most strains had a relatively high DPP activity. No relationship was observed between the DPP activity and the origin of each strain. With few exceptions, all strains were highly active in degrading hCM-7; of the 29 *B. bifidum* strains, 20 showed 100% degradative capability (Figure 2B). The activity was lower with bCM-7 and GC-7 than with hCM-7. MCC1319 and MCC1870 showed the strongest degradative capabilities (Figure 2C and 2D).

4. Discussion

Some food-derived peptides have been reported to cause adverse events, such as allergies (Balakireva and Zamyatnin, 2016; Benede *et al.,* 2015; Wal, 2004), type 1 diabetes (Chia *et al.,* 2017), celiac disease (Hardy and Tye-Din, 2016; Kumar *et al.,* 2017), and mental disorders (Sokolov *et al.,* 2014). With regard to the management of celiac disease, enzyme therapies have been used to enhance gluten digestion (Cornell *et al.,* 2005; Gass *et al.,* 2007; Lahdeaho *et al.,* 2014); in addition, avoiding foods that contain such peptides can mitigate celiac disease (Anania *et al.,* 2017;

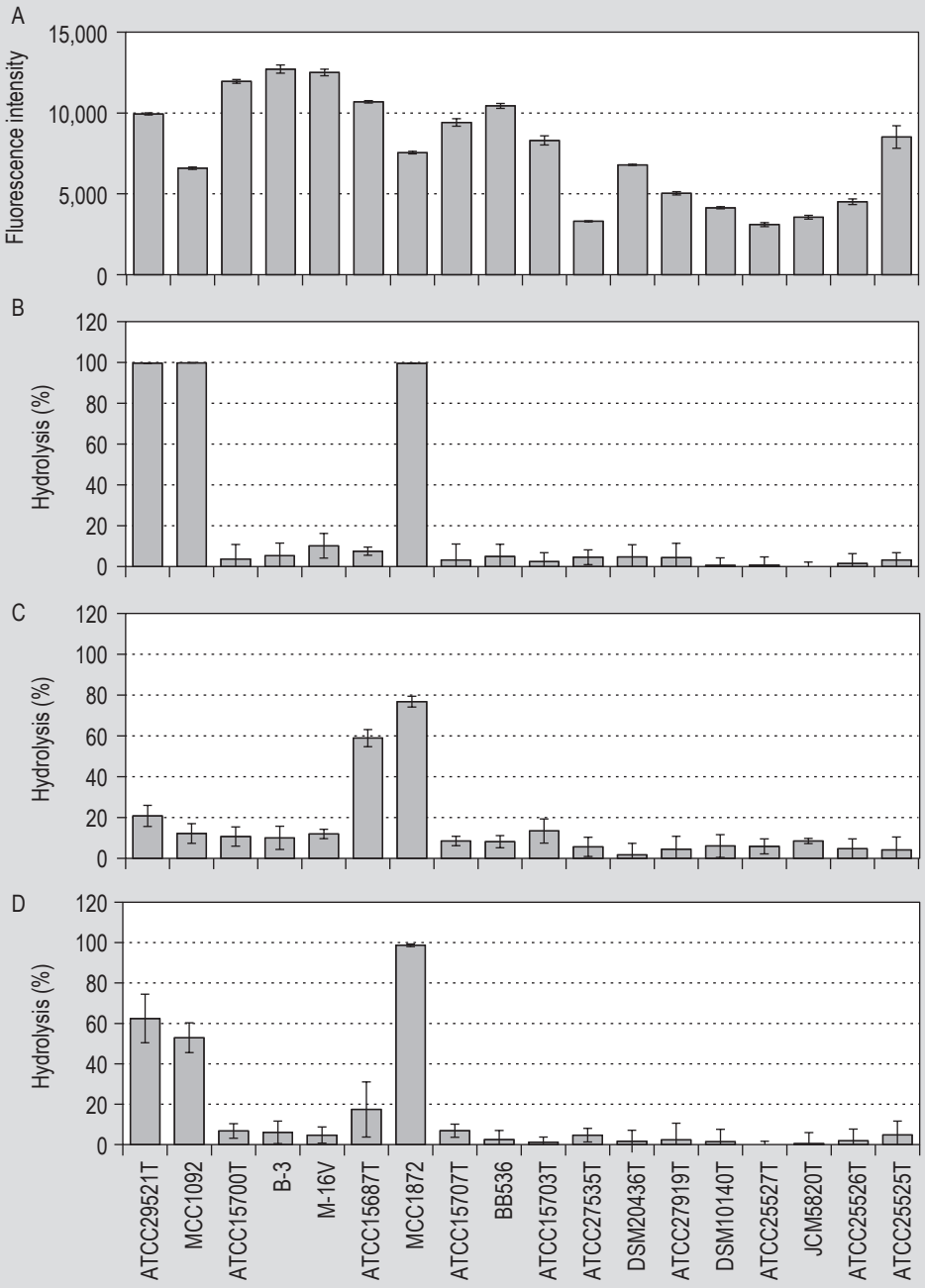

Figure 1. Dipeptidyl peptidase activity (A) and degradative capability of bifidobacterial strains in food-derived opioid peptides: (B) human casomorphin-7, (C) bovine casomorphin-7, and (D) α-gliadorphin-7. Fluorescence intensity and percent hydrolysis values are expressed as mean ± standard deviation.

Brietzke *et al.,* 2017; Posner and Bhimji, 2017). Although lactic acid bacteria have been investigated as sources of enzymatic supplements (Sanz and Toldra, 2001; Ustun and Ongen, 2012), little has been known whether bifidobacterial strains can be used to this extent.

In this report, we examined the DPP activity and degradative capability of 18 bifidobacterial strains in three different food-derived opioid peptides (hCM-7, bCM-7, and GD-7). Human infant-derived bifidobacterial strains showed a relatively higher peptidase activity than the other strains. However, regarding the degradative capability in food-derived opioid peptides (hCM-7, bCM-7, and GD-7), only some *B. longum* subsp. *infantis* and *B. bifidum* strains showed consistent degradative capabilities. These results suggest that DPP activity is not completely related to the

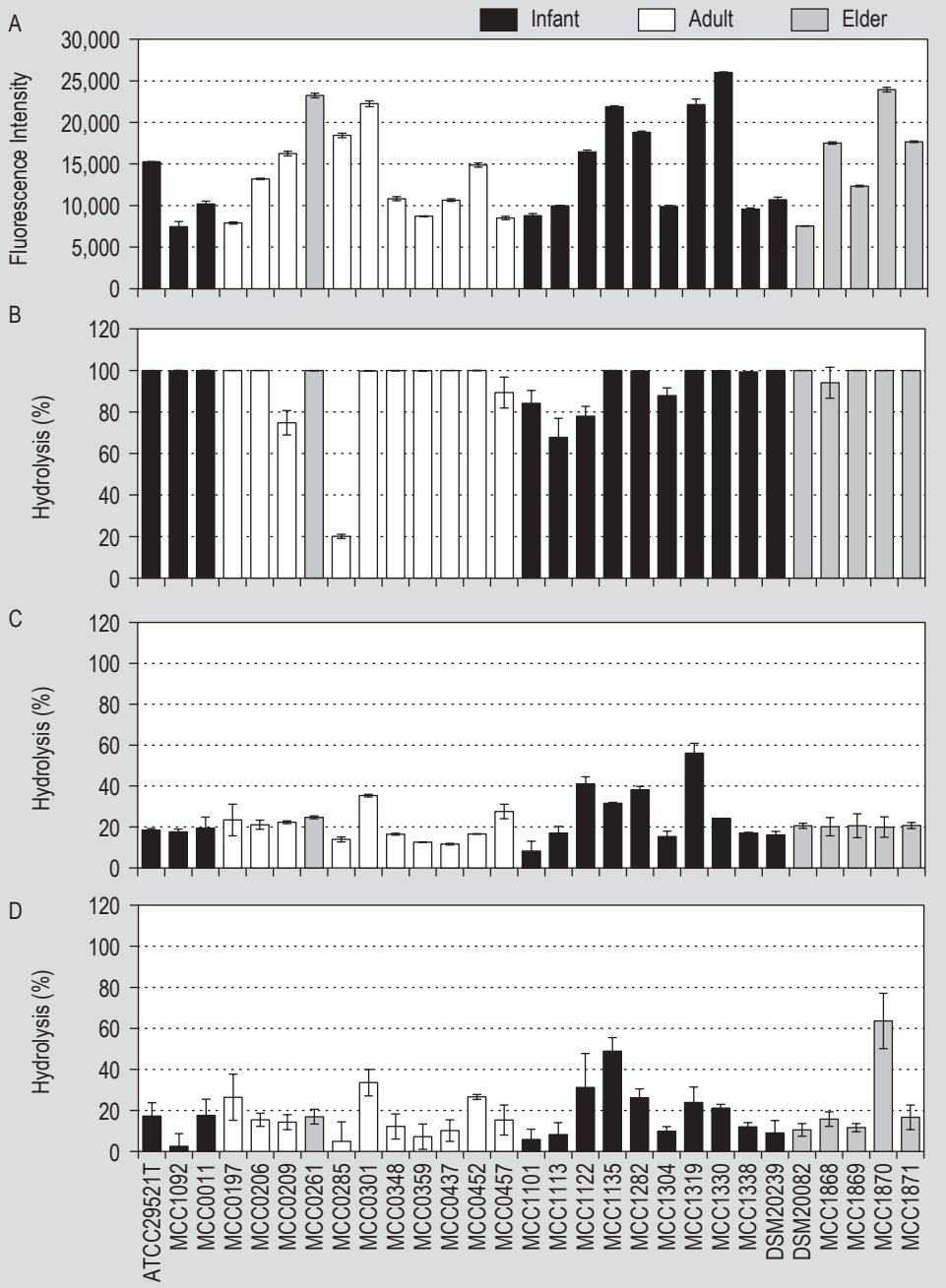

Figure 2. Dipeptidyl peptidase activity (A) and degradative capability of *Bifidobacterium bifidum* strains in food-derived opioid peptides: (B) human casomorphin-7, (C) bovine casomorphin-7, and (D) α-gliadorphin-7. Fluorescence intensity and percent hydrolysis values are expressed as mean ± standard deviation. Hosts were classified as infant- (aged, 0-1 years), adult- (aged, 20-60 years), and elder- (aged, >100 years) derived.

degradable capability in food-derived opioid peptides. We speculate that there are other enzymes, such as X-prolyl-dipeptidyl peptidase, that degrade food-derived opioid peptides (Sanz and Toldra, 2001). These results suggest that although synthetic substrates are ideal for performing rapid large-scale screening, practical examinations using target peptides are needed to assess their degradative capabilities.

We further evaluated 29 *B. bifidum* strains that showed the highest activity in degrading food-derived opioid peptides during the first screening and found differences among the strains. Our previous report suggested that the distribution of bifidobacterial species changes as the host ages (Kato *et al.,* 2017), and it has been proposed that a cause for this age-related change is dietary habits (Odamaki *et al.,* 2016). Therefore, we hypothesised that degradative capabilities

differ among strains isolated from different stages of life (infant, adult, or elderly). As shown in Figure 2, the strains isolated from infants seemed more active, although the difference was not as marked as we having expected in regard to the origin of these *B. bifidum* strains.

The degradation of food-derived opioid peptides by bifidobacteria was assessed by the amounts of the intact peptides after treatment. We failed to measure the degraded fragments in our experiments. We suppose that the degraded peptides might have been assimilated consecutively by bifidobacteria.

In conclusion, we examined the peptidase activity and degradative capability of bifidobacteria in food-derived opioid peptides and found that some *B. longum* subsp. *infantis* and *B. bifidum* strains have potential for eliminating food-derived opioid peptides. Our results showed the possible role of *Bifidobacterium* in the intestines of infants to eliminate food-derived opioid peptides and contribute to host health. The results also implied a novel probiotic property of selected bifidobacteria as an enzyme supplement for improving the health of humans.

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